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To the Graduate Council:

I am submitting herewith a dissertation written by William Nathan Cude entitled "Genetic and Ecological Characterization of Indigoidine Production by Phaeobacter sp. strain Y4I." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

Alison Buchan, Major Professor

We have read this dissertation and recommend its acceptance:

Jeffrey M. Becker, Shawn R. Campagna, Todd B. Reynolds, John P. Biggerstaff

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

Genetic and Ecological Characterization of Indigoidine Production by *Phaeobacter* sp. Strain Y4I

A Dissertation Presented for the

Doctor of Philosophy Degree

The University of Tennessee, Knoxville

William Nathan Cude

August 2013

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For Mom, Dad,

Mimi, Gala, and Karli

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and digging in the dirt for weeks at a time wasn't really for me, I have to appreciate the fact that my interest in dinosaurs shaped who I was to become. I had decided to be a scientist before I even left elementary school. So, thank you dinosaurs. May you be an inspiration to aspiring scientists for generations to come! And a big thank you to everyone who has been a friend and colleague throughout this process. I couldn't have done it without you all.

Abstract

The *Roseobacter* clade is a widely distributed, abundant, and biogeochemically active lineage of marine alpha-proteobacteria. Members of the *Roseobacter* lineage are prolific surface colonizers in marine coastal environments, and antimicrobial secondary metabolite production has been hypothesized to provide a competitive advantage in colonization. In this work, *Phaeobacter* sp. strain Y4I was found to produce the water soluble, blue pigment indigoidine via a nonribosomal peptide synthase-based biosynthetic pathway encoded by a novel series of genetically linked genes, termed *igiBCDFE*. Comparison of wildtype, non-pigmented, and hyper-pigmented Y4I insertional mutants demonstrated a perfect correlation between indigoidine production and the inhibition of *Vibrio fischeri* on agar plates, revealing a previously unrecognized bioactivity of this molecule. Competitive co-cultures of *V. fischeri* and Y4I showed that the production of indigoidine by Y4I significantly inhibits surface colonization of *V. fischeri*. Subsequent experiments identified a role for quorum sensing in the production of this secondary metabolite. Y4I has two independent quorum sensing systems, termed *pgaIR* and *phaIR.* Transposon insertions in each of the *phaIR* genes resulted in defects in indigoidine production. A transposon insertion in *pgaR* confers a null indigoidine phenotype. All of these quorum sensing mutants are unable to inhibit the growth of *V. fischeri* in competition experiments. These strains also have altered biofilm and motility phenotypes suggesting a role for the quorum sensing systems in regulation of these activities. Identification of the *N*-acyl homoserine lactone signaling molecules that are produced by Y4I was achieved using a combination of (AHL) bioreporters and mass spectrometry analyses. The two dominant AHLs were found to be *N*-octanoyl homoserine lactone (C8-HSL) and a putative monounsaturated *N*-3-hydroxydodecanoyl homoserine lactone (3OHC12:1-HSL) when the strain is grown on a complex medium.

Evidence is provided that AHL production is not wholly cell-density dependent in this strain. Finally, a comprehensive analysis of the *luxRI*-type quorum sensing systems in sequenced roseobacter genomes provide evidence that these genetic systems are closely related among lineage members and likely share a common ancestor.

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Introduction

I. Primary production and degradation in temperate salt marshes

Salt marshes and coastal wetlands are where terrestrial freshwater environments meet the ocean. Estuaries and salt marshes are some of the most productive ecosystems on the planet supporting a great diversity of life, including many types of macrofauna (*e.g.* shorebirds, marsh rabbits, terrapin, crustaceans, and cord grasses) (44). Key to the health of the ecosystem and these higher organisms are diverse fungal and bacterial populations that decompose and recycle organic matter (11, 31). Nutrient cycling by microbial populations is an essential part of the carbon, nitrogen, phosphorous, and sulfur cycles both locally and globally (28, 31, 34, 41, 52).

Plant-derived primary production in temperate tidal zones is often dominated by large monocultures of salt tolerant sea grasses of the *Distichilis, Phragmites,* or *Spartina* genera (31, 41, 56). Due to their status at the base of the food chain, much of the dissolved organic matter (DOM) in salt marshes is plant derived leaf litter (34, 35). Dominant fungal species use exoenzymes to degrade the hemi-cellulosic and lignocellulosic components of senescing plants, releasing large quantities of DOM which is further utilized by both bacterial and fungal species (10, 11, 29). Decomposition and remineralization of plant-derived DOM by heterotrophic bacteria and fungi releases labile nutrients into the coastal environment in the form of carbon and energy sources, such as carbohydrates and aromatics, as well as nitrogen sources, including amino acids (7, 11, 31, 34). The decomposition of plants in estuarine and salt marsh systems serves both to recycle bioavailable nutrients and to support the growth of higher organisms.

II. The *Roseobacter* clade are an ecologically relevant bacterial group in coastal environments

Generalist bacteria thrive in coastal salt marshes primarily because they can make use of many different species of essential nutrients. Organisms with a diverse repertoire of metabolic

pathways are able to respond to environmental nutrient fluxes and quickly modify their metabolism to utilize abundant biomolecules (37, 38). The *Roseobacter* clade is a phylogenetically distinct group of marine α-proteobacteria that contribute greatly to the degradation of plant-derived DOM in salt marshes (8, 15-17, 24, 53). In coastal environments, roseobacters can contribute up to 28% of all bacterial 16S rRNA sequences from seawater samples (8, 24). Within the *Roseobacter* clade, there are multiple genera possessing diverse metabolisms, including chemoheterotrophy and mixotrophy, which allows members to quickly respond to the varied organic inputs from plant degradation (4, 36, 38, 50). Because of this, roseobacters are considered generalists. Members of the *Roseobacter* clade have been shown to be important in carbon cycling through the degradation of recalcitrant lignin-derived aromatic substrates and in sulfur cycling through the oxidation of organic and inorganic sulfur compounds (18, 23, 25, 33, 51). Members of this lineage were found to be easily culturable in the laboratory. This distinguishes them from other abundant marine taxa, such as SAR11 clade, that are fairly resistant to standard culturing approaches (42). Genetic manipulation of roseobacter isolates has now allowed for more in-depth experimental investigations into how lineage members might contribute to nutrient cycles and how their diverse physiologies contribute to the collective success of the clade. (24, 40).

Roseobacters have also been shown to quickly colonize and dominate surface niches, out-competing δ- and γ-proteobacteria (15-17). A growing body of research hypothesizes that roseobacters are able to out-compete other microorganisms for surface niches through the production bioactive (antagonistic) secondary metabolites (1, 6, 14, 26, 32). Moreover, investigations have shown that many roseobacters synthesize uncharacterized secondary metabolites of unknown biological function (3-6, 26).

III. Bacterial biofilms

The means by which roseobacters colonize surfaces is through the formation of biofilms. Biofilms are complex bacterial communities encased in a slime-layer of extracellular polymeric substances (EPS) comprised of large molecular weight polysaccharides, DNA, and proteins (12, 45, 49). Biofilms are formed sequentially, with planktonic cells first physically attaching to a surface in a monolayer using pili and flagella. The cells then begin dividing and producing EPS which forms the three-dimensional mature biofilm (12, 13, 39, 49).

Biofilms are both medically and environmentally important. The formation of biofilms during infection contributes significantly to the virulence of the pathogen by allowing it to evade the host immune system and resist antibiotics (13, 21, 46). In aquatic environments, biofilms are thought to be a primary lifestyle for many bacteria, where they can be found attached to biotic and abiotic surfaces (2). There are two main hypotheses as to why bacteria form biofilms. First, in these biofilms bacteria are protected from toxic components of their environment, such as UV light, acidity, reactive oxygen, and heavy metals, due to the physical restrictions of the EPS matrix and the physiological state of the organisms (27). Secondly, bacteria within biofilms occupy a niche which is provided with nutrients from the environment by the movement of water. This flow of water can also serve to disperse the biofilm and spread it to new niches (27, 47, 48).

IV. Quorum sensing

In natural environments, the formation of biofilms requires the genetic coordination of many single celled bacteria. It has been widely reported that diverse groups of bacteria use a regulatory system known as quorum sensing to regulate and coordinated gene expression in a population density dependent manner in biofilms (19, 20, 22, 30). The mechanism by which

they control communal behavior is mediated by constitutively synthesized small molecular weight molecules that diffuse into the local environment. The concentration of these molecules directly reflects the density of the population. The regulation of gene expression in the presence of high density populations is thought to provide a competitive advantage to the group by allowing them to simultaneously express the same phenotype. Many different physiologies that can increase fitness are often controlled by quorum sensing including biofilm formation and antimicrobial production (19, 20, 22, 30, 54, 55).

Most sequenced roseobacter genome contain quorum sensing genes (Chapter 3). The ability of these organisms to quickly colonize surfaces and coordinate gene expression in biofilms is thought to be highly characteristic of the *Roseobacter* clade. It is hypothesized that the dominance of roseobacters on surfaces may contribute to their overall dominance in coastal environments due to their ability to out-compete other organisms for niches.

V. Research objectives

The overarching goal of this dissertation is to provide a genetic and ecological characterization of a bioactive secondary metabolite produced by *Phaeobacter* sp. strain Y4I. Y4I is a member of the *Roseobacter* lineage that was isolated from pulp mill effluent in a salt marsh on the coast of Georgia in the southeastern United States. Y4I was first characterized for its ability to degrade many plant-related substrates (9) and later for its ability to quickly colonize surfaces (43). When grown on complex medium agar, Y4I produces a characteristic blue pigmentation, and preliminary studies have shown Y4I can inhibit the growth of several γ-proteobacteria including *Vibrio fischeri* and *Vibrio anguillarum*. A draft genome sequence of Y4I (Genbank accession number ABXF00000000) became available publically in 2008, and was useful in linking phenotype and genotype.

The research objectives of this dissertation are divided into three parts, forming Chapters 1-3. The first chapter addresses the characterization of the production of this blue secondary metabolite by Y4I and reflects the primary questions driving this research. When and how does Y4I produce the blue pigment, and what fitness advantages does its production provide? The identification of the secondary metabolite and possible bioactivities are also addressed. The second chapter describes the communal behavior and cell-to-cell communication by Y4I. Of particular interest was the identification of the role(s) of quorum sensing in the colonization phenotype of Y4I and secondary metabolite production. The third chapter is a bioinformatic analysis of quorum sensing in the genomes from 43 publicly available roseobacters. Collectively, the work presented in this dissertation provides a much-needed framework for understanding the contributions of secondary metabolite production and quorum sensing in the ecological success of roseobacters.

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Chapter 1

The production of the antimicrobial secondary metabolite indigoidine contributes to competitive surface colonization in the marine roseobacter *Phaeobacter* sp. strain Y4I.

This section is a version of an article that first appeared in the journal *Applied and Environmental Microbiology* under the same title by W. Nathan Cude, Jason Mooney, Arash A. Tavanaei, Mary K. Hadden, Ashley M. Frank, Christopher A. Gulvik, Amanda L. May, and Alison Buchan. It is reprinted with permission from the journal.

W. Nathan Cude, Jason Mooney, Arash A. Tavanaei, Mary K. Hadden, Ashley M. Frank, Christopher A. Gulvik, Amanda L. May, and Alison Buchan. The production of the antimicrobial secondary metabolite indigoidine contributes to competitive surface colonization in the marine roseobacter *Phaeobacter* sp. strain Y4I. *Applied and Environmental Microbiology*. 2012 Jul;78(14):4771-80. doi: 10.1128/AEM.00297-12.

My contribution to this paper was the purification and isolation of indigoidine, the co-culture competitions both in liquid and in biofilms, the motility and surface colonization assays, the assessment of hydrogen peroxide sensitivity, and much of the literature review and writing.

I. Abstract

Members of the *Roseobacter* lineage of marine bacteria are prolific surface colonizers in marine coastal environments, and antimicrobial secondary metabolite production has been hypothesized to provide a competitive advantage to colonizing roseobacters. Here, we report that the roseobacter *Phaeobacter* sp. strain Y4I produces the blue pigment indigoidine via a non-ribosomal peptide synthase (NRPS)-based biosynthetic pathway encoded by a novel series of genetically linked genes: *igiBCDFE*. A Tn5-based random mutagenesis library of Y4I revealed a perfect correlation between indigoidine production by the *Phaeobacter* strain and inhibition of *Vibrio fischeri* on agar plates, revealing a previously unrecognized bioactivity of this molecule. In addition, null mutants of *igiD*, the indigoidine NRPS, were more resistant to hydrogen peroxide, demonstrated a motility defect, and colonized an artificial surface faster in comparison to the wildtype strain. Collectively, these data provide evidence for pleiotropic effects of indigoidine production in this strain. Gene expression assays support phenotypic observations and demonstrate *igiD* gene expression is upregulated during growth on surfaces. Furthermore, competitive co-cultures of *V. fischeri* and Y4I show that the production of indigoidine by Y4I significantly inhibits colonization of *V. fischeri* on surfaces. This study is the first to characterize a secondary metabolite produced by a NRPS in roseobacters.

II. Introduction

The *Roseobacter* clade is a widely distributed, abundant and biogeochemically active lineage of marine α-Proteobacteria comprised of multiple genera, including *Phaeobacter, Ruegeria, Loktanella,* and *Citreicella* (12, 20). *Roseobacter* abundance and activity is often greatest in coastal marine environments and in association with phytoplankton blooms (e.g. 7, 12, 20, 39, 58). The dominance of roseobacters under these conditions has been attributed, in part, to their ability to quickly colonize and out-compete other organisms for surface niches, including the surfaces of higher order eukaryotes, such as phytoplankton and vascular plants (5, 19, 20, 46, 52, 53). In addition to cell-density dependent regulatory mechanisms and morphological features, the production and secretion of bioactive metabolites are predicted to contribute to the colonization success of roseobacters (4, 10, 18, 26, 37). Our understanding of the chemical diversity and biological function of *Roseobacter* secondary metabolites, however, is limited.

Some of the earliest indications that *Roseobacter* representatives produce antimicrobial molecules are from studies seeking to identify probiotic bacteria for aquaculture. Hjelm et al. (2004) identified roseobacters that were antagonistic against fish larval bacterial pathogens and *Phaeobacter galleaeciensis* was demonstrated to have probiotic effects on scallop larvae (49). The genetic underpinnings as well as structural and biological characterization of secondary metabolite production in roseobacters have been most extensively studied in strains that produce the antimicrobial compound tropodithietic acid (TDA). TDA has an unusual seven-member aromatic tropolone ring backbone and its production has been demonstrated to be growth condition dependent (8, 9, 18, 23). TDA has been shown to inhibit the growth of pathogenic *Vibrio* species and aid in the competitive surface colonization of strains capable of its production

(18, 44). The ability to synthesize TDA has been demonstrated in several *Roseobacter* genera, including *Ruegeria* and *Phaeobacter* species, and is a valuable model for *Roseobacter* secondary metabolite production. Genomic investigations, however, have shown that most sequenced roseobacters lack the characterized TDA biosynthesis pathway (39). Moreover, roseobacters are collectively known to synthesize a myriad of secondary metabolites of undefined function (6, 7, 10, 12, 34, 58).

Phaeobacter sp. strain Y4I of the *Roseobacter* lineage was isolated from pulp mill effluent in the coastal southern United States and produces a dark blue, water insoluble pigment when grown on agar plates containing a complex medium (13). The production of this pigment has not previously been reported in characterized *P. daeponensis* strains, which share 99% identity of the 16S rDNA gene with Y4I; however, *Phaeobacter* sp. strain Y3F, which was isolated alongside Y4I, and isolates of *P. caeruleus* have been reported to be blue on complex media (11, 57, 62). Y4I has been characterized with respect to its ability to catabolize aromatic compounds (11, 13) and its genome sequence is available, providing additional insight into the metabolic potential of this strain (39). Preliminary studies have shown that Y4I suppresses the growth of representative marine bacteria such as *Vibrio fischeri*, *Vibrio anguillarum*, and *Ruegeria lacuscaerulensis* on agar plates (53), yet it does not have the required genes for TDA biosynthesis (39). Here, we identify the genes that encode the biosynthetic machinery that synthesizes the blue pigment and characterize biological roles of this metabolite not previously reported in roseobacters.

III. Methods and Materials

Growth conditions and media. Phaeobacter sp. strainY4I was isolated from an indulin enrichment of coastal seawater as reported previously (13, 53). Unless otherwise noted, the following culture conditions were used: Phaeobacter sp. strainY4I was maintained in YTSS broth [per liter: 2.5 g yeast extract, 4 g tryptone, 15 g Sea Salts (Sigma-Aldrich, St. Louis, MO)] at 30°C, with shaking. YTSS agar (1.5%) plates were used for streaking to isolation and also incubated at 30°C. Escherichia coli EA145 is auxotrophic for 2,4-diaminopimelic acid (DAP) and was maintained in YTSS liquid with 1 mM DAP (Acros Organics, Geel, Belgium) at 30°C, shaking (24). V. fischeri ES114 (ATCC Number: 700601) was kindly provided by Eric Stabb (University of Georgia) and maintained on YTSS broth at 23°C, shaking.

Generation of transposon mutant library. A random mini-Tn5-transposon library of Y4I was generated by conjugal transfer of plasmid pRL27::mini-Tn5- KmR-oriR6K from E. coli EA145 using previously described methods (35). Briefly, liquid mating mixtures of donor and recipient cells were prepared with 200 µl of early log phase cells ($OD_{540} \approx 0.35$) and combined in 1.5 ml microcentrifuge tubes. The cell mixture was pelleted by centrifugation and suspended in 15 µl of fresh YTSS. The mating mixture was spotted onto YTSS agar with 1 mM DAP and incubated at 30°C overnight. Using a sterile toothpick, the microbial biomass was removed and suspended in 1 ml fresh YTSS broth. The suspension was plated on YTSS agar with kanamycin (25 µg/ml) and incubated at 30°C overnight. Kanamycin resistant (KmR) colonies were patched to fresh selective media and the library was drawn from those plates. Unless otherwise stated, all subsequent growth of Tn5 mutants was done in YTSS containing 50 µg/ml kanamycin.

Screen for V. fischeri inhibition. To screen for suppression of V. fischeri, the mini-Tn5 mutant library was inoculated into 96-well plates (BD Falcon, Franklin Lakes, NJ) containing YTSS

broth, without antibiotic. The stability of the KmR marker in the absence of antibiotic selection was demonstrated for randomly selected mutants (data not shown). Mid-exponential phase V. fischeri ES114 was spread to lawn density on 22 cm x 22 cm YTSS agar plates. A 96 pin replicator (Nunc 250520, Rochester, NY) was used to spot 1 µl of broth culture from each well of the mini-Tn5 library onto the ES114 lawn. The plates were incubated at 23°C and scored after 24 h. The presence of a zone of clearing was considered indicative of inhibition.

Arbitrary PCR and DNA sequencing of mini-Tn5 transposon mutants. Arbitrary PCR was used to localize Tn5 chromosomal insertion sites in selected mutants using a previously described method (42). Briefly, genomic DNA was extracted from overnight cultures of the selected mutants using the DNeasy® MiniPrep kit (Qiagen, Valencia, CA), and 100 ng was used for the subsequent amplifications. The transposon specific forward primer TNPR13Out (5' CAG CAA CAC CTT CTT CAC GA 3') and the tagged reverse primer ARB1 (5' GGC CAC GCG TCG ACT AGT ACN NNN NNN NNN GAT AT 3') were used to amplify DNA upstream of the 5' end of the insertion. The reverse primer TNPR17Out (5'AAC AAG CCA GGG ATG TAA CG 3') and the forward primer ARB6 (5' GGC CAC GCG TCG ACT AGT ACN NNN NNN NNN ACG CC 3') were used to amplify downstream of the 5' end of the insertion. The initial amplification cycles consisted of a denaturation at 95°C for 5 min, five cycles of 30 s denaturation at 94°C, 30 s annealing at 30°C, and 1 min extension at 72°C, then 30 cycles of 30 s denaturation at 94°C, 30 s annealing at 45°C, and 1 min extension at 72°C, and a final extension at 72°C for 5 min. The products of the initial amplification were used in the subsequent nested amplification step. The forward primer TNPR13Nest (5' CTA GAG TCG ACC TGC AGG CAT 3') and the ARB2 reverse primer (5' GGC CAC GCG TCG ACT AGT AC 3') were used to remove non-specific amplification of the upstream PCR. TNPR17Nest (5' CTG ACA TGG

GGG GGT ACC 3') and ARB2 were used to clean the downstream amplification. The PCR for the nested amplification consisted of a denaturation at 95°C for 5 min, thirty cycles of 30 s denaturation at 94°C, 30 s annealing at 30°C, and 1 min extension at 72°C, and a final extension at 72°C for 10 min.

The products of the arbitrary amplification reactions were sequenced by fluorescent dye-terminator cycle sequencing at the University of Tennessee Molecular Biology Resource Facility. Homology searches (BLASTX) were conducted using the NCBI online server. The locations of transposon insertions were then manually mapped to the published draft genome of Y4I (GenBank Accession: ABXF00000000).

Purification and analysis of the blue pigment. Extraction and purification of the blue pigment was performed following methods outlined in Takahashi et al. 2007. Briefly, ~10^3 CFU/ml broth culture of a clpA::Tn5 mutant was spread on YTSS agar plates and incubated at 30ºC until large, darkly pigmented colonies formed (~48 h). The colonies were removed from plates using Cell Scrapers (Corning, Corning, NY) and cellular biomass was placed in a 50 ml conical tube (BD Biosciences, Franklin Lakes, New Jersey). The mass of the collected cells was recorded and DMSO was added at 1 ml 10 mg-1 of cells. The tube was placed on ice and the cells were lysed using a Microson Ultrasonic Cell Disrupter XL 2000 (Qsonica, Newtown, CT) with four cycles of 5s burst 5s rest. The output power was set at 8 watts. The cellular debris was pelleted by centrifugation at 6000 × g for 15 min. The supernatant was transferred to a new tube and re-centrifuged. This was repeated until no cellular pellet could be seen. The supernatant was passaged through a 0.2 µm filter. The pigment was precipitated by addition of sterile Milli-Q water at five times the volume of the DMSO. This solution was then ultracentrifuged at 50,000 × g for 1 h using a Sorvall WX Ultra 80 (Thermo Scientific, Swedesboro, NJ) at 23ºC. The

supernatant was removed and the pellet was washed with sterile Mill-Q water. The pellet was dried using a Savant SpeedVac SC110 (Thermo Scientific, Swedesboro, NJ).

The lyophilized pigment was solubilized in DMSO and diluted 1:10 for UV/Vis absorbance measurements using a Beckman Coulter DU 800 spectrophotometer (Beckman Coulter, Brea, CA). A high-resolution mass spectrum of the lyophilized pigment was measured on an AccuTOF mass spectrometer (JEOL, Peabody, MA) using a DART ion source in positive ion mode. The 1H-NMR spectrum was recorded on a Varian INOVA 500 MHz spectrometer (Varian, Palo Alto, CA) using the blue pigment dissolved in deuterated DMSO.

Motility assay. Swimming motility was assessed using 0.35% agar 10% YTSS plates [per liter: 0.25 g yeast extract, 0.4 g tryptone, 15 g Sea Salts, and 3.5 g agar] as described previously (1, 2). Ten µl of stationary phase cultures (OD540~1.5) were spotted onto the motility plates and incubated at 30°C and scored after 24 h by measuring the diameter of the swim circle.

