Isolation and Characterization of Novel Marine Bacteriophages from Santa Rosa Island, Florida, Using Local and Nonlocal Bacterial Strains as Hosts

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ABSTRACT
Bacteriophages, or phages, are viruses that infect bacteria and are the most prevalent biological entities in the world. While phage research has continued to develop, the investigation of phages within ecological biology remains in the early stages and requires an extensive database of environmentally derived phages, such as from marine ecosystems. The objectives of this research were to a) determine the local beach bacterial composition and select locally isolated strains as potential bacteriophage hosts, b) determine the efficacy of local bacteria as marine bacteriophage hosts (*Erythrobacter citreus* Pensacola AB and *Microbacterium oleivorans* Pensacola AB), and c) compare the host efficacy between locally isolated bacterial strains and non-local *Microbacterium* isolates (*Microbacterium* sp. Casco Bay and *Microbacterium foliorum* NRRL B-24224). The results suggest that of the two locally isolated bacterial strains tested, *Microbacterium oleivorans* was not a successful host for the scope of this research. In contrast, *Erythrobacter citreus* Pensacola AB was an efficient host across several trials. Of the non-local bacteria, both *Microbacterium* sp. Casco Bay and *Microbacterium foliorum* NRRL B-24224 were successful hosts at isolating phage, although not at the same consistency as *Erythrobacter citreus*. This research concluded that local bacterial strains could be effective for phage hunting and that the overall success of finding local marine phage was not dependent on using local bacterial strains. Four phages, two *M.* sp. Casco Bay phages, one *M. foliorum* phage, and one *E. citreus* phage, were selected for further purification and characterization. The four phage genomes were sequenced to characterize the molecular nature of these marine phages.

KEYWORDS
Microbiome; Bacteriophage; Marine phage; Plaque Assay; Genomic sequencing; Marine ecology; Zinc chloride ($ZnCl_2$) precipitation; PEG (polyethylene glycol) precipitation

INTRODUCTION

Bacteriophage (phage) biology has long been an important research topic in medical microbiology. Over 100 years ago, phage research was popularized by investigating the mechanisms by which phage particles destroy bacterial cells, hence the initial phase of phage therapy. This trend was halted as antibiotics became prevalent and replaced clinical phage research.[1] At the time, the bulk of phage biology was established by studying a small subset of *E. coli* phages such as lambda phage, however, there remained many gaps in the knowledge of phage biology. With the increase in bacterial antibiotic resistance,[2] interest in phage research has resurged in the 21st century.[1] It has been revealed that phages play essential roles in influencing their bacterial hosts' metabolic behaviors by utilizing phage-encoded auxiliary metabolic genes.[3] Such influence in the microbial ecosystem was especially noted in studying marine phages.[3]

The prevalence of phage in the marine environment was highly underestimated, with the original estimated virus-to-bacteria ratio (VBR) being around ten virus-like particles per bacterial cell in surface seawater. However, as technology has advanced, the VBR in marine environments has been reevaluated to be as high as 100:1.[3] As a result, a large pool of unknown marine phage genes may exist to regulate phage-bacteria interaction, thus creating an untapped reservoir for societal benefit. Societal niches potentially impacted by phage functions include, but are not limited to, applications in molecular editing,[4] drug development, [5,6] and environmental mitigation.[7,8] By characterizing new phages from the marine environment, one can better understand overall phage biology and their molecular mechanisms.

While the exact composition of bacterial communities varies throughout the oceans, the literature suggests that *Pseudomonas* sp., *Vibrio* sp., *Achromobacter* sp., *Flavobacterium* sp., and *Micrococcus* sp. are the most prevalent marine bacterial residents.[9,10] Currently, marine bacterial lineages commonly used for phage hunting efforts reside within the phyla Cyanobacteria, Proteobacteria, and Bacteroidetes.[11] To isolate phages from the local marine environment, it is crucial to understand which bacterial species represent the local microbiome within environmental samples. Metagenomic and lab culturing approaches can be used to determine the local bacterial composition, and to culture and identify bacterial strains as possible hosts for phage hunting. However, several unknowns challenge this approach, including the ability to culture marine bacteria in the local environmental sample under laboratory conditions [12, 13] and the abundance and geographical distribution of phages infecting a marine bacterial strain of interest. It is also valuable to explore the use of nonlocal hosts with local environmental samples.

