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Packaging of Genetic Material by Gene Transfer Agents (GTAs) Produced by Marine *Roseobacter* Species and Their Effect on Stimulating Bacterial Growth

Shahd Bader Aljandal
University of South Florida, Dr.sbj@hotmail.com

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by

Shahd Bader Aljandal

A thesis submitted in partial fulfillment
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Major Professor: John H. Paul, Ph.D.
Mya Breitbart, Ph.D.
Lauren McDaniel, Ph.D.

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DEDICATION

This thesis is dedicated to my parents, Bader and Eman, and my significant other, Khalid, for the endless love and encouragement they have shown throughout the challenges of being away from home and graduate school. Also, to my siblings Reem, Maha, Salem and Yousef who have always been there for me.

I would also like to dedicate this thesis to Dr. Mohammad Afzal, Dr. Husain Alawadhi, and Dr. Huda Mahmoud whose passion for teaching brought me here! It was your guidance that gave me the foundation to believe in myself.

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“Bacteria trade genes more frantically than a pit full of commodities traders on the floor of the Chicago Mercantile Exchange”

—Lynn Margulis and Dorion Sagan, 1995
What Is Life?

ABSTRACT

Horizontal gene transfer is one of the most important mechanisms for prokaryotic genome innovation and evolution. Gene Transfer Agents (GTAs) are phage-like particles that package small fragments of the genome of a GTA-producing bacterial cell. GTA chromosomal gene clusters usually contain 15-conserved open reading frames (ORFs) and are present in most of the sequenced marine alpha-proteobacteria genomes. Some marine strains have been shown to produce GTA particles that were biologically active in marine environment.

GTA particles range in size, morphology and the amount of host DNA they package. To date, the characteristics of GTAs are largely based on observations of *Rhodobacter capsulatus*, a bacterial isolate from freshwater pond and soil samples. One of the main characteristics of the GTAs produced by *R. capsulatus* is random packaging of the genetic contents of the GTA-producing strain. However, there is no evidence that marine GTAs behave in a similar manner to those produced by *R. capsulatus*.

This thesis focuses specifically on the GTAs produced by marine bacterial isolates, aiming to expand the available knowledge of how GTAs of marine bacterial strains contribute to HGT and how they affect the bacterial adaptation and fitness in the ocean. Here, the putative GTA particles produced by marine bacterial strains grown in artificial seawater media were examined to investigate the randomness of the DNA packaging and the biological effect of the GTA particles, specifically examines the effect of GTAs on stimulation of bacterial growth *in vitro*.

To reach the desired outcome, first, the DNA packaged within GTA particles produced by *Roseovarius nubinhibens* (RnGTA), *Ruegeria pomeroyi* (RpGTA) and *Roseobacter denitrificans* (RdGTA) was sequenced to determine if random portions of the bacterial genomes are packaged, similar to results shown for *R. capsulatus*, or if certain areas of the genome are preferentially packaged (overrepresented). Further, purified active GTA particles derived from each of the three marine *Roseobacter* strains were tested to determine the effect of active GTA particles on bacterial growth compared to controls containing heat-inactivated GTA particles, induced prophages (where applicable) and buffer.

In summary, the production of GTA particles produced by *R. denitrificans* was observed for the first time. Additionally, the results of sequencing, annotating and assembling the packaged DNA within GTAs from the marine *Roseobacter* strains that were studied here suggested that although there was a good representation of the whole genome packaged within the GTA particles, still there is significant enrichment (overrepresentation) of gene groups that could expand their metabolic capabilities. Also, *in vitro*, under nutrient replete conditions, GTA particles of *R. nubinhibens* (RnGTA) seemed restricted to having impact on growth to members of the same species. On the contrary, when seawater samples were treated with GTAs there was increase in viable cell counts. By closely examining the colony morphologies, there was a clear difference between the bacterial species that grew when seawater samples were treated with RpGTA and RdGTA compared to controls. The 16S rRNA identifications revealed that under the tested laboratory settings, some species belonging to phylum *Flavobacterales* are more responsive to active GTA treatment than others, causing microbial community shift in seawater samples. This study has expanded what is known about GTAs of marine origin, providing genetic and metabolic evidence that GTAs may stimulate microbial diversity and survival in the

marine environment. Knowledge gained from this study will help us understand the role of GTAs and HGT mechanisms in the ocean, therefore advancing our knowledge about the evolution and interaction of marine microbes.

INTRODUCTION

Marine Microbes

Over the vast ages since planet Earth was formed, about 4.4 billion years ago when the Earth cooled down, gasses and vapors from the molten rocks condensed and it began to rain, resulting in the birth of the oceans (1), which now covers about 71% of Earth's surface. Paleontological records suggest that life on Earth most likely began in the ocean around 3.6 billion years ago with a single microbial cell (2, 3). Ancestral marine microorganisms and the Earth evolved together, affecting the chemical properties of our planet to make it possible for us to live. Marine microbes are important as the base of marine food webs, they carry out much of primary production of organic matter setting the stage for the consumers of higher trophic levels to feed and flourish (4). The genetic diversity and different metabolic capabilities of marine microorganisms have kept the geochemical elements cycling throughout the ocean.

Marine bacteria have evolved to live in a dynamic ecosystem such as the ocean, where in many places the temperature, oxygen levels, salinity, and nutrients are continuously fluctuating. Even top predators and humans may affect the composition, diversity and structure of the oceanic microbial communities (5). Thus far, microbes can grow and continue to thrive almost everywhere from the furthest reaches of the atmosphere to the deepest trenches of the ocean, using a wide range of nutrients and energy sources.

Microbial Diversity and Adaptability

Despite their small size, microbes are the most abundant and diverse living cells on Earth. In the open ocean, it is estimated that microbes exceed 1.2E+29 cells, with an average of 5E+5 bacterial cells in every one milliliter of seawater accounting for about 90% of the total marine biomass (6). Perhaps the primary reason for this success is their high diversity and ability to occupy a wide range of ecological niches. The key, as Charles Darwin proposed, is evolution, the ability to change and adapt in the current environment (7). It is true that all living organisms have the potential to evolve, yet bacteria are the most adaptable of the forms of life on our planet. Firstly, they have a short generation time, e.g.: *Vibrio natriegens* has a doubling time of less than 10 minutes (8). When a bacterial cell divides, the result is identical daughter cells; therefore, a single bacterial cell can, under the right conditions, produce a large bacterial population in a short time. Occasionally, spontaneous gene modification or what is known as mutation occurs, altering the parental (wild-type) cell's genetic composition. Such mutations can be negative, positive or neutral. With positive selection of the trait conferred by a mutation, it will be passed to future generations by a process known as vertical inheritance (9).

With the advancement of comparative analysis of sequenced microbial genomes, it became clear that the complexity of microbial genomes and their mosaic nature is not only due to vertically inherited random mutations but also due to events of gene exchange including gene acquisition and loss, known as horizontal gene transfer (HGT; also known as lateral gene transfer) (10–15).

Along with spontaneous mutations, HGT currently provides a plausible explanation for the high microbial diversity, genetic variability and irregularities often observed in phylogenetic trees (13, 16, 17). Some experiments suggest that the rate of gene transfer in the environment can

be as low as 1E-8 to 1E-9 (transferred genes per recipient) (18, 19), but the size of microbial populations and their rapid doubling times result in HGT imparting a major force on microbial evolution and community structure (20, 21). Based on genome-wide analysis, it is estimated that between 1.6% and 32.6% of the genes in each microbial genome are acquired by HGT (22).

In general, the microbial genome is composed of what are described as a conserved set of core (essential) genes and a variable set of accessory (non-essential) genes (23). The core genes are present in all strains of a species, and typically include essential (housekeeping) genes for cell metabolism and replication. Losing these genes means the cell could no longer sustain life, (e.g.: replication genes, ribosomal genes, etc). The accessory genes are not necessarily present in all strains of a species; they are considered the flexible pool where the cells in a population may have access to them, and gaining or losing them does not affect the cell dramatically. In fact, such genes provide additional advantage to support the cell's adaptation to a specific niche (e.g.: antibiotic, heavy metals and UV resistance genes) (24–26). Based on the complexity hypothesis (27), core genes are less susceptible to HGT. Yet even accessory genes can be transferred with varying frequencies. The most abundant marine microbe on Earth, *Prochlorococcus marinus* has a core genome of less than 20% of its whole genome, indicating the effect of HGT on microbial genome composition and gene transfer in the ocean (28).

Mechanisms of Horizontal Gene Transfer

The common mechanisms of HGT between prokaryotes, listed chronologically by time of discovery are transformation, conjugation, and transduction.

Transformation is the uptake and expression of naked DNA from its surrounding environment. It was first recognized in 1928 in *Streptococcus pneumonia* (29). The process of

natural transformation usually occurs with the integration of extracellular DNA into a susceptible recipient cell, such cells are called competent cells. Gene transformation can happen in the absence of a living donor cell. During transformation the exogenous DNA may be integrated into the competent bacterial cell's chromosome or to a plasmid that co-exists with the chromosomal host DNA (30, 31).

Conjugation is a plasmid-encoded gene transfer mechanism that requires synthesis of a pilus structure and direct physical contact between cells. In 1946, conjugation was documented for the first time in *Escherichia coli* (32). Typically, the genetic material transferred via conjugation is in the form of circular piece of DNA or a plasmid. Since conjugation requires proximity between donor and recipient cells, it has been estimated that conjugation is more prevalent in biofilms than transformation or transduction (33). Gene transfer by conjugation, requires the least restriction on the relatedness of donor and recipient cells (21).

Transduction was first discovered in *Salmonella enterica* (34) and is part of the viral life strategy termed lysogeny. Lysogeny is when a bacteriophage (also known as phage; the virus that infect bacteria) integrates into a bacterial chromosome after or during infection at which point it is called a prophage (35). In this case the temperate phage takes a lysogenic instead of a lytic pathway and the prophage remains dormant and replicates when the host cell does until prophage induction is triggered. In the event of induction, the prophage switches from a lysogenic to lytic cycle. By definition, transduction happens when a lytic phage and/or a prophage activates to the lytic cycle and as a packaging error the produced phages transfer host genes from one bacterium to another by packaging it in the viral capsid. This happens in one of two ways: generalized or specialized transduction (34).

In generalized transduction, the bacteriophage packages and transfers an arbitrary set of host genes to a recipient cell. In contrast, specialized transduction, is specific, transferring the genes adjacent to the phage's genome insertion site on the bacterial chromosome. Studies have demonstrated that transduction is an important HGT mechanism in the ocean (19), and that is due to the high abundance of the viruses in the ocean, especially temperate viruses (36, 37). Other studies suggested that, about 1E+25 phage mediated gene transfer events happen globally every second (38, 39).

In addition to the three widely investigated mechanisms of HGT described above, there have been studies documenting several other mechanisms of horizontal gene transfer, to name few: gene transfer agents (GTAs) (40–42), membrane vesicles (43–45), and genomic islands (46). This thesis focuses specifically on gene transfer mediated by GTAs.

Gene Transfer Agents

What is known about GTAs so far is based on early work with the GTA particles produced by *Rhodobacter capsulatus* (termed RcGTA). RcGTAs were described as phage-like particles controlled by and released from the donor host bacteria, packaging random linear (~4.5 kb) host genomic dsDNA fragments (40, 41). GTAs are only produced if the host genome contains the gene cluster representing the GTA structure (47). Unlike lytic viruses or prophages, due to the small amount of DNA they package, GTAs cannot package enough genes to encode the proteins of the particles themselves (~13-16 kb). This prevents the particles from packaging the complete GTA gene cluster that is responsible for the particles' production (42, 47–50).

GTAs have been identified in multiple prokaryotic species: first recognized in *R. capsulatus* (40), then demonstrated in *Desulfovibrio desulfuricans* (51), *Brachyspira*

hyodysenteriae (52), *Methanococcus voltae* (53). With the sequencing of marine bacterial genomes, GTA gene clusters analogous to *R. capsulatus* were observed in many marine Rhodobacterales, including *Ruegeria pomeroyi* (54).

Based on sequence alignments (48, 55), a typical chromosomal GTA-encoding gene cluster is organized in 15 predicted genes or open reading frames (ORFs, Figure 1). The first ORF is usually the terminase large subunit gene, which is responsible for packaging the host genome. In most viruses containing terminases, a small subunit gene accompanies the large subunit gene. The terminase small subunit gene determines the specificity of the DNA packaged by the large subunit terminase gene (56). The absence of the terminase small subumit gene in GTAs is hypothesized to explain the random packaging of host DNA in GTAs (55). ORFs 3 to 5 are portal, prohead protease and major capsid proteins. Tail proteins including a head to tail adapter protein, a major tail protein and sometimes a tail tape measure protein are usually encoded in ORFs 6 to 11. ORF 14 encodes a putative cell wall hydrolase (responsible for boring holes in the host cell enabling injection of the GTA-packaged DNA). ORF 15 is the largest ORF in the GTA gene clusters and is sometimes annotated as a host specificity protein although its exact function has not been conclusively determined (57). Commonly, the first gene after the 15 ORFs is the bacterial metabolic gene serine O- acetyltransferase, providing evidence that the GTA encoding gene clusters are vertically inherited and not inserted in random loci on the chromosomal DNA as in the case of some prophages (55).