Surface colonization assay. Surface colonization was measured in 96-well vacuum-gas plasma treated polystyrene plates (BD Falcon, Franklin Lakes, NJ) with 20% YTSS broth [per liter: 0.5 g yeast extract, 0.8 g tryptone, 15 g Sea Salts]. 20% YTSS was used to minimize flocculation by some of the mini-Tn5 mutants (data not shown). The Y4I variants were inoculated in triplicate at a concentration of ~1 x 106 CFU/ml. A sterile 4 mm glass bead (Walter Stern, Post Washington, NY) was added to each well and the plates incubated under stationary conditions at 27°C. Samples were taken at 2, 4, 6, 10, 12, 14, 24, 48 and 72 h post inoculation. Attached cells were removed from the beads by a modified version of the Leriche and Carpentier 1995 method that has been used previously for roseobacters (18, 36). At each time point, 3 beads were removed and placed in glass screw-top vials with 940 µl YTSS broth and 60 µl 10% (v/v)

TWEEN20 (Fischer Scientific, Pittsburg, PA). Samples were ultrasonicated at 40 kHz in a water bath sonicator (Branson, Danbury, CT) for 6 min and vortexed for 30 s. Samples were then serial diluted and plated for CFU counts. The addition of TWEEN20 was to limit cell clumping evident under phase contrast microscopy of early trials. Preliminary experiments using the wildtype and igiD::Tn5 strains showed no significant loss of viability due to the treatment (data not shown).

Co-culture competition assays. For broth culture assays, 50 µl of stationary phase wildtype or mutant cultures of Y4I (~5×10^9 CFU/ml) were inoculated into 10 ml YTSS broth lacking antibiotic with 50 µl overnight V. fischeri culture (~5×10^9 CFU/ml) in triplicate. V. fischeri and Y4I variant monoculture control cultures were prepared and run in parallel. The cultures were incubated for 20 h at 27°C due to the similar growth rates of both strains at that temperature (data not shown). Aliquots were removed and serially diluted for CFU counts. Plates were incubated at 34°C, a prohibitive temperature for V. fischeri, for Y4I viable counts and at 20°Cfor V. fischeri viable counts. The two strains can be differentiated at 20°C because of the delayed emergence of visible Y4I colonies relative to V. fischeri at this temperature. In order to address the question of whether a density advantage is necessary to see evidence for a competitive interaction between the Y4I variants and V. fischeri, competition assays were also performed in which the initial concentration of V. fischeri was 104 CFU/ml and Y4I was 106 CFU/ml.

For competition assays on surfaces, overnight broth cultures of the Y4I strains and V. fischeri were diluted to ~1×10^6 CFU/ml in 20% YTSS and added to 96-well plates with glass beads. Monocultures of each strain were used as no competition controls. All competitions and controls were performed in triplicate. The co-cultures were incubated for 24 h at 27ºC in 20% YTSS and then ultrasonicated to liberate the cells for CFU counts, as described above.

Assessment of HOOH sensitivity. Strains were exposed to hydrogen peroxide in broth using a method similar to that previously reported for D. dadantii (48). Stationary phase Y4I broth cultures (~10^9 CFU/ml) were exposed to 250 mM hydrogen peroxide in YTSS broth, which has been shown in the lab to be inhibitory to wildtype (data not shown). The strains were exposed in 1 ml aliquots for 10 and 60 min at 30°C, with shaking. The cultures were then serially diluted and plated for CFU counts. All experiments were done in triplicate.

Peroxide resistance was also monitored in surface attached cells by exposing biofilms on glass beads to 75 mM hydrogen peroxide, a concentration experimentally determined to be inhibitory to wildtype Y4I biofilms (data not shown). Wildtype Y4I biofilms were grown on glass beads for 24 h in 20% YTSS, as described above. Biofilms of the igiD mutant were incubated for 4 h, to ensure comparable numbers of cells per bead (~5 x 10^5 CFU/bead). The beads were then removed from the growth medium and exposed to 75 mM hydrogen peroxide in 20% YTSS for an immediate exposure and 2, 5, 10, 15, 30, and 60 min incubations. The immediate exposure was a transfer to and immediate removal from the hydrogen peroxide solution. Biofilms were then removed as described earlier and serially diluted for CFU plate counts. CFU counts were compared to a no hydrogen peroxide control. All experiments were done in biological and technical triplicate.

RNA extraction. RNA was extracted from colonies grown on agar plates, cells in broth cultures, flocking cells from broth cultures, and biofilms grown on polycarbonate. Collected cells were briefly centrifuged at 5,000 × g to pellet. The supernatant was removed and the cells were resuspended in 200 µl RNAlater RNA Stabilization Reagent (Qiagen, Germantown, MD). The RNA was extracted using the Qiagen Rneasy Mini Kit. After extraction of the RNA, DNA

was removed using the TURBO DNA-free Kit (Ambion, Austin, TX). Samples were checked for the presence of DNA by PCR after Dnase treatment to ensure purity.

Reverse transcription and quantitative PCR. The resulting RNA sample was converted to cDNA using M-MLV Reverse Transcriptase and random hexamers (Invitrogen, Carlsbad, CA). 0.5 µl of the random primers (500 ng/µl), 1.0 µl dNTPs (10 mM), and 10.5 µl of the RNA sample (6 ng/µl) were heated to 65°C for 5 min and then chilled on ice. Four µl 5X First Strand Buffer [250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM MgCl2], 2 µl 0.1 M dithiothreitol, and 1µl Rnase Out (Invitrogen) was then added. The solution was mixed and incubated at 37°C for 2 min. One µl M-MLV RT (200 units/µl) was added to the solution and incubated 10 min at 25°C followed by 50 min at 37°C. The enzyme was inactivated by heating 15 min at 70°C.

RT-qPCR assays were used to measure gene expression of indigoidine biosynthesis genes as well as reference genes. Three reference genes (*map, rpoC,* and *alaS*) were selected using previously described criteria (40). Primers were designed for the three reference genes and the indigoidine biosynthesis NRPS (*igiD*) using the Primer3 online software tool (http://frodo.wi.mit.edu/) and the Y4I genome sequence (NCBI Reference Sequence: NZ_ABXF00000000.1). All primer sets were tested on genomic Y4I DNA and yielded a single band of the expected size. Primers for the RT-qPCR are as follows: *igiD* forward (5' GGT CAG AAA GGA CGC GTC GCG G 3') and reverse (5' AGC GCG CGA TGC CGA GCT GAT C 3'), *map* forward (5' GTG TTC CAC GCC CCG CCC AAC 3') and reverse (5' CCC GGC CGG TGA CAG GGT GAA 3'), *rpoC* forward (5' CGG CGC TGA AGC GAT CCG TGA 3') and reverse (5' CCG GAC GGT TGC CCG ATT CCA 3'), and *alaS* forward (5' GCT GTG GGC GGA GGG GCA ATG 3') and reverse (5' GCC GAT CGA ACC GCC GGT GAC 3'). Optimum qPCR amplification conditions for each primer set were determined by performing

control runs using varying concentrations of forward and reverse primer (from 100-1500 nM) and a set genomic DNA concentration of 2.5×10^5 genomes per reaction. The primer concentration that gave the lowest threshold cycle (Ct) value and had the highest efficiency was chosen as the optimum concentration for the following reactions. All primer sets yielded a product between 160 and 240 bp. The PCR was done in a Bio-Rad DNA Engine Opticon 2 real-time PCR detector (Bio-Rad, Hercules, CA) with SYBR Green PCR reagents (Invitrogen, Carlsbad, CA). Twenty-five µl reactions were prepared in 0.2 ml skirted 96-well qPCR plates (Thermo Scientific, Waltham, MA) with 12.5 µl SYBR green Pre-Mix 2X, 7.5 µl dH_2O, 1.25 µl forward primer, 1.25 µl reverse primer, and 2.5 µl of the sample cDNA. Plates were thermocycled for 95°C for 15 min, 40 cycles of 95°C for 45 s, 65°C for 45 s, 72°C for 15 s, and followed by 72°C for 5 min. Melting curves were generated after each assay to verify the specificity of the amplification by heating the samples from 50°C to 100°C at 1°C/s and taking fluorescence measurements every 1.0°C. These melt curves consistently showed a single peak indicating high specificity of the primer sets.

Statistical analyses. ANOVA mean separations were done using the Tukey's Honestly Significant Difference test in SigmaPlot 11.0 (Systat Software, Inc., Chicago, IL). RT-qPCR data analysis and the normalized relative transcript quantity was calculated using the qBASE method which permits the use of multiple reference genes to guarantee reference gene stability as well as the use of biological replicates to guarantee experiment reproducibility (29). These data were normalized to the three reference genes and relative to late exponential broth cultures of wildtype.

IV. Results and Discussion

The antagonistic behavior of *Phaeobacter* sp. strain Y4I is correlated with pigmentation.

As some secondary metabolites in roseobacters have been found to be antimicrobial in nature (e.g. (10, 38)), we sought to determine if the previously reported antagonistic behavior of Y4I towards *Vibrio fischeri* (53) was correlated with the production of a blue pigment characteristic of Y4I growth on complex agar media (Fig. 1). To that end, we generated a 6048-member mini-Tn5-random transposon mutant library of Y4I and screened for mutants with alterations in the *V. fischeri* inhibition phenotype. Forty-five Tn5 mutants were unable to inhibit *V. fischeri*. A direct correlation was observed between lack of pigmentation and failure to inhibit the growth of *V. fischeri* in plate assays. Five hyper-pigmented mutants were also isolated and demonstrated enhanced inhibition of *V. fischeri* (Fig. 1). The insertion site of the mini-Tn5 cassette was determined for all 50 of these mutants using arbitrary PCR. Twelve non-pigmented mutants had transposon insertions in either a gene predicted to encode a non-ribosomal peptide synthetase (NRPS), annotated as an indigoidine synthase (*igiD*), or two adjacent genes, designated *igiB* and *igiC*, that form a putative operon with *igiD* and three additional genes (Table 1, Fig. 2). Among the eight *igiD* mutants, four independent insertion sites were represented. Two independent mutants were obtained for both *igiB* and *igiC*. The randomly inserted mini-Tn5 cassette has been shown previously to allow read through to downstream genes (35), suggesting that *igiB, igiC, and igiD* are all necessary for pigmentation in this strain. Polar effects, however, cannot be ruled out and additional studies are required to determine this conclusively. Other non-pigmented mutants were found to have transposon insertions in each of two *luxRI*-like quorum sensing systems annotated in the Y4I genome, suggesting quorum sensing is involved in pigment production. The remaining 28 non-pigmented mutants were localized to genes encoding poorly

characterized transcriptional and response regulators, methyltransferases, and hypothetical proteins (Table 3). The five hyper-pigmented mutants that showed enhanced *V. fischeri* inhibition all had insertional disruptions in a gene encoding the universal regulatory chaperone protein ClpA. Within those five mutants, three independent insertion sites were represented. In *E. coli*, ClpA has been shown to be involved in the degradation of abnormal and regulatory proteins, suggesting an insertion in *clpA* may enhance pigment production due to alterations in gene regulation (25, 32). Representative *igiD*::Tn5 and *clpA*::Tn5 mutants were selected for further phenotypic characterizations described below; none of these mutants displayed growth abnormalities in broth cultures.

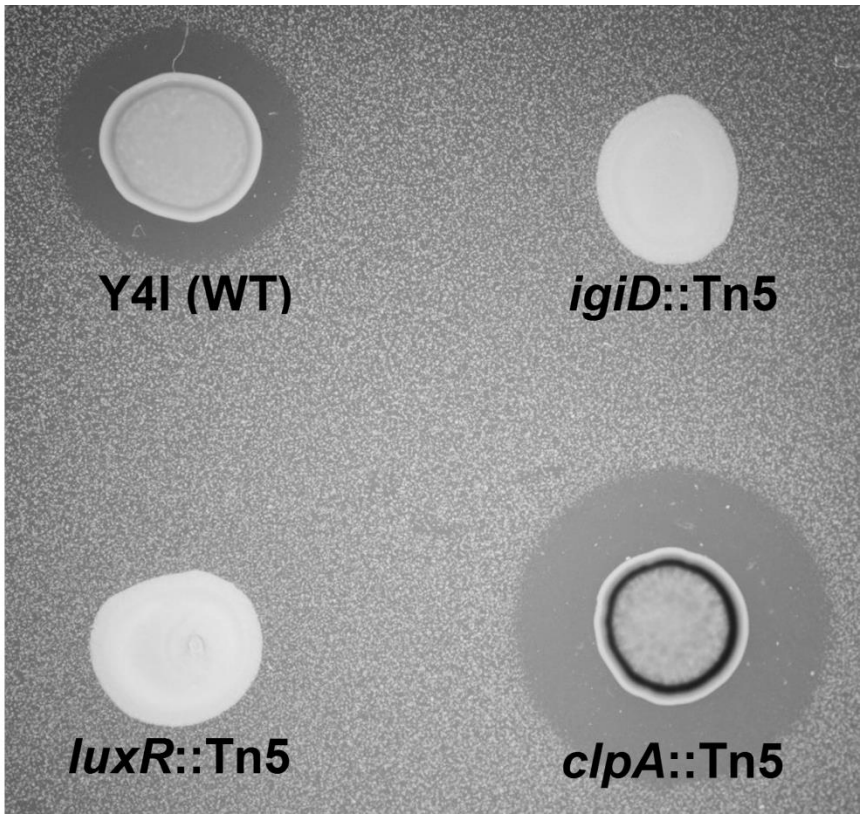


Figure 1. Qualitative screen for *V. fischeri* inhibition by *Phaeobacter* sp. strain Y4I. Wildtype and Tn5 insertional mutants are shown on a lawn of *V. fischeri. igiD* encodes the indigoidine NRPS; *clpA* encodes a regulatory protein protease; and *luxR* encodes a regulatory protein involved in quorum sensing.

Table 1. Representative *Phaeobacter* sp. strain Y4I Tn5 insertional mutants.

Interrupted gene (GenBank locus tag)	Position of mini-Tn5 cassette[a]	Pigmentation[b]	*V. fischeri* inhibition[b]
igiB (RBY4I_1554)	586 (1)	-	-
	606 (1)	-	-
igiC (RBY4I_3713)	362 (1)	-	-
	498 (1)	-	-
igiD (RBY4I_2890)	595 (2)	-	-
	1692 (2)	-	-
	2968 (2)	-	-
	2496 (2)	-	-
luxR (RBY4I_1689)	269 (1)	-	-
	373 (1)	-	-
luxI (RBY4I_3631)	N/A (0)	N/A	N/A
luxR (RBY4I_1027)	429 (1)	-	-
	154 (1)	-	-
luxI (RBY4I_3464)	373 (1)	-	-
clpA (RBY4I_3424)	443 (3)	++	++
	446 (1)	++	++
	447 (1)	++	++

[a]Nucleotide position within the designated gene; the number of individual mutants isolated with an insertion at that position are indicated in parenthesis. [b]Pigmentation and inhibition scores are relative to wildtype, with (-) indicating no pigmentation and no inhibition and (++) indicating enhanced pigmentation and inhibition, as determined by plate assays. N/A indicates that no mutants with insertions in that gene were found in the library.

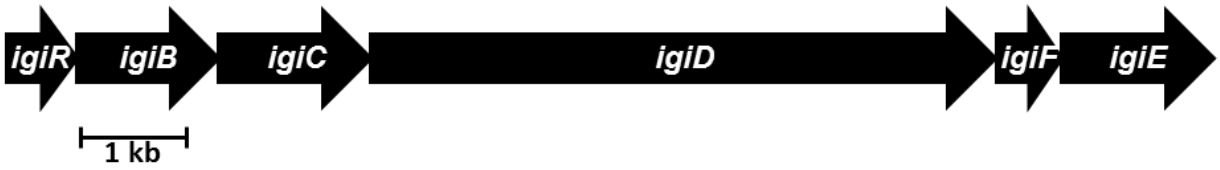


Figure 2. *Phaeobacter* sp. strain Y4I indigoidine biosynthesis operon. The organization of the genes within the operon (RBY4I_313, RBY4I_1554, RBY4I_3713, RBY4I_2890, RBY4I_618, and RBY4I_1719) was obtained from the published genome sequence. The genes are represented by block arrows, which indicate orientation and are shown to scale. Gene product names are given in Table 2.

Pigment characterization. Identification of interrupted genes in the non-pigmented mutants suggested the blue compound produced by Y4I was indigoidine, a bicyclic 3,3'-bipyridyl molecule synthesized by the cyclization of two glutamine molecules (55). This was further confirmed by analysis of the purified pigment. The absorbance spectrum of the purified compound in DMSO has a peak at 612 nm, which has been previously reported as the absorbance maxima of indigoidine (33). Profiles of the Y4I variant show approximately 5 times higher absorbance at this wavelength for the *clpA*::Tn5 mutant compared to the wildtype strain (Fig. 7). MS analysis yielded a $[M+H]^+$ peak at 249.06110 *m/z* (Fig. 3A) which is consistent with the chemical formula $C_{10}H_9N_4O_4$ for indigoidine with an ionizing proton (21). The ^{1}H-NMR spectra contained three singlets at 11.29, 8.18, and 6.47 ppm that had relative integrations of 1, 1, and 2 protons, respectively (Fig. 3B). These chemical shifts, splitting patterns, and integrations are consistent with those expected for the -NH, -CH, and -NH_2 bonds of indigoidine, respectively, and are identical to previously published reports (55).

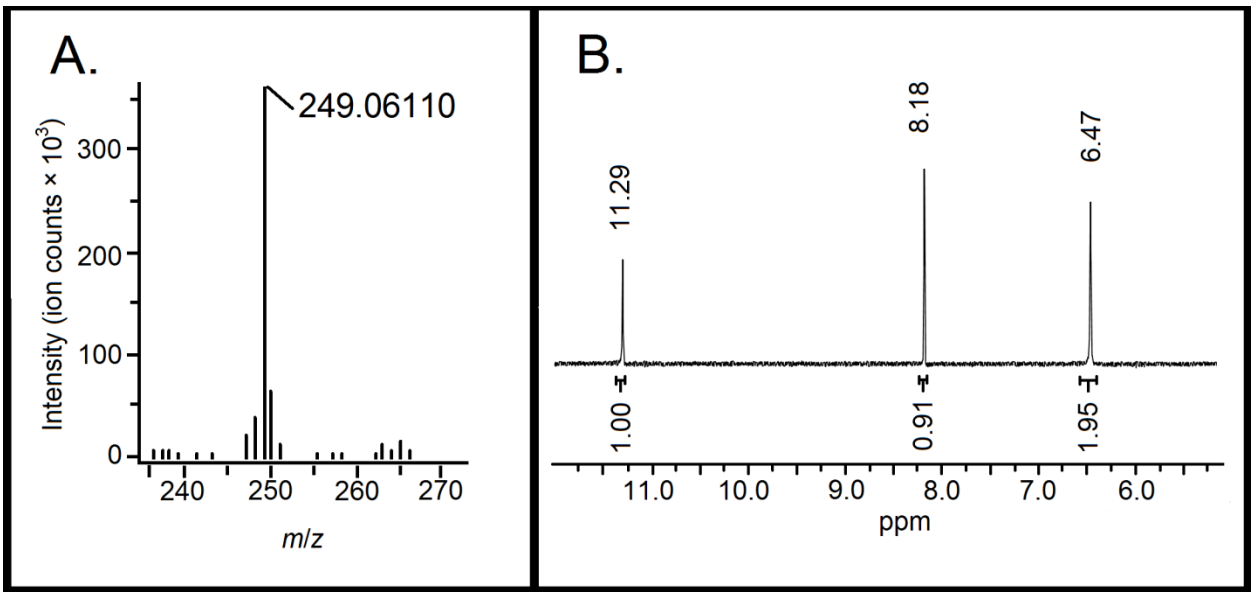


Figure 3. Chemical analyses of purified pigment. (A) Mass spectrum analysis by HRMS-DART. The peak m/z at 249.06110 is consistent with protonated indigoidine ($C_{10}H_9N_4O_4$). (B) ^{1}H-NMR spectrum with peaks at 11.29 (NH), 8.18 (CH), and 6.47 (NH_2) which is consistent with the published spectrum of indigoidine (55).

Genes encoding indigoidine biosynthesis. Indigoidine is synthesized by a NRPS encoded by a gene typically annotated as either igiD or indC (Fig. 4, Table 2) (48, 55). Previous studies of the chemistry of indigoidine focused on its redox activity and deep blue pigmentation for use as a possible redox state sensor or industrial dye (21, 30, 33, 54). Its biological role as a secondary metabolite, however, remains poorly characterized. Indigoidine is found in two redox states, an oxidized blue form which is insoluble in water and a reduced colorless form which is water soluble and historically referred to as leucoindigoidine (21, 30, 33, 47, 54). It is currently hypothesized that indigoidine is synthesized in the oxidized form and subsequent reduction is biologically mediated (48, 55), possibly by the oxalocrotonate tautomerase encoded by igiF (Table 2, Fig 3), though this has not been experimentally confirmed. Given the solubility issues of the oxidized compound, it is anticipated that the reduced state is the biologically relevant form for the inferred antimicrobial properties. This is supported by assays performed with the isolated oxidized compound in either a lyophilized state or solubilized in DMSO (data not shown). Unfortunately, reduction of purified indigoidine requires a strong acid with reoxidation occurring readily under non-reducing conditions (21, 54). These features prevent biological assays with the isolated compound in a reduced state. Thus, it could be argued that indigoidine needs to be in the appropriate biological context in order to function as predicted and that this is an important area for future investigation.

Indigoidine biosynthesis genes have been identified in a phylogenetically diverse group of microbes, including α-, β-, and γ-Proteobacteria, as well as several *Streptomyces* species (14, 21, 30, 48, 55). The biosynthesis operon of Y4I shares the most homology with putative indigoidine biosynthesis genes from the β-Proteobacterium *Vogesella indigofera* (AF088856) that are deposited in GenBank but are not described in the literature (Fig. 9).

The single common gene present in all characterized indigoidine producing strains is the aforementioned NRPS-like indigoidine synthase, designated either *igiD* or *indC*. Sequence similarity alone indicates that the IgiD of Y4I and the IndC of the plant pathogen *Dickeya dadantii* 3937 (formally *Erwinia chrysanthemi*) are functionally homologous, sharing 50% identity and 63% similarity across the 1,314 residues of the sequence. Genes adjacent to that encoding the NRPS likely serve to modify either substrate or product or to produce co-factors necessary for indigoidine synthesis; these modifiers typically differ between lineages (Table 2).

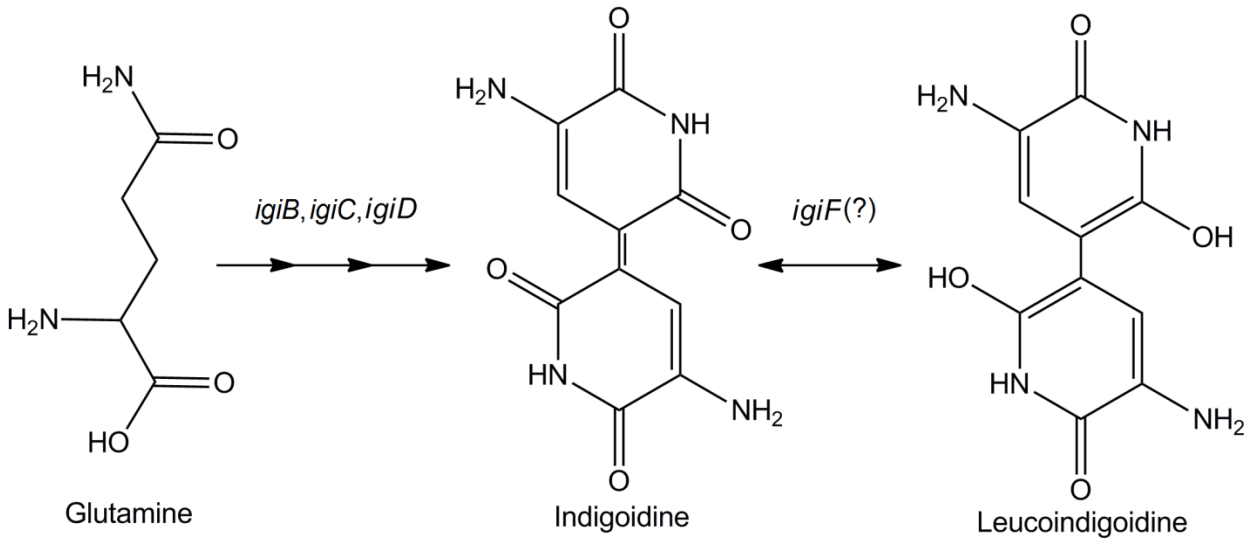


Figure 4. Proposed mechanism of indigoidine biosynthesis by *Phaeobacter* sp. strain Y4I. Indigoidine and leucoindigoidine structures are derived from references (48, 55).

