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The Role of Gac/Rsm Signal Transduction Pathway in the Biology of Antagonistic Marine Bacterium *Gynuella sunshinyii* 449

by

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ABSTRACT

Gynuella sunshinyii is a newly characterized bacterium with a remarkable ability to produce multiple secondary metabolites that act as potential antibiotics for medical applications. Currently, little is known about gene regulation in this unusual microorganism. In 2018, studies conducted of rhizosphere microbiomes of tidal marsh grasses on Deer Island, MS, resulted in multiple isolates of halophilic, aerobic, Gram-negative bacteria with antagonistic properties. One of the most active antagonists was identified as *Gynuella sunshinyii* 449. The genome of strain 449 was sequenced with Illumina MiSeq, assembled with Unicycler and SPAdes, and annotated with RASTtk. The annotation revealed a 6.09 Mb genome that encodes 52 RNAs and 5,608 proteins, 2,822 of which had functional assignments. The analysis also revealed that the strain harbored genes for the production of numerous polyketides with potential cytotoxic and antibacterial activities. In many *Gammaproteobacteria*, the production of secondary metabolites, surface motility, and biofilm formation are regulated by the GacS/GacA system. The *G. sunshinyii* 449 genome encodes well-conserved homologs of GacA and GacS, and we hypothesized that these genes might be involved in the motility and ability to produce bioactive metabolites. To test this hypothesis, we constructed a knockout plasmid by cloning the *gacA* gene of strain 449. This plasmid will be used in the future to create an isogenic mutant that will be tested for alterations in the motility and ability to produce polyketides. This study provides insights into pathways conferring the regulatory and metabolic flexibility in a species that serves as a rich source of bioactive metabolites.

Keywords: *Gynuella sunshinyii* 499, Gac/Rsm signal transduction pathway, secondary metabolites

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LIST OF ABBREVIATIONS

ACP	Acyl Carrier Protein
BLAST	Basic Local Alignment Search Tool
CFU	Colony Forming Units
GacA	Global Activation of Antibiotic
GacS	Global Activation of Cyanide Synthesis
LB	Luria Bertani
Mbp	Megabase pair
MIC	Minimal Inhibitory Concentration
PCR	Polymerase Chain Reaction
PKS	Polyketide Synthases
TCS	Two Component System
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
UBC	University of British Columbia

CHAPTER I: INTRODUCTION

Antibiotics used for the treatment of infectious diseases are among the most important tools of modern medicine. Many currently used antibiotics are classified as microbial secondary metabolites or their semi-synthetic and synthetic derivatives (Demain & Fang, 2000), and since 1995, of the 12,000 antibiotics, 55% are derived from filamentous bacteria described in the genus *Streptomyces* (Quinn et al., 2020). Although the discovery and characterization of antibiotics have been successful in controlling diseases, self-medication and overuse of antibiotics have resulted in resistance and reducing the lifetime of antibiotics (Procópio et al., 2012). The United States Centers for Disease Control and Prevention (CDC) estimate that, despite the billions of dollars used to preserve the efficacy of antibiotics and research for the development of new drugs, antibiotic-resistant bacteria infect over 2.8 million people annually, causing more than 35,000 deaths (CDC https://www.cdc.gov/drugresistance/about.html). Emerging antibiotic-resistant pathogenic bacteria will continue to pose a challenge as the urgency for research and production of new antibiotics increases. There is a need for the isolation and characterization of organisms that may serve as a source of new antimicrobials.

Gynuella sunshinyii is a Gram-negative, halophilic bacterium of the order *Oceanospirillales*. The species has an unusual ability to produce multiple antibacterial and antifungal secondary metabolites, which could potentially contribute to the application and production of pharmaceutical drugs (Ueoka et al., 2018; Chung et al., 2015). In many Gram-negative bacteria, the expression of genes involved in the production of secondary metabolites, motility, stress tolerance, formation of biofilms, and virulence are controlled by the Gac/Rsm signaling pathway (Latour, 2020). In response to

environmental signals, this system activates the transcription of small RNA (sRNA) genes, upregulating the production of several proteins whose production is repressed by RsmA/CsrA proteins (Lapouge et al., 2008). The preliminary genetic analysis of *G. sunshinyii* revealed the presence of well-conserved homologs of GacA and GacS. However, their role in regulating social traits and synthesis of secondary metabolites by this interesting organism is unknown. Accordingly, the aims of my study were to i) assemble, annotate, and analyze the genome of antibiotic-producing strain *G. sunshinyii* 449, and ii) start the analysis of its Gas/Rsm signaling pathway to gain insights into the role of these global regulators in a species that serves as a rich source of bioactive metabolites. We hypothesized that *G. sunshinyii* 449 encodes multiple pathways involved in the synthesis of bioactive metabolites and that these genes are controlled by the Gac/Rsm pathway.

CHAPTER II: LITERATURE REVIEW

2.1 *Gynuella sunshinyii*

Gynuella sunshinyii is a Gram-negative, facultatively anaerobic, moderately halophilic, rod-shaped marine bacterium. It belongs to the order *Oceanospirillales* and the family *Saccharospirillaceae*, which contains three genera, *Gynuella*, *Saccharospirillum,* and *Salinispirillum* (Ling et al., 2020). The only studied *Gynuella* strain, YC6258^T, was isolated in 2015 from the rhizosphere of the halophyte *Carex scabrifolia* that grows on tidal flats and coastal dune areas of Namhae Island, Korea (Chung et al., 2015). The organism forms pale yellow, flat, and circular colonies and is sensitive to chloramphenicol, penicillin, streptomycin, and tetracycline but is resistant to ampicillin. The 16S rRNA-based and phenotypic analyses identified YC6258^T as a member of a new genus comprised of just one species, *G. sunshinyii* (Chung et al., 2015). The original and subsequent studies of YC6258^T revealed an extensive antagonistic activity against a variety of fungi and bacteria, including plant pathogens *Botrytis cinerea* and *Pythium ultimum* (Chung et al., 2015). Genome-directed studies linked these antagonistic properties with the ability of *G. sunshinyii* to produce multiple classes of bioactive polyketide metabolites (Ueoka et al., 2018; Ueoka et al., 2020). Polyketides comprise a large group of secondary metabolites with antibacterial, antiviral, anticancer, immune-suppressing, and anti-inflammatory activities (Risdian et al., 2019).

Despite the overall progress, *Gynuella* remains poorly studied and was featured only in a handful of studies, all of which involved the type strain, YC6258^T. In 2018, several new *Gynuella* strains were recovered from the roots of tidal marsh grasses collected on Deer Island, MS (Mavrodi et al., 2018). These organisms were isolated by

dilution plating of rhizosphere soil suspensions and identified by 16S rRNA sequencing as *G. sunshinyii*. Among these strains was *G. sunshinyii* 449, which exhibited strong antagonistic activity and suppressed the mycelial growth of phytopathogenic *Fusarium* spp., but the genetic basis for this activity remained unexplored (Mavrodi et al., 2018).

2.2 Secondary Metabolites

In microorganisms, metabolism is comprised of a network of biochemical processes responsible for the production of intermediates and pathway substrates, collectively known as 'metabolites' (Gokulan et al., 2014). These metabolites can further be classified into primary and secondary. The primary microbial metabolites serve as the main source of energy and are involved in the essential biochemical and physiological functions of cells. On the other hand, secondary metabolites are not involved in cell growth and development but serve in competition, nutrient acquisition, defense, differential effectors, and establishment of symbiotic and parasitic interactions with eukaryotic hosts (Demain & Fang, 2000).

Polyketides represent an extensive and diverse class of secondary microbial metabolites of utmost practical importance to developing pharmaceuticals for human and animal applications (Singh et al., 2019). These compounds are assembled by three classes of polyketide synthase enzymes (PKS I, II, III). The PKS I are large multifunctional enzymes comprised of modules, each of which recognizes specific substrates to ultimately synthesize the final product (Tsai & Ames, 2009). In the PKS II enzymes, similar functions are performed by different proteins that iteratively catalyze the assembly of a polyketide. Unlike PKS type I and II, the PKS III enzymes (chalcone synthases) lack ACP (acyl-carrier protein) domains and use activated acyl-CoA

derivative substrates (Tsai & Ames, 2009). The type strain of *G. sunshinyii* carries multiple PKS modules and produces several polyketide metabolites, including the tetracyclic alkaloid alteramide A and cytotoxins lobatamide A and lacunalide (Ueoka et al., 2018, Ueoka et al., 2020). Although the biological function of these *G. sunshinyii* metabolites is unknown, in other groups of bacteria, polyketides help to repel bacterivorous protozoa (Mazzola et al., 2009), help bacteria to move on solid surfaces by reducing surface tension (Kinsinger et al., 2003), or solubilize hydrophobic carbon sources (Gutiérrez-Chávez et al., 2021).