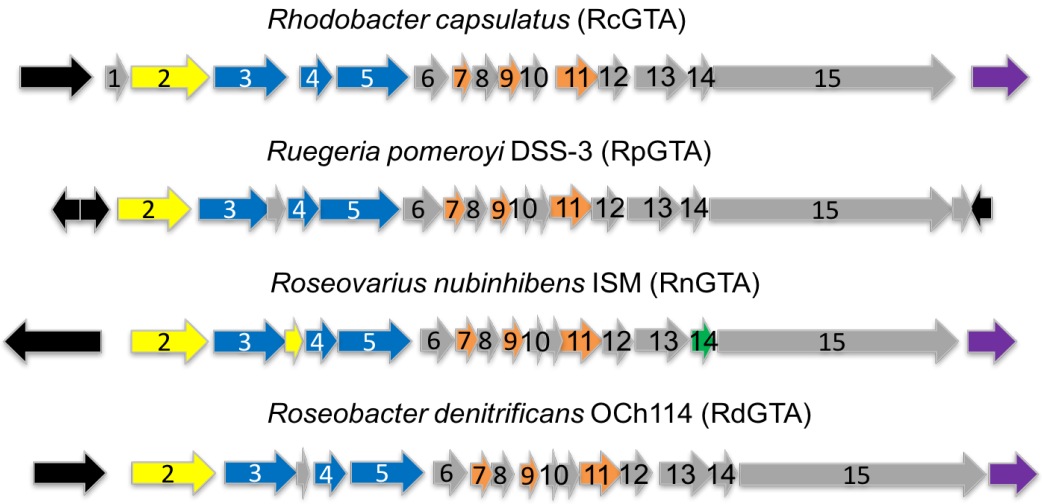

Figure 1. Typical GTA Genomic Gene Cluster. ORF arrows 1 to 15 are color coded: Yellow, terminase large subunit; Blue, portal, capsid protease, and major capsid proteins; Orange, tail associated proteins; Grey, unknown or conserved hypothetical proteins; Green, lytic enzyme; Purple, serine O- acetyl transferase (Host); Black, host.

Compared to bacteriophages, GTA particles are unique because they are produced after the host strain enters the stationary growth phase (58). In addition, the particles' production is completely under the control of the donor host cell (48). The production of GTA particles involves multiple regulatory host systems (47–50). What makes gene transfer mediated by GTAs different than generalized transduction is that in transduction only a few particles package and transfer fragments of the host genome to a recipient cell (49). Also, GTAs do not form plaques in cultures, nor are they induced by UV or Mitomycin C (40, 41).

Still, the mechanism of production and release of the GTA particles remains undetermined. Recent studies found that, in addition to the RcGTA primary structural gene cluster, there are more GTA related genes in separate locations on the host genome that are involved in the process of the particles' production. These loci include holin and endolysin genes that are vital for the release of the RcGTA from the producing host cell (49, 50, 59, 60).

Other studies have shown that the production of RcGTA particles requires cell cycle regulatory proteins that are part of the essential CckA-ChpT-CtrA phosphorelay system (48). The disruption of *ctrA* and/or *cckA* genes diminishes GTA production and transduction capabilities in the host cell. Without CtrA the host cells could not initiate transcription and did not express the RcGTA structural gene cluster during the stationary growth phase, leading to the failure to produce particles (61). Additionally, recipient host cells lacking *ctrA* could not receive the GTA-packaged DNA (62). While the lack of CckA had a different effect on the host, the cell did express the RcGTA structural gene cluster but was not able to express the genes that encodes for the lytic system (48, 59, 60, 63, 64). The translation of *ctrA* is regulated by the quorum sensing system, involving GtaI and GtaR proteins that are important for the maximal expression of the RcGTA structural gene cluster in the donor host cell and for the capsule synthesis in the recipient cell (60, 65–67).

Up until 2015, scientists described GTAs as a novel method of HGT that has features resembling transduction (40, 49). However, after studying in depth the bacterial cell regulators controlling the ability of the host to receive RcGTA packaged DNA (68), which is regulated by CtrA and GtaI/quorum sensing (61, 62, 68), it became clear that RcGTA-mediated gene transfer also requires a transformation-related system including *comEC, comF, comM* and *dprA* (68) that is usually found in naturally competent bacteria. These results suggest that at least in RcGTA, the process of GTA mediated gene transfer combines features of both transduction and natural transformation to transfer GTA-packaged genes to a recipient cell (68).

Since the GTA-encoding gene clusters are vertically inherited, the gene arrangement appears to be conserved in almost all sequenced genomes of the Rhodobacterales order of alpha-proteobacteria (55, 57). The most highly conserved genes are the major capsid protein gene (*g5*)

and terminase large subunit genes (55, 57). The *Roseobacter* clade within Rhodobacterales comprises a significant fraction of the marine microbial populations (~20%) (69–72). Due to the high conservation of GTA genes a study by Zhao *et al*. (73) used alignments of the GTA *g5* gene to design primers as diagnostic tool to investigate the presence of Rhodobacterales in the Chesapeake Bay aquatic environment. Based on the sequences from PCR amplifying the *g5* gene, they have successfully shown amplification of 26 members of this order, and the results of this study and others were consistent with 16S rRNA-based phylogenies (74, 75).

As for many mobile genetic elements, at present, there is no direct evidence to reveal the origin of GTAs, but some suspect that GTAs might have evolved from mutant defective prophages (49). Support for this hypothesis was provided by recent reports of the genome sequences of some Rhodobacterales phages, including roseophage RDJLϕ1 that infects *Roseobacter denitrificans* OCh114 (76), and phage DSS3ϕ8 that infects marine *Ruegeria pomeroyi* DSS-3 (77). These phages contain four GTA-related genes including GTA ORF 12 to 15. These findings illustrate a possible evolutionary route between phages, prophages and GTAs (49, 77).

In a previous study by McDaniel *et al*. (78), some strains belonging to marine order Rhodobacterales that are known to contain the GTA gene cluster were observed for the production of GTA-like particles and gene transfer activity. When cell-free, DNase and RNase treated GTA particles from the genetically marked donor strains containing transposon *Tn5* were introduced to the wild-type recipient strains or natural bacterial assemblages, comparison of antibiotic resistance rates between controls and GTA particle treated samples provided evidence for GTA particles mediated the transfer of the *Tn5* genes. This finding was confirmed by PCR amplification and sequencing that documented recovery of *Tn5* genes from the recipient bacteria.

This study suggested that GTA mediated gene transfer in different marine environments was higher than measured frequencies of transformation and transduction (78).

Importance of HGT

HGT mechanisms are hypothesized to expand the existing metabolic capabilities of the recipient, enabling such bacteria to survive in a new ecological niche (22, 79). In addition, HGT helps to explain how bacteria adapt rapidly to changing environments. For example, the association of plasmids in marine *Vibrio spp*. is involved in expanding the ecological and pathogenic niches with the host that it infects (80). As a HGT mechanism, GTA-related gene transfer events have been viewed as an adaptive mechanism to provide metabolic flexibility in response to the continuously changing marine environment (54, 78).

Thesis Overview

In this thesis, the main goal is to expand the available knowledge of how GTAs of marine bacterial strains function, especially in comparison to the type strain *R. capsulatus*. Do marine GTAs package host DNA similarly to *R. capsulatus*? how do they contribute to HGT? and do they affect bacterial adaptation and bacterial fitness in the ocean?

First, the assumption that the DNA packaged within GTA particles is random was investigated. The strains *R. nubinhibens, R. pomeroyi* and *R. denitrificans* all contain the typical chromosomal GTA gene cluster and 2 out of the 3 strains have been documented to produce particles consistent with GTAs. Additionally, these host cells have been sequenced and their genomes are publicly available. The GTA-packaged DNA produced by these strains was

sequenced to determine if random portions of the bacterial genomes are packaged, similar to results shown for *R. capsulatus*, or if certain areas of the genome are preferentially packaged.

Second, purified active GTA particles derived from each of the marine three *Roseobacter* strains were tested for biological activity. Previous experiments done in our laboratory have shown that besides GTA particle-mediated transfer of antibiotic resistance genes, an interesting and unexpected stimulation of growth or cultivability occurred when natural populations and wild-type strains were treated with cell-free GTAs in the absence of selective pressure. Here, the effect of active particulate GTA treatment on stimulating bacterial growth was investigated. Heat-inactivated GTA particles, induced prophage (where applicable) and SM (saline magnesium) buffer were used as negative controls and compared to treatment with active, cell-free, purified GTAs added to different recipient cultures to determine if the presence of GTA particles affects growth of the strain they are derived from or other, related strains.

TESTED HYPOTHESES

Hypothesis (1): Packaging of GTA Particles

H_0: GTA particles package random host genes in *R. nubinhibens, R. pomeroyi,* and *R. denitrificans*.

H_a: GTA particles preferentially package certain group of host genes in *R. nubinhibens, R. pomeroyi* and *R. denitrificans*.

Hypothesis (2): Biological Activity of GTA Particles

H_0: GTA particles of marine *R. nubinhibens* (RnGTA), *R. pomeroyi* (RpGTA), *R. denitrificans* (RdGTA) do not influence the growth rate or other growth parameters of recipient marine bacteria.

H_a: GTA particles of marine *R. nubinhibens* (RnGTA), *R. pomeroyi* (RpGTA), *R. denitrificans* (RdGTA) influence the growth rate or other growth parameters of recipient marine bacteria.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

In this study, the GTAs of *Roseovarius nubinhibens* ISM (RnGTA), *Ruegeria pomeroyi* DSS-3 (RpGTA), and *Roseobacter denitrificans* OCh114 (RdGTA), three marine bacteria known to contain a complete GTA gene cluster were screened.

To verify the identity of the strains prior to experimentation, each strain was thawed from frozen stocks and grown in peptone and yeast extract artificial seawater medium ASWJP+PY (see Appendix A) at 26 ^{O}C with shaking at 50 rpm on an orbital shaker. DNA was extracted using the QIAGEN DNeasy Blood and Tissue kit according to the manufacturer's protocol for gram-negative bacteria (www.qiagen.com). The DNA yields were quantified by Nanodrop® spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE). The extracted DNA was PCR amplified using standard 16S rDNA primers (81), sequenced and its identity was confirmed by BLAST (https://www.ncbi.nlm.nih.gov). Glycerol stocks of each strain were prepared and stored at -80ºC to preclude contamination during the project. For experiments, each strain was revived from one of the aliquots of verified frozen stocks in 25% filter-sterilized glycerol.

Growth curves of all species along with spontaneous viral particle (putative GTAs) production were monitored. For the growth curve, replicates of 1% inoculum (vol/vol) in ASWJP+PY (and YTSS media as well for *R. pomeroyi*) of each strain were monitored by measuring the optical density (OD) at wavelength 600 nm using Spectronic™ GENESYS 20®

spectrophotometer every 2 hours until late log phase, then the OD was measured every 12 hours until stationary phase. Also, to monitor the GTA-like particle production with time, a sub-sample of each strain was taken at 24-hour intervals after start of growth, centrifuged, 0.2 μm filtered, prepared for SYBR® Gold visualization and virus-like particle counts as described below.

Prophage Induction

The wild-type isolate of *R. nubinhibens,* and *R. pomeroyi* were tested for prophage induction in replicates, as follows: two ml of overnight culture were inoculated into 48 ml of ASWJP+PY media while the OD at 600 nm of the growth was monitored. When the cells reached the log-phase (OD of 0.4-0.6), the culture was split into two equal volumes. The test volume was inoculated with Mitomycin C for a 0.5 μg ml^{-1} concentration and the control culture was inoculated with the same volume of sterile DI water (82). Both flasks were incubated overnight at 26 ºC with shaking. A one ml sub-sample from test and control samples were precipitated by centrifugation at 16,000× g for 5 minutes, 0.2 μm filtered, and immediately stained with SYBR® Gold to enumerate the particles and compare both samples.

GTA Particles Concentration

The GTA particles were harvested at the time of maximal production for each of the selected strains, by centrifuging the cultures at 9,500 × g for 10 minutes, followed by 0.2 μm filtration to remove the host cells. Then, the particles were concentrated with either: polyethylene glycol (PEG) precipitation (83) or by Amicon® Ultra-15 centrifugal filter devises (Millipore Corp., Bedford, MA, USA) according to the manufacturer's instructions.

PEG Precipitation

Sodium chloride was added to the filtrate at 1 M final concentration, followed by 10% (wt/vol) Polyethylene glycol (PEG) 6000, incubated on ice overnight, then centrifuged at 9,500 × g for 20 minutes at 4 ^{O}C to precipitate and concentrate the particles according to standard protocols (84). To ensure maximal particles recovery, the pellet containing the concentrated particles was suspended with 500 µl of SM buffer and incubated at room temperature for 1 hour. After that, the suspension was pipetted into a Phase-Lock Gel™ tube (5 Prime, Gaithersburg, MD, USA), then equal volume of chloroform was added to eliminate membrane vesicles (85) followed by mixing and centrifugation at 1,500 × g for 5 minutes at room temperature. The organic phase (in the bottom) was discarded and the aqueous phase was transferred to a clean sterile tube. After that, 2.5 units DNase and 0.1 units RNase per milliliter of the sample were added to degrade any non-encapsidated nucleic acids and to eliminate the possibility of natural transformation.

Amicon® Ultra-15 Centrifugal Filtration

According to manufacturer's instructions using Amicon® Ultra-15 50 kDa filter units, for maximum concentrate recovery using fixed angle centrifugation, the recommended sample starting volume is 12 ml, centrifuged at 5,000 × g for 15 minutes at room temperature.

GTA Particles Visualization

Epifluorescence Microscopy

One ml of the culture at each time point was centrifuged at 16,000 × g for 5 minutes, filtered using 0.2 µm × 33 mm Millex® filter units, diluted 1:100 with SM buffer, then

immediately stained in dark with 1:10 diluted SYBR® Gold nucleic acid stain on 0.02 µm, 25 mm diameter Acrodisc filters according to standard protocols (86) and viewed using an ZEISS Axio scope A1 and AxioCam MRm digital imaging system (www.zeiss.com).