Table 2. Comparison of genes in indigoidine biosynthesis operons.

Y4I operon	**Annotation**	**Homolog in γ-Proteobacteria[a] or *Streptomyces* operons**	**% aa identity to *V. indigofera* operon**	**% aa identity to *Dickeya dadantii* 3937 operon**
N/A	DNA binding transcriptional regulator	*pecS*	39[c]	100
N/A	membrane bound transcriptional regulator	*pecM*	44[c]	100
N/A	pseudouridine-5'-phosphate glycosidase family	*indA*	0	100
N/A	phosphoglycolate phosphatase	*indB*	0	100
igiR	*tetR* family regulator	N/A	40	N/A
igiA[b]	4'-phosphopantetheinyl transferase	N/A	40	N/A
igiB	6-phosphogluconate dehydrogenase family	N/A	54	N/A
igiC	glutamate racemase	N/A	58	N/A
igiD	indigoidine synthase	*indC*	54	49
igiF	4-oxalocrotonate tautomerase	N/A	N/A	N/A
igiE	Major facilitator family transporter	N/A	58	N/A

[a]The γ-Proteobacteria included in this table are *D. dadantii*, *Serratia proteamaculans*, and *Photorhabdus asymbiotica* (NC_014500.1, NC_009832.1, and NC_012962.1, respectively). *Streptomyces* included in this table are *S. albus* and *S. lavendulae* (NZ_ABYC00000000.1 and AB240063, respectively). [b]In Y4I, *igiA* is not located in the indigoidine biosynthesis operon, but is found in a different area of the chromosome. [c]*pecS* and *pecM* are hypothesized to be required for indigoidine biosynthesis in *V. indigofera* (AF088857.1), but are found together in a different area of the chromosome from the *igi* operon. N/A indicates that no homolog of that gene was found in the respective organism.

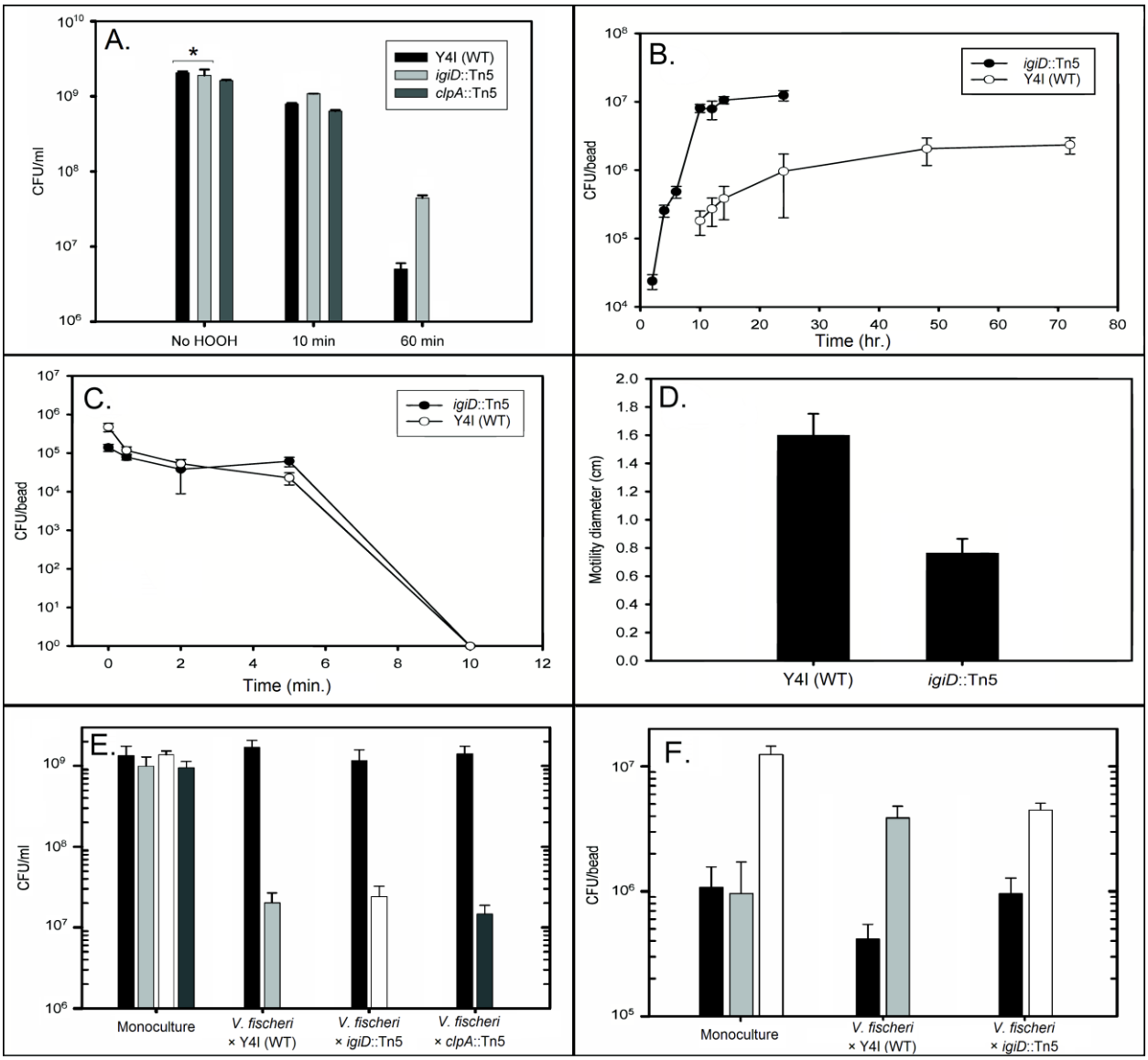
Phenotypic characterization of indigoidine mutants. It has been suggested that indigoidine provides protection to *D. dadantii* through the neutralization of reactive oxygen species generated by the plant during bacterial infection (48). As this was the only reported biological role for indigoidine to date, we sought to address whether a similar protection was afforded to Y4I. Thus, hydrogen peroxide resistance of wildtype and the *igiD*::Tn5 and *clpA*::Tn5 mutants was assayed. Surprisingly, both wildtype and the *clpA*::Tn5 mutant were much more sensitive to hydrogen peroxide exposure than the *igiD*::Tn5 mutant ($p=<0.001$; Fig. 5A). Given the broad range of regulatory proteins predicted to be effected by ClpA mutations, it is unclear whether the *clpA*::Tn5 strain has enhanced susceptibility due to over production of indigoidine or an unrelated physiological change. Therefore, the *clpA*::Tn5 mutant was omitted from further assays, with the exception of the competition assays in broth where constitutive indigoidine production could be evaluated.

Given that Y4I has been demonstrated to form prolific biofilms (53) and that indigoidine production in this strain is most visually apparent when cells are grown on agar surfaces, sensitivity to hydrogen peroxide was also assayed on cells grown in biofilms. Interestingly, while there was no difference in the growth rate of wildtype and the *igiD*::Tn5 mutant in broth cultures, differences were seen in the rates of surface colonization. The *igiD*::Tn5 mutant colonized surfaces significantly earlier and to a higher cellular density than wildtype (Fig. 5B; $p=<0.001$). Due to the difference in colonization density and rate, biofilms were allowed to develop for a period of time that allowed for comparable cellular densities between wildtype and the mutant (wildtype=24 h, *igiD*::Tn5=4 h). Under these conditions, there was no significant difference in hydrogen peroxide sensitivity between the strains (Fig. 5C). The discrepancy between broth culture and biofilms is likely due to the poor penetration of reactive oxygen in

biofilms, limiting cellular exposure (16, 17). Moreover, the innate resistance of Y4I to hydrogen peroxide exposure is considerably higher than that of *D. dadantii* (>150 mM hydrogen peroxide [data not shown] vs. ~10mM (48), respectively). Thus, it may be the inherent differences in sensitivity to hydrogen peroxide that influences the role, or lack thereof, of indigoidine as a protectant in these two organisms.

The difference in surface colonization evident between the wildtype and the *igiD*::Tn5 mutant was unexpected, yet intriguing. Given that flagella are essential for surface colonization in many proteobacteria (e.g. (28, 41)) the *igiD*::Tn5 mutant was assayed for defects in motility. Motility of the *igiD*::Tn5 mutant was inhibited in soft agar assays relative to wildtype (Fig. 5D; p=<0.001), but showed signs of swimming when viewed microscopically. Twitching and swarming motilities showed no significant difference in comparison to wildtype (data not shown). Collectively, the motility and colonization assays suggest basal indigoidine production contributes to swimming motility and the surface attachment rate of Y4I. In this way, indigoidine biology may share features with that of a structurally similar group of redox-active, nitrogen-containing heterocyclic secondary metabolites known as phenazines (43). In pseudomonads, alterations in motility, surface attachment, and biofilm formation have all been associated with defects in phenazine production (41, 43, 45, 59). Similarly, indigoidine may play a role as an intracellular signaling molecule. This is not unprecedented, as secondary metabolites in other roseobacters have been shown to have signaling properties. For example, in *Ruegeria* (formerly *Silicibacter*) sp. TM1040, TDA is an autoinducer of the genes necessary for its biosynthesis and transport (22).

Figure 5. Phenotypic characterization of *Phaeobacter* sp. strain Y4I and mutants with alterations in indigoidine biosynthesis. (A) Viable cell counts of planktonic Y4I variants after exposure to 250 mM hydrogen peroxide for 0 (No HOOH), 10, and 60 minutes. The asterisk denotes cell counts that are the same ($p=<0.001$); all others are statistically different from each other ($p=\leq 0.05$). (B) Viable counts of wildtype (WT) Y4I and the *igiD*::Tn5 strain as they colonize a glass surface overtime. (C) Viable cell counts of attached Y4I (WT) and the *igiD*::Tn5 strain after exposure to 75 mM hydrogen peroxide from 0 to 10 minutes. (D) Diameter of swim zone of Y4I (WT), *igiD*::Tn5, and *clpA*::Tn5 strains on soft agar motility plates. Viable counts of competitions between planktonic Y4I variants and *V. fischeri* in broth culture (E) and in biofilms (F). Bar coding is as follows: Black = *V. fischeri*, light gray = Y4I (WT), white bars = *igiD*::Tn5 and dark gray = *clpA*::Tn5 viable cell counts, unless otherwise noted. Error bars represent the standard deviation of biological and technical triplicates.


A.
*
Y4I (WT)
igiD::Tn5
clpA::Tn5
CFU/ml
No HOOH
10 min
60 min
B.
igiD::Tn5
Y4I (WT)
CFU/bead
Time (hr.)
C.
igiD::Tn5
Y4I (WT)
CFU/bead
Time (min.)
D.
Motility diameter (cm)
Y4I (WT)
igiD::Tn5
E.
CFU/ml
Monoculture
V. fischeri × Y4I (WT)
V. fischeri × igiD::Tn5
V. fischeri × clpA::Tn5
F.
CFU/bead
Monoculture
V. fischeri × Y4I (WT)
V. fischeri × igiD::Tn5

Regulation of indigoidine biosynthesis. Previous studies have demonstrated that indigoidine biosynthesis is under transcriptional regulation and that its production is influenced by environmental factors, including growth medium (30, 33, 47, 54, 55). Among the organisms that produce indigoidine, however, the characterized mechanisms of regulation and the conditions that favor indigoidine biosynthesis are quite varied. In *S. lavendulae*, the induction of indigoidine production is only evident upon addition of a synthetic analog of lactone-based quorum sensing molecules, γ-nonalactone (55). In the plant pathogen *D. dadantii*, expression of the indigoidine biosynthesis gene cluster is mediated by the PecS-PecM transcriptional regulatory system, which similarly controls the production and secretion of extracellular pectinases and cellulases that are required for invasion of the plant host (47, 48). Interestingly, in both *S. lavendulae* and *D. dadantii*, discovery of indigoidine biosynthesis was serendipitous, as neither strain produces the metabolite when grown under standard laboratory conditions. This is not the case for Y4I where blue colonies on agar plates are diagnostic of the strain and pigmentation is not evident in aerated broth cultures, except where there is biofilm formation at the liquid-air interface on the wall of the growth vessel. Thus, the regulation of indigoidine biosynthesis in Y4I is in response to different stimuli than those characterized for *S. lavendulae* and *D. dadantii* and more consistent with conditions described to induce TDA production in *Ruegeria* and *Phaeobacter* strains (4, 22, 23).

Indigoidine production in *Phaeobacter* sp. strain Y4I is associated with growth on surfaces. The antagonistic behavior exhibited by Y4I against *V. fischeri* on agar plates was not evident when strains were inoculated in broth co-cultures at equal starting densities (Fig. 5E), nor when the Y4I strains had an initial 100-fold density advantage (Fig. 8). Viable counts of *V. fischeri* following co-culture with Y4I or either mutant in broth showed no significant inhibition of *V.*

fischeri versus controls lacking Y4I strains (p=>0.200). In fact, all three Y4I variants were significantly outcompeted nearly 100 fold by *V. fischeri* (p=<0.001; Fig. 5E) when the cultures were seeded at equal initial cell densities. The inability of the hyper-indigoidine producing strain (*clpA*::Tn5) to inhibit *V. fischeri* in broth cultures, even when provided a density advantage, suggests the production of indigoidine does not provide a competitive advantage when cells are growing planktonically. This result could be due to the redox state of indigoidine in agitated broth cultures, which was oxidized and visually evident by the blue color of co-cultures containing the *clpA*::Tn5 strain. An alternative explanation is that *V. fischeri* susceptibility to indigoidine is growth condition dependent.

The gene expression data presented for the wildtype strain show upregulation of *igiD* when the strain grows as colonies on agar surfaces and in biofilms compared to broth cultures (Fig. 6). The hyper-pigmented *clpA*::Tn5 mutant showed the highest levels of *igiD* gene expression. In all cases, phenotypic observations of pigmentation support the gene expression data (Fig. 6). Low levels of *igiD* gene expression in high density (~10^9 CFU/ml) broth cultures and flocs of cells in wildtype Y4I suggest high cellular densities alone are insufficient to induce indigoidine production. Tn5 mutants with insertions in either of the two LuxIR quorum sensing systems present in Y4I, however, also lack indigoidine production, suggesting a role for both systems in its biosynthesis (Table 1).

Given the evidence for upregulation of indigoidine biosynthesis in biofilms, we investigated whether indigoidine production by wildtype Y4I could inhibit the colonization of *V. fischeri* in biofilms. Monoculture controls of *V. fischeri* biofilms were compared to co-culture biofilms with wildtype Y4I and the *igiD*::Tn5 mutant. While both Y4I strains were able to inhibit *V. fischeri* colonization, wildtype inhibited its colonization by 57 ± 14% more than the

indigoidine null mutant (p=0.002). In fact, wildtype Y4I was able to colonize surfaces to a greater extent in the presence of *V. fischeri* than alone. Conversely, the Δ*igiD* mutant was impaired in surface colonization when grown with *V. fischeri* (Fig. 5F). These data provide evidence that indigoidine biosynthesis of surface attached Y4I cells may provide a significant advantage in colonizable niches. Biofilms are made of densely packed microcolonies that contain multiple microenvironments (16, 17). Such environments could act to increase the local concentration of indigoidine to levels that would be inhibitory to the growth or attachment of competing organisms.

Regulatory hierarchies are common and complex in bacteria. In multiple species, the biosynthesis of secondary metabolites is primarily controlled by quorum sensing, but environmental factors such as temperature, pH, and nutritional requirements can supersede cell density dependent regulation (3, 15, 43, 50, 60). As *igiD* gene expression is elevated in Y4I colonies and biofilms, we hypothesize that surface attachment and high cell densities are both required for indigoidine production: after a surface niche is colonized and quorum is reached, indigoidine biosynthesis is upregulated to suppress the colonization of competing organisms. Studies in TDA-producing *Phaeobacter* strains have shown biosynthesis occurring at measureable levels in shaking and stagnant broth cultures and on agar plates (22, 44). This is the first report of antimicrobial production by a *Roseobacter* in response to surface colonization; however, several studies with unclassified marine isolates have shown similar results (31, 56, 61). Furthermore, particle-associated representatives of the *Roseobacter* clade have been previously demonstrated to be >10 times more likely to produce antimicrobial compounds than free-living members (37).

Antagonistic behavior is widespread throughout the *Roseobacter* clade and is thought to be due to the synthesis of antimicrobial secondary metabolites, which have been hypothesized to contribute to the success of these organisms in natural systems. (6, 8, 10, 12, 26, 27, 37, 38, 44, 51, 58). With the exception of TDA, little is known of the structural and biological nature of these secondary metabolites. We show here that indigoidine production by *Phaeobacter* sp. strain Y4I is directly related to the inhibition of the model marine bacterium *V. fischeri.* Moreover, we show that indigoidine biosynthesis is upregulated during growth on a surface and its production provides a significant advantage to Y4I in competitive surface colonization. Preliminary investigations in our lab have shown that indigoidine producing strains of Y4I are also able to suppress the growth of *V. anguillarum, Ruegeria lacuscaerulensis,* and *Candida albicans,* suggesting indigoidine may act on multiple competing species in the environment (data not shown). The full spectrum of organisms that indigoidine is effective against is not yet realized, but may be quite broad. Similarly, the physiological roles of indigoidine production by *Phaeobacter* spp. likely extend beyond those reported here and might be expected to provide the organism with a competitive advantage in the varied environmental niches it encounters.

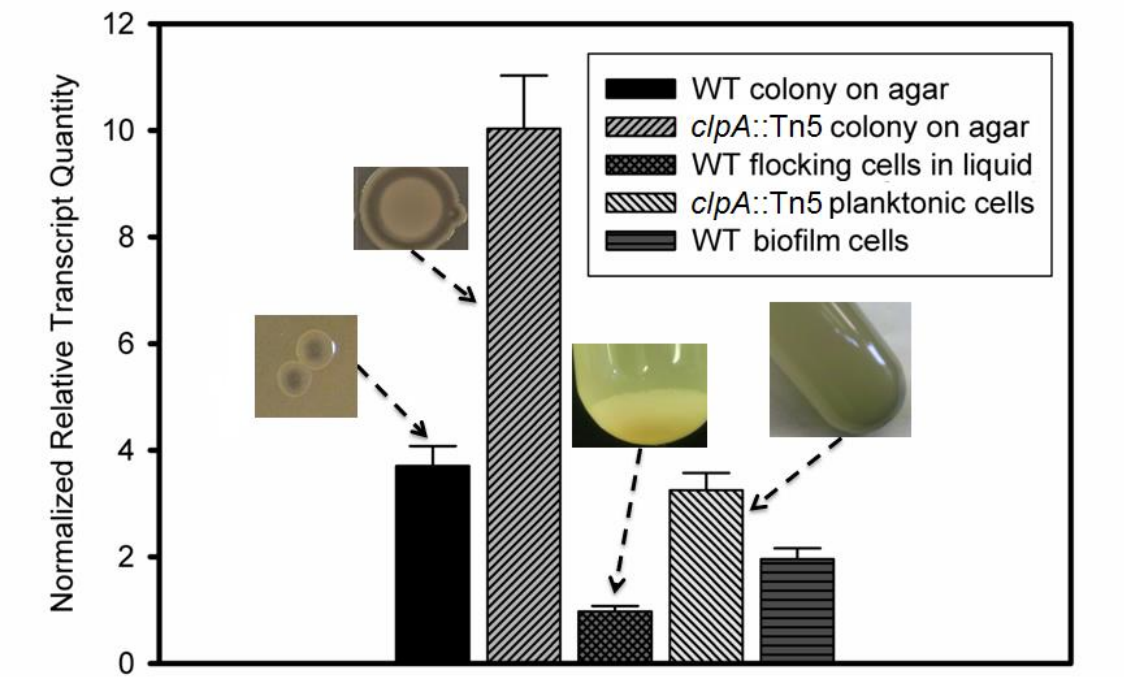


Figure 6. *igiD* gene expression of *Phaeobacter* sp. strain Y4I (wildtype) and the *clpA*::Tn5 mutant under different culture conditions. Expression levels are normalized to three reference genes and relative to late exponential (16.5 h) wildtype planktonic cells. Error bars represent the standard error of the mean. Images show the phenotype of each strain in the culture condition measured, WT = wildtype.

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VI. Appendix 1

Table 3. Additional Tn5 insertion sites that cause non-pigmented mutants.

Y4I gene locus	Putative function (NCBI annotation)	Number of mutants in library
RBY4I_3509	flagellar biosynthetic protein FliR	2
RBY4I_3858	oxidoreductase, NAD-binding/iron-sulfur cluster-binding protein	2
RBY4I_3417	conserved hypothetical protein	1
RBY4I_3376	conserved hypothetical protein	4
RBY4I_2499	transcriptional regulator, XRE family	1
RBY4I_3334	conserved hypothetical protein	1
RBY4I_3601	sulfate adenylyltransferase	2
RBY4I_3739	phosphoadenosine phosphosulfate reductase	1
RBY4I_559	Ppx/GppA phosphatase	2
RBY4I_615	conserved hypothetical protein	2
RBY4I_3810	conserved hypothetical protein	2
RBY4I_3671	putative transposase	2
RBY4I_3612	hypothetical protein	1
RBY4I_2168	short-chain dehydrogenase/reductase SDR	1
RBY4I_3336	Helix-turn-helix domain protein	2
RBY4I_1027	response regulator receiver protein, putative	2

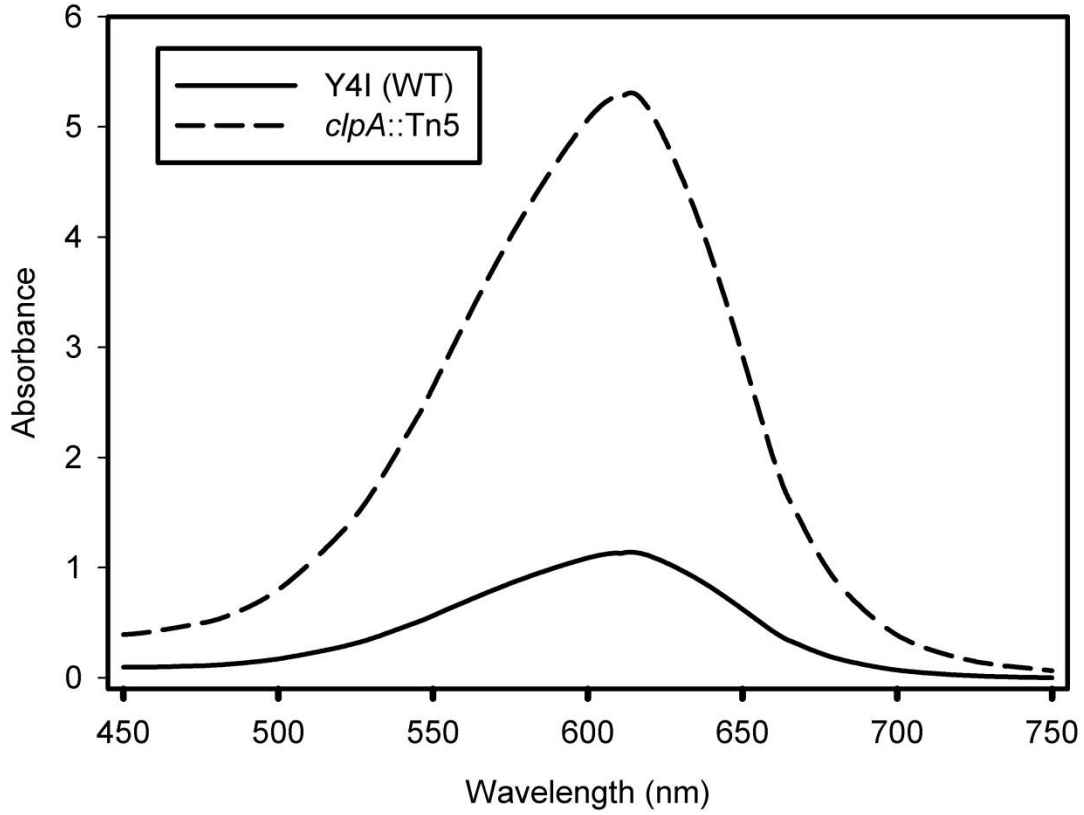


Figure 7. UV/Vis absorbance spectrum of the crude indigoidine extract in DMSO from the hyper-pigmented (*clpA*::Tn5) and wildtype cultures. Extracts were normalized to 10 mg of cellular material per 1 ml of DMSO. Absorbance numbers were calculated from diluted indigoidine extracts. The documented absorbance peak of indigoidine is the same as the isolated pigment at 612 nm (1).