2.3 The Gac/Rsm Pathway

In some *Gammaproteobacteria*, the production of secondary metabolites, surface motility, and biofilm formation are regulated by the GacS/GacA signaling system (Lapouge et al., 2008). The Gac/Rsm cascade also plays an essential role in managing carbon storage and expression of virulence and biocontrol factors. Although the exact arrangement depends on the bacterial species, the Gac/Rsm pathways have three components (Figure 1). The first component is a two-component signal transduction system that includes an inner membrane sensor kinase and a cytosolic cognate response regulator (Latour, 2020). Upon coming into contact with an environmental signal, the sensor (known as S) undergoes autophosphorylation, which activates the response regulatory (known as the response activator, or A) by phosphotransfer. In *Pseudomonas*, the two-component system is known as GacS/GacA, which stands for global activation of cyanide synthesis and antibiotic, respectively. Other groups of *Gammaproteobacteria* also carry the GacS/GacA homologs, although they are named differently. Studies in *Pseudomonas* revealed that the activated GacA response regulator binds to promoters

containing a conserved upstream element termed the GacA box (Lapouge et al., 2008). When induced, the GacA activates the transcription of several sRNA genes (termed *rsmX*, *rsmY*, and *rsmZ* in *Pseudomonas* and other related bacteria). These sRNAs bind and titrate off the RsmA/CsrA protein(s), which ultimately derepresses and upregulates the target pathways.

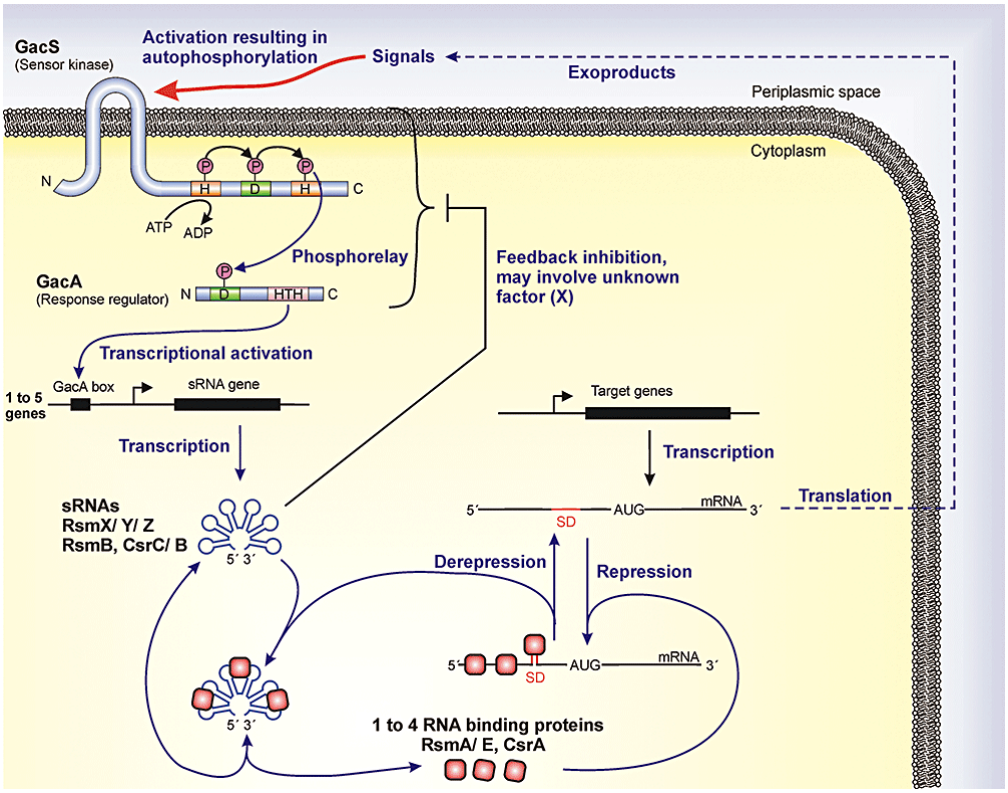


Figure 1. General overview of the Gac/Rsm signal transduction pathway in Gammaproteobacteria. The scheme was adapted from (Lapouge et al., 2008).

2.4 The GacS/GacA Two-Component Signal Transduction System

In this two-component signal transduction system, the GacS histidine kinase serves as the two-component system (TCS) membrane sensor of the regulatory Gac/Rsm signal transduction pathway (Haas & Keel, 2003). In a highly populated microbiome, the

GacS signal can also be triggered to switch from primary to secondary metabolite production and change in bacterial lifestyle. Although the exact signal perceived by GacS is unknown, it was noted that the response is triggered by nutritional stress and competition (Latour, 2020). The importance of the GacA/GacS system for the expression of various traits in *Gammaproteobacteria* was demonstrated in numerous studies with *gacS/gacA* mutants that show a reduction in virulence factor production and are less virulent than their wildtype counterparts in various host-pathogen systems (Lapouge et al., 2008; Rahme et al., 1995). In biocontrol agents, the inactivation of *gacS/gacA* strongly decreases the production of secondary metabolites and lytic enzymes and reduces the ability to antagonize plant pathogens. The GacS/GacA mutant of *P. fluorescens* Pf-5 lost the ability to produce exoproteases, antibiotics, quorum-sensing signals, and exotoxins (Driscoll et al., 2011; Hassan et al., 2010). Similar effects were observed in the Gac mutant of *Serratia plymuthica* (Ovadis et al., 2004).

The GacS/GacA system is essential in swarming motility, which is known as flagella-driven movement over solid surfaces (Kearns, 2010; Song et al., 2016). One benefit of swarming is allowing the bacteria to disperse and occupy new habitats, thereby expanding their bacterial community (Song et al., 2016). As observed in *P. protegens* Pf-5, the loss of production of the biosurfactant orfamide A in a Gac mutant abolished the ability to swarm (Song et al., 2016). Results of that study showed that repeated rounds of swarming by the wild type Pf-5 drive the accumulation of *gacS/gacA* spontaneous mutants on the swarming edge. While these mutants were unable to swarm on their own due to lack of production of the biosurfactant orfamide A, they did co-swarm with the wild type.

In several species, the GacS/GacA two-component system also regulates the formation of biofilms. For example, in *Pseudomonas putida* KT2442, the inactivation of the GacS component resulted in defects related to biofilm formation (López-Sánchez et al., 2016). The authors suggested that the Gac system controls the expression of two adhesins, LapA and LapF, which are critical for the surface attachment and formation of biofilms. In the Gram-negative human pathogen *Vibrio vulnificus*, the deletion of *gacA* resulted in reduced cytotoxicity and the ability to cause skin or systemic infections in mice (Gauthier et al., 2010).

2.5 The Csr (Rsm) system

The ultimate targets of the GacS/GacA system are CsrA/RsmA proteins that act as global regulators of post-transcriptional gene expression in a variety of proteobacteria pathogens. The function of CsrA/RsmA homologs is best understood in pathogens, where these regulators integrate gene expression networks that govern bacterial physiology, metabolism, and virulence. Specifically, these proteins bind to specific mRNAs and alter the translation, turnover, and/or elongation of transcripts encoding virulence factors (Vakulskas et al., 2015). The expression of CsrA/RsmA is modulated by small, noncoding RNAs containing multiple binding sites, which allow them to sequester CsrA homodimers from their target mRNA (Vakulskas et al., 2015).

The opportunistic human pathogen *P. aeruginosa* infects patients with cystic fibrosis and causes acute respiratory infections that frequently become chronic (Sakidot et al., 2005). The recurring episodic infections often become septic and nearly impossible to treat with antibiotics. Several studies demonstrated that RsmA mediates the switch between acute and chronic infections by positively regulating genes associated with acute

virulence (type III secretion system, type IV pili) and negatively regulating factors associated with chronic virulence, including biofilm formation and type VI secretion system (Vakulskas et al., 2015) (Figure 2). Studies in the mouse model of acute respiratory infection showed that a *rsmA* mutation led to a reduction in colonization by *P. aeruginosa* during the first few stages of infection. At the same time, the complete deletion of the gene favors persistent infections (Mulcahy et al., 2008).