Transmission Electron Microscopy

Ten microliters of purified, PEG concentrated (as above) GTA particles, were spotted on either a 200 or 400 mesh carbon coated grid (Electron Microscopy Sciences, www.emsdiasum.com) and air-dried for 25 minutes. Then, the grids were stained for 30 seconds in 2% aqueous uranyl-acetate and rinsed with 0.2 µm filtered DI water. The grids were then allowed to air dry for 1 hour. Stained grids were viewed using a Hitachi 7100 electron microscope, with a 100 keV accelerating voltage. Micrographs were obtained with a Gatan Orius high-resolution digital camera.

GTA Particles Purification

Due to the need for highly purified particles for DNA extraction and sequencing, concentrated particles were purified with cesium chloride density gradient, where a CsCl gradients of 1.3, 1.5 and 1.7 g/ml were layered, then the concentrated GTA prep was carefully placed on the top. The gradient was centrifuged in an ultracentrifuge at 100,000 × g for 2 hours at 4 °C. The purified GTA prep was recovered from the 1.3-1.5 interface and SYBR® Gold stained to verify the presence of the particles.

GTA-Packaged DNA Extraction, Amplification and Sequencing

The nucleic acids were extracted from the purified particles using formamide disruption of the capsids and ethanol precipitation as previously described (83). Since the GTA particles package about 4.5 kb (42), before proceeding to the amplification step, the extracted nucleic acid was Covaris® fragmented to 300-400 bp fragments to unify the template size according to the manufacturer's instructions. The dsDNA fragments were amplified using Accel-NGS® 1S Plus DNA Library and Indexing kits (Swift Biosciences, Ann Arbor, MI, USA) according to manufacturer's instructions (www.swiftbiosci.com). After amplification, three microliters of the extracted DNA were stained with ethidium bromide and visualized on a 2% agarose gel to verify amplification. Gel images were taken using an Alpha Imager EC (www.alphainnotech.com).

The purified, extracted and amplified DNA from the GTA particles was sequenced using the Illumina MiSeq sequencing platform. Using Circos software (87), the sequences of the DNA packaged within the GTA particles were mapped against the whole-genome of the GTA producing host wild-type strain to determine the relative coverage of different areas of the genome.

Biological Activity of GTA Particles

Spontaneous Antibiotic Resistant Mutant Strains Activity of RpGTA and RdGTA

For initial biological activity experiments, spontaneous streptomycin resistant mutant strains were created by growing wild-type strains on selective ASWJP+PY agar plates with streptomycin (1 mg ml^{-1}), then the antibiotic resistant mutant colonies were picked and used as donor host cells grown to maximal production day to harvest GTAs containing the selective marker. Then, the particles were PEG concentrated (as mentioned above).

Recipient cells (same wild-type strain or seawater sample) at log phase were concentrated on 0.2 μm × 47 mm polycarbonate filters. The filters were placed on ASWJP+PY agar plates, incubated with the concentrated GTA preps, heat-inactivated GTA preps (by heating the active GTAs in the microwave for about 20 seconds) and SM buffer controls overnight at 26 °C. After the incubation, the cells on the filters were re-suspended, diluted, and plated on non-selective ASWJP+PY and selective ASWJP+PY with streptomycin (1 mg ml^{-1}) agar plates, selectively aiming to detect the occurrence of transduction by supporting the growth of the cells containing the streptomycin marker. Finally, all plates were incubated at 26 °C for 3 days.

RnGTA Effect on Different Wild-Type Recipient Strains: Viable Counts

To determine the RnGTA treatment effect on stimulating the growth of different recipients in their exponential growth phase including: (*R. nubinhibens* ISM, *R. pomeroyi DSS-3, R. denitrificans* OCh114 and *Vibrio parahaemolyticus* St 16), cell free preparation of RnGTA particles was collected at the time of maximal production, concentrated using the viral concentration methods (as described above) then added to each recipient. The concentrated particles were also plated on plain ASWJP+PY media to verify the absence of host cells.

The recipient populations and RnGTA preparations were enumerated to calculate the multiplicity of infection (MOI) (the total number of GTA particles (concentration * volume) divided by the total number of recipient cells (concentration * volume)). To achieve estimated (low, mid, high) MOIs, replicates of the recipient cells were treated with active RnGTA particles or heat-inactivated particles and SM buffer controls (as described above). Additionally, since *R. nubinhibens* is known to contain "hidden" prophages in its genome (88), Mitomycin C induced prophages (as described above) were also used as a control.

Verification of RnGTA Particles Effect on Bacterial Growth Stimulation: Growth Curve, Viable Counts and Metabolic Activity Measurements

After adding active RnGTA and controls to the recipient cells, the growth was monitored by measuring the OD at 600 nm every 2 hours for the first 24 hours, then absorbance was measured every 4 hours in late stationary phase then 12 hours when the absorbance readings started to decline. At the 6-hour time point, subsamples were taken to measure the metabolic activity of the recipient cells when treated with RnGTA and the controls was measured by using the CellTiter 96® Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA) per manufacturer's instructions (https://www.promega.com). This assay is based on the concept that metabolically active cells will convert the soluble tetrazolium dye to an insoluble formazan product. The absorbance at 570 nm was measured for each replicate of all treatment and controls. In conjunction, at the same time point, 100 µl subsamples were diluted and plated on agar plates for viable count calculations.

RESULTS AND DISCUSSION

Confirmation of GTA Particles Production

Previous experiments have successfully demonstrated that under the tested conditions some, but not all Rhodobacterales strains containing identifiable GTA-like gene clusters produce GTA particles under laboratory conditions. *R. pomeroyi* and *R. nubinhibens* contain the GTA gene cluster and have been documented to produce functional GTA particles consistent with RcGTA (54, 89). In this study, these two species as well as *R. denitrificans* were selected and studied to determine their DNA packaging specificity and the biological effect of their GTA particles on the growth of the wild-type producing strain and/or other marine bacterial strains.

In order to achieve the goals of this study it is important to understand the characteristics that define GTAs and distinguish them from prophages or lytic phages. In the type strain *R. capsulatus* it is known that the GTA genes are maximally expressed and the particles are maximally produced in stationary phase *in vitro* (66). Hence, it is critical to track the cells and construct growth curves to monitor when the cells enter the stationary phase and when the host cells spontaneously start producing GTA particles. Figures 2 and 3 represent the growth curve (optical density at 600 nm vs. time in hours) of *R. pomeroyi* and *R. denitrificans*. The time of maximal GTA production of *R. nubinhibens* has already been determined (78).

In the case of *R. pomeroyi* (Figure 2), two growth media were tested to assess which growth media yields more cells and whether or not it makes a difference in GTA production. Growing the cells in ASWJP+PY media led to an increase in cell concentrations (absorbance).

The maximum particle production was estimated at approximately 96 hours of the growth period for all species and this support the results from *R. capsulatus* showing that GTA production is maximum after attainment of the stationary phase (58). The measurements were relative not quantitative, due to difficulty obtaining countable particle preparations. This is most likely due to the small size and limited DNA content of GTA particles.

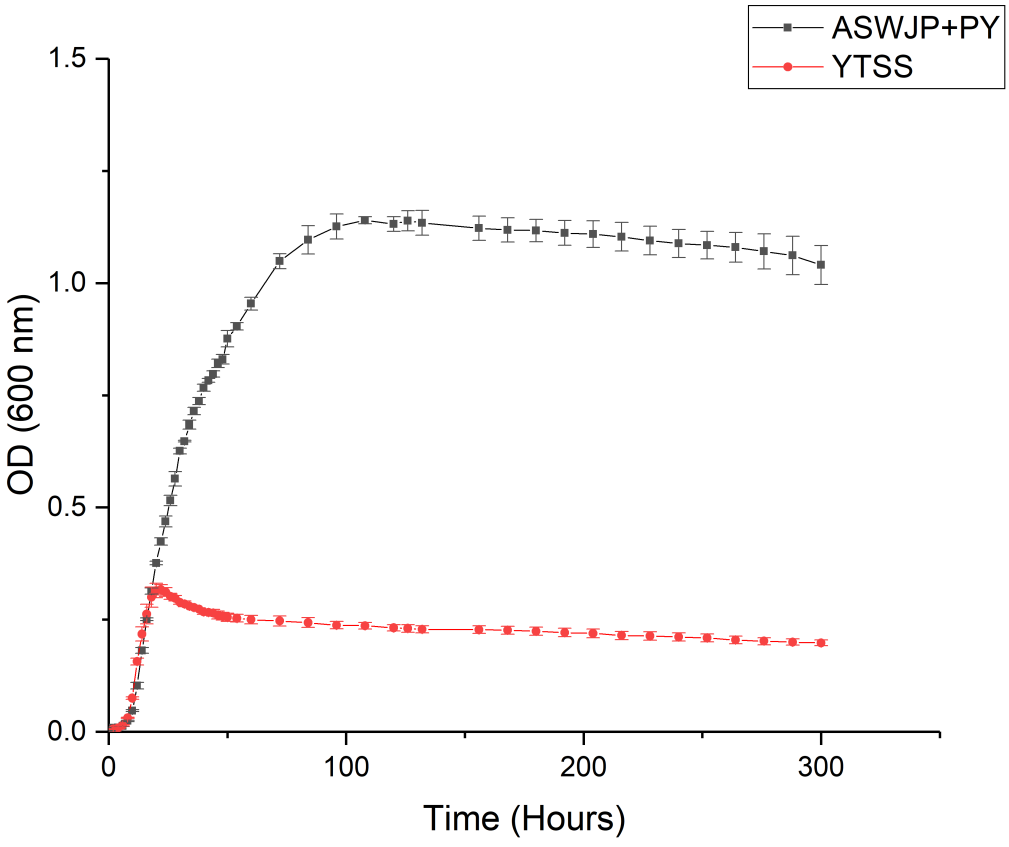

Figure 2. Growth Curve of *R. pomeroyi* Performed in Two Different Culture Media. Error bars represent standard deviation between triplicate OD measurements.

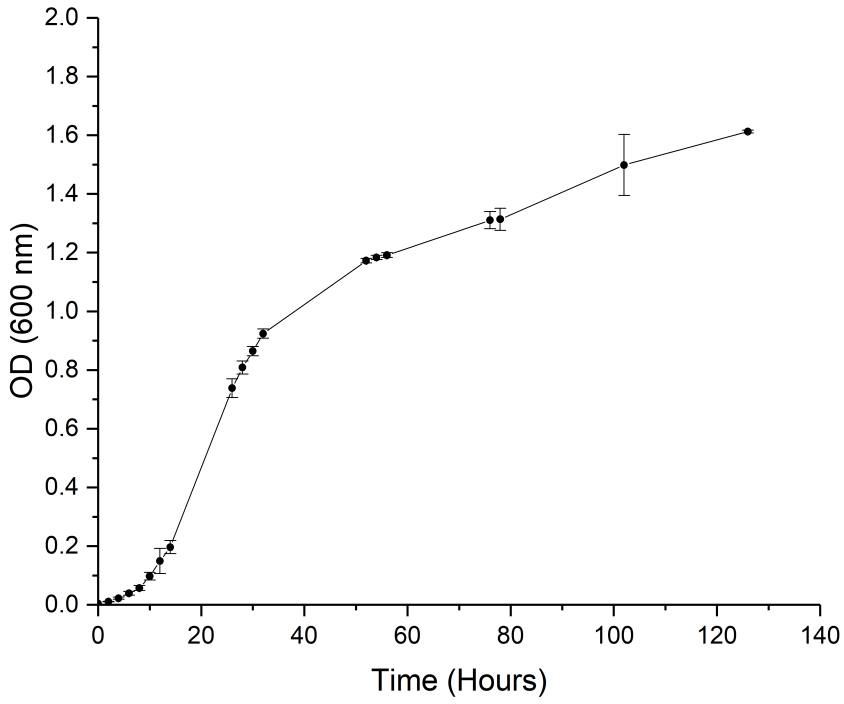

Figure 3. Growth Curve of *R. denitrificans* in ASWJP+PY Culture Media. Error bars represent standard deviation between triplicate OD at 600 nm measurements.

To support that the particles are in fact GTAs, *R. pomeroyi* and *R. nubinhibens* were screened for inducible prophages (Figures 4 and 5). For *R. pomeroyi*, the prophage induction experiment resulted in a non-significant (P = 0.0704) difference between the control replicates (2.28E+6 VLPs/ml) and replicates treated with Mitomycin C (2.91E+6 VLPs/ml), meaning that *R. pomeroyi* doesn't have an experimentally inducible prophage in its genome. While, *R. nubinhibens* resulted in significant (P = 0.00281) increase in VLPs (Viral-like particles) counts after Mitomycin C treatment, indicating the presence of inducible prophages, where replicates treated with Mitomycin C resulted in an average of 3.65E+8 VLPs/ml compared to the control replicates 1.42E+8 VLPs/ml. In both experiments, the high VLP counts in controls could be due

to spontaneous induction and/or spontaneous GTA particles production. In the case of *R. nubinhibens*, the presence of experimentally induced prophages must be considered in further experiments.

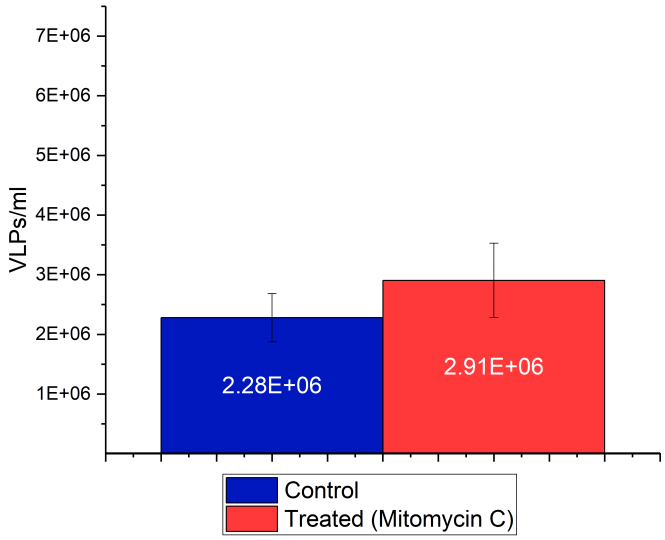

Figure 4. Prophage Induction by Mitomycin C in *R. pomeroyi*. Error bars represent standard deviation between triplicate epifluoresence microscopy counts. Non-significant *t*-test between counts (P = 0.0704).