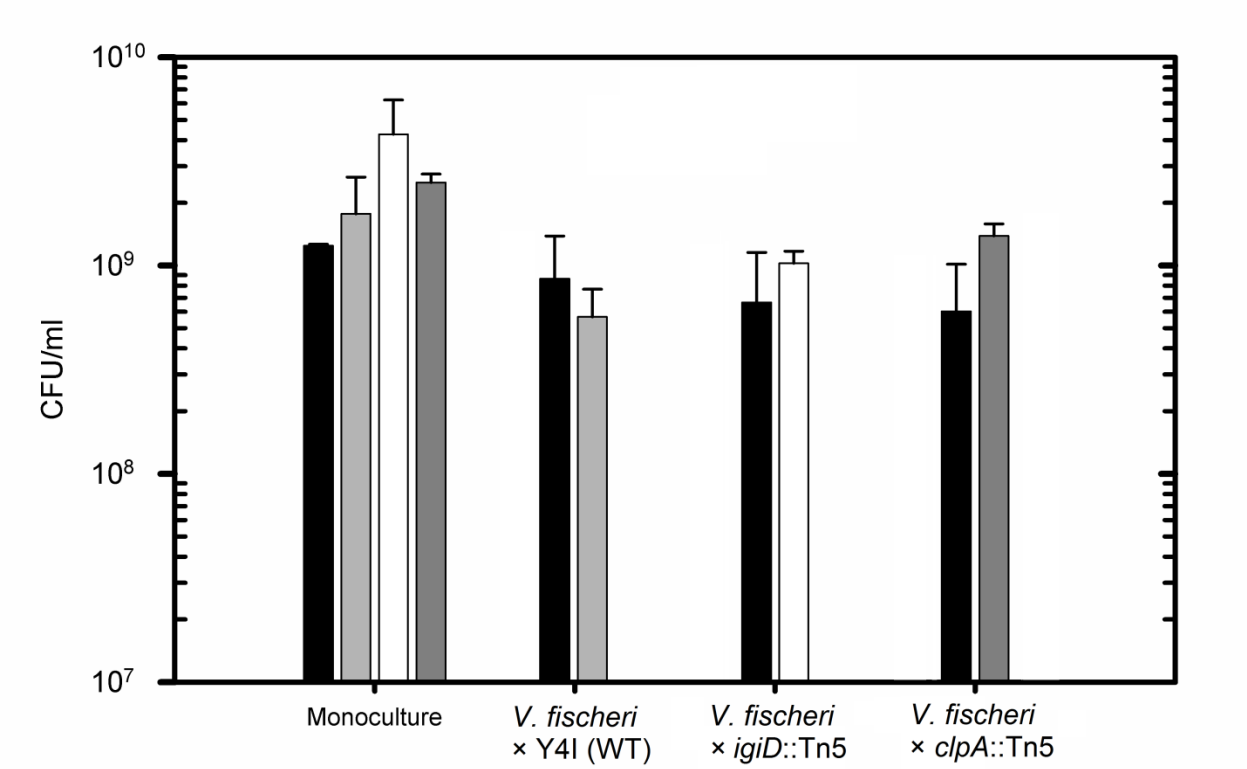
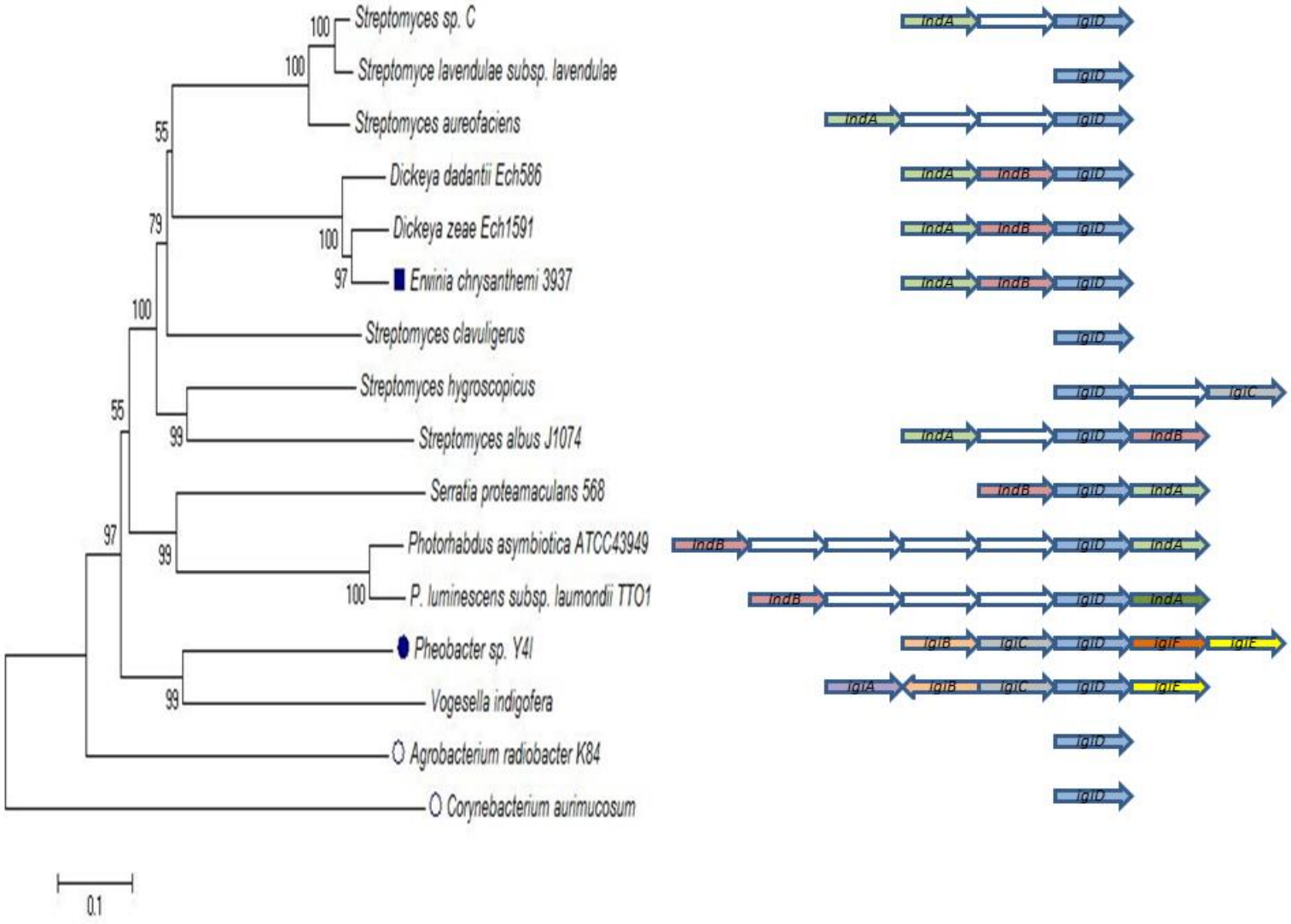


Figure 8. Liquid co-culture competitions with an initial cellular density advantage of the Y4I variants over *V. fischeri*. *V. fischeri* cultures were diluted 10^{-2} prior to inoculating with un-diluted overnight Y4I cultures. Black bars indicate *V. fischeri* CFU counts. Light gray bars indicate Y4I wildtype CFU counts. White bars indicate *igiD*::Tn5 CFU counts. And dark gray bars indicate *clpA*::Tn5 CFU counts.

Figure 9. Neighbor-joining phylogenetic tree of the indigoidine biosynthetic NRPS (IgiD). The closed square indicates *Dickeya dadantii* (formally *Erwinia chrysanthemi*). The closed circle indicates *Phaeobacter* strain sp. Y4I. The open circle indicates the *igiD* gene is plasmid borne in that organism. The putative gene organization of the indigoidine biosynthetic operon is shown to the right of each organism. The annotation for each gene can be found in Table 2.


Streptomyces sp. C
Streptomyce lavendulae subsp. lavendulae
Streptomyces aureofaciens
Dickeya dadantii Ech586
Dickeya zeae Ech1591
Erwinia chrysanthemi 3937
Streptomyces clavuligerus
Streptomyces hygroscopicus
Streptomyces albus J1074
Serratia proteamaculans 568
Photorhabdus asymbiotica ATCC43949
P. luminescens subsp. laumondii TTO1
Pheobacter sp. Y4I
Vogesella indigofera
Agrobacterium radiobacter K84
Corynebacterium aurimucosum
100
100
55
79
100
97
100
55
99
97
99
100
99
0.1
IndA
IglD
IndB
IglC
IglB
IglF
IglE
IglA

References

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Chapter 2

Phaeobacter sp. strain Y4I uses two separate quorum sensing systems to control the production of the antimicrobial secondary metabolite indigoidine.

This section is a collaborative project with the Campagna laboratory in the Department of Chemistry and it is in preparation for submission. The tentative title is:

W. Nathan Cude, Carson W. Prevatte, Amanda L. May, Shawn R. Campagna, and Alison Buchan. *Phaeobacter* sp. strain Y4I uses two separate quorum sensing systems to control the production of the antimicrobial secondary metabolite indigoidine. [In preparation]

My contribution to this chapter was the growth of bacterial cultures, bioreporter assays, RT-qPCR, motility assays, attachment assays, AHL extractions (with the aid of C.W.P.), and literature review and writing.

I. Abstract

The marine roseobacter *Phaeobacter* sp. strain Y4I synthesizes the antimicrobial secondary metabolite indigoidine when grown in biofilms or on agar plates. The production of this antimicrobial allows Y4I to outcompete *Vibrio fischeri* for surface colonization. Tn5 insertional mutations in each of two separate *luxRI*-like quorum sensing systems, known as *pgaRI* and *phaRI*, results in pigmentation defects, as well as motility defects and thicker biofilms. Here, we investigated the role of quorum sensing in the control of indigoidine production. Y4I synthesizes two dominant *N*-acyl homoserine lactones (AHLs) at different points in its growth cycle. The AHL produced during exponential phase is likely an isomer of *N*-3-oxododecanoyl homoserine lactone (3OC12-HSL), which we suggest is a monounsaturated *N*-3-hydroxydodecanoyl homoserine lactone (3OHC12:1-HSL). We hypothesize that this AHL is synthesized by PhaI. As a batch culture reaches stationary phase, 3OHC12:1-HSL concentrations go down and *N*-octanoyl homoserine lactone (C8-HSL) concentrations, produced by PgaI, increase quickly. Here we show that both quorum sensing systems play a role in the control of indigoidine biosynthesis. We postulate that this may allow Y4I to maintain tight control of secondary metabolisms so as to conserve energy and produce antimicrobials only when it is most beneficial to the community.

II. Introduction

Bacteria are able synchronize gene expression in response to population density by sensing and responding to small diffusible molecules synthesized by individuals. This form of cell-to-cell communication is known as quorum sensing and is utilized by many bacterial species to coordinate group behavior. Often this group behavior allows a population of organisms to work together to achieve goals greater than what could be achieved by an individual bacterium.

Examples of this include the formation of biofilms, the suppression of immune responses during infection, exoenzyme production, swarming motility, colonization of host by a symbiont, and antimicrobial production (9, 11, 51, 52).

The most investigated form of quorum sensing is that mediated by *N*-acyl-homoserine lactones (AHLs) which are synthesized by proteobacteria (4). The established model of quorum sensing is rooted in investigations of the bioluminescent squid symbiont *Vibrio fischeri*. In this model an AHL is synthesized constitutively in low concentrations by the AHL synthase LuxI. AHLs cross the cell membrane and enter the local environment. As the local concentration of AHLs increases due to increased population density, the AHL binds the regulator protein LuxR and activates it. LuxR upregulates the genes required for coordinated group behavior. LuxR also upregulates *luxI* transcription and a positive feedback loop occurs resulting in increased expression of LuxI, higher levels of AHL synthesis, and stronger activation of LuxR in nearby individuals (9, 11, 36). AHLs have also been shown to activate regulators different from their cognate LuxR, and the AHL concentration has shown to change throughout the growth phase of some bacteria (18, 21, 37). This suggests the type and concentration of AHLs may convey information about the metabolic state of the population as well as population density (16).

AHLs are thought to be species- or group-specific based on their structural features. AHLs produced by multiple species have been shown to have different acyl chain lengths (C4-C18), variable degrees of oxidation at the third acyl carbon (-H, -OH, or =O), a terminal methyl branch, and one or multiple sites of unsaturation in the acyl chain. The modifications in structure may make the AHLs target specific (5, 46). While cross communication between species has been observed and is an active area of research, most studies have focused on intraspecies responses to quorum sensing and AHLs (9, 28, 44, 50, 52).

Roseobacters are an abundant and biogeochemically active group of marine α-proteobacteria found in coastal environments (2, 14, 15, 35). Members of the *Roseobacter* clade have been shown to produce multiple AHLs and these compounds have been linked to biofilm formation, motility, and antimicrobial production (1, 17, 25, 42, 49, 55). A member of the *Roseobacter* lineage, *Phaeobacter* sp. strain Y4I, produces the blue antimicrobial secondary metabolite indigoidine which suppresses the growth of *V. fischeri*, as well as other organisms, on agar plates and in biofilms. Previous studies suggested that transposon insertional mutations in quorum sensing genes can affect the production of indigoidine and the inhibition of *V. fischeri* (7). Figure 10 shows the two separate *luxRI*-like quorum sensing systems, *pgaRI* (RBY4I_1689 and RBY4I_3631) and *phaRI* (RBY4I_1027 and RBY4I_3464), in the Y4I genome (GenBank accession number ABXF00000000). Here, the role of quorum sensing in indigoidine production and the AHLs produced by Y4I was investigated.

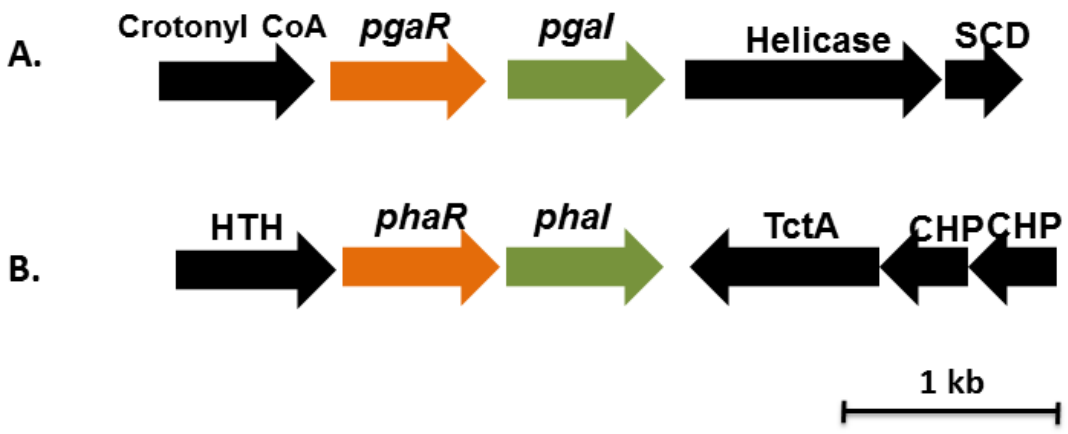

Figure 10. Gene orientation for the two putative quorum sensing systems in Y4I. Orange arrows indicate *luxR*-like genes, and green arrows indicate *luxI*-like genes. Black arrows represent adjacent genes. Abbreviations used: Crotonyl CoA - Crotonyl CoA reductase, CHP – conserved hypothetical protein, SCD – short chain dehydrogenase, TctA – TctA family transmembrane transporter, HTH – helix-turn-helix motif containing gene.

III. Methods and Materials

Growth conditions and media. The following culture conditions were used, unless otherwise noted: *Phaeobacter* sp. strain Y4I and mutants were maintained in YTSS broth [per liter: 2.5 g yeast extract, 4 g tryptone, 15 g Sea Salts (Sigma-Aldrich, St. Louis, MO)] at 30°C, with shaking (200 rpm). YTSS agar (1.5%) plates were used for streaking to isolation and also incubated at 30°C. The *Agrobacterium tumefaciens* NTL4 (pCF218)(pCF372) AHL bioreporter strain was maintained in AT medium [per liter: 10.7 g KH_2PO_4, 4 g $(NH_4)_2SO_4$, 0.078 g $MgSO_4$, 0.0076 g $CaCl_2$, 0.005 g $FeSO_4 \cdot 7H_2O$, 0.0022 g $MnSO_4 \cdot H_2O$, 5% w/v glucose] at 28°C with streptomycin (50 µg/ml) and tetracycline (5 µg/ml). *Escherichia coli* MG4 (pQF5016b.bmaR1)(pBD4) is a *N*-octanoyl-L-homoserine lactone (C8-HSL) bioreporter (10) and was maintained on LB medium (per liter: 10 g tryptone, 5 g yeast extract, and 10 g NaCl) with ampicillin (100 µg/ml) and gentamicin (20 µg/ml).

Attached biomass and motility assays. Wildtype Y4I, *pgaR*::Tn5, *phaR*::Tn5, and *phaI*::Tn5 were screened for total attached biomass using the crystal violet assay (38). Briefly, each culture was diluted to 10^6 CFU/ml and inoculated in 100 µl volumes to a 96-well polypropylene plate (Costar, Corning, NY) in octuplet. The plates are incubated at 27°C for 18 hours. 25 µl of crystal violet was added to each well and allowed to stain the biomass for 15 min. The wells were then washed 3 times with deionized water, and the crystal violet was solubilized in 95% ethanol for 1 hour. Absorbance readings were measured using a plate reader (BioTek, Winooski, VT) at 600nm.

Motility was assessed using 1/10 YTSS 0.35% agar plates (per liter: 0.25 g yeast extract, 0.4 g tryptone, 15 g Sea Salts, and 3.5 g agar). 20 µl of stationary phase wildtype Y4I,

pgaR::Tn5, *phaR*::Tn5, and *phaI*::Tn5 was spotted to the center of a motility agar plate. The plates were incubated at 30°C for 48 h. The diameter of the swim circle was measured.

RT-qPCR for *igiD* expression. . RT-qPCR assays of *igiD* expression were done on Y4I and mutant colonies grown on agar plates for 24 hours using a method previously described (7). Briefly, primers were designed to amplify products between 160 and 240 bp of three reference genes (*alaS, map,* and *rpoC*) as well as the gene encoding the indigoidine non-ribosomal peptide synthetase (NRPS) *igiD*. The colonies were removed from the plate and resuspended in RNA*later* (Qiagen, Germantown, MD) and RNA was extracted using the RNeasy extraction kit (Qiagen, Germantown, MD). The qPCR was performed on a Bio-Rad DNA Engine Opticon 2 real-time PCR detector (Bio-Rad, Hercules, CA) with SYBR green PCR reagents (Invitrogen, Carlsbad, CA). RT-qPCR data analysis and the normalized relative transcript quantity were calculated using the qBASE method (19).

AHL bioreporters for AHL identification and relative quantification. AHL production over time was measured using the *traR-lacZ* fusion AHL bioreporter strain *A. tumefaciens* NTL4 (pCF218)(pCF372) in the form of β-galactosidase activity. Y4I was inoculated to YTSS broth medium (OD_{540} ~0.05) in triplicate. Aliquots of the culture were removed over a growth curve at time points 2.5, 5, 7, 20 and 24 h and filter sterilized. Aliquots of 1 ml were removed after 24 h and filter sterilized. 100 µl of the filtrate was added to 2 ml pre-induced *A. tumefaciens* NTL4 bioreporter cultures in AT medium with no antibiotics. Synthetic C8-HSL was added to the NTL4 culture at a final concentration of ~10µM as a positive control. Sterile dH_2O was used as a negative control. Cultures were incubated at 25°C until they reached an OD_{600} ~0.50 (~16 h) at which time the cells were lysed and β-galactosidase activity was calculated using a previously reported method (26, 27). Separately, liquid cultures of wildtype Y4I, *pgaR*::Tn5, *phaR*::Tn5,

and *phaI*::Tn5 were inoculated in triplicate and incubated at 30°C. Aliquots were taken at 24 h and filter sterilized. The β-galactosidase activity of these cultures, as well as positive and negative controls, was assessed as above.

AHL Identification and Deuterated AHL synthesis. 200 ml of Y4I culture were grown for 5 and 24 h in triplicate. At the end of the incubation, the entire culture was pelleted by centrifugation at 3700 x *g* using a Sorvall Legend RT (Thermo Fisher, Waltham, MA) for 20 min. The supernatant was filtered with a 47 mm 0.45 µm nylon filter (GE Osmonics, Feasterville-Trevose, PA). The filtrate was extracted twice with 150 ml in ethyl acetate acidified with 1% v/v acetic acid. The organic layer was removed and concentrated *in vacuo* to give an off-white solid, which was reconstituted with 300 µl ethyl acetate and transferred to an autosampler vial.

Analysis was performed using a previously developed high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method optimized for AHL detection (32). Briefly, 10 µl of the concentrated extract was injected into a reverse-phase C18 core-shell column (Phenomenex Kinetex, Torrance, CA) by an Electron Surveyor autosampler (Thermo Fisher Scientific, Waltham, MA) at 25°C. HPLC was performed with an electron surveyor MS pump at a flow rate of 200 µl/min and the effluent was applied to a TSQ Quantum Ultra Triple Stage Quadrupole mass spectrometer (MS) for ion detection (Thermo Fisher Scientific, Waltham, MA). Samples were subjected to an electrospray ionization chamber of the triple quadrupole with a spray voltage of 4500 V. Detection was done in positive ion mode with nitrogen as a sheath gas and argon as the collision gas. Samples were analyzed with selected reaction monitoring (SRM) with a scan time of 0.05 s and a scan width of 0.1 *m*/*z*. AHLs present in the extracts were determined by HPLC retention time and m/z. Deuterated AHL standards of

(D_2)C8-HSL and *N*-3-oxododecanoyl homoserine lactone ([D_2]3OC12-HSL) were synthesized for quantification of AHLs as previously reported (13, 32).

E. coli MG4 (pQF5016b.bmaR1)(pBD4) C8-HSL bioreporter was grown at 37°C in LB medium supplemented with 0.25% v/v arabinose to induce plasmid expression until it reached OD600 ~0.25. 100 µl of the induced culture was spread on YTSS plates supplemented with 0.25% arabinose and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal; 40 µg/ml). The plates were allowed to dry at room temperature in a laminar flow hood and 20 µl of stationary phase Y4I, *pgaR*::Tn5, *phaR*::Tn5, and *phaI*::Tn5 was spotted to the center of the plate. The plates were incubated at 30°C for 48 hours.