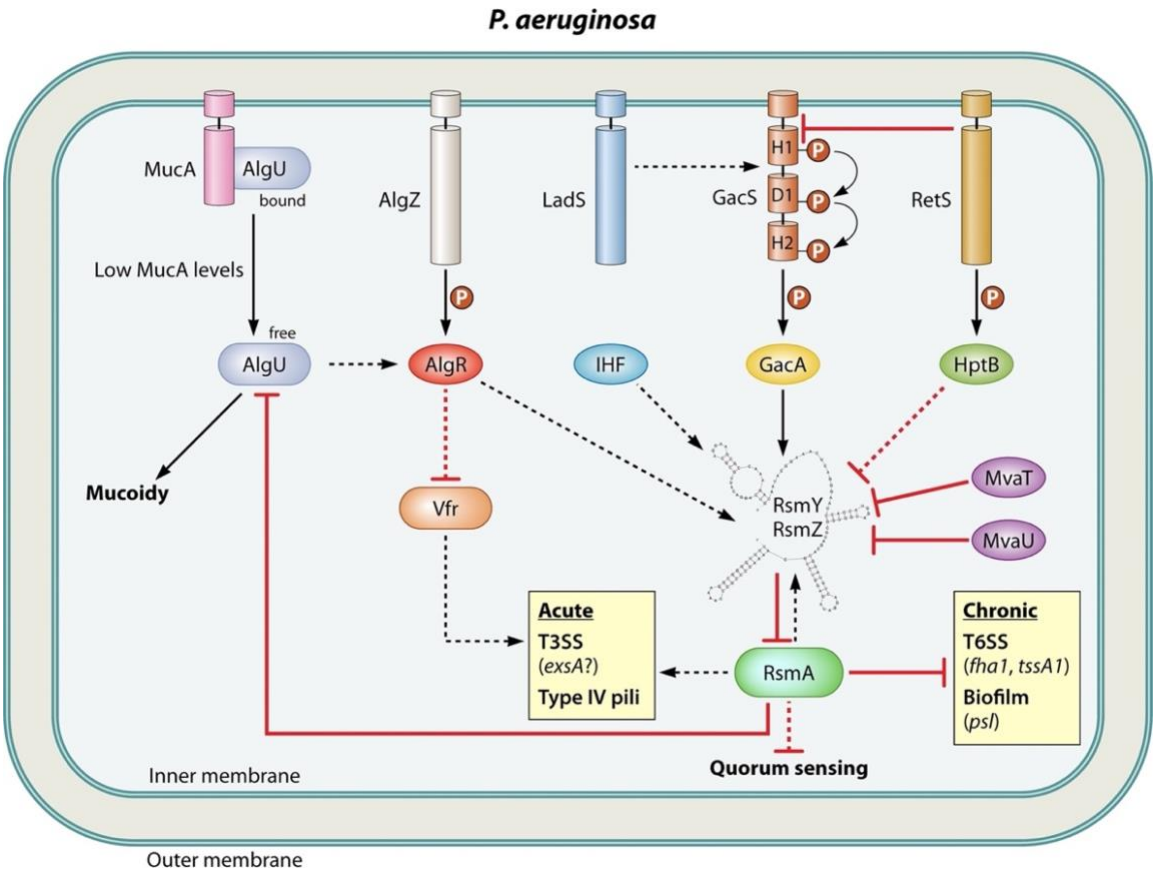


Figure 2. The Gac/Rsm regulatory pathway of P. aeruginosa. This scheme was adapted from (Vakulskas et al., 2015).

Recent studies revealed that fluorescent pseudomonads differ in the number of RsmA paralogs. For example, in addition to RsmA, *P. fluorescens* encodes a protein

called RsmE, while *P. aeruginosa* also carries RsmF (Vakulskas et al., 2015). Interestingly, pseudomonads also differ in the number and types of inhibitory sRNAs. Both *P. aeruginosa* and *P. fluorescens* possess the sRNAs *rsmY* and *rsmZ*, but *P. fluorescens* also has a third sRNA called *rsmX* (Vakulskas et al., 2015). In both bacteria, knocking out the GacA-controlled sRNA genes results in a phenotype similar to that of a *gacA* mutant. This observation suggests that the GacS/GacA system and its homologs drive the expression of sRNA genes (Lapouge et al., 2008; Kay et al., 2006).

CHAPTER III: MATERIALS AND METHODS

3.1 Genome Analysis of *G. sunshinyii* 449

The isolation and identification of *G. sunshinyii* 449 are described by Mavrodi et al. (2018). In this study, the strain was cultured in Tryptic Soy broth containing 15% SW30 artificial saltwater (Dyall-Smith, 2009). After 48 h of growth at 27°C, the cells were pelleted, and high molecular weight DNA was isolated using the cetyltrimethyl ammonium bromide (CTAB) miniprep protocol (Ausubel et al., 2002). The DNA samples were shipped to the Molecular and Genomics Core Facility at the University of Mississippi Medical Center (Jackson, MS) and sequenced with a MiSeq instrument using v.3 chemistry (Illumina, San Diego, CA). The raw reads were filtered with FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and assembled with Unicycler (Wick et al., 2017) and SPAdes v.3.12.0 (Bankevich et al., 2012). The genome assemblies were annotated with RASTtk (Brettin et al., 2015) implemented in the Pathosystems Resource Integration Center (PATRIC) (Wattam et al., 2014). The predicted proteome of *G. sunshinyii* 449 was compared to that of the type strain *G. sunshinyii* YC6258^{T} by all-against-all BLAST searches (Altschul et al., 1990) using cutoff e-value of 1e-6, identity of 40%, and coverage of 60%. Circular genome maps were created with CGview server (Stothard et al., 2019). The annotated 449 genome was also screened with antiSMASH v. 5.0 (Blin et al., 2015), which predicts secondary metabolite biosynthesis gene clusters such as those involved in the production of acyl-amino acids, ß-lactones, fungal ribosomally synthesized and post-translationally modified peptides (RiPPS), streptide-like thiother-bond RiPPs (RaS-RiPPs), polybrominated diphenyl ethers, C-nucleosides, ketones, and lipolanthines.

3.2 Additional Analyses of GacS/GacA and Cra Homologs

The structures of GacS, GacA, and Cra proteins from *G. sunshinyii* 449 were also explored with Phyre2, which uses advanced homology threading methods to predict folds and ligand binding sites and analyzes the effect of amino acids substitutions in the protein sequence (Kelley et al., 2015). In addition, protein sequences were screened for the presence of signal peptides and transmembrane helices using Protter (Omasits et al. 2014), a web-based tool that helps to integrate and visualize annotated protein sequence features and experimental proteomic data.

3.3 Antibiotic Susceptibility Assays

A Kirby-Bauer disk diffusion susceptibility test was performed with *G. sunshinyii* 449 using the following antibiotics: ampicillin, 10 μg/mL, tetracycline, 30 μg/mL, streptomycin, 10 μg/mL, chloramphenicol, 30 μg/mL, and kanamycin 30 μg/mL. The BBL Sensi-Disc antibiotic discs were purchased from Becton-Dickinson (Franklin Lakes, NJ). Minimal inhibitory concentrations (MICs) for selected antibiotics were determined using the microtiter plate broth dilution assay of Wiegand et al. (2008) and methods from the Hancock Lab at University of British Columbia (UBC) (http://cmdr.ubc.ca/bobh/method/mic-determination-by-microtitre-broth-dilution-method/). The final inoculum size for the broth dilution was 5×10^5 colony forming units (CFU)/mL. For each antibiotic, the starting concentration was 128 μg/mL that was serially diluted to 0.06 μg/mL. The assays were performed in CytoOne untreated 96-well plates (USA Scientific, Ocala, FL). For growth control, the bacteria were inoculated in the broth without antibiotics. The sterility control contained only culture medium. The

cultures were incubated statically at 27°C and bacterial growth was scored by measuring absorbance at 600 nm ($O.D._{600}$) at 48 hours.

3.4 The Construction of GacA Gene Knockout Plasmid

Genome fragments flanking the *gacA* gene of *G. sunshinyii* 449 were amplified by PCR using Q5 High-Fidelity 2X Master Mix (New England Biolabs, Ipswich, MA) and oligonucleotide primers gacA1, gacA2, gacA3, and gacA4 (Table 1, Figure 3). The amplification was perfomed using the following protocol: denaturation at 98°C for 30 sec; 6 cycles of 98°C for 10 sec, 57°C for 10 sec, 72°C for 30 sec; followed by 30 cycles of 94°C for 10 sec, 65°C for 10 sec, 72°C for 30 sec; final elongation at 72°C for 2 min and hold at 16°C. The predicted sizes of the gacA1/gacA2 and gacA3/gacA4 amplicons were 614 bp and 636 bp, respectively.

Table 1. Oligonucleotide primers used in this study.