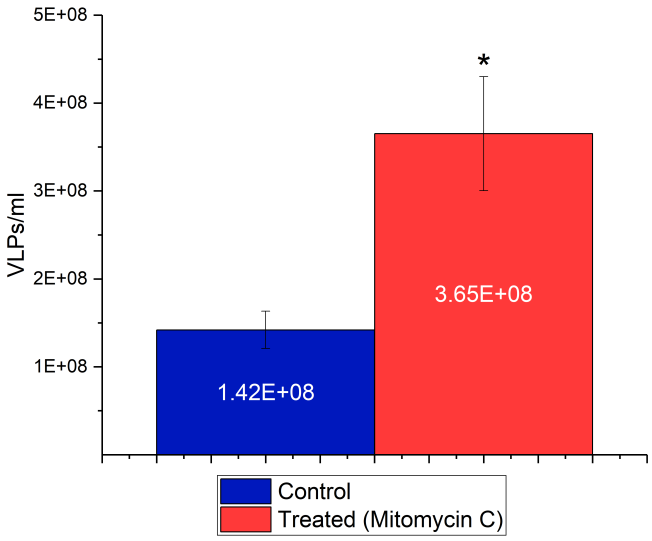

Figure 5. Prophage Induction by Mitomycin C in *R. nubinhibens*. Error bars represent standard deviation between triplicate epifluoresence microscopy counts. Significant *t*-test between counts (P = 0.00281).

The presence of GTA particles was also verified by visualizing the particles produced from *R. pomeroyi* and *R. nubinhibens* by SYBR® Gold staining (Figure 6A) and transmission electron microscopy (TEM) (Figure 6B and Figure 7). The TEM micrographs were used to assess the morphology, where both strains seem to produce morphologically similar GTA particles as the head size of the virus-like tailless RpGTA and RnGTA particles is about 50 nm in diameter, compared to the size of the tailed GTA particles produced by *R. capsulatus* which is about 30 nm (42).

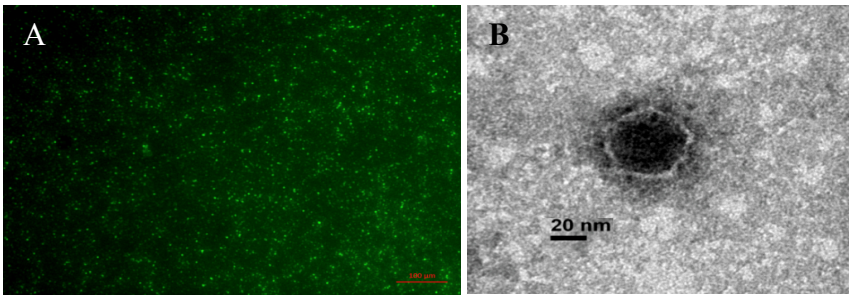

Figure 6. Visualization of RpGTA Assessed by Epifluorescence Microscopy Stained with SYBR® Gold (A) and Electron Microscopy (B).

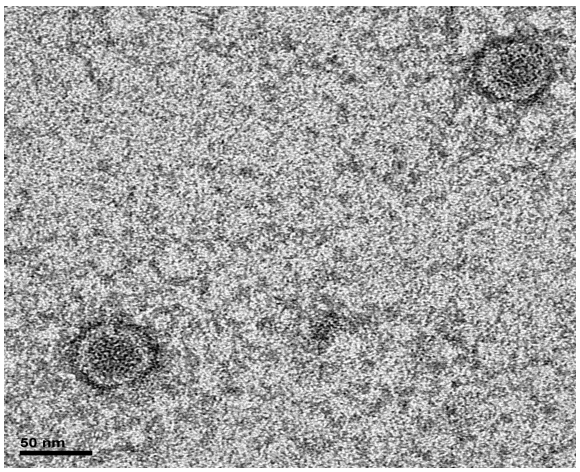

Figure 7. Visualization of RnGTA Assessed by Electron Microscopy.

Packaging of GTA Particles

Prior to this study, the only GTA particles whose contents have been sequenced are those of RcGTA (41, 42, 50). Here the genomic content of GTA particles produced by marine strains *R. nubinhibens, R. pomeroyi* and *R. denitrificans* were sequenced.

In the type strain *R. capsulatus*, RcGTA randomly package about 4.5 kb of host DNA (41, 42, 47). After purifying and concentrating GTA particles from each of the three strains used in this study, the DNA was extracted from two independent particle preparations of each strain then attempted to visualize the extracted nucleic acids with gel electrophoresis (2% agarose) (Figure 8A). Since the particles are known to package low concentrations of small DNA fragments, it is often difficult to visualize the DNA on gel electrophoresis. To further proceed in this experiment, the DNA was amplified to get a better yield. Considering that the GTA particles package random host genes, it is challenging to amplify the DNA within the GTA particles with familiar amplification methods such as polymerase chain reaction (PCR), which requires prior knowledge of the genetic target. To combat this problem the approach was to use Accel-NGS® 1S Plus DNA Library Kit (Swift Biosciences, Ann Arbor, MI), a unique amplification kit that eliminates the requirement of using target-specific primers, instead it allows addition of an adapter oligonucleotide onto the 3′ end of the DNA strand in a template independent manner resulting in the amplification of random fragments of DNA (Figure 8B). Since only 3 µl of each post amplification sample with different concentrations were used, the bands in well 5 and well 7 (Table 1 and Figure 8B) appear to be faint in comparison to the other amplicons due to their low starting DNA concentrations. The gel bands are consistent with the expected size of the sheared template DNA.

Table 1. Concentrations of DNA Extracted from GTA Particles Before and After Amplification. Wells 2 & 3, 4 & 5, and 6 & 7 are true replicates (see Figure 8).

		Qubit concentrations (ug/ µl)	
Sample	**Well #**	**Before amplification**	**After amplification**
R. pomeroyi (RpGTA)	2	0.686	11.20
R. pomeroyi (RpGTA)	3	0.350	9.48
R. denitrificans (RdGTA)	4	0.322	11.00
R. denitrificans (RdGTA)	5	0.132	2.36
R. nubinhibens (RnGTA)	6	<0.50	6.08
R. nubinhibens (RnGTA)	7	<0.50	2.78

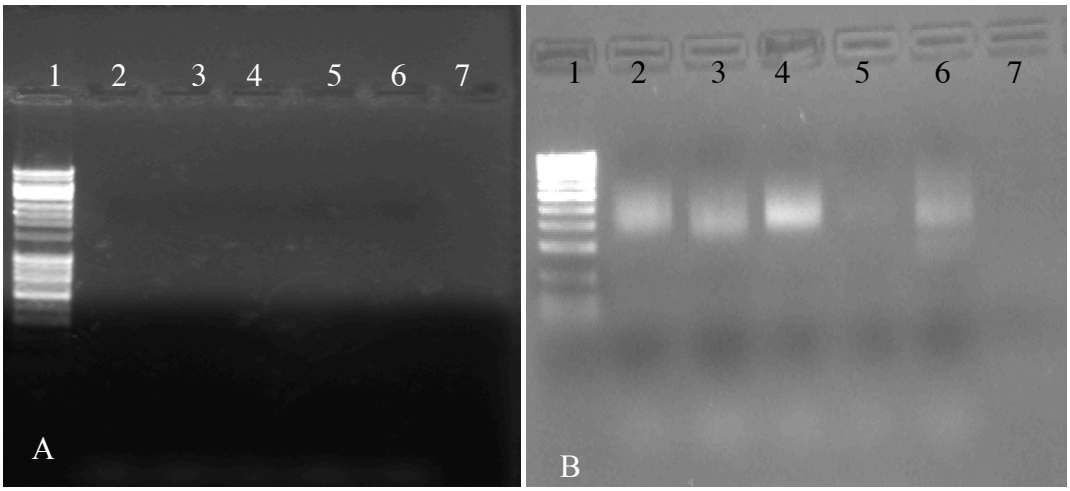

Figure 8. 2% Agarose Gel Electrophoresis of Formamide Extracted DNA from GTA Particles. (A) GTA packaged DNA before amplification, well 1: 100 bp ladder, well 2-3: *R. pomeroyi* true duplicates, well 4-5: *R. denitrificans* true duplicates, well 6-7: *R. nubinhibens* true duplicates. (B) GTA packaged DNA after amplification, well 1: 100 bp ladder, well 2 – 3: *R. pomeroyi* true duplicates, well 4-5: *R. denitrificans* true duplicates, well 6-7: *R. nubinhibens* true duplicates.

Each of the three sequenced bacterial genomes used in this study was screened for possible prophages using PHASTER (PHAge Search Tool Enhanced Release, www.phaster.ca) (90). Since induced prophages and GTA particles can co-purify, the sequences of the GTA contents were filtered to remove sequences originating from inducible prophages identified by PHASTER. The remaining sequences were mapped against the wild-type reference genomes (Figures 9, 10, and 11).

Finally, the cutoff value to determine the overrepresented and highly mapped genes was set at 90th percentile of gene frequencies. Those genes were annotated and analyzed for enrichment in biological processes and molecular functions using topGO (91). The results revealed that the marine GTA particles of all 3 strains studied here were enriched in group of genes encoding for processes playing a role in lipids and amino acid biosynthesis, transporters, signal transduction and other processes involved in metabolic flexibility (Tables 2, 3, and 4). In future studies, a control should be included to exclude any amplification or sequencing biases, this could be achieved by preparing a bacterial DNA using the same library preparation methods that were used to prepare the GTA libraries. Also, another control could be following the same amplification and sequencing protocol to analyze RcGTA packaged DNA.

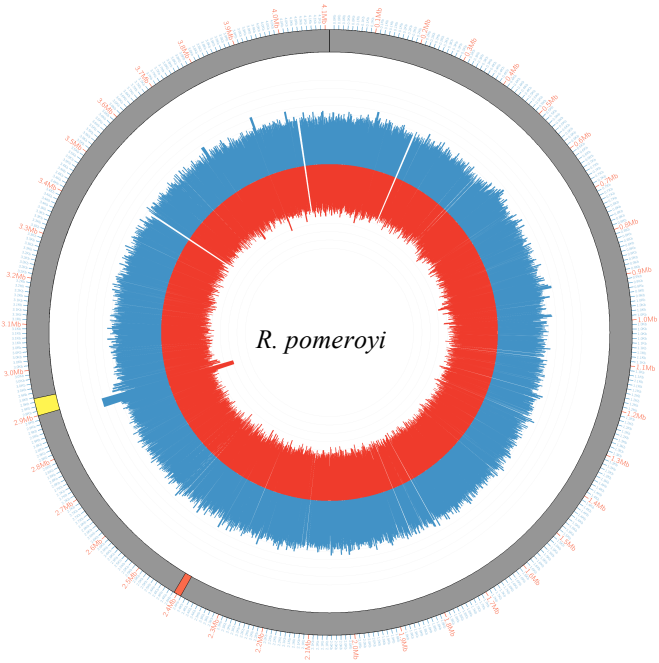

Figure 9. *R. pomeroyi* GTA-Packaged DNA (RpGTA) Mapped Against Its Whole Bacterial Genome. The outer grey circle represents the bacterial chromosome (0 to 4.1 Mb) and the inner histograms represent GTA reads, true replicates are represented in blue and red (0 to 5 mapped reads). The orange band indicates the GTA gene cluster and the yellow band indicates possible identifiable prophages.

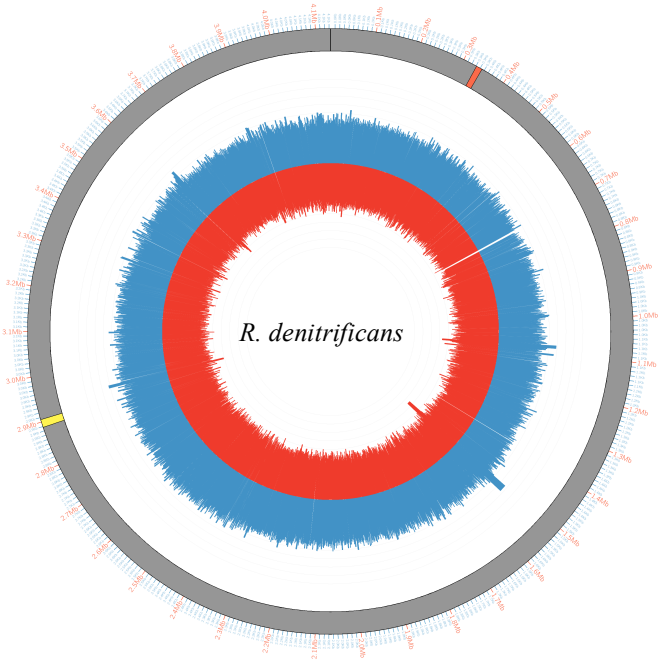

Figure 10. *R. denitrificans* GTA-Packaged DNA (RdGTA) Mapped Against Its Whole Bacterial Genome. The outer grey circle represents the bacterial chromosome (0 to 4.1 Mb) and the inner histograms represent GTA reads, true replicates are represented in blue and red (0 to 5 mapped reads). The orange band indicates the GTA gene cluster and the yellow band indicates possible identifiable prophages.