AHL quantification over time. 500 µl of an overnight Y4I culture was inoculated to multiple 10 ml YTSS broth tubes and incubated at 30°C with shaking. 1800 µl aliquots were removed at 2, 4, 6, 12, 14, 20, 22 h time points. OD_{540} measurements were taken at each time point, and diluted and recalculated if over ~1.000. (D_2)C8-HSL and (D_2)3OC12-HSL internal standards were added to each aliquot at a final concentration of 1.1 µM, and cells were pelleted by centrifugation at 16100 × *g* for 2 min. 1700 µl of the supernatant was transferred to an autosampler vial and analyzed by HPLC-MS/MS as above. AHL concentrations from extracts were determined by integrating the peaks from ion chromatograms. Ion counts were converted to concentration (nM) by integrating the area under the curve and using the following formula: (signal AHL/signal (D_2)AHL) × (concentration (D_2)AHL).

Statistical analysis. ANOVA mean separations were done using the Holm-Sidak method in SigmaPlot 11.0 (Systat Software, Inc., Chicago, IL).

IV. Results and Discussion

Indigoidine production by *luxIR*-like mutants. Indigoidine is synthesized by the cyclization and condensation of two glutamine residues by a non-ribosomal peptide synthetase encoded by the *igiD* gene (7, 40, 45). When grown on agar plates for six days, *pgaR*::Tn5, *phaI*::Tn5, and *phaR*::Tn5 show differences in pigmentation when compared to wildtype. The wildtype shows significant pigmentation overnight. The *phaI*::Tn5 and *phaR*::Tn5 mutants pigment later (~3 days) and to a lesser degree than wildtype. The *pgaR*::Tn5 mutant never pigments and appears similar to the indigoidine biosynthesis insertional mutant *igiD*::Tn5 (Fig. 11). These changes in pigmentation also result in an inability of the strains to inhibit *V. fischeri* (7). The pigmentation phenotype shown in these strains suggests a direct connection between quorum sensing and the production of indigoidine. Similar to Y4I, antimicrobial production has shown to be tied to AHL-based quorum sensing in a closely related roseobacter *Phaeobacter gallaeciensis.* In *P. gallaeciensis N*-3-hydroxydecanoyl homoserine lactone (3OHC10-HSL) is produced by a protein homologous to PgaI. 3OHC10-HSL was shown to control tropodithietic acid (TDA) production at high cell densities in liquid cultures (1). In the case of Y4I, phenotypic observations suggest that both quorum sensing systems are required for wildtype pigmentation.

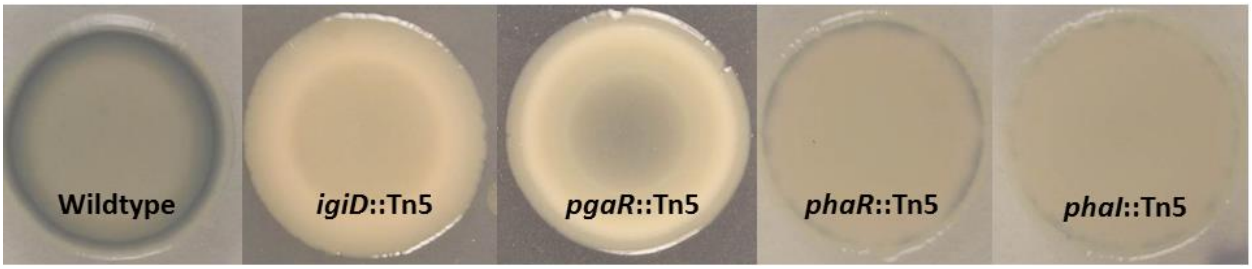

Figure 11. Phenotype of quorum sensing mutants when compared to wildtype and an indigoidine biosynthesis mutant (*igiD*::Tn5) after six days of grown on rich medium agar plates. The *pgaR*::Tn5 never pigments, while both the *phaR*::Tn5 and the *phaI*::Tn5 mutants show less pigmentation than wildtype.

Indigoidine biosynthesis machinery expression in quorum sensing mutant backgrounds. The lack of pigmentation in the quorum sensing insertional mutants (Fig. 11) led to an investigation in the expression levels of the indigoidine biosynthesis machinery. RT-qPCR of colonies of each of the mutants showed significant differences in the levels of indigoidine expression (Fig. 12). *igiD* expression in wildtype was significantly higher than the quorum sensing mutants (P=<0.001). As depicted in the phenotypes, both the *phaR*::Tn5 and the *phaI*::Tn5 insertional mutants show mid-range *igiD* expression levels. The levels are statistically indistinguishable (P=0.747), though significantly more than the *pgaR*::Tn5 strain (P=≤0.008). The *pgaR* insertional mutant showed significantly less *igiD* expression than the wildtype and the *phaRI* mutants (P=<0.001). Moreover, the *pgaR*::Tn5 strain showed an *igiD* expression level of 0.50 ± 0.05, which is lower than the wildtype liquid culture used to relativize the data and considered the baseline of *igiD* expression. This suggests very strong repression of indigoidine biosynthesis in this mutant background, and that PgaR is required to activate *igiD*.

The production of secondary metabolites diverts energy from cellular division (23, 43, 47). Thus, tight control of biosynthesis may allow for antimicrobial production only when it would increase the fitness of the community. It has been previously shown that surface colonization is required for indigoidine production by Y4I (7). Utilizing quorum sensing in connection with surface colonization may provide Y4I a means of tightly controlling indigoidine production until its production is most beneficial to the group.

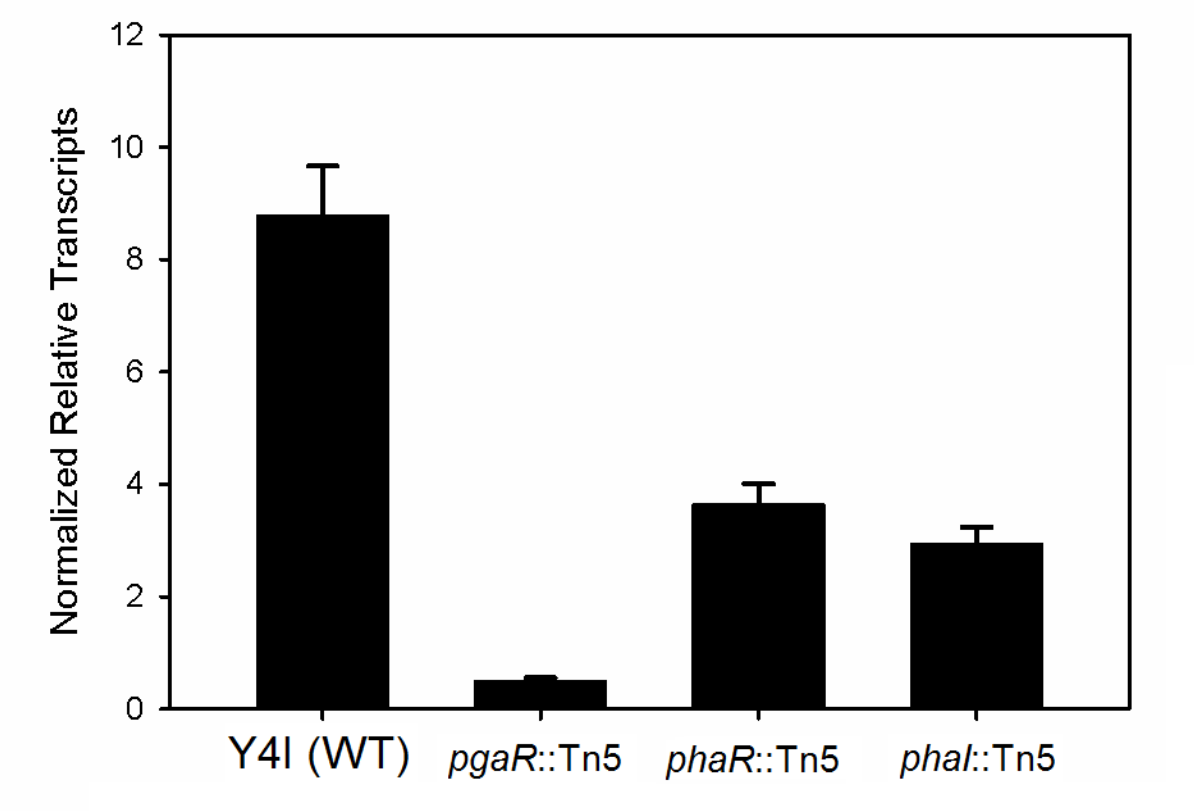

Figure 12. Normalized relative transcript quantity of *igiD* from colonies grown on agar plates in the quorum sensing mutant backgrounds. All quorum sensing mutants show significantly less pigmentation than the wildtype (P=<0.001). Error bars represent the standard error of the mean of biological and technical triplicates.

Biofilm formation and motility by quorum sensing mutants. All three transposon insertional mutants showed significantly (P=<0.001) more attached biomass than the wildtype (Fig. 13). It has previously been reported in another closely related roseobacter, *Ruegeria* sp. KLH11, that mutations in quorum sensing systems lead to increased surface colonization rates and defects in motility. It has been suggested that quorum sensing in this organism may control the lifestyle switch between the sessile (biofilm) and planktonic forms (55). Mutations in both quorum sensing systems in Y4I also cause significant defects in motility (P= <0.001) when compared to the wildtype. Moreover, the *pgaR*::Tn5 mutant is significantly less motile than the other quorum sensing mutants, suggesting that the *pgaRI* quorum sensing system may influence motility more than the *phaRI* system (Fig. 14). The quorum sensing mutants are not totally immotile, but this defect could lead to less dispersion and thus thicker biofilms. Y4I is an obligate aerobe (data not shown), so it may be disinclined to produce dense biofilms that could critically limit oxygen concentrations (6, 53). Therefore, quorum sensing may be used to induce dispersion once the biofilm becomes too dense, leading to the less attached biomass seen in the wildtype.

Surface colonization, motility, and indigoidine production are all related to quorum sensing. It has been shown that constitutive indigoidine biosynthesis by planktonic cells does not significantly inhibit *V. fischeri*, and it was therefore hypothesized that indigoidine production was limited to within biofilms (7). Indigoidine has two forms, an oxidized form and a reduced form, known as leucoindigoidine. Leucoindigoidine is thought to react with atmospheric oxygen and spontaneously oxidize to indigoidine (7, 20, 29). Leucoindigoidine is also thought to be the antimicrobial form of the molecule, as it is water soluble and indigoidine is not (7).). Y4I may be regulating biofilm density in order to control oxygen limitation. The oxygen concentration needs to be low enough inside the biofilm so high concentrations of leucoindigoidine can be

maintained to suppress the colonization by competing organisms, but it also needs to be high enough so as to not critically inhibit aerobic respiration. Utilizing two quorum sensing systems to control both indigoidine production and biofilm dispersal may allow Y4I to maintain this homeostasis.

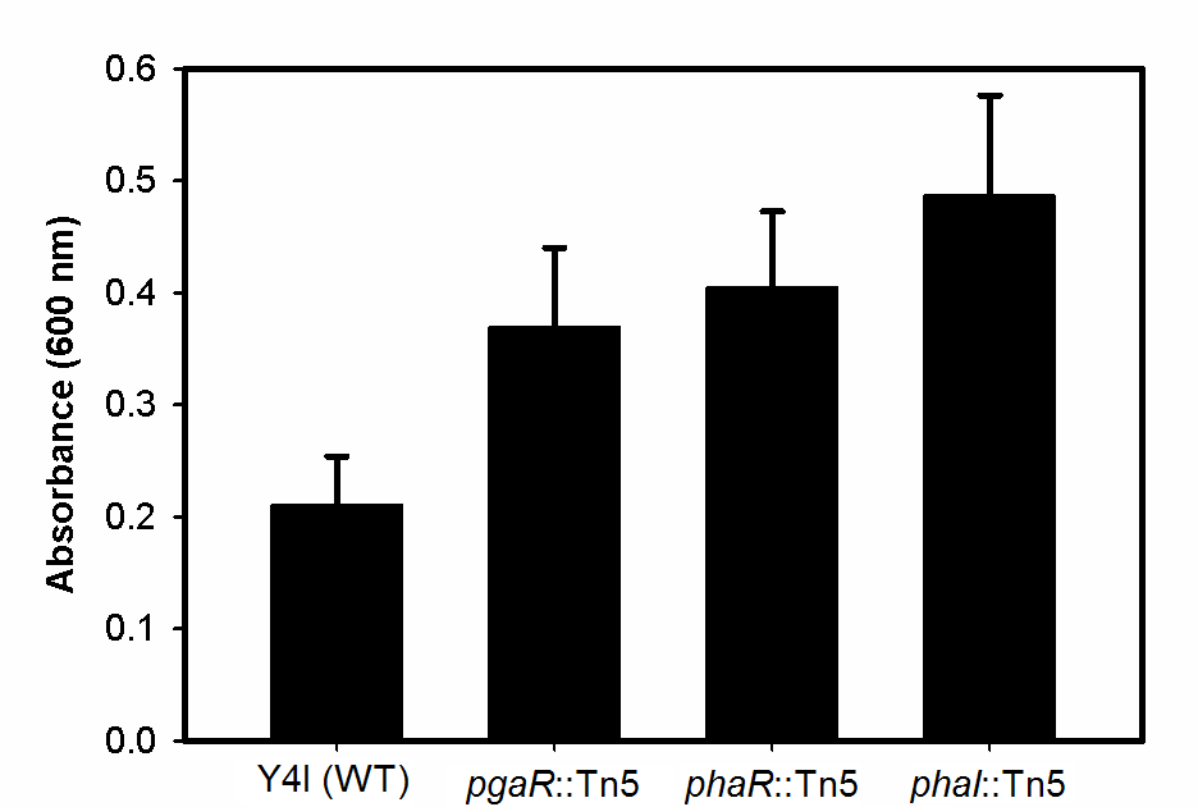

Figure 13. Absorbance of solubilized total attached biomass for Y4I and the quorum sensing mutants. Error bars represent the standard deviation of octuplets.

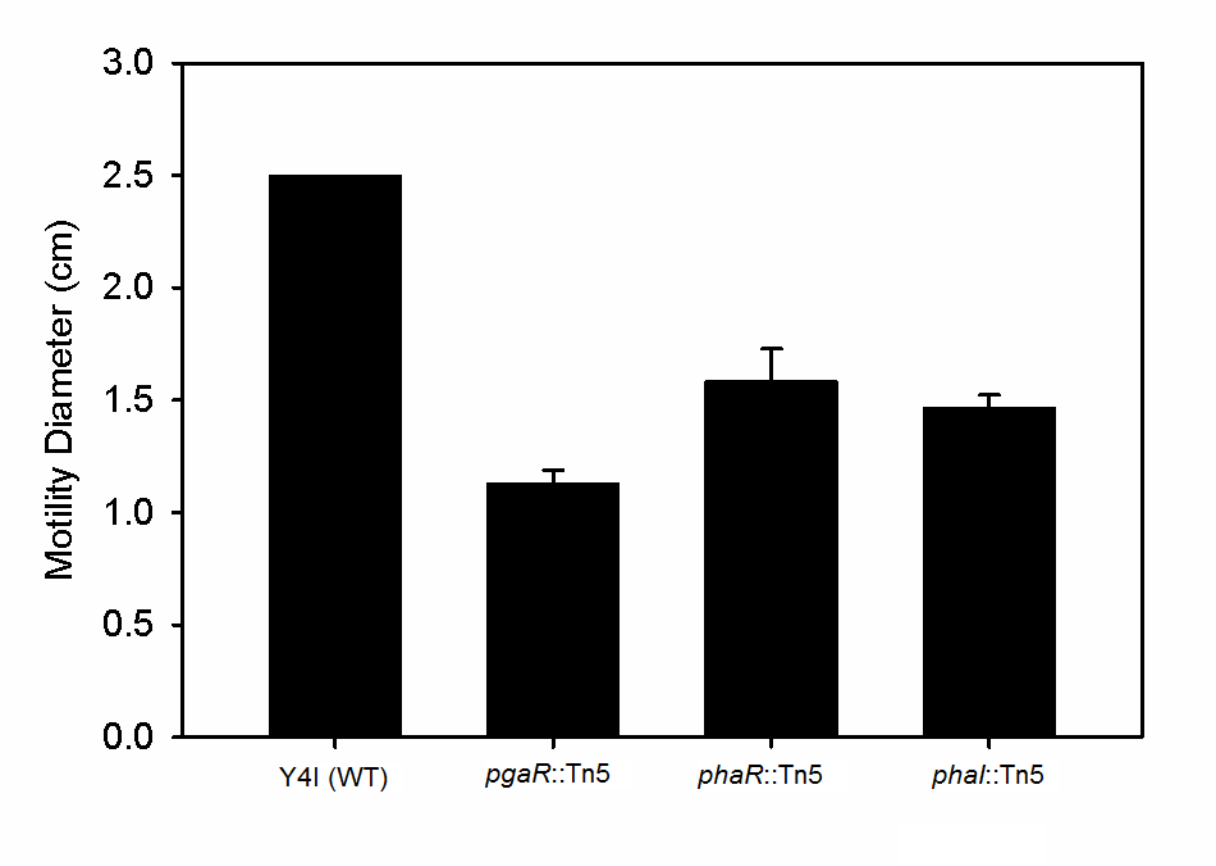

Figure 14. Motility diameter of wildtype Y4I and the quorum sensing mutants. Transposon insertions in each of three quorum sensing genes causes significant, but not total, inhibition of motility. Error bars represent the standard deviation of biological triplicates.

Identification AHLs produced by Y4I. Mass spectrometry (MS) and *lacZ*-bioreporters were used to identify the AHLs being produced by Y4I. Preliminary MS analyses of the AHLs involved the extraction of large cultures (200 ml) with acidified ethyl acetate and a ~1000 fold concentration. HPLC-MS/MS analyses of these samples showed two AHLs at high ion counts. These AHLs had ionized parent masses of 228 and 298, and retention times of 7.50 – 7.75 and 7.65 – 8 min, respectively (retention times of concentrated extracts not shown). Based on known retention times of synthetic AHLs (32) and the structures of AHLs that have been previously identified in proteobacteria (10, 22, 24, 30) it was hypothesized that Y4I produced C8-HSL and 3OC12-HSL. Deuterated C8-HSL and 3OC12-HSL were synthesized (32) to be used as internal standards for quantifying AHL production without concentrating the samples. Internal standard spiked cell supernatants were run on the triple quad HPLC-MS/MS. This method greatly narrows the retention time range. Analyses of 20 h cultures clearly show the C8-HSL eluting at the same time as the deuterated standard. This confirms the production of C8-HSL by Y4I. A difference in the HPLC retention time of the 298 parent mass AHL from Y4I and that seen with (D_2)3OC12-HSL (7.77 versus 8.08, respectively) indicates that Y4I does not produce 3OC12-HSL, but possibly an isomer of this molecule with similar polarity. We hypothesize that Y4I may be making a monounsaturated 3OHC12:1-HSL (Fig. 15). Studies are on-going to identify the structure of this AHL.

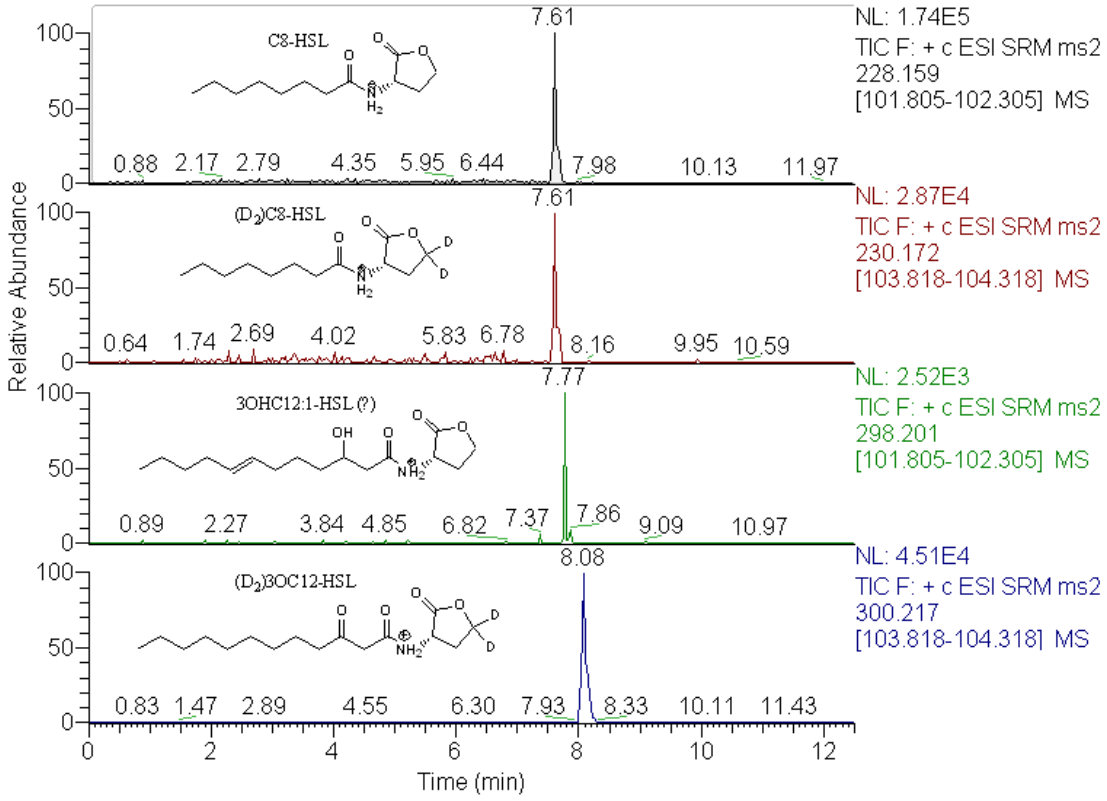

Figure 15. Chromatograms of the elution time and ion counts of AHLs found in internal standard spiked Y4I liquid culture supernatants. Parent masses were filtered for desired products by identifying the lactone ring mass (102 for biological AHLs and 104 for deuterated AHLs) after fragmentation in the triple quad MS/MS. Maximum ion counts are represented to the right of the graphs as NL. The black lined graph represents the C8-HSL with a parent mass of 228. The redlined graph represents the deuterated C8-HSL internal standard with a parent mass of 230, which elutes at the same time as the Y4I sample. The green lined graph represents an unidentified AHL with a parent mass equal to that of the 3OC12-HSL (298), but with a different retention time. The blue lined graph represents the deuterated 3OC12-HSL with a parent mass of 300. The structures of each of the AHLs are shown. The unknown AHL is hypothesized to be an isomer of 3OC12-HSL. The hypothesized structure is suggested but the location of the carbon-carbon double bond in the acyl chain is unknown and arbitrary in this diagram.

To further confirm the MS results for the C8-HSL, a specific *lacZ* bioreporter was used. *Burkholderia mallei* synthesizes C8-HSL as a primary AHL using the enzyme BmaI1. Duerkop *et al*. used the regulator and synthase genes from *B. mallei* to generate an *E. coli* based C8-HSL *lacZ* fusion bioreporter (10).). Using this bioreporter, we were able to confirm the production of C8-HSL by Y4I on agar plates (Fig. 16). Moreover, observations of the radius of bioreporter activation we were able to show that the *pgaR*::Tn5 mutant makes considerably less C8-HSL than the wildtype and other quorum sensing mutants. This suggests that C8-HSL may be made by PgaI, of which we do not currently have a mutant. In many quorum sensing systems the AHL-bound response regulator will upregulate the production of the synthase which leads to higher concentrations of AHL (8, 12, 31, 39). These bioreporter results suggest that inactivation of *pgaR* leads to lower concentrations of C8-HSL, which may be tied to *pgaI* expression.