Primer	Sequence
gacA1	5′CAGGGTTTTCCCAGTCACGACTCTTGATTGTGGACGAGGCTC 3'
gacA2	5'CGACGCAGATGTTACTCACTTACTGGACAAGCTTTGGCGATCT 3'
gacA3	5′TAAGTGAGTAACATCTGCGTCGTATGCCAGACAGCAATGGTCG 3′
gacA4	5′GGATAACAATTTCACACAGGAGCCAGGAAATGTTTGATCGGA 3′
rM13f	5'GTCGTGACTGGGAAAACCCTGGCG 3'
rM13r	5'TCCTGTGTGAAATTGTTATCCGCT 3'

The gene replacement vector pEX18Ap (Hoang et al., 1998) was prepared by inverse PCR with primers rM13f and rM13r (Table 1) using the following amplification protocol: denaturation at 98°C for 1 min; 30 cycles of 98°C for 10 sec, 68°C for 10 sec, 72°C for 2.5 min; final elongation at 72°C for 5 min and hold at 16°C. The amplified

vector was digested with *Dpn*I (New England Biolabs) to remove any residual template and purified with the GeneJet PCR Purification Kit (Thermo Fisher Scientific, Waltham, MA).

The *gacA* shoulder fragments were cloned into pEX18Ap using the NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs). Briefly, the shoulder fragments were mixed with the vector at 1:2 ratio and combined with an equal volume of the NEB HiFi Master Mix. The mixture was incubated at 50°C for 15 min, and 2 µL of

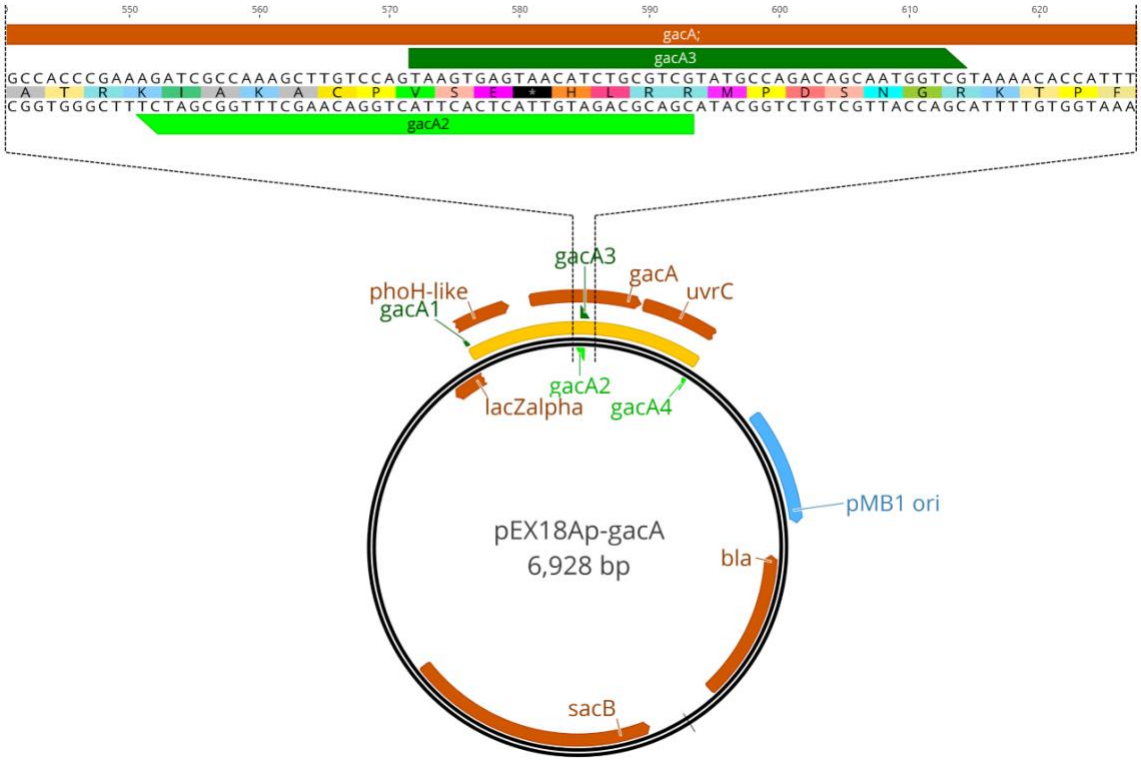


Figure 3. Physical map of the pEX18Ap-gacA knockout plasmid showing the location of relevant genes and primers. The top panel shows the deletion and internal stop codon that were introduced to interrupt the *gacA* gene.

the assembled product were used to transform the chemically competent *E. coli* NEB 5-alpha competent cells (New England Biolabs). The transformants were selected by plating on Difco LB (Luria Bertani) agar amended with 100 μg/mL of ampicillin, and the inoculated plates were incubated overnight at 37°C.

To identify recombinants, 25 colonies from the overnight plates were picked and patched on fresh LB-Amp plates and grown for 4-5 hours. These colonies were then screened by PCR with *gacA*1 and *gacA*4 primers and DreamTaq DNA polymerase (Thermo Fisher Scientific) using the following protocol: denaturation at 95°C for 2 min; 30 cycles of 95°C for 20 sec, 52°C for 15 sec, 72°C for 1.5 min and hold at 16°C. The plasmid DNA of pEX18Ap was used as one of the negative controls. Plasmids carrying inserts were extracted with GeneJet Plasmid Miniprep Kit (Thermo Fisher Scientific) and their integrity was verified by sequencing with *gacA*1 and *gacA*4 primers at Eurofins Genomics (Louisville, KY).

3.5 Electroporation of *G. sunshinyii* 449

Different approaches were used to prepare electrocompetent cells of *G. sunshinyii* 449. First, we grew the strain overnight on TSA-SW30 agar at 27°C, scraped bacterial cells from the agar surface, and washed twice by resuspension in 10% glycerol and centrifugation at 4°C for 5 min at 7,000 rpm. Fifty microliters of the prepared electrocompetent cells were transferred into 1-mm gap electroporation cuvets and mixed with 300-500 ng of the pEX18Ap-*gacA* plasmid DNA. The electroporation was performed with a 2510 Electroporatorator (Eppendorf, Germany) at 1.8 kV, 10 μF, and 600 Ω. The electroporated cells were pregrown at 27°C with shaking at 220 rpm in 950 μL of TSB-SW30 media for 2.5 hours and plated on TSA-SW30-ampicillin plates. The

same electroporation protocol was modified by adding a centrifugation step after the second wash at 7,000 rpm for 8 min, for better cell pellet collection. 100 μL of washed cells were used for electroporation.

A second approach for preparing electrocompetent cells was based on a modified procedure by Harris et al. (2016). Briefly, three 50-mL cultures of bacterial cells were grown overnight at 27°C, 250 rpm, and harvested at OD_{600} of 0.4. The cells were pelleted at 5,000xg for 10 min and washed twice with room temperature 300 mM sucrose. One hundred microliters of electrocompetent cells were aliquoted into 1-mm gap cuvettes, mixed with the pEX18Ap-*gacA* plasmid, and electroporated and processed as described above. The protocol was modified by growing and harvesting bacteria at OD_{600} of 0.08. We also reduced the volume of sucrose and centrifugation time during washes.

For the negative control, 950 μL of TSB-SW30 media and 50 μL of electrocompetent cells were mixed and grown along with the electroporated cells. To measure the transformation efficiency, the competent cells were electroporated with 20 ng of the broad host range plasmid vector pBBR1MCS (Kovach et al., 1995). The pBBR1MCS transformants were plated on TSA-SW30 amended with 8, 10, 12, and 15 μg/mL of chloramphenicol, respectively.

CHAPTER IV: RESULTS

4.1 *Gynuella sunshinyii* 449 Genome Statistics

The genome of the bacterial strain *G. sunshinyii* 499 was sequenced using Illumina MiSeq, which generated about 2.6 million high-quality 150-bp paired-end reads. Results of bacterial strain genome analysis are summarized in Table 2. The annotation of the genome assembled using Unicycler revealed a 6.09 Mbp genome that encoded 52 RNAs and 5,674 proteins, 2,786 of which had functional assignments. The annotated genome assembled using SPAdes revealed a 6.11 Mbp genome that encoded 48 RNAs and 5,618 proteins, 2,635 of which had functional assignments. The functional analysis revealed 272 subsystems comprised of 1,694 genes involved in metabolism, protein processing, energy, stress response, membrane transport, cellular processes, DNA and RNA processing, cell envelope, regulation and cell signaling (Figure 4A). The rest of the genes were not in subsystems and were classified as non-hypothetical (1,785) or hypothetical (2,635). The *G. sunshinyii* 499 genome also encoded 39 genes associated with antibiotic resistance (vs. 36 genes in YC6258^{T}) and four CRISPR arrays with a total of 224 repeats and 220 spacers.