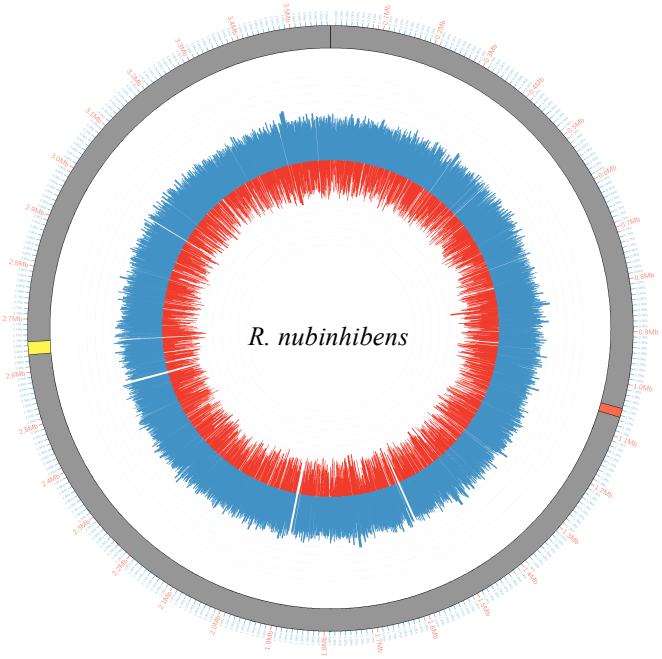

Figure 11. *R. nubinhibens* GTA-Packaged DNA (RnGTA) Mapped Against Its Whole Bacterial Genome. The outer grey circle represents the bacterial chromosome (0 to 3.5 Mb) and the inner histograms represent GTA reads, A true replicates are represented in blue and red (0 to 5 mapped reads). The orange band indicates the GTA gene cluster and the yellow band indicates possible identifiable prophages.

Examining the sequences revealed that the packaging process is not completely random as described previously (41, 42, 50). Rather, it looks like some group of genes were enriched or preferentially packaged over other group of genes and the processes represented appears to overlap across strains. While it's clear that certain group of genes are preferentially packaged, there is also a good representation of packaging the whole host genome.

A hypothesis by *Kristensen et al*. (92) suggested that GTAs might preferably package "cloud" genes or the least conserved genes. Cloud genes are the least conserved genes that are categorized as non-essential to the cell, when knocked out the cell is still viable but loses an accessory function. Non-essential genes are defined as the genes that are redundant and has a dispensable function (93). In this study while examining the DNA packaged in GTA particles, the data seem to provide a valid evidence for this hypothesis.

A study examining the sequences of double stranded DNA (dsDNA) viral-like particles showed that there are about 2500 viral genotypes in 100 liters of seawater (94). Many of the sequences had no homology with any of the known bacterial or other bacteriophage genes (94–96). These findings led some scientists to hypothesize that known and characterized viruses may not be representative of marine viral metagenomes (also known as viromes). In this light, a study bioinformatically analyzing the marine dsDNA virome, suggested that it might be dominated by viral-like particles possibly including GTAs (92), moreover, suggesting that GTA-packaged DNA is specifically a collection of poorly conserved, or "cloud", bacterial genes.

Escherichia coli is the most well-known and extensively studied bacterium and, researchers have studied every gene of its genome and the associated functions. The profiling of *E. coli* chromosome (PEC) database (https://shigen.nig.ac.jp/ecoli/pec/) characterizes the *E. coli* genome according to the function of each gene. This database also classifies each gene as

essential (302 genes) or non-essential (4,439 genes) based on evidence from published experiments and other criteria based on general function. Since the PEC database classifies the *E. coli* genes in a GO format, a search of some of the genes that are significantly enriched within the GTA particles in this study was performed against the *E. coli* profiling database. This analysis was conducted to see if the overrepresented functions within GTA-packaged genes are considered essential or non-essential. One example is the gene of lysine biosynthesis (GO ID: 0009089), which has multiple homologs in *E. coli*'s genome, mostly of which are classified as non-essential genes.

Here, the results demonstrate that under laboratory settings the significantly enriched or overrepresented group of genes packaged within the GTA particles of the marine isolates *R. nubinhibens, R. pomeroyi* and *R. denitrificans* are mostly non-essential genes, providing some preliminary support for the hypothesis that marine GTAs may preferentially package non-essential or "cloud" genes (92) (Tables 2, 3, and 4). The sequencing of the DNA packaged within GTAs of *R. capsulatus* failed to support this hypothesis (50). This difference may exist because this species originates from a different, non-marine environment, living under different selective pressures. On the contrary, this research provides an evidence that marine GTAs may not behave in a similar manner to those produced by *R. capsulatus*.

Considering all evidence, the enrichment in the packaging process with such groups of cloud genes that are metabolically significant demonstrate that GTAs could provide a mechanism of gene exchange that supplies the marine community with metabolic flexibility to access a wider verity of non-essential genes to overcome fluctuations in the marine environment.

Table 2. GO Term Analysis of Significantly Overrepresented Gene Categories Packaged by RpGTA Showing Enrichment in Biological Processes and Molecular Functions. The analysis includes the 90th percentile of gene frequencies across true duplicates combined, based on Fisher's test (P-value cutoff less than 0.05) to assess whether the annotated gene with known GO term is significantly higher than expected by chance taking in consecration all genes that were packaged.

R. pomeroyi					
Biological Processes					
GO ID	**GO Term**	**Annotated**	**Significant**	**Expected**	**P-value**
GO:0009061	anaerobic respiration	11	4	1	0.0064
GO:0031460	glycine betaine transport	3	2	0.27	0.0232
GO:0009089	lysine biosynthetic process via diaminopimelate	8	3	0.73	0.0294
GO:0000160	phosphorelay signal transduction system	49	9	4.45	0.0297
GO:0016539	intein-mediated protein splicing	4	2	0.36	0.0436
GO:0044209	AMP salvage	4	2	0.36	0.0436
GO:0006355	regulation of transcription, DNA-templated	266	33	24.17	0.0482
Molecular Function					
GO:0008863	formate dehydrogenase (NAD+) activity	7	4	0.63	0.0018
GO:0047121	isoquinoline 1-oxidoreductase activity	2	2	0.18	0.0081
GO:0008134	transcription factor binding	6	3	0.54	0.0118
GO:0004642	phosphoribosylformylglycinamidine synthase activity	3	2	0.27	0.0228
GO:0015418	quaternary-ammonium-compound-transporting ATPase activity	3	2	0.27	0.0228

Table 3. GO Term Analysis of Significantly Overrepresented Gene Categories Packaged by RdGTA Showing Enrichment in Biological Processes and Molecular Functions. The analysis includes the 90th percentile of gene frequencies across true duplicates combined, based on Fisher's test (P-value cutoff less than 0.05) to assess whether the annotated gene with known GO term is significantly higher than expected by chance taking in consecration all genes that were packaged.

R. denitrificans					
Biological Processes					
GO ID	**GO Term**	**Annotated**	**Significant**	**Expected**	**P-value**
GO:0009245	lipid A biosynthetic process	7	4	0.61	0.0016
GO:0044208	'de novo' AMP biosynthetic process	2	2	0.17	0.0076
GO:0006817	phosphate ion transport	6	3	0.52	0.0108
GO:0055129	L-proline biosynthetic process	3	2	0.26	0.0215
GO:0001407	glycerophosphodiester transport	3	2	0.26	0.0215
GO:0015794	glycerol-3-phosphate transport	3	2	0.26	0.0215
GO:0009306	protein secretion	15	4	1.31	0.0359
GO:0006935	chemotaxis	10	3	0.87	0.0498
Molecular Function					
GO:0000156	phosphorelay response regulator activity	4	3	0.34	0.0023
GO:0051082	unfolded protein binding	11	4	0.94	0.0108
GO:0050567	glutaminyl-tRNA synthase (glutamine-hydrolyzing) activity	3	2	0.26	0.0208
GO:0005525	GTP binding	27	6	2.32	0.0238
GO:0016787	hydrolase activity	530	53	45.52	0.0346
GO:0003984	acetolactate synthase activity	4	2	0.34	0.0392
GO:0003924	GTPase activity	16	4	1.37	0.0422

Table 4. GO Term Analysis of Significantly Overrepresented Gene Categories Packaged by RnGTA Showing Enrichment in Biological Processes and Molecular Functions. The analysis includes the 90th percentile of gene frequencies across true duplicates combined, based on Fisher's test (P-value cutoff less than 0.05) to assess whether the annotated gene with known GO term is significantly higher than expected by chance taking in consecration all genes that were packaged.

R. nubinhibens					
Biological Processes					
GO ID	**GO Term**	**Annotated**	**Significant**	**Expected**	**P-value**
GO:0006779	porphyrin-containing compound biosynthetic process	13	3	0.39	0.005
GO:0006428	isoleucyl-tRNA aminoacylation	1	1	0.03	0.03
GO:0043335	protein unfolding	1	1	0.03	0.03
GO:0019509	L-methionine biosynthetic process from methylthioadenosine	1	1	0.03	0.03
GO:0019357	nicotinate nucleotide biosynthetic process	1	1	0.03	0.03
GO:0046901	tetrahydrofolylpolyglutamate biosynthetic process	1	1	0.03	0.03
Molecular Function					
GO:0016887	ATPase activity	126	8	3.68	0.027
GO:0008780	acyl-[acyl-carrier-protein]-UDP-N-acetylglucosamine O-acyltransferase activity	1	1	0.03	0.029
GO:0004516	nicotinate phosphoribosyltransferase activity	1	1	0.03	0.029
GO:0008691	3-hydroxybutyryl-CoA dehydrogenase activity	1	1	0.03	0.029
GO:0008534	oxidized purine nucleobase lesion DNA N-glycosylase activity	1	1	0.03	0.029
GO:0004754	saccharopine dehydrogenase (NAD+, L-lysine-forming) activity	1	1	0.03	0.029
GO:0004612	phosphoenolpyruvate carboxykinase (ATP) activity	1	1	0.03	0.029
GO:0003870	5-aminolevulinate synthase activity	1	1	0.03	0.029
GO:0004326	tetrahydrofolylpolyglutamate synthase activity	1	1	0.03	0.029
GO:0017061	S-methyl-5-thioadenosine phosphorylase activity	1	1	0.03	0.029
GO:0008381	mechanically-gated ion channel activity	1	1	0.03	0.029
GO:0016887	ATPase activity	126	8	3.68	0.027

Biological Activity of GTA Particles

Once it was confirmed that *R. nubinhibens*, *R. pomeroyi* and *R. denitrificans* not only have the GTA gene cluster but also produce particles consistent with GTAs during stationary growth phase, it is appropriate to investigate the function of GTAs in the marine environment.

In this section, the intention was primarily to study the overall function of RpGTA and RdGTA particles, but due to difficulties counting the particles and determining the MOI (GTAs dose), RnGTA particles were also studied in following sections.

Initial gene transfer experiments via the technique of filter mating were conducted by transferring cell-free purified GTAs to recipient cultures. The particles were derived from

chromosomal streptomycin resistant spontaneous mutants of *R. pomeroyi* and *R. denitrificans* and harvested at the time of maximal production then DNase treated to eliminate the possibility of natural transformation. Here, the recipient cultures were either wild-type strain cells or natural marine communities (seawater samples) to observe the potential and function of GTAs. In both cases, the recipient cultures were divided into replicates of treatments (active GTAs) and controls (heat-inactivated GTAs and SM buffer). Finally, all samples were plated on both selective (ASWJP+PY media with streptomycin) and nonselective media (ASWJP+PY only) to determine the total culturable or viable cell abundance as colony forming units (CFUs) per ml.

Within Species Treatment: Antibiotic Resistant Strain as Donor and Wild-Type Strain as a Recipient

By comparing the effect of RpGTA treatment (derived from streptomycin resistant mutant *R. pomeroyi*) on total CFUs (number of viable cells) of wild-type strain *R. pomeroyi* plated on selective and nonselective media, there was a significant viable colony increase on both media (P = 1.30739E-7, 0.00018, respectively). Most importantly, there was a statistically significant difference between active GTA treatment and heat-inactivated control on total CFUs when the cells were grown on nonselective and selective media (P = 0.0004, 0.00003, respectively). Additionally, the heat treatment successfully inactivated the RpGTA particles as there was no significant difference (P = 0.83045) between the counts of cells treated with heat-inactivated RpGTA particles and the SM buffer controls on both media. Under selective pressure, the total CFUs on the selective media was elevated which means the cells were able to thrive in the presence of streptomycin, indicating that the GTA particles may have successfully packaged the spontaneously introduced streptomycin resistance marker (Figure 12).

Additionally, when the same experiment was performed to test the effect of RdGTA on its wild-type producing host cells, interestingly, there was a significant increase in CFUs with the presence of the RdGTA particles (P = 0.00003) while there was no significant increase in CFUs between any of the treatment groups in the presence of streptomycin selective pressure (P = 0.20397) (Figure 13). This response was also observed in an unpublished study (McDaniel *et al*.), where GTA particles of *Ruegeria mobilis* 45A6 (RmGTA) with an antibiotic marker stimulated the bacterial viable plate counts in the absence of antibiotic selection in media. Since the viable bacterial counts are observed to be elevated and stimulated when the recipient cells receive GTA treatments compared to the controls in the absence of antibiotic pressure, more investigation is clearly needed in this area.

Between Species Treatment: Antibiotic Resistant Strain as Donor and Natural Marine Communities as a Recipient

Based on previous experiments documenting that GTAs have a large potential to catalyze HGT in the ocean (78), here, the effect of GTA particle treatment on natural marine bacterial assemblages was examined.

For this experiment, GTA particles again were derived from streptomycin resistant mutants of *R. pomeroyi* and *R. denitrificans* and were harvested at the time of maximal production for each strain. Active GTA particles along with heat-inactivated GTAs and SM buffer controls were used to treat the bacterial assemblages in seawater samples by the filter mating technique.