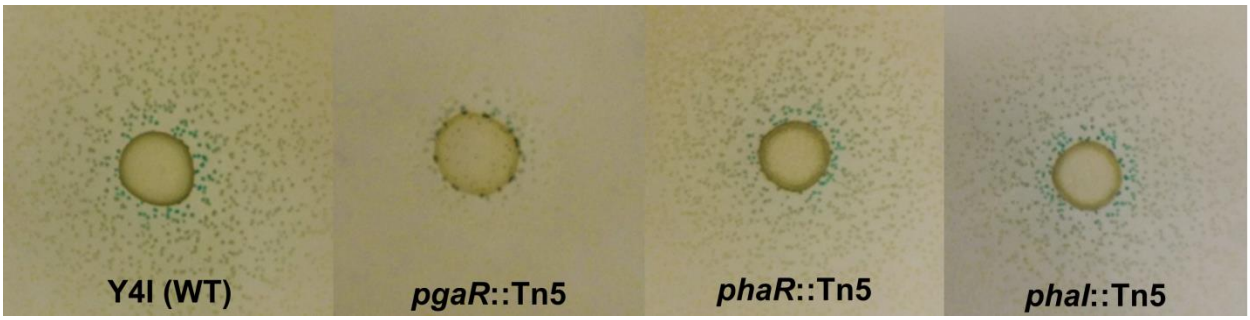

Figure 16. C8-HSL bioreporter response to Y4I strain colonies on agar plates. Lawns of a BmaR1-LacZ C8-HSL bioreporter (10) show LacZ activity around all Y4I spots, but there is a considerably smaller blue halo around the *pgaR*::Tn5 mutant, suggesting less C8-HSL biosynthesis in that strain.

AHL production over the Y4I life cycle in batch culture. The general AHL bioreporter strain *A. tumefaciens* NTL4 (pCF218)(pCF372) recognizes C6-C12 straight chain AHLS with any modification at the third carbon (-H, -OH, or =O). Depending on the AHL structure and third carbon modification the limit of detection for straight chain AHLs is between 5 pM – 0.3 μM (27, 34, 41). NTL4 has also been shown to respond to monounsaturated AHLs, but the limits of detection for these are unknown (34, 55). To determine whether NTL4 can respond to Y4I AHLs, aliquots of liquid culture filtrate at five time points over a 24 h growth curve were tested for β-galactosidase activity. It was shown that NTL4 can significantly detect Y4I AHLs when compared to a negative control as early as 5 h ($P=<0.001$; Fig. 17).

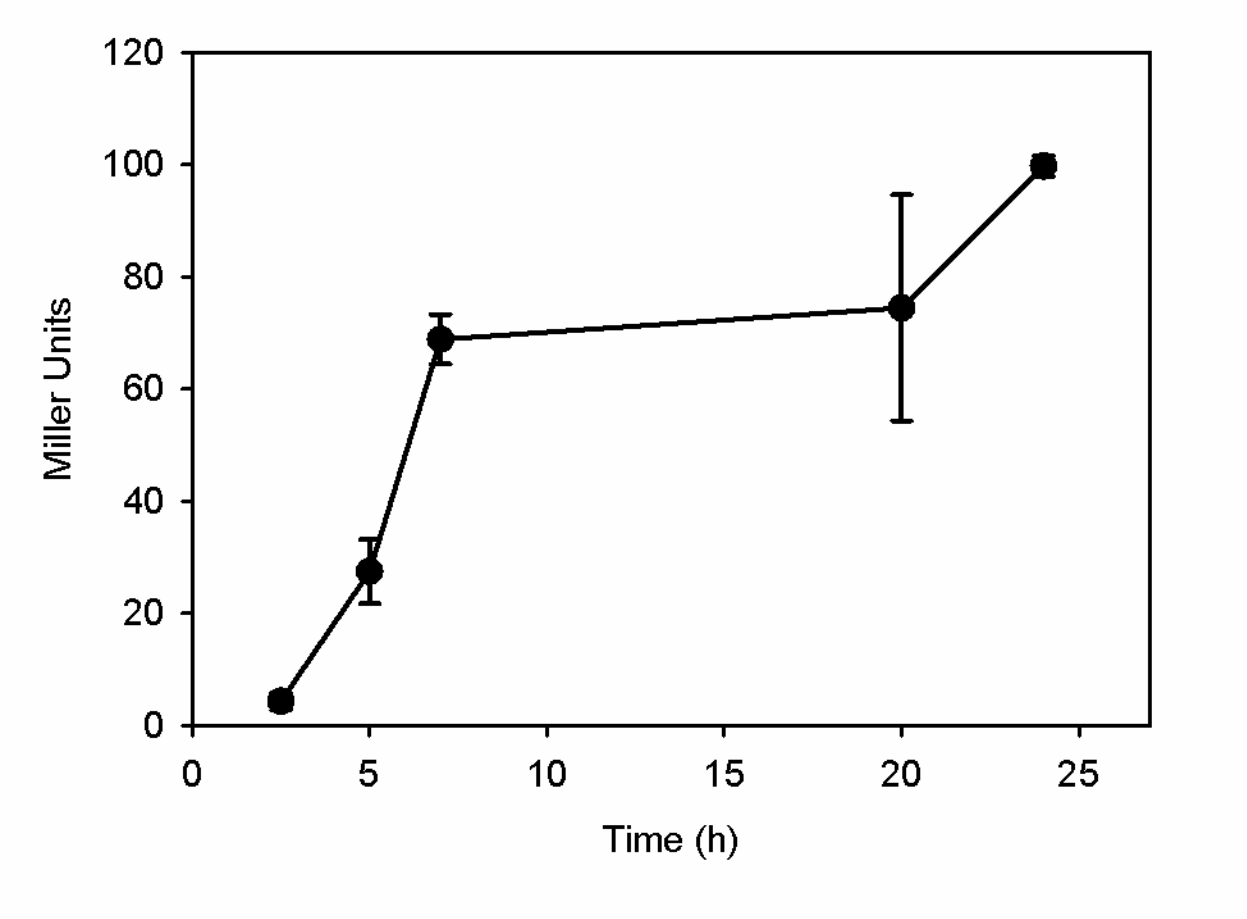

Figure 17. *A. tumefaciens* NTL4 AHL bioreporter induction by filtrates of wildtype Y4I cultures over time. NTL4 can significantly report AHL production in Y4I cultures by 5 h (P= <0.001). Error bars represent the standard deviation of biological triplicates.

Due to the high induction of NTL4 at 24 h by the wildtype, the bioreporter was used to assess AHL production by the quorum sensing mutants at that time point. The wildtype strain induced the bioreporter significantly more than all of the mutants ($P=\leq 0.001$) and similar to the positive control (Fig. 18), suggesting maximum induction. The relative drop in AHL production correlates with the pigmentation phenotypes of these mutants. The intermediate induction of the bioreporter by the *phaR* and *phaI* mutants corresponds to less pigmentation of these strains when grown on agar plates (Fig. 11), suggesting the putative 3OHC12:1-HSL contributes significantly to wildtype pigmentation. The *pgaR* mutant showed no induction of the bioreporter, and was indistinguishable from the negative control ($P=0.549$). This also correlates with the pigmentation phenotype of the *pgaR*::Tn5 strain, which produces no detectable indigoidine (Fig. 11), and suggests that C8-HSL may be required for indigoidine production. These data support the idea that the AHLs synthesized by quorum sensing systems contribute to *igiD* activation.

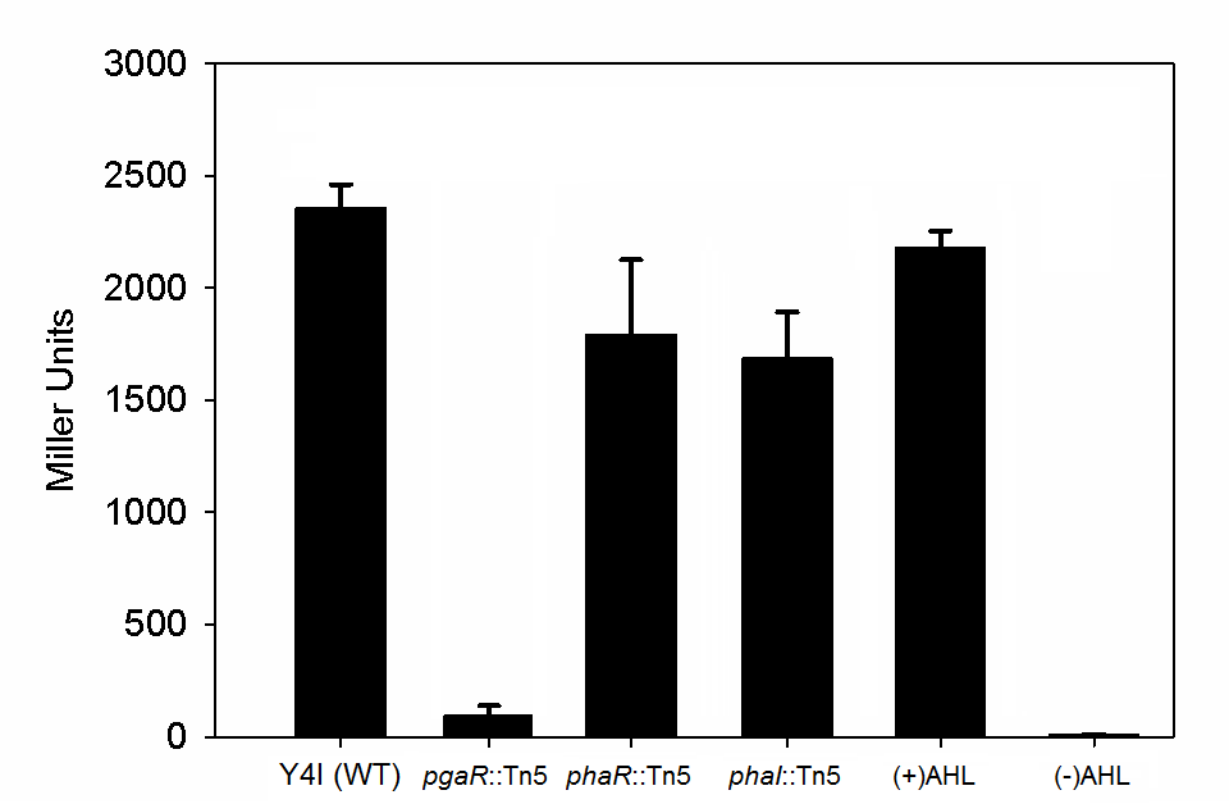

Figure 18. β-galactosidase activity of *A. tumefaciens* NTL4 AHL bioreporter in response to incubation with filtrate of liquid cultures of Y4I strains. Error bars represent the standard deviation of biological triplicates. The (+)AHL treatment was incubated ~10 µM C8-HSL. The (-)AHL treatment had no added AHLs.

AHL concentrations at seven time points over a 26 h growth curve were measured using HPLC-MS/MS. The C8-HSL and the putative 3OHC12:1-HSL concentrations were measured using deuterated standards that corresponded to the parent masses. As the structure is unknown for the putative 3OHC12:1-HSL, the 3OC12-HSL standard was used and only relative concentrations were obtained. The putative 3OHC12-HSL is synthesized at nM levels as the population enters exponential phase (~2-4 h) and then begins decreasing from mid to late log phase (~6-16 h; Fig. 19). As the culture enters mid-stationary phase (~20 h), C8-HSL is synthesized at μM levels (Fig. 19). Interestingly, it appears that neither of these AHLs is produced in concentrations directly relative to population density. As the population increases through exponential phase, the putative 3OHC12:1-HSL actually diminishes in concentration, and only when the population reaches a maximum density in stationary phase is the C8-HSL produced. It is possible that the threshold concentration needed to activate a response by the putative 3OHC12:1-HSL is still being met, and the cells are producing more than necessary during exponential phase. More investigation of the kinetics of the response regulators and cognate AHLs could elucidate the threshold concentration. It is clear that there is a sequential production of AHLs, with C8-HSL being produced much later than the putative 3OHC12:1-HSL. Because it has been shown that an intact *pgaR* gene is required for pigmentation, and it is thought that PgaI may synthesize C8-HSL, this switch in AHL production may be the signal that induces indigoidine biosynthesis in biofilms. It must be noted, however, that there may be differences in AHL production when Y4I is growing attached to a surface rather than planktonically, and future investigation of AHLs in biofilms are needed in this strain.

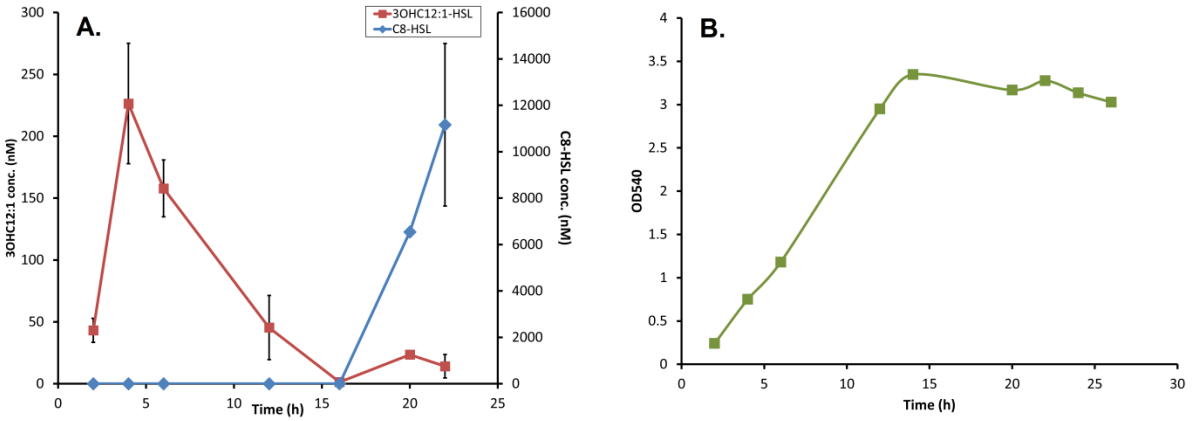

Figure 19. Quantification of AHLs over a growth curve. A. The concentration of the putative 3OHC12:1-HSL and C8-HSL from wildtype Y4I supernatants over the course of a growth curve. B. Optical density of the Y4I culture over the growth curve.

Y4I uses two separate quorum sensing systems to control multiple phenotypes.

Collectively, the pigmentation phenotypes (Fig. 11), *igiD* gene expression levels (Fig. 12), and bioreporter expression levels (Fig. 18) indicate that both quorum sensing systems (and the AHLs produced by these systems) are required for wildtype motility, surface colonization, and indigoidine production in Y4I.

Two separate, but connected, quorum sensing systems in Y4I are hypothesized to control the production of indigoidine. Similar sequential and multi-tiered regulatory networks have been shown in *V. fischeri,* where quorum sensing is used to modulate early and late colonization factors and bioluminescence (3, 31). Like Y4I, *V. fischeri* has two AHL-based quorum sensing systems, known as the *lux* and *ain* systems. LuxI and AinS synthesize different AHLs, which are recognized by their cognate regulator, LuxR and AinR, respectively. LuxI synthesizes *N*-3-oxo-hexanoyl homoserine lactone (3OC6-HSL), and AinS synthesizes C8-HSL. When C8-HSL concentrations are low, AinR inhibits the production of the activator LitR through a phosphorelay. When C8-HSL concentrations reach a threshold concentration, C8-HSL binds AinR and it no longer inhibits LitR. LitR then activates LuxR, and LuxR induces the production of LuxI. LuxI produces 3OC6-HSL which further activates LuxR, causing a positive feedback loop. The activated LuxR induces the *lux* operon leading to bioluminescence (3, 31).

Such complicated quorum sensing regulatory cascades are commonly used in bacteria to control energy intensive processes. Using these data we hypothesize that there are similarly complicated cascades in Y4I which control motility, biofilm formation, and indigoidine production. Multiple regulatory criteria must be met to have wildtype indigoidine production. It has been shown that surface colonization supersedes quorum sensing as the primary criterion. Even when quorum is reached in liquid cultures indigoidine is not produced (7). After a surface

is colonized, we hypothesize that PhaI first synthesizes the putative 3OHC12:1-HSL, then changes in the metabolism as the colony reaches stationary phase, such as intracellular fatty acyl availability, and AHL concentrations induce the *pgaRI* system which synthesizes C8-HSL. When C8-HSL meets the threshold concentration, PgaR and PhaR coordinate to upregulate indigoidine production. In this way, Y4I is able to tightly control the energy intensive process of secondary metabolite production until it reaches a niche where its production would be of best benefit to the fitness of the group. Future MS analyses with the *pgaR*::Tn5 and *phaI*::Tn5 mutant are necessary to obtain supporting data for this hypothesis.

It cannot be ignored that both *V. fischeri* and Y4I produce C8-HSL, and C8-HSL has been shown to be required for indigoidine production. Further, of the strains tested, *V. fischeri* is the most susceptible to the antimicrobial effects of indigoidine. Cross-talk between these organisms when grown in co-culture is likely. Investigations into whether the C8-HSL produced by *V. fischeri* can increase the amount of indigoidine produced by Y4I would have very interesting natural implications for the roles of interspecies communication and antagonism. C8-HSL is produced by many organisms (10, 11, 30, 33, 41, 48, 54), and Y4I may use the production of this AHL to respond to not only its own population, but competing populations as well by producing the antimicrobial indigoidine.

VI. Acknowledgments

We would like to acknowledge Amy Schafer at the University of Washington for providing us with the BmaR1 C8-HSL bioreporter strain and Clay Fuqua for providing us with the NTL4 AHL bioreporter strain. We would also like to acknowledge Mohammed Moniruzzaman for photodocumentation.

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Chapter 3

Quorum sensing in the *Roseobacter* clade: complex cell-to-cell communication controls multiple physiologies that contribute to environmental dominance

This section is a review that was written in preparation for submission to *Frontiers in Aquatic Microbiology*. The word limit for mini-reviews in this journal is 3000 words, which is reflected in the length of this chapter. The tentative title is:

W. Nathan Cude and Alison Buchan. Quorum sensing in the *Roseobacter* clade: complex cell-to-cell communication controls multiple physiologies that contribute to environmental dominance [In preparation].

I. Abstract

Bacteria have been widely reported to use small diffusible metabolites and genetic regulatory systems, known as quorum sensing (QS) systems, to coordinate gene expression in a population density dependent manner. In the Proteobacteria, the most common small molecules used for QS are acyl-homoserine lactones (AHL). While the model of QS comes from the γ-proteobacterium *Vibrio fischeri*, emerging studies have suggested that the α-proteobacteria may be the dominant AHL producers in the world's oceans. The *Roseobacter* lineage is an abundant group of α-proteobacteria known to dominate coastal environments and produce AHLs. Here, we review the state of quorum sensing research in the *Roseobacter* clade. Moreover, there are 43 publically available roseobacter genomes, and we review quorum sensing gene homology and putative operon topography found in the genomes.

II. Introduction

As individuals, bacterial cells have limited capabilities to influence their local environment, but when acting as coordinated communities, much larger effects can be achieved. It has been widely reported that diverse groups of bacteria use genetic regulatory systems, known as quorum sensing (QS) systems, to coordinate gene expression in a population density dependent manner (10, 19, 24, 36, 48). Physiologies that are often controlled by QS include virulence, biofilm formation, and coordinated motility, including swarming and swimming (19, 21, 24, 36, 70, 71, 73). Density dependent gene regulation has been hypothesized to provide these organisms with a competitive advantage in various environmental niches.

The chemical mediators of QS systems are small molecular weight diffusible molecules termed autoinducers (11, 24). A well-characterized type of QS system uses acyl-homoserine

lactones (AHL) as autoinducers and is only found in the Proteobacteria. Canonical AHL-QS systems produce and respond to AHLs using two gene products that regulate signal production and response, LuxI and LuxR-like proteins, respectively (50, 56). The genes encoding these two proteins are typically immediately adjacent to one another in bacterial chromosome (11, 25, 28). LuxI-like proteins are AHL synthases that mediate autoinducer production by cyclization of *S*-adenosyl methionine into a lactone ring and covalent bonding an acylated carbon chain from cellular fatty acid pathways. Chain length and modification of the side chain at the third carbon (either H, OH, or O) allow for species or group specificity (24, 59). LuxR-like proteins are response regulators that regulate genes required for the communal behavior, as well as *luxI*, in response to intracellular concentration of cognate AHLs (25, 26). Activated LuxR proteins upregulate *luxI* transcription, increasing the rate of AHL synthesis, thus causing a positive feedback loop resulting in higher AHL concentrations and increasing the rate of induction of regulators (10, 24, 25, 28).

Proteobacteria dominate coastal marine systems where QS is prevalent (32, 70, 73). One of the most abundant groups of marine α-proteobacteria is the *Roseobacter* clade (9, 16, 30). Here, we describe the current state of research in QS of the *Roseobacter* clade as well as areas of future investigation.

III. Roseobacters and Quorum Sensing

AHL-based QS was first described in the γ-proteobacterium *Vibrio fischeri* for its involvement in bioluminescence in the light organ of the bobtail squid (*Euprymna scolopes*) (50). Recent studies have suggested that α-proteobacteria also harbor diverse and complex QS systems and may be the dominant AHL producers in marine environments (1, 13, 21, 42, 69). There are no marine models of QS systems within the α-proteobacteria, but QS in better

characterized soil α-proteobacteria, such as *Agrobacterium tumefaciens* and *Sinorhizobium meliloti*, have been shown to be complex and involve multiple synthases and regulators. In these bacteria, QS plays important roles in plant colonization and interspecies interactions (5, 27, 33, 45, 47, 63, 74).

Roseobacters have been shown to be the primary surface colonizers and can comprise up to 30% of the total 16S rRNA genes in coastal environments (16-18) where they are predominant decomposers of plant biomass (9, 31). Further, roseobacters have been shown to produce diverse AHL structures ranging from eight to eighteen carbon acyl chains and with all three oxidative modifications at the third carbon position (3, 13, 32, 49, 69, 73). Of the 43 publically available roseobacter genomes, only five lack annotated *luxI* autoinducer synthase homologs, *Oceanicola batsensis* HTCC2597, *Oceanicola* sp. S124, *Pelagibaca bermudensis* HTCC2601, Rhodobacterales bacterium HTCC2255, and *Ruegeria* sp. TM1040 (www.roseobase.org). Further, the production of AHLs has been detected by LuxR-LacZ fusion bioreporters and mass spectrometry in many isolates (3, 4, 6, 32, 46, 62, 67, 69, 73). Emerging studies suggest that the roles of QS in roseobacters are varied and complex and may play an important role in the many phenotypes that may contribute to abundance of group members in the world's oceans.

AHL-based QS has been shown to control multiple physiologies in roseobacters including surface colonization, antimicrobial production, and motility (3, 6, 12, 15, 29, 73). QS was first reported in roseobacters associated with marine snow and tied to their ability to colonize particulate matter in the ocean (32). Because roseobacters are primary surface colonizers in coastal environments, coordinated biofilm formation is likely to be a widespread theme throughout the clade (16, 17, 62). Since this original observation, however, QS has been almost exclusively studied in the *Ruegeria-Phaeobacter* branch of the *Roseobacter* clade and it

is unclear whether discoveries made in this subgroup are readily transferable to other lineage members.