This research tested the hypotheses that the local beach bacterial composition could be determined through environmental DNA (eDNA) analysis, that locally isolated marine bacterial strains would have associated phage in the environmental samples, and that the locally isolated bacteria are more efficient in isolating marine phages than non-local bacterial strains. Sand samples from various locations along Santa Rosa Island, Florida, were used to culture eight bacterial strains. Two non-pathogenic bacterial strains of the eight strains isolated were chosen as local phage hunting hosts, *Microbacterium oleivorans* Pensacola AB (*M. oleivorans*) and *Erythrobacter citreus* Pensacola AB (*E. citreus*). Additionally, two non-local bacterial strains were also chosen as phage hunting hosts, *Microbacterium foliorum* NRRL B-24224 (*M. foliorum*) and *Microbacterium* sp. Casco Bay [14] (*M.* sp. Casco Bay). The efficiency of phage hunting using these hosts was compared by infecting these bacterial strains with various environmental samples. Plaques are observed within a bacterial lawn of the host bacteria to confirm the presence of phage. A plaque is a clear zone on a bacterial lawn that is formed when a single phage particle infects, replicates, and lyses bacteria during the incubation time. The morphology and the number of plaques formed on the plate are good indicators to determine the efficacy of bacterial host and to characterize phage morphology during the initial stage of the phage hunting process.[15] At the end of this research in the summer of 2023, four phages were further isolated and characterized by their morphological, physiological, and genomic features.

METHODS AND PROCEDURES

Profiling the local bacterial composition

Wet intertidal sand was collected from Pensacola Beach near the fishing pier at 30.3304928, -87.1409214 on January 23, 2022. Environmental DNA was extracted from a 0.5 g sand sample using a Qiagen DNEasy® PowerSoil® isolation kit. A metagenomic library was prepared using Oxford Nanopore Technologies' 16S Barcoding Kit, which utilizes barcoded 27F and 1492R primers [16] and quantitative PCR amplification. The amplified 16S rRNA segments were then sequenced in situ using MinION MK1C flow-through sequencer with an R9 flow cell (Oxford Nanopore Tech Inc.), and the data returned was further analyzed using EPI2ME 16S workflow software (Oxford Nanopore Tech Inc.) to identify the bacterial taxa in the metagenomic sample.

Bacteria isolation and identification

A 20 g sample of the sand taken from Pensacola Beach was combined with 50 mL sterile water and shaken at 33 RPM in an incubator at 37 °C for 24 hours to isolate bacterial cultures for use as phage hosts. The resulting supernatant was serially diluted from 10^0 to 10^{-5}. Using sterile microbiology techniques, duplicate 5 mL aliquots from each dilution factor were spread onto Luria-Bertani (LB) and Marine 2216 nutrient agar plates, incubated at 25 °C, and monitored daily for growth. Colonies were viewed under a microscope to determine isolation (i.e., not touching other colonies) and uniqueness based on morphological factors such as color, form, elevation, and margin. Single colonies were then inoculated in 500 µl of appropriate liquid nutrient media (LB or Marine 2216) and purified twice by streaking plates, picking single colonies, and inoculating. Single colonies from the second round of purification were used to produce a final uncontaminated streak plate. A Qiagen DNEasy® Blood & Tissue spin-column isolation kit was used to extract DNA from a single pure colony of each cultured bacterium. Subsequently, using non-barcoded 27F and 1492R primers [16], the 16S rRNA genes from each bacterial DNA were amplified; the resulting amplicons were sent to North Carolina State University's Genomic Sciences Laboratory for Sanger DNA sequencing. The returned sequence data from each sample were then compared to the NCBI database using the nucleotide BLAST tool to identify the species name of the isolated bacterial strain.[17]

Culturing hosts on multiple growth media

The two non local hosts *Microbacterium* sp. Casco Bay and *Microbacterium foliorum* were acquired through other labs. *M.* sp. Casco Bay[14] was gifted to us by the Tarbox Lab at Maine Community College and originally discovered from marine mud samples in South Portland, Maine. This strain is obtainable from Bigelow Laboratory National Center for Marine Algae and Microbiota as *Microbacterium* sp. NCMA B81. *Microbacterium foliorum* NRRL B-24224 was a stock strain obtained from the SEA-PHAGES stock center. Each of the chosen bacterial hosts (*M. oleivorans, M. foliorum, M.* sp. Casco Bay*, and E. citreus*) was tested using streak plates and liquid cultures on four different medium types. The bacterial growth was analyzed after 48 hours at 28 °C.[15] The media types used were peptone yeast calcium agar media (PYCa) with 0.01 g/L cycloheximide (CHX) and a 0.1% dextrose concentration,[15]

Marine 2216 agar (BD Difco), and mock seawater made with 25 g/L Instant Ocean salt, 0.5 g/L yeast, 0.5 g/L peptone, 0.5 g/L Casamino acids (Bacto™ Casamino Acids), and 1.2 g/L glycerol in water.#

Collecting environmental samples
Environmental samples were collected in two different locations on Santa Rosa Island: Pensacola Beach, Florida, near the fishing pier (30.3304928, -87.1409214), and Navarre Beach, Florida (30.38034, -86.85560). Sample types included dry beach sand, wet intertidal sand, underwater sand, Gulf of Mexico seawater, seagrass of unknown species, coquina clams (*Donax variabilis*), and Atlantic sand crabs (genus *Emerita*).