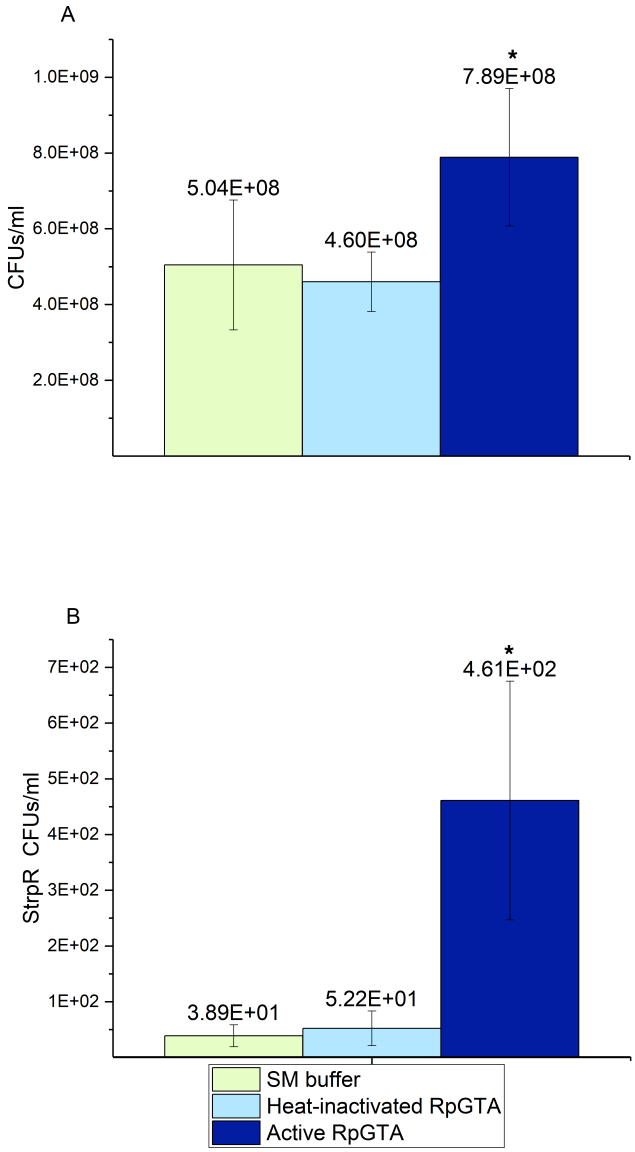

Figure 12. Within Species Treatment: Antibiotic Resistant Strain as Donor (RpGTA) & *R. pomeroyi* Wild-Type Strain as a Recipient. RpGTA treatment effect on *R. pomeroyi* wild-type strain (A) Colony counts on nonselective, (B) Selective (Streptomycin) agar plates.

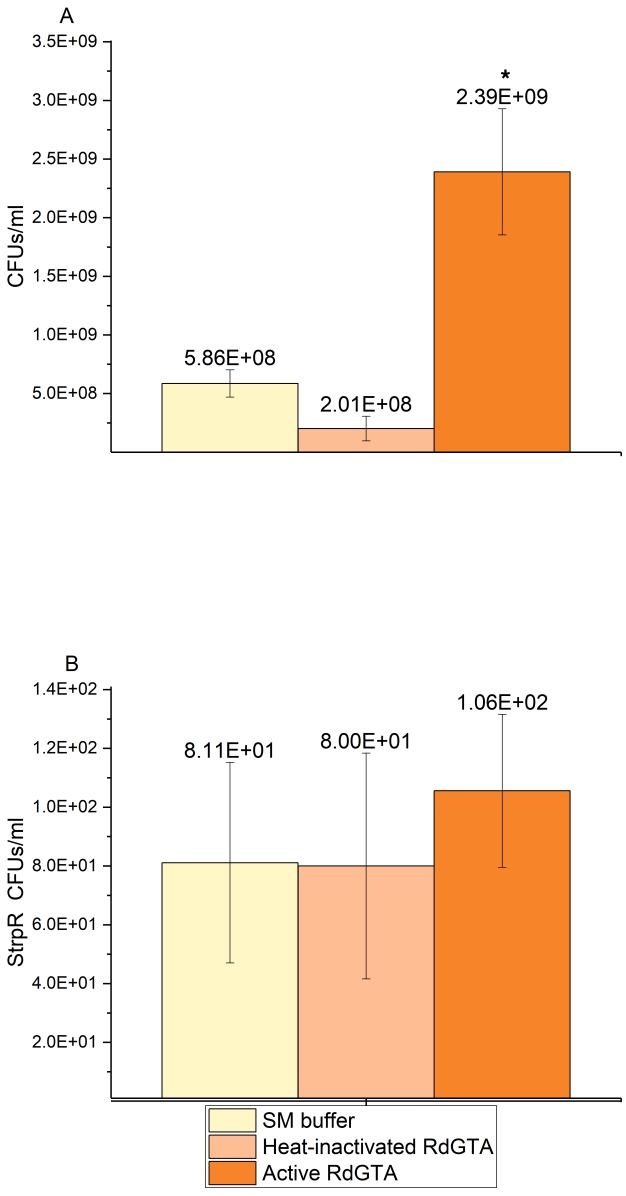

Figure 13. Within Species Treatment: Antibiotic Resistant Strain as Donor (RdGTA) & *R. denitrificans* Wild-Type Strain as a Recipient. RdGTA treatment effect on *R. denitrificans* wild-type strain (A) Colony counts on nonselective, (B) Selective (Streptomycin) agar plates.

By looking only at the total bacterial abundances as CFUs when the seawater samples were incubated with RpGTA then plated on selective and nonselective media, there was a significant difference among the replicates of RpGTA treated, heat-inactivated and SM buffer control (P = 9.57085E-6, 1.77E-17, respectively). After RpGTA treatment, a decrease in viable counts was observed when the cells were grown without antibiotic in their growth media, however, in the presence of antibiotic in the growth media there was an increase in the viable counts. This observation indicates the importance of a selective pressure detected by stimulation and/or prevention of the growth of certain species but not others (Figure 14).

Again, when a similar experiment was performed using a natural microbial assemblage incubated with RdGTA particles (Figure 15), there was no significant relationship between total CFUs of samples incubated with active RdGTAs and controls on selective media (P = 0.14615) and a significant relationship between CFUs of RdGTA treated and controls in the absence of the selective pressure among the groups (P = 0.00219). While it is clear that the GTA particles are having a functional effect, we cannot determine the mechanism based only on total CFUs because we are dealing with a complex natural community containing different species with differing functional capacities.

By closely examining the colony morphologies, there was a clear difference between the bacterial species that were culturable when seawater samples were treated with RpGTA and RdGTA compared to both controls. Therefore, 16S rRNA gene amplification and sequencing were performed on each distinct colony morphotype to estimate the identify the culturable populations. Table 5 lists all species that grew with each treatment. Here, under the tested laboratory settings, it was observed that some species belonging to phylum *Flavobacterales* were more responsive to active GTA treatment than others, causing microbial community shift in

seawater samples (Table 5 and Figure 16). A similar observation was recorded in a previous study (78), providing support for these findings.

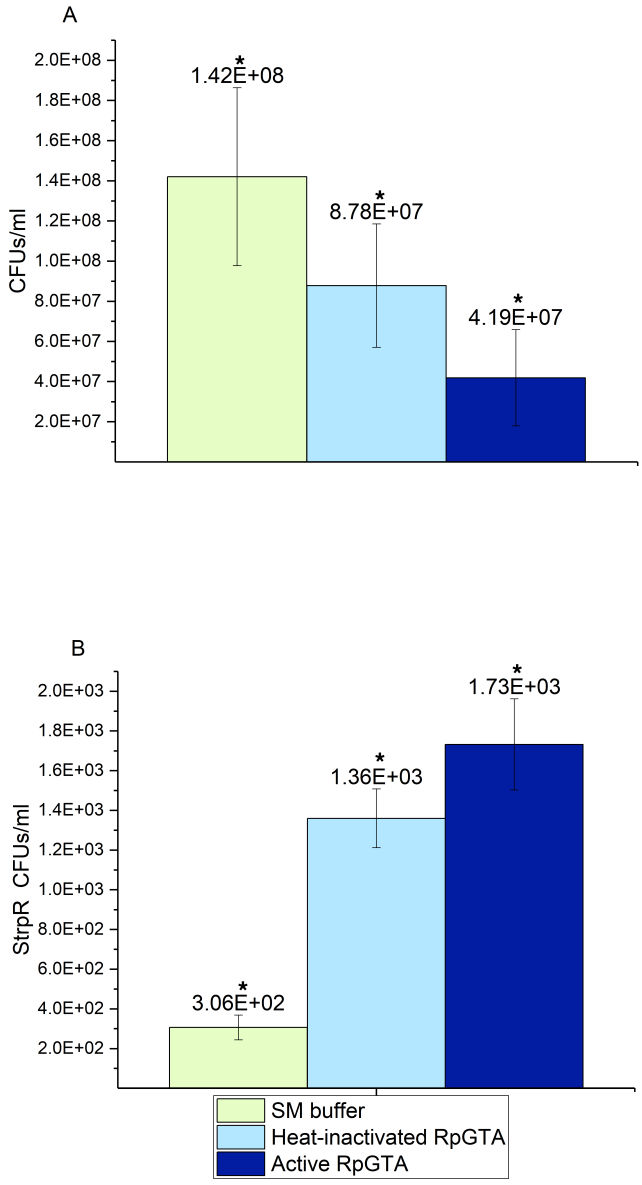

Figure 14. Between Species Treatment: Antibiotic Resistant Strain as Donor (RpGTA) & Natural Marine Communities as a Recipient. RpGTA treatment effect on natural marine communities**,** (A) colony counts on nonselective, (B) selective (Streptomycin) agar plates.

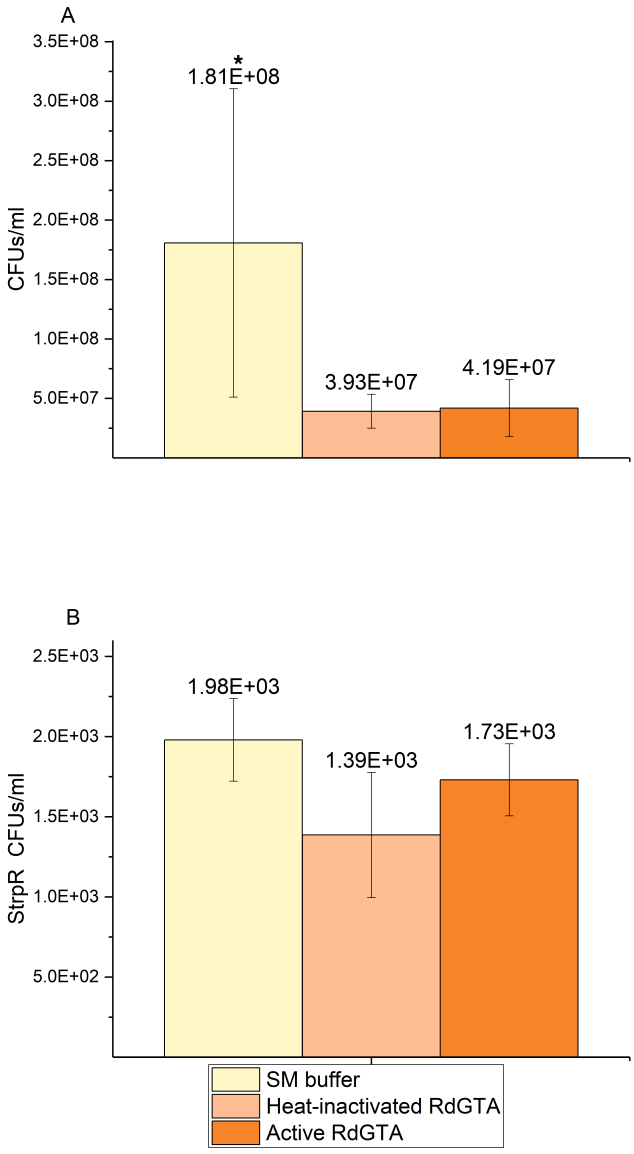

Figure 15. Between Species Treatment: Antibiotic Resistant Strain as Donor (RdGTA) & Natural Marine Communities as a Recipient. RdGTA treatment effect on natural marine communities (A) colony counts on nonselective, (B) selective (Streptomycin) agar plates.