Further analysis of the annotated Unicycler assembly by antiSMASH revealed potential pathways for the production of a macrodioloide polyketide(s) of the tartrolon class, a lacunalide-like polyketide, and dihydromaltophilin, which is a macrocyclic lactam containing a tricyclic skeleton with tetramic acid moiety (Table 3). Other polyketide synthase genes partially matched known pathways involved in the synthesis of asukamycin, glidobactin, and phosphinothricin-tripeptides. Like many other marine bacteria, the strain carries genes for the production of ectoine, which acts as a compatible

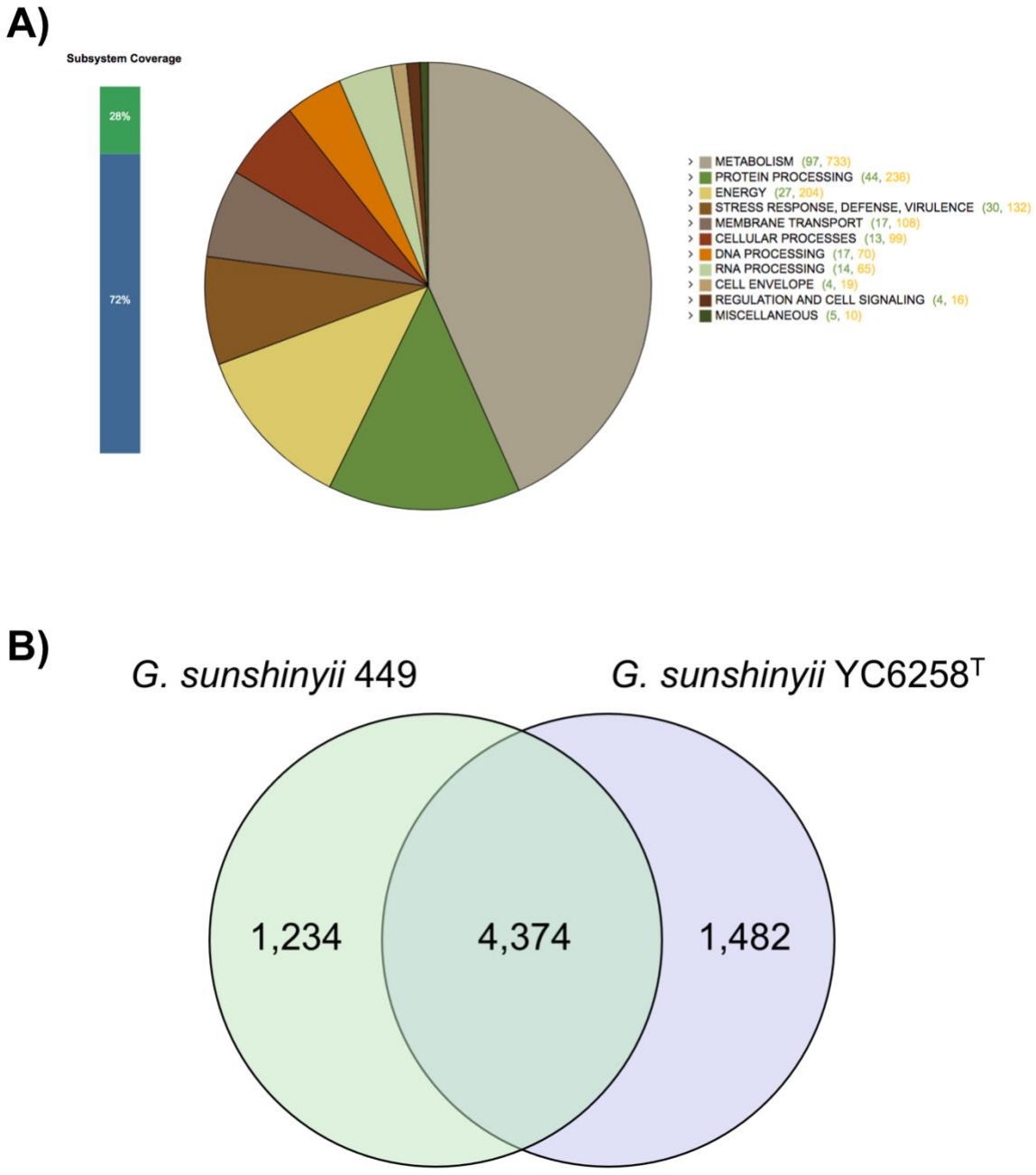


Figure 4. The distribution of G. sunshinyii 449 genes into functional categories. Categories as defined by RASTk subsystems (A), and Venn diagram summarizing the comparison of predicted proteomes of strains 449 and YC6258T (B). The proteome comparison was conducted by all-against-all BLATSp using the cutoff e-value of 1e-6, identity of 40%, and coverage of 60%.

solute that provides protection from osmotic stress (Hermann et al., 2020). The gene content comparison of strains 449 and YC6258^{T} identified 4,374 shared genes, which constituted 75-77% of each proteome (Figures 4B and 5). In addition, strains 449 and YC6258^{T} carried, respectively, 1,234 and 1,482 unique genes corresponding to 22% and 25% of the strain's proteome.

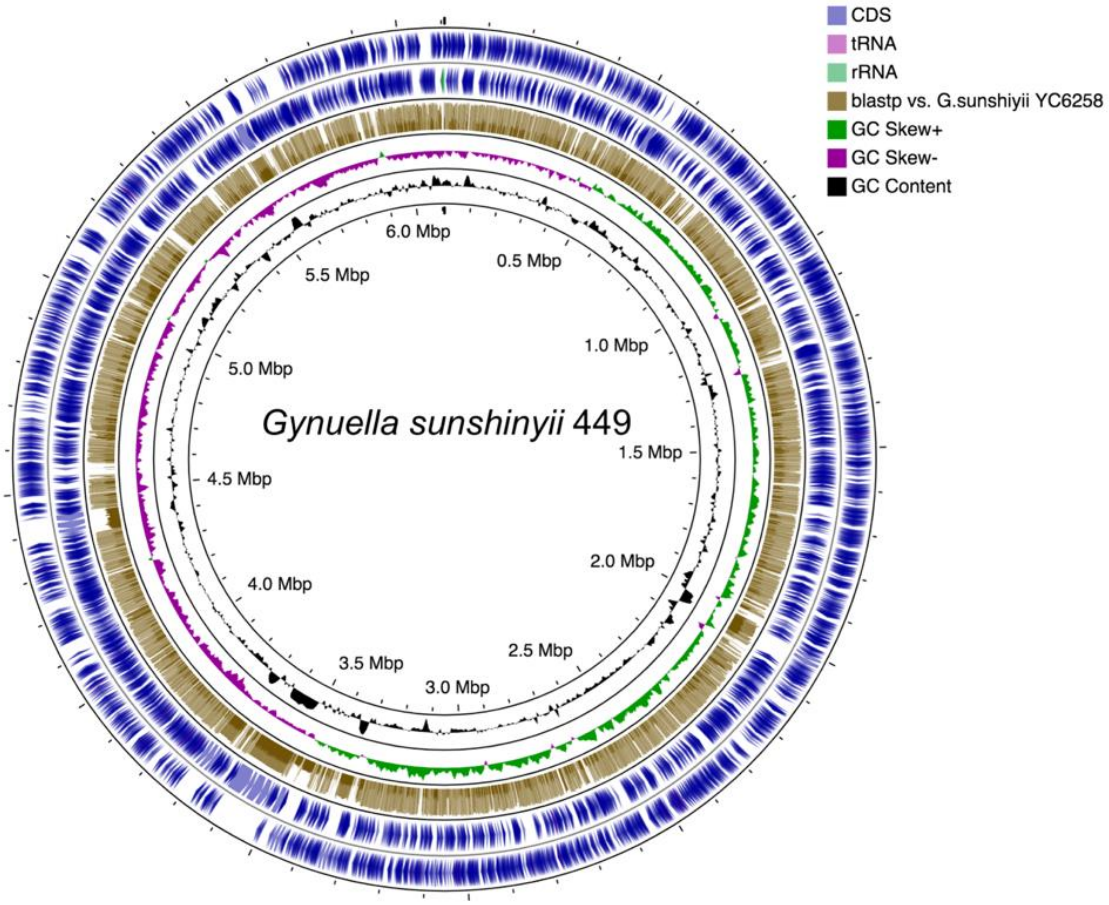


Figure 5. Genome map of G. sunshinyii 449. The concentric rings represent (starting from the inside): scale in Mbps, GC content, GC skew, results of the BLASTp proteome comparison (e-value of e-5) against *G. sunshinyii* YC6258T, complementary strand genes, and forward strand genes. The map was created with CGview server (Stothard et al., 2019).

Table 2. Genome features of *G. sunshinyii* 449.

Assembler	Fold coverage (x)	Genome size (bp)	No. of contigs	N_{50} values (bp)	G+C (%)	Total no. of genes	No. of RNA genes	Total no. of CDSs[a]	No. of PECs[b]	No. of hypothetical proteins
Unicycler	63.6	6,089,278	85	193,877	47.98	5,660	52	5,674	1,076	2,786
SPAdes	63.4	6,107,650	61	205,561	47.97	5,666	48	5,618	1,084	2,983

[a] CDSs, coding sequences with proteins. [b] PECs, proteins with Enzyme Commission numbers.