Roseobacters have been shown to use QS to form biofilms and become symbionts of several higher eukaryotes, including marine sponges and algae (38, 61, 73). Culture studies of endosymbionts of marine sponges have revealed that roseobacters are the dominant AHL producers and that these AHLs are structurally diverse (66). The Fuqua group has established a model of sponge-associated roseobacters using Rhodobacterales bacterium KLH11 (also known as *Ruegeria* sp. KLH11). Proteomic analyses of KLH11 demonstrated that a wide array of protein expression levels change as AHL concentration and population density increases, suggesting many metabolic processes may be completely or partially controlled by QS (49). KLH11 has been shown to contain two sets of *luxRI* homologs, designated *ssaRI* (RKLH11_1559 and RKLH11_2275) and *ssbRI* (RKLH11_1933 and RKLH11_260), as well as a recently discovered orphan *luxI* designated *sscI* that is not present in publically available genomes. *sscI* is thought to be a recent duplication of *ssbI* based on sequence homology (73). Heterologous expression of SsaI and SsbI in *E. coli* showed that each predominantly produces long chain saturated and unsaturated AHLs (C12-16), and that SsaI produces 3O-HSL variants whereas SsbI and SscI produce 3OH-HSLs (73). The modification at the third carbon has been shown in other organisms to cause specificity of signaling molecules to LuxR homologs and may allow KLH11 to finely tune its metabolism to cellular density and AHL diversity (37). Experiments on swim agar showed that KLH11 mutants deficient in QS had impaired motility compared to wildtype, and RT-qPCR analyses showed significant downregulation of genes encoding flagella biosynthesis machinery. Counter intuitively, the QS and motility impaired mutants formed drastically thicker biofilms, suggesting when motility or QS is retarded, biofilm

formation is induced (73). This may also suggest that biofilm formation may not be directly controlled by QS, but that when quorum is achieved motility and biofilm dispersion is induced. A similar phenotype has been seen in other roseobacters, and this trend may extend to the rest of the *Ruegeria-Phaeobacter* subgroup and contribute to their dominance on surfaces (7, 15, 20).

QS in roseobacters has also been closely tied to antimicrobial production. Berger *et al.* showed that *Phaeobacter gallaeciensis* DSM17395 produces *N*-3-hydroxydecanoylhomoserine lactone (3OHC10-HSL) using the LuxI homolog PgaI. Increases in the concentration of 3OHC10-HSL due to population density activated the adjacent regulator PgaR which led to the upregulation of a tropodithietic acid (TDA) biosynthetic operon (3). TDA has been shown to be a broad spectrum antimicrobial produced by multiple roseobacters (8, 53, 54). TDA, therefore, is produced when there are high *P. gallaeciensis* densities, allowing for a higher TDA concentration and inhibiting the growth of competing organisms (53). Interestingly, in a Δ*pgaI* mutant background, where no AHLs were produced, exogenous TDA could also upregulate the TDA biosynthesis machinery, suggesting that TDA may be an autoinducer as well as an antimicrobial (3). In the TDA-producing roseobacter *Ruegeria* sp. TM1040, which lacks any *luxI* homologs and does not produce AHLs, exogenous TDA upregulates the TDA biosynthesis machinery and induces its production (29). This opens the possibility that roseobacters may use novel autoinducers in tandem with AHL-based QS systems.

Similar to *P. gallaeciensis*, in *Phaeobacter* sp. strain Y4I, the production of the antimicrobial compound indigoidine has been shown to be connected to cellular density, as well as surface colonization. Transposon insertions in genes in each of two separate *luxRI*-like systems led to an inability of the Y4I mutants to produce indigoidine or inhibit the growth of *V. fischeri*, suggesting quorum must be met in both systems to lead to the phenotype (15). The

presence of multiple QS systems in the genomes and the antagonistic effects of many roseobacters suggest that this multi-layered control may be used to reserve antimicrobial biosynthesis to only when it would be most beneficial to the fitness of the group.

Table 4. LuxRI homologs in the 38 sequenced roseobacters. Homologs of LuxI and LuxR-encoding genes were determined using blastn and blastp on Roseobase (www.roseobase.org). Gene orientations are represented in Figure 22.

Strains	Gene orientation	*luxR* gene #	*luxI* gene #
Rhodobacterales bacterium HTCC2083	A	RB2083_3272	RB2083_3255
Rhodobacterales bacterium KLH11	A	RKLH11_1559	RKLH11_2275
Roseovarius sp. 217	A	ROS217_18272	ROS217_18267
Roseovarius sp. TM1035	A	RTM1035_10475	RTM1035_10485
Ruegeria lacuscaerulensis ITI-1157	A	SL1157_2477	SL1157_2476
Ruegeria pomeroyi DSS-3	A	SPO2286	SPO2287
Ruegeria sp. TW15	A	RTW15_010100013877	RTW15_010100013872
Citreicella sp. 357	B	C357_10197	C357_10192
Citreicella SE45	B	CSE45_4055	CSE45_4054
Roseobacter denitrificans OCh 114	B	RD1_1638	RD1_1639
Sagittula stellata E-37	B	SSE37_11169	SSE37_11164
Ruegeria pomeroyi DSS-3	B	SPO0371	SPO0372
Dinoroseobacter shibae DFL 12	B1	DSHI_2852	DSHI_2851
Loktanella sp. SE62	B1	LSE62_0618	LSE62_0617
Phaeobacter gallaeciensis 2.10	B1	PGA2_c03430	PGA2_c03440
Phaeobacter gallaeciensis DSM 17395	B1	PGA1_c03880	PGA1_c03890
Phaeobacter gallaeciensis ANG1	B1	ANG1_1316	ANG1_1315
Phaeobacter sp. Y4I	B1	RBY4I_1689	RBY4I_3631
Rhodobacterales bacterium KLH11	B1	RKLH11_1933	RKLH11_260
Rhodobacterales bacterium HTCC2150	B1	RB2150_14426	RB2150_14421
Roseobacter sp. AzwK-3b	B1	RAZWK3B_04270	RAZWK3B_04275
Roseobacter sp. GAI101	B1	RGAI101_376	RGAI101_3395
Roseobacter sp. MED193	B1	MED193_10428	MED193_10423
Ruegeria lacuscaerulensis ITI-1157	B1	SL1157_0613	SL1157_0612
Ruegeria sp. R11	B1	RR11_2850	RR11_2520
Ruegeria sp. TW15	B1	RTW15_010100017779	RTW15_010100017784

Strains	Gene orientation	*luxR* gene #	*luxI* gene #
Roseobacter sp. R2A57	B2	R2A57_2403	R2A57_2404
Thalassiobium R2A620	B2	TR2A62_3165	R2A57_2405 TR2A62_3166 TR2A62_3167
Maritimibacter alkaliphilus HTCC2654	B3	RB2654_09024	RB2654_09014
Rhodobacterales bacterium HTCC2083	B4	RB2083_3265	RB2083_730
Roseobacter litoralis Och 149	B4	RLO149_c030690	RLO149_c030680
Dinoroseobacter shibae DFL 12	C	DSHI_0311	DSHI_0312
Jannaschia sp. CCS1	C	JANN_0619	JANN_0620
Loktanella vestfoldensis SKA53	D	SKA53_05835	SKA53_05830
Loktanella sp. SE62	D1	SKA53_05840 LSE62_3230	LSE62_3231
Oceanicola granulosus HTCC2516	D1	LSE62_3229 OG2516_02284	OG2516_02294
Octadecabacter antarcticus 307	D1	OG2516_02289 OA307_2044	OA307_4586
Roseobacter sp. CCS2	D1	OA307_3216 RCCS2_02083	RCCS2_02078
Octadecabacter arcticus 238	D2	RCCS2_02088 OA238_4151 OA238_3367	OA238_2886
Roseobacter sp. SK209-2-6	E	RSK20926_22079	RSK20926_22084
Sulfitobacter NAS-14.1	E	NAS141_01141	NAS141_01136
Maritimibacter alkaliphilus HTCC2654	F	RB2654_20053	RB2654_20048
Roseovarius sp. 217	G	ROS217_01405	ROS217_01410
Roseobacter litoralis Och 149	G1	RLO149_c036220	RLO149_c036210
Sulfitobacter NAS-14.1	H		NAS141_00695
Sulfitobacter sp. EE-36	H		EE36_01635

Strains	Gene orientation	*luxR* gene #	*luxI* gene #
Roseovarius nubinhibens ISM	I		ISM_03755
Oceanibulbus indolifex HEL45	I		OIHEL45_00955
Ruegeria sp. R11	J		RR11_2017
Roseobacter sp. MED193	J		MED193_08053
Ruegeria sp. TW15	J		RTW15_010100005486
Dinoroseobacter shibae DFL 12	K		DSHI_4152
Phaeobacter gallaeciensis 2.10	L	PGA2_c18970	PGA2_c18960
Phaeobacter sp. Y4I	L1	RBY4I_1027	RBY4I_3464
Phaeobacter gallaeciensis 2.10	M		PGA2_c07460
Phaeobacter gallaeciensis DSM 17395	M		PGA1_c07680
Rhodobacterales bacterium HTCC2150	N	RB2150_11281	RB2150_11291
Roseobacter litoralis Och 149	O		RLO149_c036590
Roseobacter sp. AzwK-3b	P		RAZWK3B_19371
Roseobacter sp. SK209-2-6	Q	RSK20926_15126	RSK20926_15131
Ruegeria lacuscaerulensis ITI-1157	R		SL1157_1706
Ruegeria sp. Trich CH4B	S		SCH4B_1938

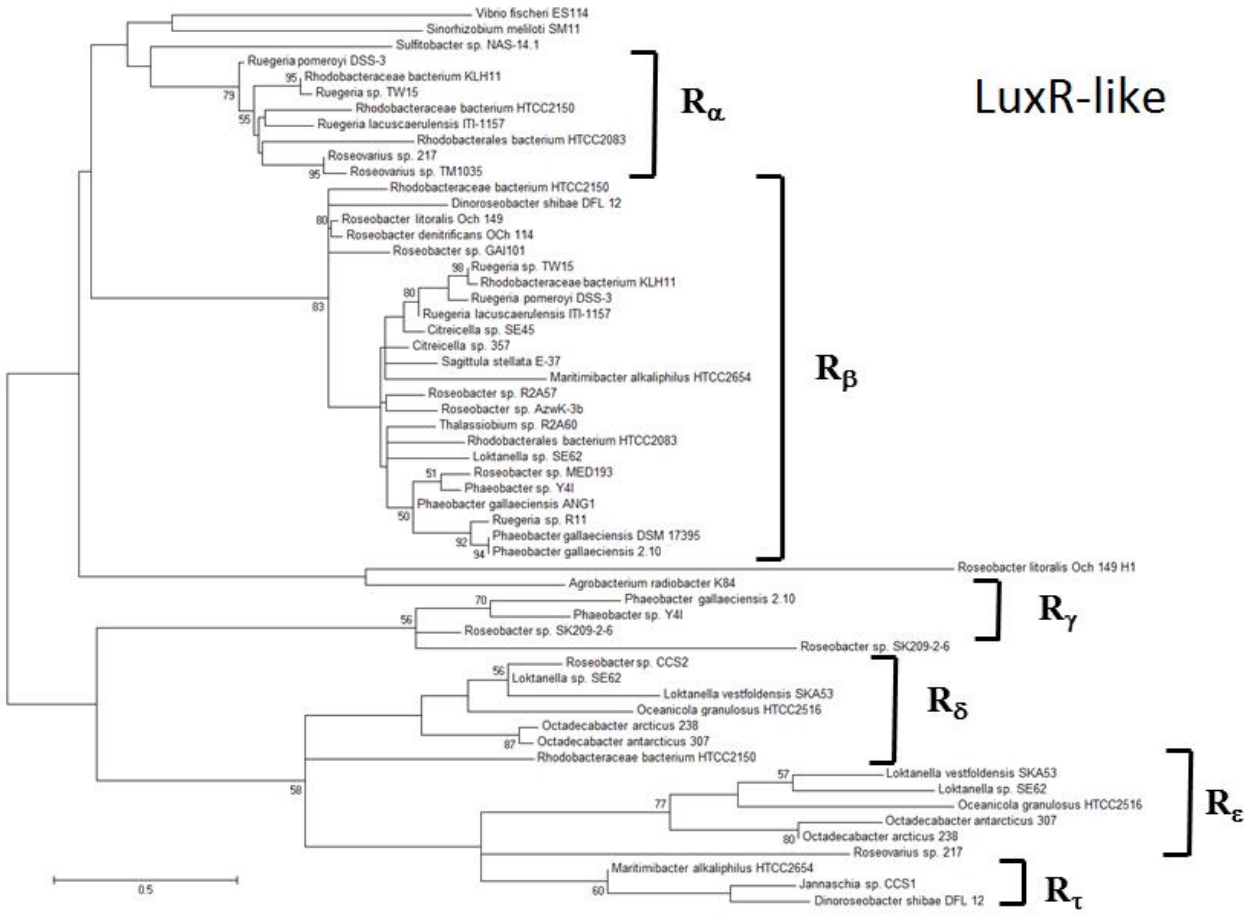


Figure 20. Proposed designations of LuxR groups in roseobacters. Maximum likelihood phylogenetic tree of LuxR-like genes from 38 published roseobacter genomes. Protein alignments of the LuxR homologs were done using T-COFFEE Expresso (www.tcoffee.org) with default parameters (2). Maximum-likelihood phylogenetic trees were generated using MEGA 5.1 with the WAG model of amino acid substitution and 1000 bootstrap replications (65). Groups were divided and defined by natural divisions in the tree and gene topography in the genome (Fig. 22). The LuxR, SinR, and TraR proteins of *Vibrio fischeri*, *Sinorhizobium meliloti*, and *Agrobacterium tumefaciens*, respectively, were used as out groups for the alignment and tree formation.

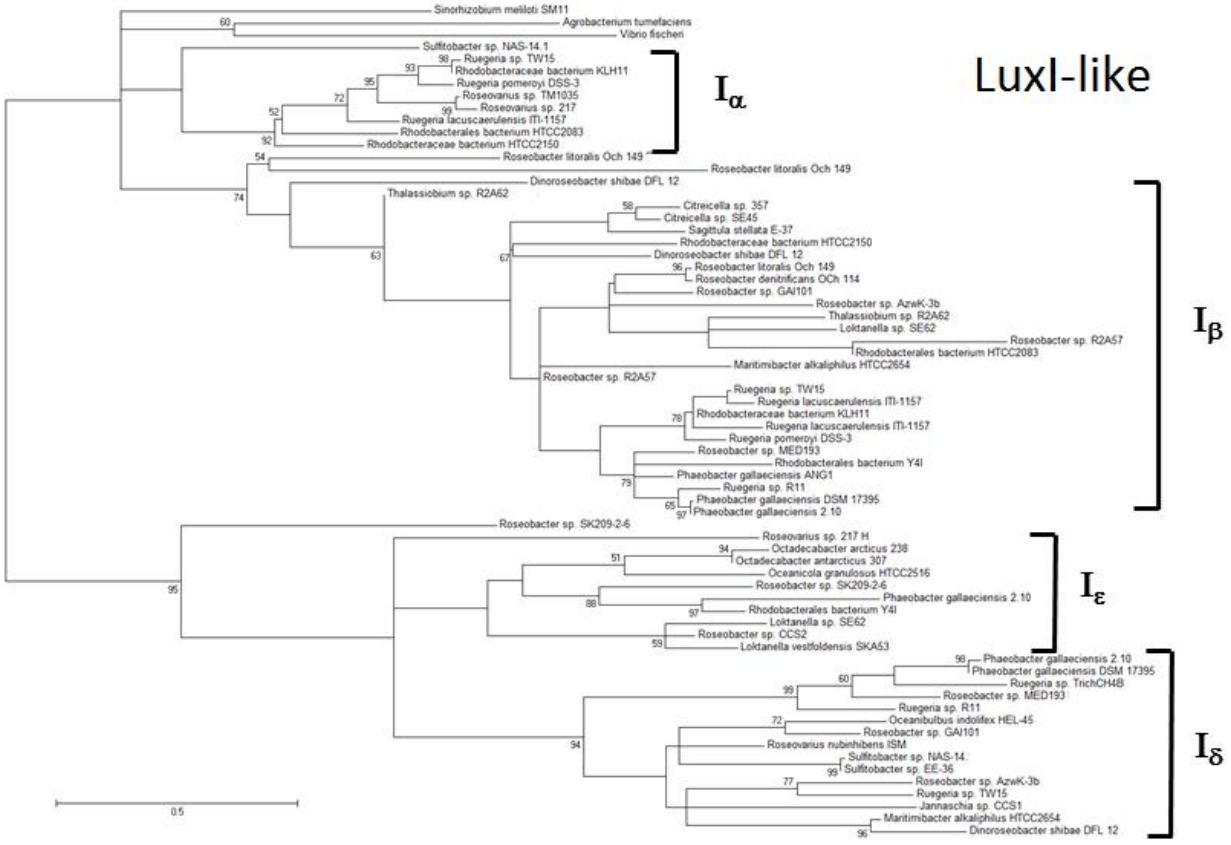


Figure 21. Proposed designations of LuxI groups in roseobacters. Maximum likelihood phylogenetic tree of LuxI-like genes from 38 published roseobacter genomes. Protein alignments of the LuxI homologs were done using T-COFFEE Expresso (www.tcoffee.org) with default parameters (2). Maximum-likelihood phylogenetic trees were generated using MEGA 5.1 with the WAG model of amino acid substitution and 1000 bootstrap replications (65). Groups were divided and defined by natural divisions in the tree and gene topography in the genome (Fig. 22). The LuxI, SinI, and TraI proteins of *Vibrio fischeri*, *Sinorhizobium meliloti*, and *Agrobacterium tumefaciens*, respectively, were used as out groups for the alignment and tree formation.

IV. Quorum Sensing Gene Homology and Topography

Not surprising due to the close evolutionary relatedness between members of the *Roseobacter* clade, many of the LuxI- and LuxR-like protein sequences found in roseobacters share common lineages and can be grouped together based on homology (Figs. 20 and 21). Using phylogenetic trees, we can show that there are six different LuxR-like (R_α, R_β, R_γ, R_δ, R_ε, and R_τ) and four different LuxI-like homologous proteins (I_α, I_β, I_γ, and I_δ) that are found in the majority of the sequenced roseobacters. Beyond the sequence conservation of LuxR and LuxI, the genomes also show multiple conserved QS operon topographies that can allow for further grouping and classification. All variations in operon topography in sequenced roseobacters have been identified and classified into groups and subgroups in Figures 22 and Table 4, including both conserved and unique topographies.

The most conserved gene topographies are the A and B groups, of which 28 different roseobacter genomes contain one of the orientations, and three genomes contain both. Genomes that contain the A topography have highly homologous LuxI and LuxR sequences and share a Trigger Factor (TF) encoding gene directly upstream from *luxRI* (Figs. 20, 21, and 22). The similarity of the LuxI and LuxR proteins suggest the operon found in these different organisms shares a common ancestor (Figs. 20 and 21). The LuxI and LuxR of the A topography have been dubbed I_α and R_α, respectively (Fig. 22). The location of this TF is conserved in seven genomes. In *Vibrio cholera* TFs play a role in the folding and secretion of newly translated proteins, and, though its function is unknown in roseobacters, it may have a similar role for proteins that are under the control of QS (43).

The B topography is the most highly conserved and is found in four variations in 24 genomes (Table 4). Like the A topography, the LuxI and LuxR protein sequences are

homologous between the organisms that contain the B topography and may share a common ancestor (Figures 20 and 21). The LuxI and LuxR of the B topography have been labeled I_β and R_β, respectively (Fig. 22). The conserved regions of the B topography include a gene encoding a crotonyl-CoA reductase preceding *luxRI* and a putative ATP-dependent helicase encoding gene following *luxRI*. In streptomycetes where the protein was characterized, crotonyl-CoA reductase converts unsaturated crotonyl-CoA to saturated butyryl-CoA by reducing the double bond and thus provides a precursor to fatty acid biosynthesis). It is possible that the presence of this reductase before the *luxRI* locus increases the concentration of saturated fatty acids used by the LuxI in order to synthesize straight chain AHLs. The following helicase has not been conclusively characterized but does contain an ATPases associated with diverse cellular activities domain (AAA) which can contribute to DNA repair, protein degradation, or gene regulation (64). Further investigation is needed to determine the role of this helicase in QS. The most conserved within the B group is the B1 subgroup which contains a short-chain dehydrogenase following the helicase. This gene orientation is conserved in 14 roseobacter genomes. Short-chain dehydrogenases are a large family of proteins that are responsible for modification in carbon chain structures of many different substrates (35). The protein encoded by this gene may function to modify AHL biosynthesis substrates before or after AHL production.

Variations of the D topography are found in six roseobacter genomes. As seen with the A and B topographies, the LuxI and LuxR protein sequences are homologous among those organisms that share this topography (Figs. 20 and 21). The LuxI and LuxR of the D topography have been designated I_δ and R_ε, respectively (Fig. 22). This topography shares two genes in common between the variations, *fliG* in the opposite orientation upstream of *luxRI* and an

adenylosuccinate lyase encoding gene downstream. In *E. coli* FliG is the flagellar motor switch which controls the spin direction of flagella (55). The characterized role of QS and motility in roseobacters has been addressed above, but none of the organisms containing the D topography have been investigated for their QS and motility characteristics. The direct connection between QS and flagellar machinery may be a very interesting avenue for future investigation. The other gene in this orientation putatively encodes an adenylosuccinate lyase, which is very important in the *de novo* purine biosynthetic pathway and in controlling the levels of AMP and fumarate inside the cell (68). The position of an adenylosuccinate lyase encoding gene directly following *luxRI* indicate that organisms with the D orientation may respond to reaching quorum by increasing purine biosynthesis. Further, they may increase ATP generation through fumarate entering the citric acid cycle and the recycling of AMP to ATP. Though no studies have addressed the role of this protein in roseobacters, it is possible that this activity may prepare the planktonic cell for communal behavior by increasing energy production.

The only other orientation that is shared in more than two roseobacter genomes is the J topography, and is also highly similar to the H and I orientations. In all three of these orientations, there is a lone *luxI*-like gene that is homologous and has been denoted as I_{δ}. Linked *luxR-luxI* gene orientations appear in 48 of 62 loci in sequenced roseobacters, but the appearance of orphan *luxI* genes appears common, especially in the *Sulfitobacter*, *Ruegeria*, and *Phaeobacter* genera (Table 4).