Table 5. List of 16S rRNA BLAST Predicted Identities Obtained for Culturable Bacterial Populations in Seawater Samples in the Absence of Selective Pressure. (see Figure 16)

		16S identification	Phylogeny (Class)	Identity %	Morphology (Pigmentation)
RpGTA	**Active**	No amplification			Orange
		Tenacibaculum mesophilum	Flavobacteria	99%	Yellow
		No amplification			Orange
		Tenacibaculum mesophilum	Flavobacteria	98%	Yellow
		Pseudoalteromonas piscicida	Gamma Proteobacteria	98%	Orange
	SM buffer	*Marinomonas basaltis*	Gamma Proteobacteria	96%	Ivory
		Vibrio alginolyticus	Gamma Proteobacteria	99%	Cream-yellowish
		Aliivibrio fischeri	Gamma Proteobacteria	98%	Ivory
		Vibrio alginolyticus	Gamma Proteobacteria	98%	Cream-yellowish
	Heat-inactivated	*Vibrio alginolyticus*	Gamma Proteobacteria	97%	Cream-yellowish
		Vibrio pelagius	Gamma Proteobacteria	98%	Ivory
		Vibrio alginolyticus	Gamma Proteobacteria	98%	Cream-yellowish
		Vibrio chagasii	Gamma Proteobacteria	97%	Ivory
		Aliivibrio fischeri	Gamma Proteobacteria	98%	Ivory
RdGTA	**Active**	*Shewanella fidelis*	Gamma Proteobacteria	99%	Orange
		No amplification			Orange
		Tenacibaculum mesophilum	Flavobacteria	98%	Yellow
		No amplification			Orange
		Tenacibaculum mesophilum	Flavobacteria	98%	Yellow
		Pseudoalteromonas shioyasakiensis	Gamma Proteobacteria	98%	Ivory
	SM buffer	*Vibrio sp. strain MS1002*	Gamma Proteobacteria	97%	Ivory
		Vibrio alginolyticus	Gamma Proteobacteria	97%	Ivory
		Vibrio parahaemolyticus	Gamma Proteobacteria	97%	Ivory
		Uncultured bacterium isolate DCPISO3	Gamma Proteobacteria	99%	Ivory
		Vibrio natriegens	Gamma Proteobacteria	96%	Ivory
	Heat-inactivated	*Pseudoalteromonas spongiae*	Gamma Proteobacteria	99%	Pale orange
		Vibrio campbellii	Gamma Proteobacteria	97%	Ivory
		Vibrio harveyi	Gamma Proteobacteria	96%	Ivory
		Vibrio mexicanus	Gamma Proteobacteria	96%	Ivory
		Vibrio pelagius	Gamma Proteobacteria	98%	Ivory

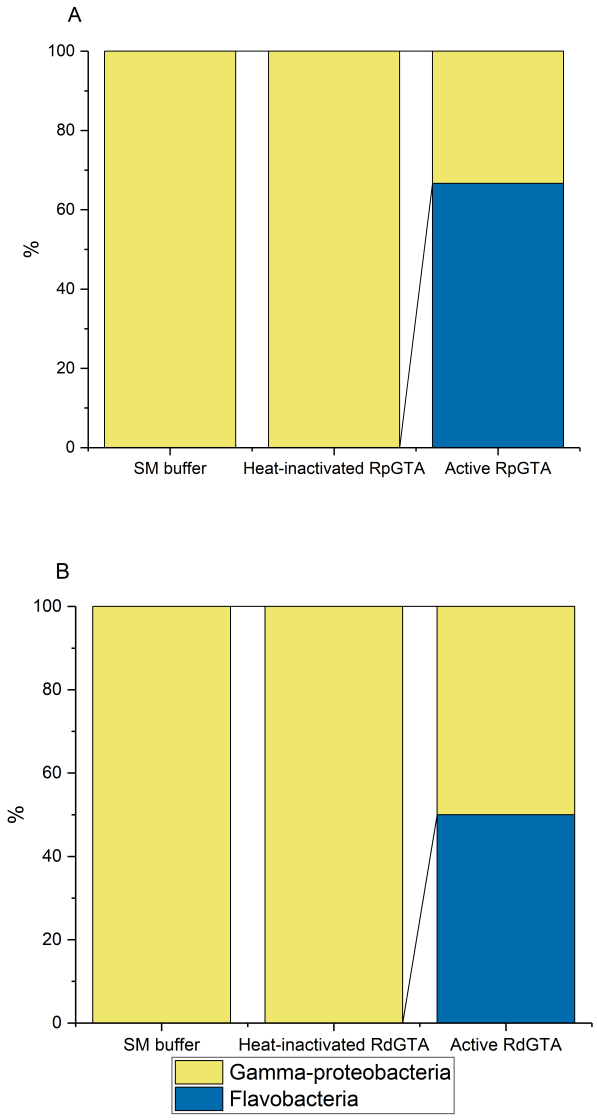

Figure 16. Representation of 16S rRNA Sequences Relative Abundance at Class Level. (A) GTAs produced by *R. pomeroyi* (RpGTA), (B) GTAs produced by *R. denitrificans* (RdGTA).

Multiple Recipient Species Treatment: Testing the Effect of RnGTA Treatment on Stimulating the Growth of Multiple Recipients in Their Exponential Growth Phase

Initial experiments were performed using GTA particles produced by *R. pomeroyi* and *R. denitrificans*. The GTA particles were visible by SYBR® Gold staining but were very faint because of their small size, hence, impossible to count. This limitation precluded the accurate calculation of the GTA dose, or multiplicity of infection (MOI) used in experiments. Due to the ability to enumerate the RnGTA particles produced by the wild-type strain *R. nubinhibens* enabling us to calculate MOI, RnGTA particles were utilized in further studies.

After examining the trends and the preliminary findings in this study**,** the data suggested that the effect of GTAs on marine bacterial communities is not arbitrary; rather the effect of GTAs is selective with some species/classes having more ability to respond to GTAs treatment than others. Additionally, the effect was observed on viable colony counts in the absence of antibiotic selection. To investigate this phenomenon further, GTAs produced by *R. nubinhibens* wild-type strain were harvested at the time of maximal production to test the effect of RnGTA treatment on stimulating the growth of different single recipients in their exponential growth phase including: (*R. nubinhibens* ISM*, R. pomeroyi DSS-3, R. denitrificans* OCh114 and *Vibrio parahaemolyticus* St 16). This was achieved using the same approach described above to determine the bacterial CFUs representing viable and culturable bacteria. The effect of GTA dose, or estimated MOI was also investigated.

Since *R. nubinhibens* is found experimentally to contain inducible prophages, additional control of induced prophages was used to rule out any possibility that an observed effect could be due to the presence of prophages instead of RnGTA. The recipient cultures were divided into replicates of treatments (active RnGTA) and controls (heat-inactivated RnGTA, induced prophages and SM buffer).

Figures 17 to 20 show that when RnGTA particles were transferred to *R. pomeroyi*, *R. denitrificans*, and *V. parahaemolyticus* there was no statistically significant difference in viable cell counts for any of the recipients at any of the tested three RnGTA doses (Table 6), (P = 0.20887, 0.86618, and 0.95761, respectively). In contrast, when RnGTA particles were added to its producing wild-type strain *R. nubinhibens*, there was statistically significant increase in viable cell counts in both true duplicate experiments at mid MOI (3.18-4.82) (P = 0.00361 and 0.00217, respectively). Also, all results indicate that the effect is not due to the inducible prophages since the P-value for this treatment = 0.88757, which is not significantly different compared to the other controls and is significantly different compared to the active RnGTA treatment (P = 0.01403).

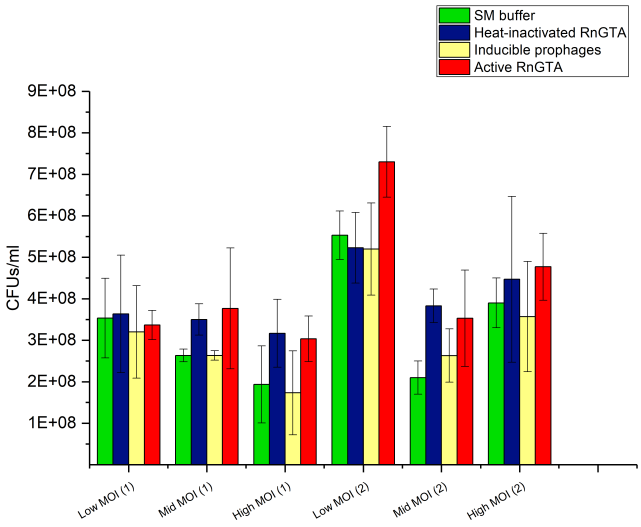

Figure 17. RnGTA Treatment Effect on *R. pomeroyi* Wild-Type Strain. Representing colony counts on nonselective agar plates (CFUs). True duplicate experiments (1) and (2) did not result in a significant difference (P = 0.20887) in viable counts compared to controls at any of the tested MOI.

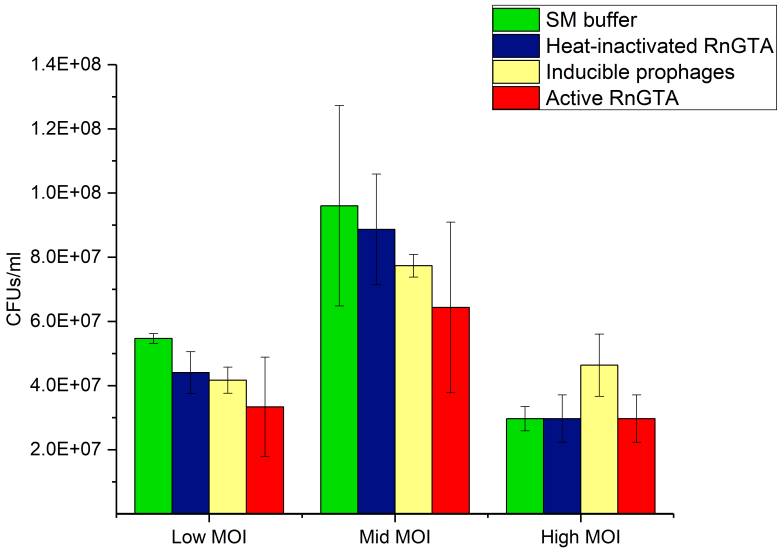

Figure 18. RnGTA Treatment Effect on *R. denitrificans* Wild-Type Strain. Representing colony counts on nonselective agar plates (CFUs). This experiment did not result in a significant difference (P = 0.86618) in viable counts compared to controls at any of the tested MOI.

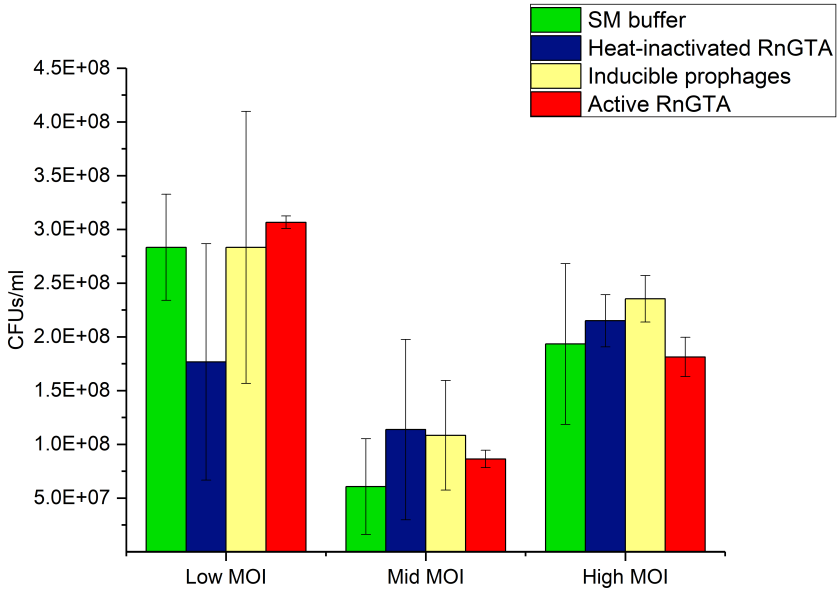

Figure 19. RnGTA Treatment Effect on *V. parahaemolyticus* Wild-Type Strain. Representing colony counts on nonselective agar plates (CFUs). This experiment did not result in a significant difference (P = 0.95761) in viable counts compared to controls at any of the tested MOI.

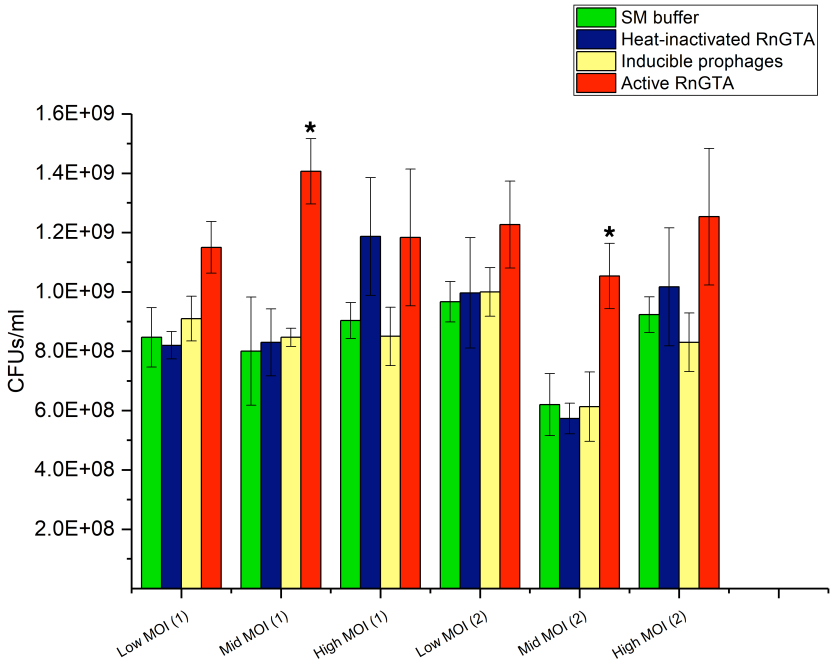

Figure 20. RnGTA Treatment Effect on *R. nubinhibens* Wild-Type Strain. Representing colony counts on nonselective agar plates (CFUs/). True duplicate experiments (1) and (2) resulted in a significant difference in viable counts at Mid MOIs (P = 0.00361 and 0.00217, respectively) in compared to controls at any of the tested MOI.

Since treating the cells with RnGTA resulted in statistically significant growth stimulation of the producing strain *R. nubinhibens*, further investigations were done to verify this effect. This was achieved by tracking the growth of *R. nubinhibens* wild-type strain when the cells were treated with active RnGTA particles or controls; the cell's optical density (OD) was measured at 600 nm every two hours until the growth declined (Figure 21). The growth curves were significantly diverged at 4 hours and 14 hours of growth. (P = 0.02086 and 0.03736, between 4 and 14 hours).

Table 6. Experimental MOIs. MOI = (GTA counts * volume) divided by (recipient cell counts * volume).