Table 3. Secondary metabolite clusters predicted in the genome of *G. sunshinyii* 449 by antiSMASH.

Region	Type[a]	From	To	Most similar known cluster[b]		Similarity
Region 1.1	PUFA, hglE-KS	323,233	374,849	asukamycin	Polyketide:Type II	4%
Region 1.2	RiPP-like	424,080	434,925			
Region 1.3	T1PKS, NRPS	478,609	526,299	N-tetradecanoyl tyrosine	Other	6%
Region 3.1	NRPS	247,071	288,685	A40926	NRP:Glycopeptide + Saccharide:Hybrid/tailoring	3%
Region 7.1	transAT-PKS	8,875	93,025	tartrolon	Polyketide	80%
Region 8.1	transAT-PKS, hserlactone, NRPS, ladderane	79,876	155,995	glidobactin	NRP + Polyketide	15%
Region 9.1	RiPP-like	106,283	117,179			
Region 10.1	NRPS	96,920	141,471			
Region 12.1	NRPS	1	35,994			
Region 13.1	thioamide-NRP	1,445	50,833			

Table 3 (continued):

Region 13.2	phosphonate	114,222	155,100	phosphinothricintripeptide	NRP	6%
Region 17.1	NRPS, transAT-PKS-like	57,260	150,206			
Region 18.1	transAT-PKS-like, transAT-PKS	1	132,375	lacunalides	Polyketide	47%
Region 26.1	T1PKS, NRPS	10,783	60,211	dihydromaltophilin (heat-stable antifungal factor)	NRP + Polyketide	50%
Region 31.1	transAT-PKS-like, NRPS	1	63,899	weishanmycin	NRP + Polyketide	6%
Region 32.1	NRPS-like	1	38,379			
Region 34.1	NRPS	1	56,127	pyoverdin	NRP	1%
Region 36.1	NRPS	1	50,313			
Region 41.1	NRPS	4,749	42,254			
Region 43.1	ectoine, arylpolyene	11,010	33,845	ectoine	Other	83%
Region 44.1	NRPS	1	23,951			
Region 49.1	NRPS	1	16,392			
Region 57.1	NRPS	1	6,206			
Region 58.1	NRPS	1	5,775			
Region 68.1	NRPS	1	2,238			
Region 75.1	NRPS	1	1,393			

[a] Trans AT-PKS, trans-acyl-transferase polyketide synthase; NRPS, non-ribosomal peptide synthetase cluster; T1PKS, Type I polyketide synthase. [b] NRP, non-ribosomal peptide.

4.2 Predicting Structures of GacS/GacA and Cra

The analysis of the *G. sunshinyii* 449 genome also revealed the presence of a well-conserved Gac/Rsm pathway. We identified homologs of GacS and GacA, as well as a single-copy gene encoding the homolog of Cra protein. The attempts to locate the sRNA component of the pathways were unsuccessful. No potential targets could be identified in BLASTn searches using known sRNAs from *Pseudomonas*, *Vibrio*, and *E. coli*. We further visualized the protein structure of GacS/GacA and Cra proteins.

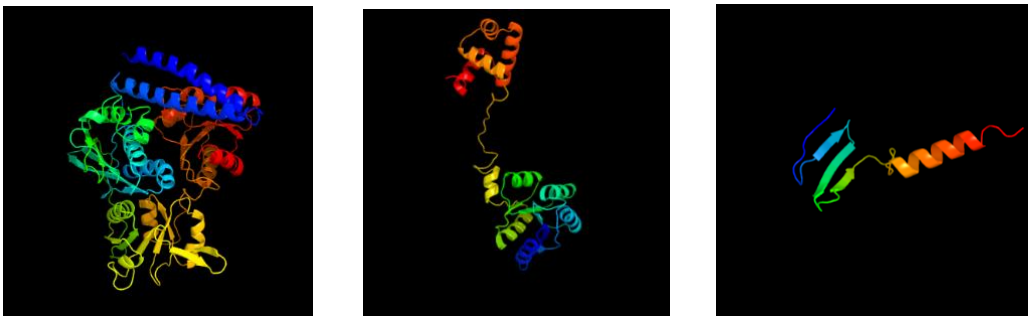


Figure 6. Predicted protein folds of GacS (left), GacA (center), and Cra (Rsm) from G. sunshinyii 499. Protein folds were determined using the Phyre2 server.

The analysis of GacS/GacA and Cra with the protein fold recognition server Phyre2 produced potential structures for all three proteins (Figure 6). The predicted structure of GacS was similar (100% prediction confidence, 49% coverage) to that of the hybrid kinase ShkA of *Caulobacter vibrioides* CB15 (Protein Data Bank structure 6QRJ). For GacA, the predicted structure closely resembled (100% prediction confidence, 96% coverage) the VraR response regulator from *Enterococcus faecium* SD3B-2 (PDB structure 5HEV). The fold of the Cra protein of *G. sunshinyii* 449 was very similar (99.9% prediction confidence, 91% coverage) to the structure of a putative carbon storage

regulator protein CsrA of *P. aeruginosa* (PDB structure 1VPZ). Finally, and as expected, we also identified two well-conserved transmembrane helices in the N-terminal part of GacS (Figure 7).

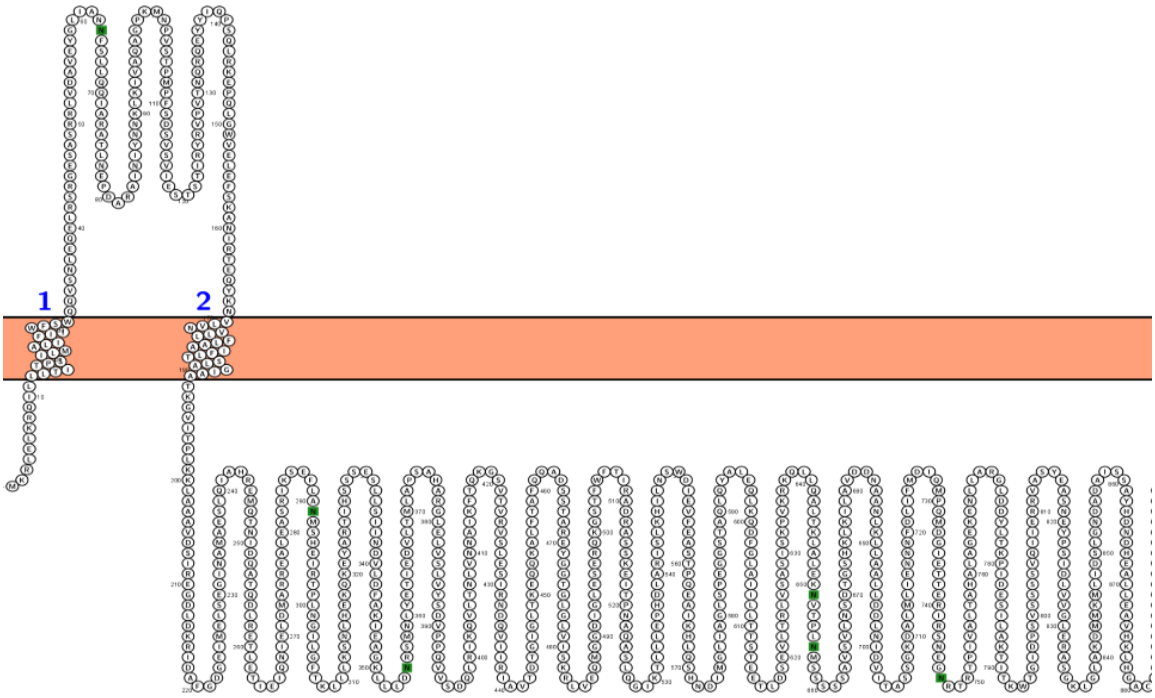


Figure 7. N-terminal transmembrane helices predicted in the GacS sensor kinase of G. sunshinyii 499. The green squares represent putative N-glycosylation motifs. The analysis was conducted with Protter (Omasits et al., 2014).

4.3 Antibiotic Susceptibility Assays

Before constructing the *gacA* mutant of *G. sunshinyii* 449, we had to decide which gene replacement vector and antibiotic selection markers to use for these experiments. We screened strain 449 for susceptibility to several antibiotics that have corresponding resistance genes available in knockout vectors to identify a suitable selection marker. The Kirby-Bauer screening with commercially available antibiotic discs demonstrated that *G. sunshinyii* 449 was resistant to streptomycin but susceptible to ampicillin,

tetracycline, and chloramphenicol (Figure 8). We selected these antibiotics for further work and performed MIC assays to identify the lowest concentrations needed to inhibit the growth of strain 449. The results of MIC assays revealed a susceptibility to ampicillin



Figure 8. Testing G. sunshinyii 449 for antibiotic sensitivity by Kirby-Bauer assay. The antibiotics used in this test were kanamycin (30 µg/mL), chloramphenicol (30 µg/mL), ampicillin (10 µg/mL), tetracycline (30 µg/mL), and streptomycin (10 µg/mL).