Shared among the H, I, and J topographies are different types of partially homologous histidine kinase (HK) encoding genes upstream of the *luxI*. These genes are in the same direction as the *luxI* as in H and I and in the opposite orientation as in J (Fig. 22). Using the Simple Modular Architecture Research Tool (SMART; http://smart.embl.de/) domain annotation

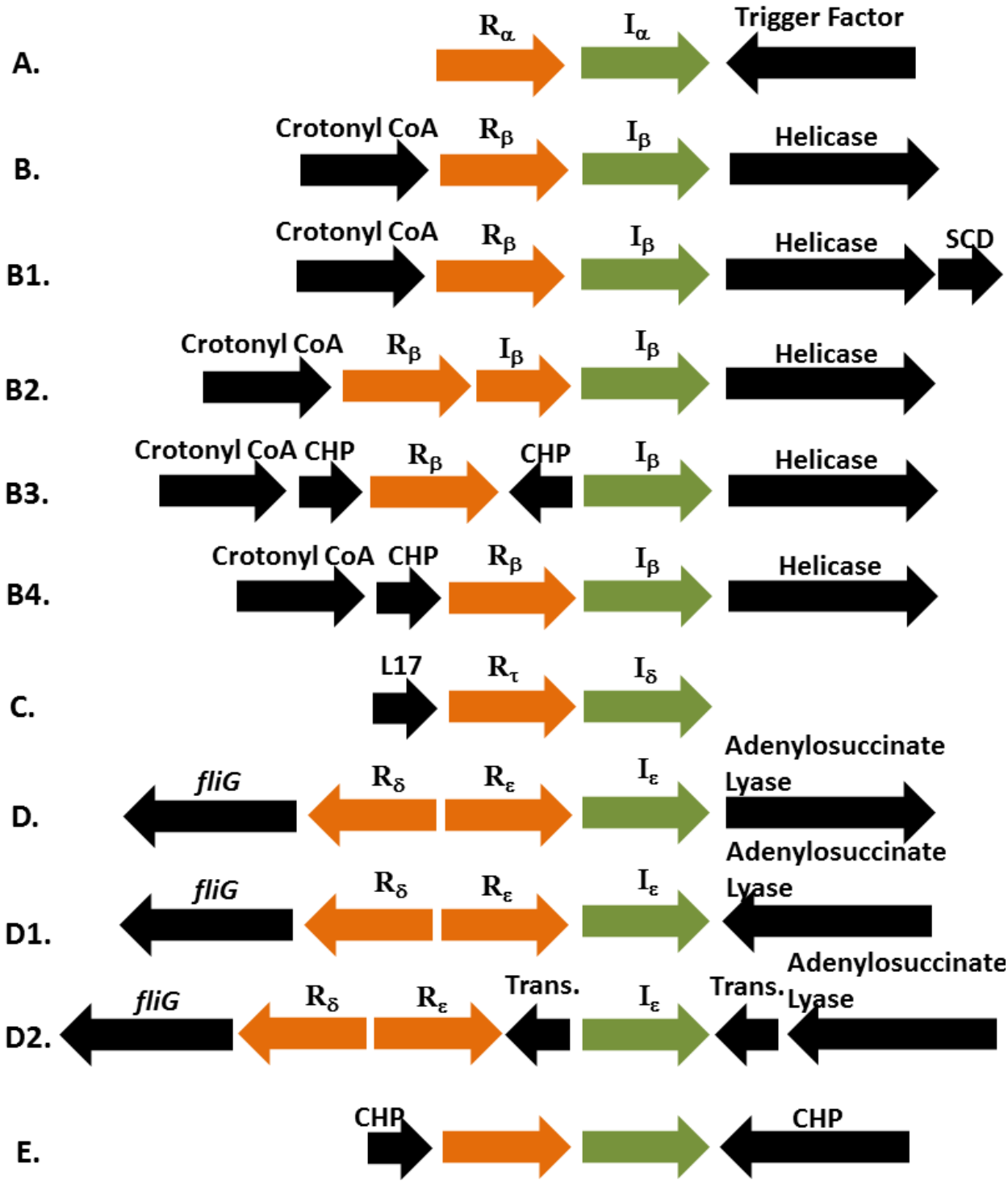
software (41, 60), the HK found in the H topography contains the classical histidine kinase A domain and several putative transmembrane domains, suggesting the protein is part of a two-component phosphorylation pathway and is membrane bound. Based on this limited annotation, the stimuli required to induce the phosphorelay, the target of the phosphorylation, and whether QS molecules are involved is unknown. In the I topography, the transmembrane HK is annotated as a hybrid histidine kinase that is fused to an uncharacterized response regulator (RR). Similarly, SMART analysis of the J topography shows that it also contains a transmembrane hybrid HK; however, this hybrid HK is fused to a REC regulatory domain. The REC superfamily consists of many different response regulators that contain a DNA binding motif (51). In hybrid HKs, the HK portion of the protein is thought to autophosphorylate to activate the RR or REC domain. Proteins containing a fused RR domain may then act to control the expression of genes or the phosphorylation of proteins, but those containing the REC domain are thought to exclusively regulate transcription (22, 72). In *Vibrio harveyi* and other model QS organisms, hybrid HK relays have been implicated in the activation of gene circuits that lead to coordinated behaviors such as bioluminescence (23, 39), suggesting similar regulatory systems may be present in these roseobacters.

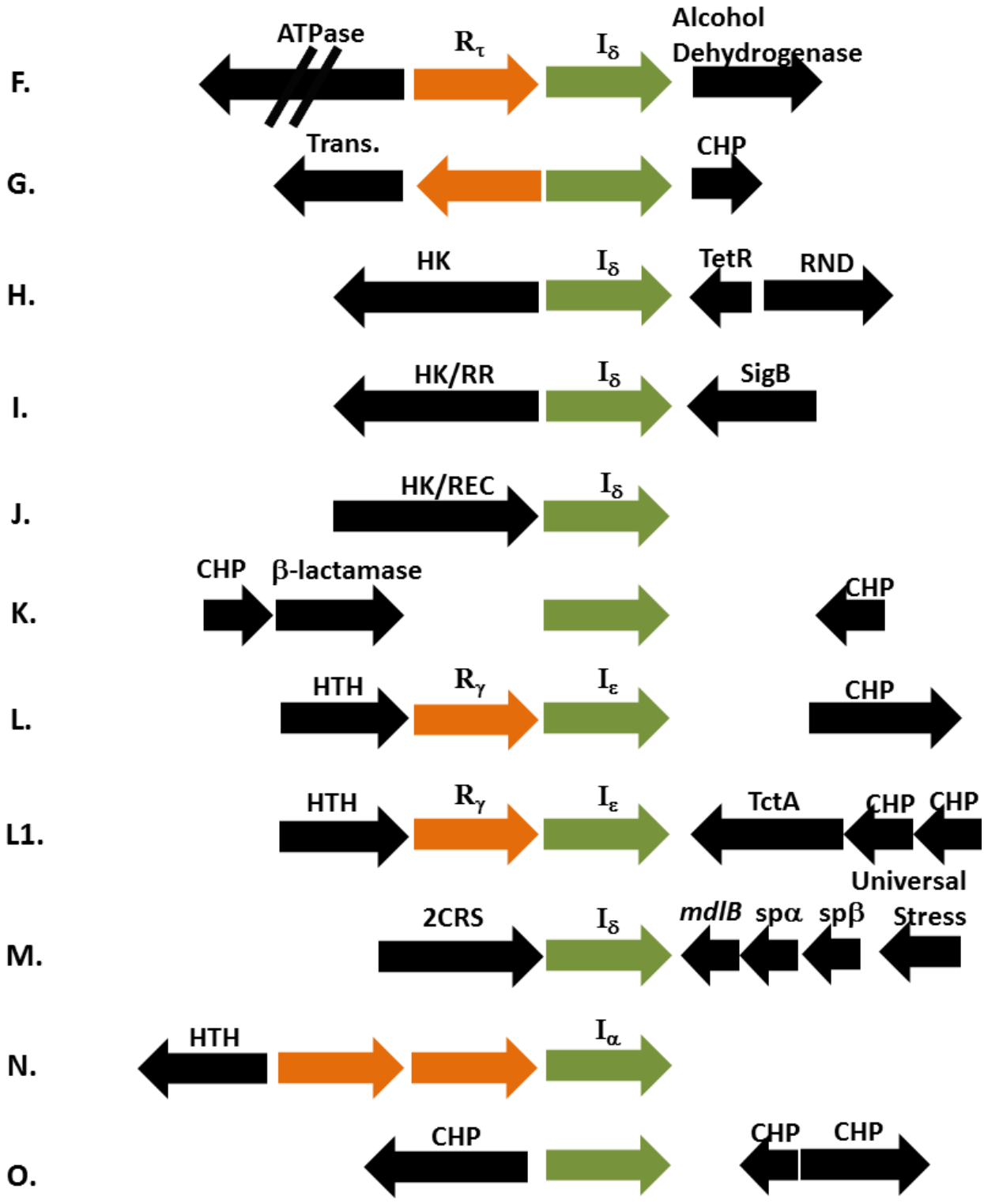
What separates the H, I, and J topographies more specifically are the genes that follow the I$_\delta$ *luxI* in the putative operons. In the H topography, the downstream genes are a *tetR*-like DNA binding regulator in the opposite orientation of the *luxI* and a RND family multidrug efflux pump in the same orientation of the *luxI*. Multiple other transporter encoding genes can also be identified surrounding the QS loci in the genomes of the two H topography isolates, *Sulfitobacter* NAS-14.1 and *Sulfitobacter* sp. EE-36. It is unclear what is being transported in and out of the cell by these putative gene products, but it can be hypothesized that many of these transport

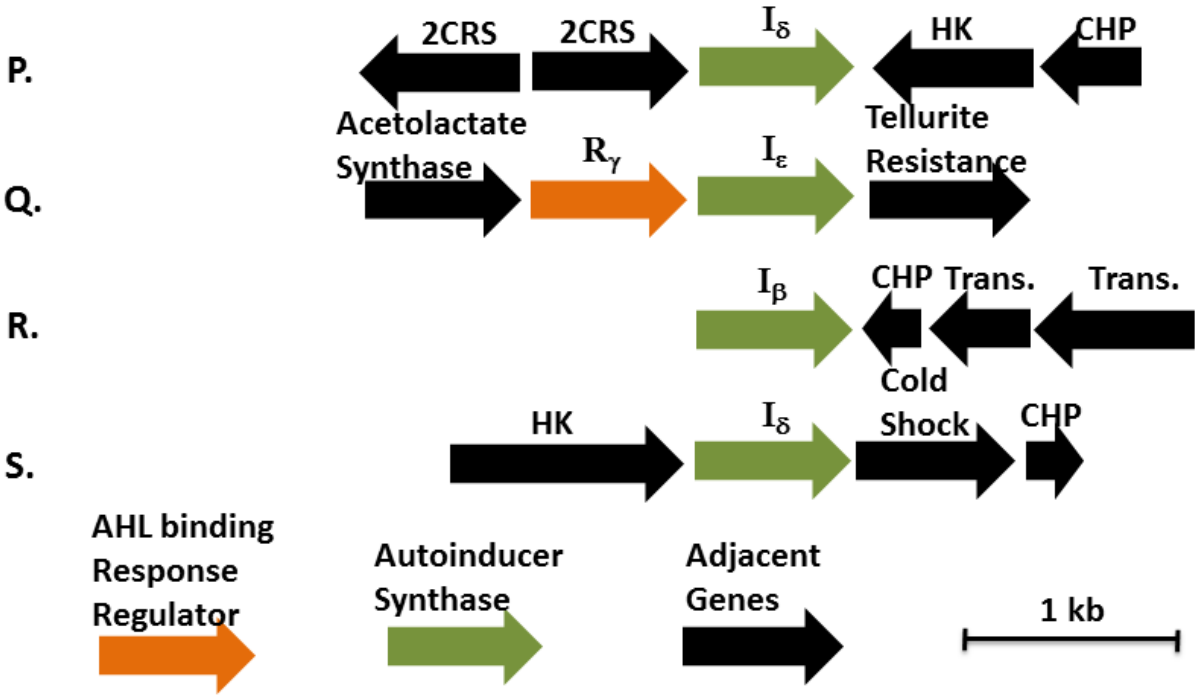
systems could be affected by QS due to their proximity. The gene following the I_{δ} *luxI* homolog in the I orientation is annotated as a putative sigma B factor response regulator encoding gene. Like the HK/REC in orientation J, this putative gene product also contains a DNA binding motif, suggesting transcriptional regulation of unknown genes. Finally, while the J topography shows conservation in the preceding HK/REC gene but, there appears to be no conservation of the genes downstream of the *luxI*.

Topography investigations clearly show the close relatedness of the *Roseobacter* lineage with respect to QS gene orientation. The conservation of *luxR* and *luxI* and the surrounding genes may allow for targeted studies to develop better models of QS in roseobacters. Similarity of gene sequence does not directly predict regulatory cascades or phenotypes, but the development of model systems for each of these topographies will provide a strong foundation for gaining further insight into how roseobacters behave as communities.

Figure 22. The gene orientation of all putative quorum sensing operons in 38 roseobacter genomes adapted from Cicirelli *et al*. 2008 to include 24 roseobacter genomes that have become available since that publication. Light gray arrows represent autoinducer synthase encoding genes, dark gray arrows represent AHL binding response regulators, and black arrows represent adjacent genes. Abbreviations used: Crotonyl CoA - Crotonyl CoA reductase, HK – histidine kinase, HK/RR – hybrid histidine kinase/response regulator, HK/REC – histidine kinase with REC domain, CHP – conserved hypothetical protein, RND – RND multidrug efflux pump, Sig B – sigma B factor, SCD – short chain dehydrogenase, Trans. – transposase, L17 – L17 component of the 50S ribosomal protein, TctA – TctA family transmembrane transporter, *mdlB* – mandelate dehydrogenase *mdlB*, spαβ – α and β subunits of sulfopyruvate decarboxylase. R_x and I_x designations above the response regulators and autoinducer synthases indicate their corresponding phylogentic subgroupings in Figures 20 and 21, respectively. Those without R_x and I_x designations indicate unique sequences not found in the conserved groupings.


A.
R_α
I_α
Trigger Factor
B.
Crotonyl CoA
R_β
I_β
Helicase
B1.
Crotonyl CoA
R_β
I_β
Helicase
SCD
B2.
Crotonyl CoA
R_β
I_β
I_β
Helicase
B3.
Crotonyl CoA
CHP
R_β
CHP
I_β
Helicase
B4.
Crotonyl CoA
CHP
R_β
I_β
Helicase
C.
L17
R_τ
I_δ
D.
fliG
R_δ
R_ε
I_ε
Adenylosuccinate Lyase
D1.
fliG
R_δ
R_ε
I_ε
Adenylosuccinate Lyase
D2.
fliG
R_δ
R_ε
Trans.
I_ε
Trans.
Adenylosuccinate Lyase
E.
CHP
CHP


F.
ATPase
R_τ
I_δ
Alcohol Dehydrogenase
G.
Trans.
CHP
H.
HK
I_δ
TetR
RND
I.
HK/RR
I_δ
SigB
J.
HK/REC
I_δ
K.
CHP
β-lactamase
CHP
L.
HTH
R_γ
I_ε
CHP
L1.
HTH
R_γ
I_ε
TctA
CHP
CHP
M.
2CRS
I_δ
mdlB
spα
spβ
Universal Stress
N.
HTH
I_α
O.
CHP
CHP
CHP


P.
2CRS
2CRS
I_δ
HK
CHP
Q.
Acetolactate Synthase
R_γ
I_ε
Tellurite Resistance
R.
I_β
CHP
Trans.
Trans.
S.
HK
I_δ
Cold Shock
CHP
AHL binding Response Regulator
Autoinducer Synthase
Adjacent Genes
1 kb

V. Conclusions and Future Directions

It is clear that QS plays vast and important roles in the life cycles of roseobacters and may contribute to the ability of roseobacters to dominate coastal environments. Beyond species-specific communication, co-culture experiments with multiple roseobacters or other marine organisms will shed light on how QS works in the natural world. Preliminary experiments have shown that *Roseobacter denitrificans* alters the expression of multiple genes when grown in the presence of *Pseudomonas aeruginosa,* suggesting more environmentally relevant and complex interactions in response to co-cultures are worthy of study (14). Moreover, the close association roseobacters have with plants as they colonize and digest plant-derived compounds could suggest possible inter-kingdom communication such as that found in the bacteria *A. tumefaciens* and *S. meliloti* and their plant hosts (34).

The role of orphan *luxI*-like genes is another area that is not well understood. The presence of orphan *luxR*-like genes has been widely described, and their gene products have been shown to respond to AHLs produced by other organisms or allow for multiple responses from the same AHL (40, 44, 52, 57). Studies of orphan *luxI*-like gene systems, however, are not prevalent in the literature. It can be hypothesized that either novel non-LuxR-like proteins are responding to AHLs or proteins encoded by genes in a different area of the genome are responding to the AHLs. Characterization of these systems could lead to a better understanding of these novel gene orientations and possible roles of orphan *luxI* that may be present in other organisms.

Recent investigations into novel non-fatty acyl-HSLs have shown that at least one roseobacter, *Ruegeria pomeroyi* DSS-3, is capable of producing *p*-coumaroyl-HSL when grown in the presence of the aromatic compound *p*-coumaric acid, a lignin derivative (58). This

discovery opens the door to many novel HSLs that could be produced by roseobacters in response to local nutrient availabilities. The production of these newly found signaling molecules and the metabolic responses caused by their production is unknown in the many cultured roseobacter representatives known to quorum sense and degrade aromatic compounds. This, coupled with the use of TDA as a QS molecule, suggests that roseobacters can utilize different types of QS systems including AHL-based systems, novel autoinducer-based systems, or a mixture of both.

In a broader sense of the *Roseobacter* clade as a whole, further investigation of putative QS systems in the isolates outside of the *Ruegeria-Phaeobacter* subgroup is needed. It is apparent from genomic investigations that the ability to quorum sense is present in the genomes of most of the cultured and sequenced isolates, but little attention has been paid to these organisms. The *Roseobacter* clade is known to dominate coastal environments, but the clade represents many organisms from many genera. There may be very interesting QS responses in the less characterized roseobacter isolates. Future studies should focus on not only expanding our knowledge or characterized systems, but on also broadening our understanding of novel systems within the clade.

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Conclusion

Summary

The *Roseobacter* lineage of α-proteobacteria has been shown to dominate coastal surfaces and waters (5, 6). The notion that the production of novel antimicrobials contributes to the dominance of roseobacters on surfaces in marine coastal environments was first proposed over a decade ago (9). There were no studies, however, that attempted to directly address the concept of antimicrobial production and competitive surface colonization. The research presented in this dissertation set out to address this fundamental gap in knowledge. The overriding objective of this dissertation research was to characterize secondary metabolite production, specifically as it relates to surface colonization and quorum sensing, in the roseobacter *Phaeobacter* sp. strain Y4I. This strain was selected for these studies because it is a member of one of the more abundant roseobacters sublineages (*Phaeobacter-Ruegeria*), it was isolated from a coastal salt marsh, it colonizes surfaces rapidly (14), and it produces a novel secondary metabolite, indigoidine, not before investigated as an antimicrobial (Chapter 1).

Y4I was shown to inhibit the growth of *Vibrio fischeri* when grown on agar plates (Fig. 1). Using random transposon mutants it was possible to identify the antimicrobial as indigoidine (Fig. 4) and then characterize indigoidine production in biofilms. In this study it was found that when in co-culture biofilms Y4I strains that produce indigoidine are able to inhibit *V. fischeri* colonization ~60% more than an indigoidine null mutant (Fig. 5F). This study provides evidence that the production of antimicrobials gives roseobacters an advantage when competing for surface niches. This was also the first study to show that indigoidine has antimicrobial properties.

The studies presented here also demonstrate that Y4I has two separate quorum sensing systems and that both are required for wildtype indigoidine production. Mass spectrometry and bioreporter systems were used to identify the two dominant acyl homoserine lactones (AHLs) quorum sensing molecules (Fig. 15) produced by this strain. These data contribute to the growing repertoire of signal molecules collectively produced by roseobacters. Further investigations, however, are needed to confirm the structure of one of the AHLs (Fig. 15). Transposon insertions in genes encoding either quorum sensing system result in a loss of the ability to produce indigoidine and inhibit *V. fischeri* (Table 1). A temporal analysis of the two dominant AHLs demonstrate that they appear at different points in the growth of Y4I (Fig 19) suggesting a disconnect between AHL concentration and cell density that warrants further study. In fact, these data provide evidence that sequential regulation, which is found in other bacteria (3) but has not been described in roseobacters, may control the production of indigoidine. A model of complex multi-tiered gene regulation is proposed so as to allow Y4I to tightly control indigoidine production. The presence of multiple quorum sensing systems is widespread in roseobacter genomes (Table 4), and by understanding the connection of antimicrobial production and quorum sensing we can gain knowledge of the complex stimuli these organisms use to sense the world around them and modify metabolism.

Multiple studies have been published recently describing the roles of quorum sensing in different roseobacter species (2, 16, 17, 20). Moreover, 43 roseobacter genomes are publically available and provide a great resource for bioinformatic analyses. It was shown that there are multiple conserved homologous quorum sensing proteins found in roseobacters (Figs. 20 and 21), and a classification system was developed for roseobacter quorum sensing protein sequences based on sequence homology. In addition to conservation at the protein level, synteny of genes

adjacent to annotated quorum sensing genes was evident among *Roseobacter* lineage members (Fig. 23). Taken together, these findings suggest multiple and distinct quorum sensing systems are an ancestral trait of roseobacters.

Future investigations of Y4I and indigoidine could focus on several aspects begun in this dissertation. Studies are on-going at the time of this submission to identify the structure of the AHL produced by Y4I during exponential phase with a mass of 298. Once the structure is confirmed by mass spectrometry, it will be possible to synthesize internal standards to better quantify the production of the AHL overtime. Further time points could be taken throughout the growth curve to better model the dynamics of each AHL overtime.

Because indigoidine is only produced when grown on a surface (Chapter 1), to tie the AHL production to indigoidine production, studies are needed to address the concentrations of AHLs produced by surface attached cultures. It is possible this could be done by allowing cultures to grow on agar plates and then extracting the AHLs from the agar after the cells have removed. This offers several technical difficulties, however, such as the loss of cell-associated AHLs and an unknown extraction efficiency. Results from a study such as this could directly tie a specific cascade of AHLs to the upregulation of indigoidine biosynthesis machinery and the production of indigoidine in a biofilm-like environment.

The role of the putative monounsaturated *N*-3-hydroxydodecanoyl homoserine lactone (3OHC12:1-HSL) also warrants further investigation. Evidence from our mass spectrometry studies (Chapter 2) indicate that the AHL is not produced in a population density dependent manner, and in fact, it is at its highest concentration at the beginning of stationary phase. While we know this AHL and its corresponding quorum sensing system are required for wildtype

indigoidine production, the specifics of this interaction are unclear. This is especially interesting as it appears that very little of the putative 3OHC12:1-HSL appears to be made in stationary phase, when we would hypothesize indigoidine production would be highest. This would suggest that the induction of the early quorum sensing system primes the population for indigoidine production, but it is only when the *N*-octanoyl homoserine lactone (C8-HSL) concentrations reach quorum that the indigoidine biosynthesis machinery upregulated.

The intracellular effects of indigoidine production may be of particular interest for future investigations, specifically the roles of cyclic diguanylate (c-di-GMP) and glutamine. c-di-GMP is an intracellular signaling molecule known to play an important role in many different phenotypes in bacterial species, including the switch between surface colonization and motility (1, 4, 7, 8, 10, 12, 13, 18, 19). At high intracellular concentrations of c-di-GMP, biofilm production is induced and motility is repressed, and at low concentrations, motility is induced and biofilm formation is repressed (7, 8).

c-di-GMP is synthesized by a well described class of enzymes known as diguanylate cyclases using two guanosine triphosphates (GTP) as substrates. The Y4I genome sequence has two annotated diguanylate cyclases (RBY4I_1416 and RBY4I_448). Guanosine monophosphate (GMP), a precursor of GTP, and indigoidine both require glutamine for biosynthesis (11, 15). This suggests that the production of indigoidine may lead to a drawdown of internal glutamine concentrations, thus also drawing down the concentrations of c-di-GMP inside the cell. In a biofilm setting, this could lead to cells that are producing high levels of indigoidine switching to a motile life cycle, dispersing from the biofilm, and subsequently repressing indigoidine biosynthesis. This hypothesis may explain the motility and colonization phenotypes seen in the *igiD*::Tn5 mutant (Figs. 5B and 5D). The inactivation of the indigoidine biosynthesis machinery

in this mutant causes it to be less motile and to colonize a glass surface an order of magnitude higher than the wildtype. It is possible that the competition between c-di-GMP biosynthesis machinery and indigoidine biosynthesis machinery is not present in this mutant, and thus the lack of indigoidine production leads to a more sessile lifestyle. Metabolomic investigations of intracellular glutamine, c-di-GMP, and indigoidine concentrations could shed light onto whether this interplay is causing the observed pleiotropic effects.

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Vita

Nathan Cude was born in Nashville, Tennessee on March 22, 1986, the son of Connie M. Cude and William Jay Cude. He attended elementary and middle school in Oak Ridge, Tennessee before moving back to Middle Tennessee and attending Gallatin High School in Gallatin, Tennessee. Nathan graduated from high school in 2004. While still in high school Nathan met his future wife, Karli Groves. Nathan received his Bachelor's degree in Biology from the University of Tennessee in 2008. Later in 2008, he returned to the University of Tennessee to pursue a graduate degree in the Department of Microbiology. Karli and Nathan were married June 20, 2009. If you are actually reading this in the future, it means Nathan received his PhD in April 2013.