Figure # / Recipient	MOI	GTA counts * vol	GTA conc. method	Cell counts * vol
Figure 17 *R. pomeroyi* (1)	Low 0.18	1.27E+07	PEG	6.90E+07
Figure 17 *R. pomeroyi* (1)	Mid 1.84	1.27E+08	PEG	6.90E+07
Figure 17 *R. pomeroyi* (1)	High 18.4	1.27E+09	PEG	6.90E+07
Figure 17 *R. pomeroyi* (2)	Low 0.19	8.67E+06	Amicon	4.50E+07
Figure17 *R. pomeroyi* (2)	Mid 1.93	8.67E+07	Amicon	4.50E+07
Figure 17 *R. pomeroyi* (2)	High 19.2	8.67E+08	Amicon	4.50E+07
Figure 18 *R.denitrificans*	Low 0.24	1.27E+07	PEG	5.33E+07
Figure 18 *R.denitrificans*	Mid 2.38	1.27E+08	PEG	5.33E+07
Figure 18 *R.denitrificans*	High 23.8	1.27E+09	PEG	5.33E+07
Figure 19 *V. parahaemolyticus*	Low 0.13	8.67E+06	Amicon	6.90E+07
Figure 19 *V. parahaemolyticus*	Mid 1.26	8.67E+07	Amicon	6.90E+07
Figure 19 *V. parahaemolyticus*	High 12.5	8.67E+08	Amicon	6.90E+07
Figure 20 *R.nubinhibens* (1)	Low 0.32	5.53E+06	PEG	1.74E+07
Figure 20 *R.nubinhibens* (1)	Mid 3.18	5.53E+07	PEG	1.74E+07
Figure 20 *R.nubinhibens* (1)	High 31.7	5.53E+08	PEG	1.74E+07
Figure 20 *R.nubinhibens* (2)	Low 0.48	3.97E+07	Amicon	8.23E+07
Figure 20 *R.nubinhibens* (2)	Mid 4.82	3.97E+08	Amicon	8.23E+07
Figure 20 *R.nubinhibens* (2)	High 48.2	3.97E+09	Amicon	8.23E+07

In conjunction with the growth curves, at time point (T = 6), when the culture density of the samples was significantly different, a sample of each flask was tested by measuring the total metabolic activity by the MTT assay that measures the conversion of a soluble tetrazolium dye to its insoluble formazan product by metabolically active cells that is measured at 570 nm (97) (Figure 22). The metabolic activity of the cells when treated with active RnGTA particles was statistically significantly higher (P = 0.00760) than the controls.

Also, at the same time point (T = 6), samples were plated to determine viable cell counts (Figure 23). The CFUs indicated significant increase when the cells were treated with active RnGTA particles (P = 0.00054). This RnGTA treatment resulted in an abundance of 2.93E+07 CFUs/ml compared to all controls that resulted in abundance ranging between 1.46-1.71E+07 CFUs/ml.

Finally, from the same RnGTA preparation, the effect of RnGTA treatment was tested on natural populations in seawater samples (Figure 24). Although the counts are elevated when the cells were treated with RnGTA, the results were statistically borderline, non-significant (ANOVA, P = 0.08596) and significant (Neuman-Keuls, 0.04134).

Overall, the results show that RnGTA particles were able to stimulate the growth within the same species, in this case *R. nubinhibens*, taking in consideration that the observations took place *in vitro* where the nutrients are available and accessible. Fortunately, the annotated sequences of the DNA packaged within the GTA particles could provide a possible interpretation for the culture based observations, since the particles seem to package the non-essential genes for metabolism flexibility advantage, hence stimulating their own growth specifically.

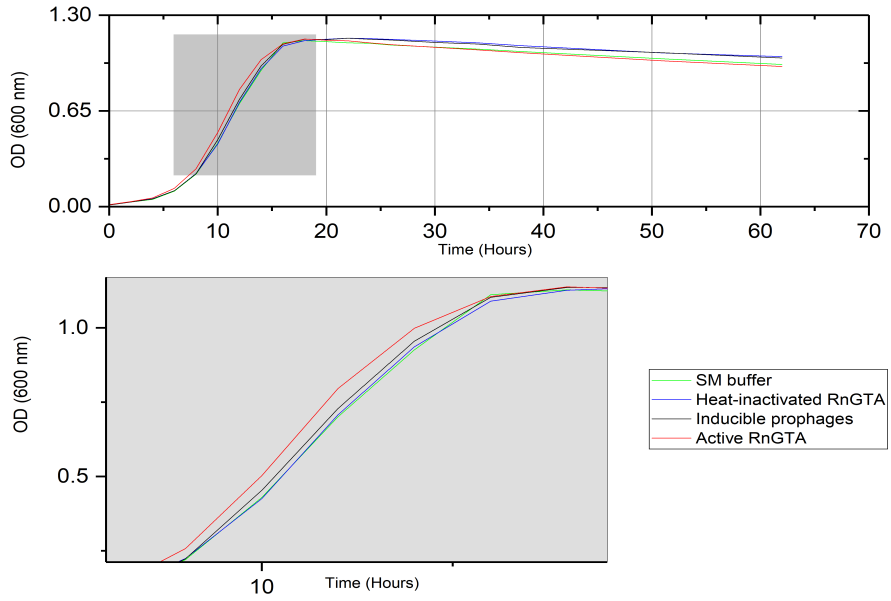

Figure 21. Growth Curve Demonstrating RnGTA Treatment Effect on *R. nubinhibens* Wild-Type Cells.

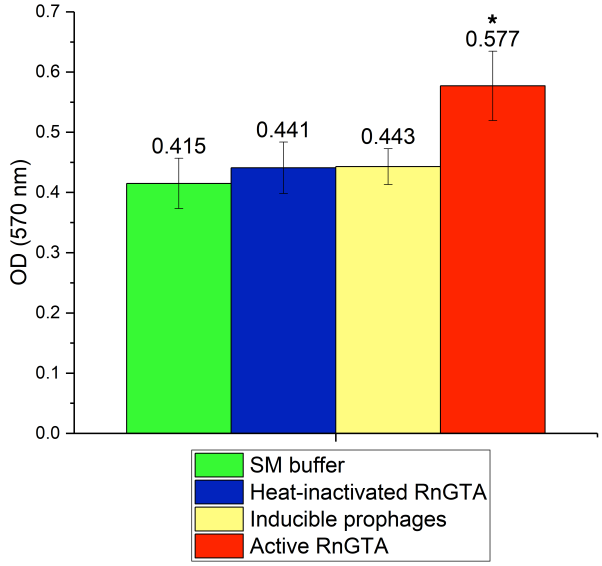

Figure 22. RnGTA Treatment Effect on *R. nubinhibens* Wild-Type Strain: Measuring the Total Metabolic Activity Using MTT Assay. Samples were collected at T = 6 from growth curve in (Figure 21). (P = 0.00760, statistically significant).

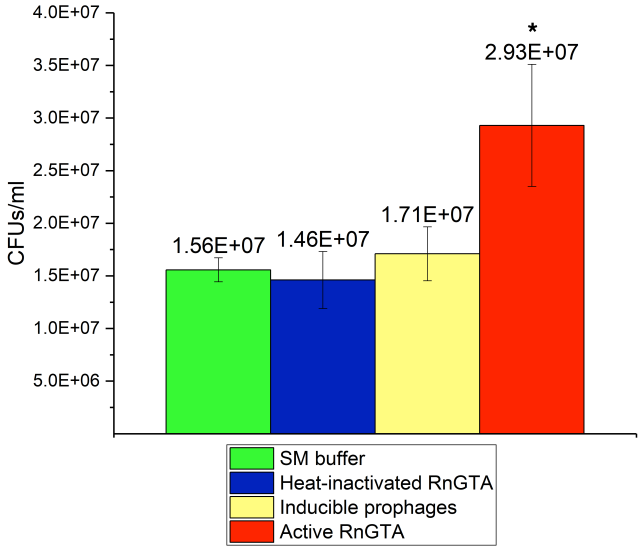

Figure 23. RnGTA Treatment Effect on *R. nubinhibens* Wild-Type Strain: Viable Cell Counts at Time Point T =6. (P = 0.00054, statistically significant).

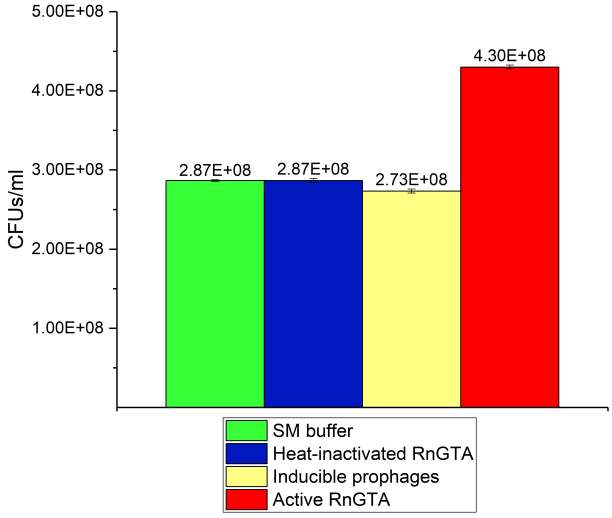

Figure 24. RnGTA Treatment Effect on Natural Populations in Seawater Samples. (P = 0.08596, statistically non-significant).

The situation could be different in natural communities e.g.: seawater, where each species is under a lot of variability. In the oligotrophic oceans, bacterial cells are facing nutrient depletion and stress, and their metabolic capacity varies depending on the environment (98). Also, an important aspect to think about when examining the natural communities, is the cell competence (the ability of a cell to take up free DNA from the surrounding environment) (31). A possible interpretation to what happened in this scenario is that under stressful conditions other species could also respond and interact with the GTA particles of other related strains. Since strains of alpha-proteobacteria that contain the GTA gene cluster make up a significant portion of marine microbiome (69, 70), these results may indicate that the presence of the GTA gene cluster and/or the ability to produce functional GTA particles in most Rhodobacterales members provides a key mechanism to overcome the fluctuations in the marine environment. Additionally, the ability of a strain to produce GTA particles may provide a regulation mechanism for maintaining population adaptation and fitness in such a constantly changing environment.

SIGNIFICANCE AND CONCLUDING REMARKS

The GTA gene cluster, initially discovered in the type strain *R. capsulatus,* has been found in the genomes of most Rhodobacterales members of the alpha-proteobacteria. It is known that GTAs are considered a mechanism of HGT by packaging and transferring random fragments of the donor host's genome. Here, three strains from marine origin that are known to contain the GTA gene cluster were studied by sequencing their GTA-packaged DNA and investigating the biological function of their particles. *R. pomeroyi* and *R. nubinhibens* strains have been already documented to produce GTA particles. In this thesis, *R. denitrificans* has been experimentally observed to produce functional GTA particles. Sequencing, annotating and assembling the GTA-packaged DNA revealed that it appears that these particles are randomly packaging chromosomal genes with significant enrichment towards non-essential group of genes or gene categories that would expand their metabolic capabilities. This means that particles of marine origin may not act like those produced by the well-studied type strain *R. capsulatus*. One possible effect of this kind of packaging was clearly observed in cultures, when the cells that were treated with active GTAs resulted in significant increase in viable cell counts and growth stimulation. Also, it was clear that under laboratory controlled conditions the effect of GTAs seemed to be restricted to members of the same species, providing a genetic and metabolic evidence that GTAs may be a key for microbial diversity and survival in the marine environment. Knowledge gained from this study will help us understand the role of GTAs and HGT mechanisms in the ocean, therefore advancing our knowledge about the evolution and interaction of marine microbes. Most

importantly, this study has expanded what is known about GTAs of marine origin by documenting that *R. denitrificans* not only has the typical GTA gene cluster but also produces particles and that GTAs from different environments could preferentially package specific portions of the genomic DNA, suggesting that it is time to invest in this topic to be able to compare what is known about GTA particles produced by strains of marine origin with GTA particles produced by the model strain *R. capsulatus*.

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APPENDIX A:

ASWJP MEDIA RECIPE

Before starting make the following stock solutions: (99, 100)

Stock	Ingredient	g / 100 ml
#1	KCl	5.5 g
	$NaHCO_3$	1.6 g
#2	KBr	0.8 g
	$ScCl_2$	0.34 g
#3	Sodium Silicate	0.4 g
#4	Sodium Fluoride	0.24 g
#5	NH_4NO_3	0.16 g
#6	Na_2HPO_4	0.8 g
#7	$CaCl_2.2H_2O$	23.8 g

For Stock solution #44 make the following:

Salt	conc. Stock	to make #44
Na_2EDTA	12 g / 200 ml	50 ml / 1000 ml
$FeCl_2.6H_2O$	3.84 g / 20 ml	2 ml / 1000 ml
$MgCl_2.6H_2O$	4.32 g / 20 ml	2 ml / 1000 ml
$CoCl_2.6H_2O$	0.2 g / 20 ml	2 ml / 1000 ml
$ZnCl_2$	0.315 g / 20 ml	2 ml / 1000 ml
$CuCl_2$	4.8 mg / 34.4 ml	2 ml / 1000 ml
H_3BO_3	3.42 g/100ml	10 ml / 1000 ml

To make ASWJP (1 Liter):

1. Add 22.05 g NaCl
   9.8 g $MgSO_4 \cdot 7H_2O$ (4.79 g anhydrous)

2. Bring up to 900 ml and dissolve.

3. Add the following:

Stock	volume
#1	10 ml
#2	10 ml
#3	1 ml
#4	1 ml
#5	1 ml
#6	1 ml
#7	10 ml
#44	10 ml

For ASWJP+PY:

Add 5 g of peptone and 1 g of yeast extract.

For plates add 15 g of bacto-agar.

Bring up to 1 liter and autoclave.