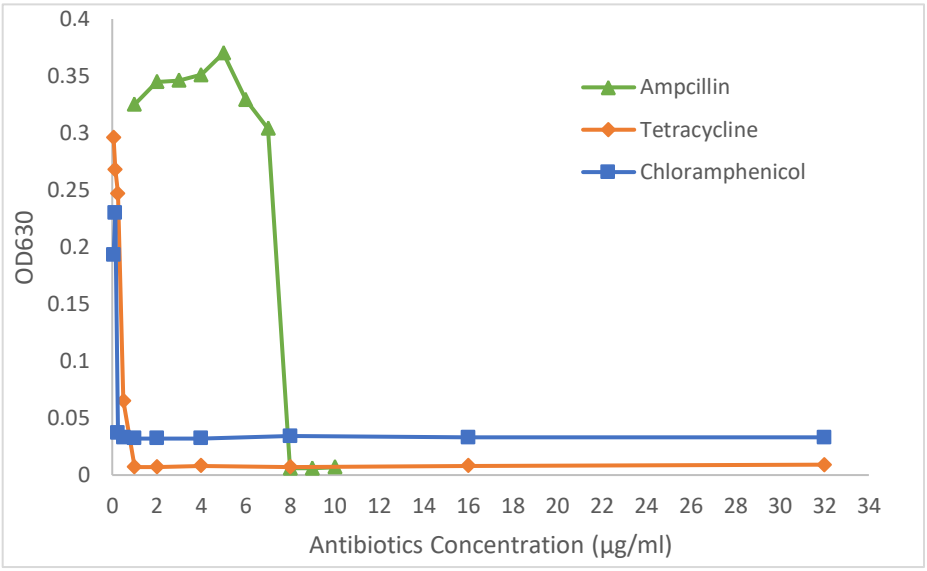


Figure 9. The results of MIC assays showing the growth of G. sunshinyii 449 in the presence of increasing concentrations of ampicillin, tetracycline, and chloramphenicol. In this case, MIC for ampicillin was determined to be 8 µg/mL, tetracycline was determined to be 1 µg/mL, and chloramphenicol was determined to be 0.5 µg/mL.

at 8 μg/mL, tetracycline at 1 μg/mL, and and chloramphenicol at 0.5 μg/mL. Based on these results, we proceeded further with the pEX18Ap knockout vector (Hoang et al., 1998) that carries an ampicillin resistance marker.

4.4 Construction of the GacA Knockout Plasmid and Mutagenesis of *Gynuella*

In order to create a *gacA* knockout plasmid, genome areas flanking the gene were amplified by PCR with oligonucleotide primer pairs gacA1/gacA2 and gacA3/gacA4, respectively. The primers were designed for Gibson cloning with identical 5'ends of the gacA2 and gacA3 primers that generated a 22-bp overlap. Fusing the gacA1/gacA2 and gacA3/gacA4 amplicons introduces in *gacA* a deletion and internal stop codon (Figure 3).

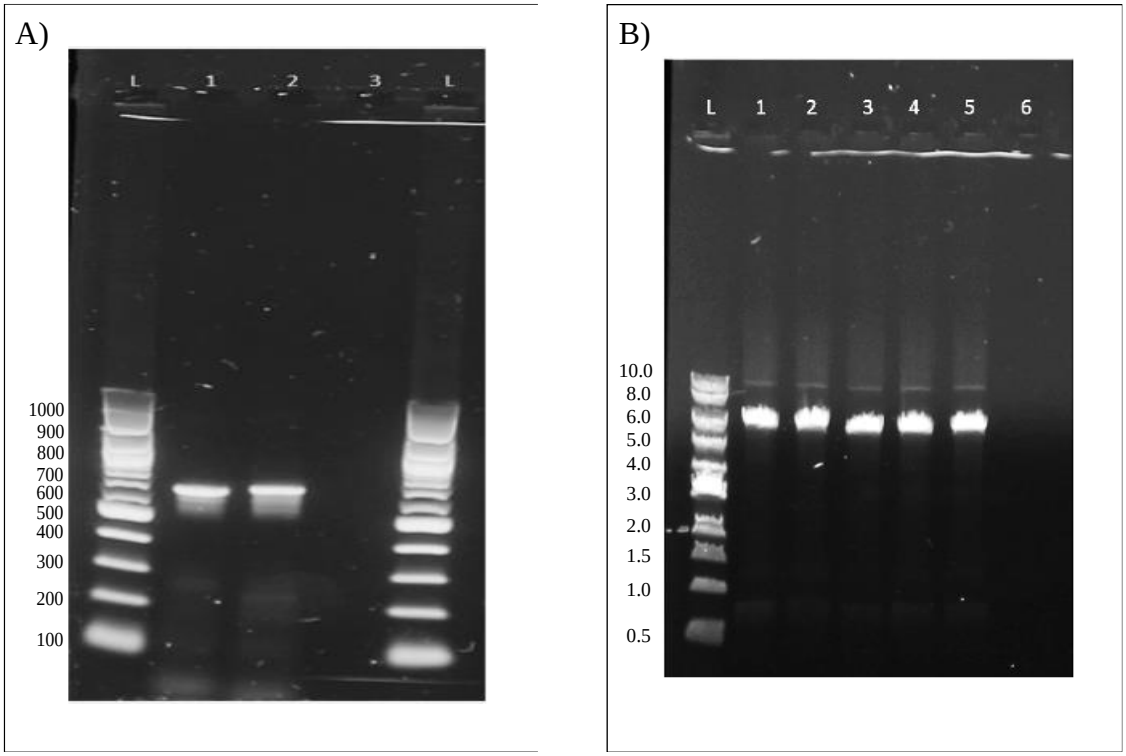


Figure 10. Agarose gel electrophoresis of gacA shoulder amplicons (A) and the amplified and DpnI-treated knockout vector pEx18Ap (B). The agarose concentration in the gel was 1%. Lanes marked with L contain 100-bp (left) or 1-kb (right) DNA ladders from. The expected size of the gacA shoulder fragments is 600 and 640 bp, respectively. The size of the linearized pEX18Ap is 5.8 kb.

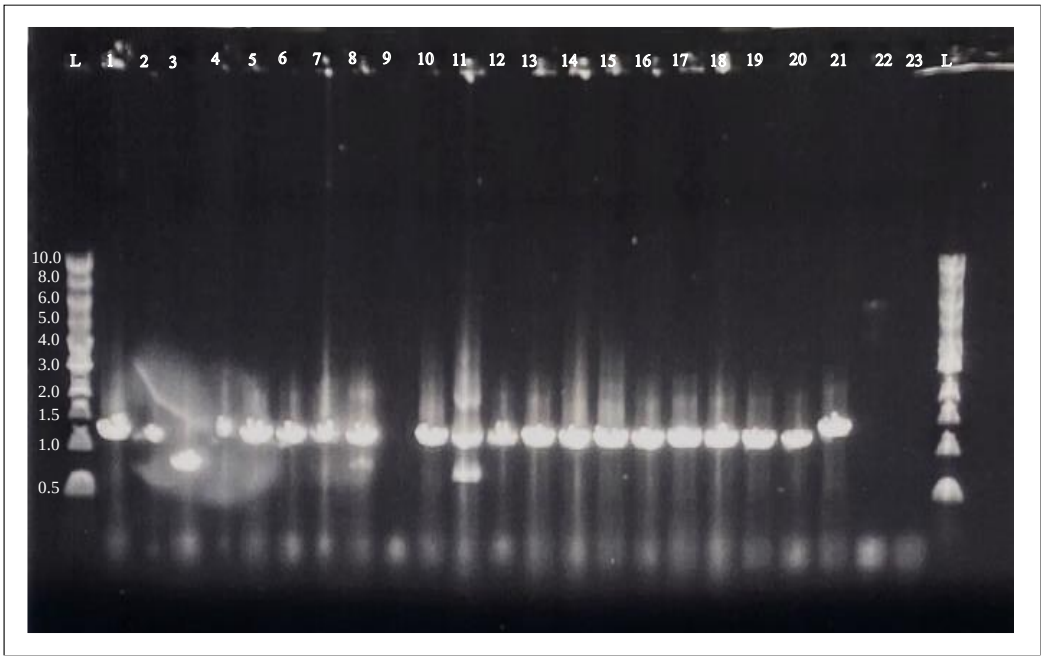


Figure 11. Agarose gel electrophoresis of the colony PCR to confirm the transformation of gene knockout plasmid in E. coli. The agarose concentration in the gel was 0.5%. Lane L has the 1-kb DNA ladder, while lanes 1-21 correspond to E. coli clones screened by colony PCR with gacA1/gacA4 primers that were to produce a 1.2-kb amplicon. Lanes 22 and 23 were negative controls.

Similarly, the 5'ends of the gacA1 and gacA4 primers were designed to overlap with regions flanking the multiple cloning site of the pEX18Ap vector. The two *gacA* shoulder regions and the cloning vector were amplified by PCR with the high-fidelity Q5 DNA polymerase yielding amplicons of the expected length (Figure 10). The amplicons were purified and used for the three-fragment Gibson assembly cloning with the NEBuilder HiFi DNA Assembly Master Mix. After the Gibson assembly, the DNA was transformed in *E. coli,* and the resultant clones were screened by colony PCR with gacA1/gacA4 primers for the presence of cloned shoulder fragments. The screening of 21 randomly selected clones yielded 15 recombinants that contained the deletion variant of *gacA* (Figure 11). Three recombinant clones (6, 13, and 18) were chosen for DNA extractions yielding plasmids of approximately 7.0 kb (the pEX18Ap backbone size is 5.8 kb, and the size of the fused *gacA* shoulder fragment is 1.2 kb) (Figure 12).

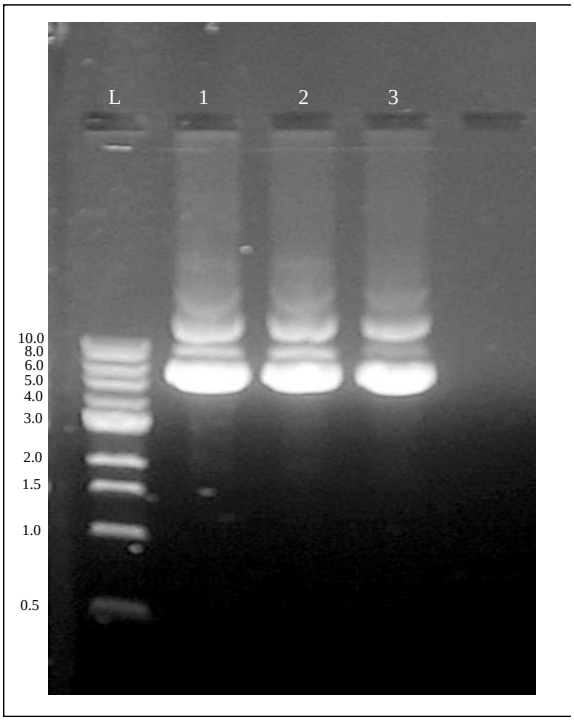


Figure 12. Agarose (0.8%) gel electrophoresis of the gene knockout plasmid pEX18Ap-gacA. Lane L contains the 1-kb DNA ladder. Lanes 1, 2, and 3 are plasmids extracted from E. coli clones #6, #13, and #18. Results revealed the presence of pEX18Ap-gacA of correct band size of ~7.0 kb.

Results of end-sequencing confirmed the integrity of recombinant plasmids and the absence of unwanted mutations introduced during the PCR step. We next attempted to generate an isogenic *gacA* mutant by electroporating *G. sunshinyii* 449 with the pEX18Ap-gacA knockout plasmid. However, these attempts failed, even though we used two different methods of preparing electrocompetent cells and varied the growth conditions, the type and volume of the wash medium (10% glycerol or 0.3 M sucrose), the centrifugation protocols, and antibiotic concentrations used to select transformants. No growth was observed in any of the plated cultures despite a week of incubation. Similarly, no transformants were recovered in controls electroporated with the stable broad host range plasmid vector pBBR1MCS.

CHAPTER V: DISCUSSION

This project analyzed the genome sequence of *G. sunshinyii* 449, which is the second sequenced genome of this species. The genome of strain 449 is slightly smaller than that of *G. sunshinyii* YC6258^T (6.1 Mbp versus 6.48 Mbp). Nevertheless, 22% of its gene content is different from YC6258^T. Of the 1,234 *G. sunshinyii* 449-specific genes, 85.7% encode hypothetical or conserved hypothetical proteins. The rest of the unique genes encode enzymes of fatty acid, carbohydrate and DNA metabolism, mobile genetic elements (transposons, prophages, retrons), transporters, stress response proteins (chloroperoxidase, alkyl hydroperoxide reductase), components of type VI protein secretion system, and CRISPR-Cas-associated proteins. The *G. sunshinyii* 449 genome also encodes almost 40 genes associated with antibiotic resistance, including multidrug efflux pumps implicated in the resistance to quinolones, fluoroquinolones, and macrolides. Unlike the type strain YC6258^T, *G. sunshinyii* 449 lacks the CatB-family chloramphenicol O-acetyltransferase, which explains its sensitivity to chloramphenicol. Similarly absent are tetracycline resistance determinants, which agrees with the sensitivity of strain 449 to this antibiotic.

The genome of *G. sunshinyii* 449 also encodes an impressive array of secondary metabolite pathways underscoring this organism's potential as a rich source of nonribosomal peptides and polyketides (Table 3). Among polyketide metabolite pathways identified in strain 449 are those involved in synthesizing the broad-range antibacterial macrodioloide tartrolon (Elshahawi et al., 2013). Also present are pathways for the synthesis of the cytotoxic polyketide lacunalide (Ueoka et al., 2018) and dihydromaltophilin. This broad range antimycotic kills fungi by interfering with the

biosynthesis of sphingolipids (Yu et al., 2007). The 449 genome also encodes an array of nonribosomal peptide synthases absent from the type strain, suggesting the capacity to produce multiple novel bioactive metabolites. Overall, the number and diversity of secondary metabolites potentially produced by *G. sunshinyii* is comparable to that of *Streptomyces* spp., which traditionally served as a primary source of antibiotics and other bioactive metabolites.

Although the unusual predicted ability of *G. sunshinyii* to produce multiple secondary metabolites has recently received considerable attention, the regulation of the corresponding biosynthesis genes remains unexplored. Like many *Gammaproteobacteria*, *Gynuella* carries a Gac/Rsm signal transduction pathway comprised of well-conserved homologs of GacA, GacA, and RsmA. Interestingly, we failed to detect the GacA-controlled small RNAs, which suggests that *Gynuella* may differ significantly from *E. coli*, *Pseudomonas*, and *Vibrio* in the type and/or the number of these important regulatory genes. Based on the high level of conservation and findings in other organisms, we hypothesized that the Gac/Rsm pathway might regulate the production of bioactive polyketide metabolites, surface motility, and biofilm growth in strain 449**.** To test this hypothesis, we attempted to construct an isogenic *gacA* mutant of strain 449. We used Kirby-Bauer and MIC assays to determine the antibiotic susceptibility profile of *G. sunshinyii* 449 and chose for further experiments the gene replacement vector pEX18Ap, which carries an ampicillin resistance marker (Hoang et al., 1998). Using a combination of high-fidelity PCR and Gibson cloning, we constructed a pEX18Ap-based plasmid designed to introduce a deletion into the *gacA* gene of *G. sunshinyii* 449. The cloning was successful and produced the desired knockout plasmid.

However, all attempts to create an isogenic mutant by electroporating strain 449 with the pEX18Ap-gacA plasmid failed.

Speculations for the failed attempts include loss of viability during the preparation of electrocompetent cells. Unfortunately, the electroporation efficiency could not be determined using pBBR1MCS. Although pBBR1MCS is based on a broad host range replicon, it is possible that this plasmid cannot replicate in *G. sunshinyii*. Another reason behind the failed electroporations could be improper optimization of the electroporation protocol (voltage, amount of DNA and cells to be electroporated, etc.) and preparation of competent cells (Kinosita et al., 1992; Tsong, 1991). Cell lysis was observed while washing biomass with 10% glycerol or 300 mM sucrose, and the prepared electrocompetent cells had a slimy consistency indicative of lysed cells. Finally, it is possible that the electroporated plasmid was damaged by endonucleases. The analysis of the *G. sunshinyii* 449 genome revealed the presence of type I, type III, and Mrr restriction-modification systems that could rapidly degrade the unmodified plasmid DNA and interfere with homologous recombination.

Other approaches will be considered for future work, including gene inactivation by mutagenesis with EZ-Tn*5* transposomes (Lucigen, Middleton, WI), which does not rely on homologous recombination. The transposon mutants will be screened for alterations in the production of secondary metabolites (plate inhibition assays with reference strains) and surface motility. Regulatory mutants will be identified by mapping transposon insertion sites through sequencing. The resultant isogenic mutants can be compared to the wild-type *G. sunshinyii* 499 for defects in the production of individual polyketide metabolites by real-time PCR.

APPENDIX A: DNA SEQUENCE OF THE pEX18A-gacA PLASMID

>pEX18Ap-gacA

TCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGAT
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