Phage Isolation and Purification
Phage isolation was conducted using *Phage Discovery Guide* methods.[15] The environmental samples tested included dry sand, underwater sand, dissected marine animals, seagrass, and mixed samples. 30 ml Marine 2216 or PYCa broth was added to each sample and shaken overnight. The supernatant was centrifuged, filtered through a 0.22 μm vacuum filter, and aliquoted for testing with direct and enriched isolation methods. Filtrates for the direct isolation method were followed by plaque assay. Filtrates for the enriched isolation method were mixed with 0.5 ml of a bacteria host culture and incubated for two days at 220 rpm, followed by filtration, before proceeding with plaque assay. Alternatively, the enriched filtrate was precipitated with PEG solution before plaque assay.

Concentrating phage particles using polyethylene glycol (PEG) and zinc chloride ($ZnCl_2$)
Each one mL of enriched sample filtrate or phage lysate of known titer was mixed with 600 μl 20% PEG/2.5M NaCl through inversion and incubated at 4 °C overnight.[15] The lysate was centrifuged, resuspended in 1/100th of the original volume in phage buffer, and plated for a plaque assay. For testing the efficiency of using $ZnCl_2$ to concentrate phage lysate, one mL of lysate was mixed with 25 μL of 2M $ZnCl_2$ through inversion and incubated at 37 °C for 10 mins.[15] The lysate was centrifuged, resuspended in 1/100th of the original volume in phage buffer, and plated for a plaque assay.

To determine the quality of phage particles post-concentration, the concentrated phage particles were examined by a plaque assay. Gel electrophoresis was used to examine the phage DNA: 2 μL of 0.8% SDS/6X loading dye was added to 10 μL of concentrated lysate and incubated at 65 °C for 5 mins; this treatment broke the phage particles to release phage DNA.

Plaque assay
A plaque assay was carried out by first incubating 250 μl of the bacterial host in broth with 500 μl of filtrate derived from the direct method or 10 μl from enriched isolation for 15 minutes. Subsequently, the sample was mixed with 3 ml of the desired top agar (1X PYCa top agar or 1.25X Marine 2216 top agar) and plated onto an appropriate agar plate. The plates were incubated at 25 °C for 24-48 hours before observing plaques.

After a plaque of interest was identified, it was picked and dipped into a tube containing 100 μl of phage buffer, generating a phage lysate containing phage particles. The phage lysate was then serially diluted and examined by plaque assay for consistency of plaque morphology to ensure the purity of the phage of interest (purification round 1). A plaque derived from this process was to go through a similar process for another round of purification. After three rounds of purification, the phage of interest was amplified using the amplification process described in the Phage Discovery Guide to produce a high-titer lysate for characterization and archiving.[15]

Phage DNA isolation, sequencing, and genome assembly
DNA was extracted from the high-titer lysates using the Norgen Phage DNA isolation kit. The quantity and quality of each sample of extracted DNA were determined using a Qubit fluorometer and gel electrophoresis, respectively. A total of 2 μg of DNA from each phage sample was sent off for sequencing and genome assembly at the North Carolina State Genomic Sciences Laboratory.

Transmission electron microscopy (TEM)
Phage lysates were shipped to Taiwan for transmission electron microscopy (TEM) analysis by the Lin lab at National Taiwan University. A high-titer lysate of interest placed on Formvar-coated copper grids was negatively stained with 1% uranyl acetate.[18] Imaging was carried out with a Hitachi H-7650 microscope operated at 75 kV and equipped with a 1kx1k CCD detection camera (Gatan 782). For each novel phage, two photos capturing two different phage particles (two individual phage particles per isolate) were analyzed using ImageJ measuring software to measure the length and width of phage capsids and tails.

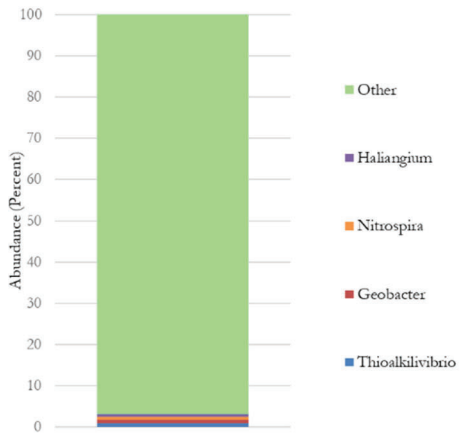
RESULTS

To test if the locally isolated bacterial strains are more efficient in isolating marine phages than non-local bacterial strains, this project began by isolating and characterizing local bacterial strains from Pensacola Beach. This project used common methodologies of phage hunting to isolate, purify, and characterize novel phages.

Isolating local marine bacterial strains and choosing hosts for phage hunting

Wet intertidal sand was collected from Pensacola Beach to determine the local bacterial composition and isolate bacterial strains (see Methods and Procedures). The former relied on metagenomic information based on profiling 16S rRNA data derived from the sand sample, and the latter utilized microbiological culturing techniques and subsequent 16S rRNA sequences of isolated bacterial strains.

1A.



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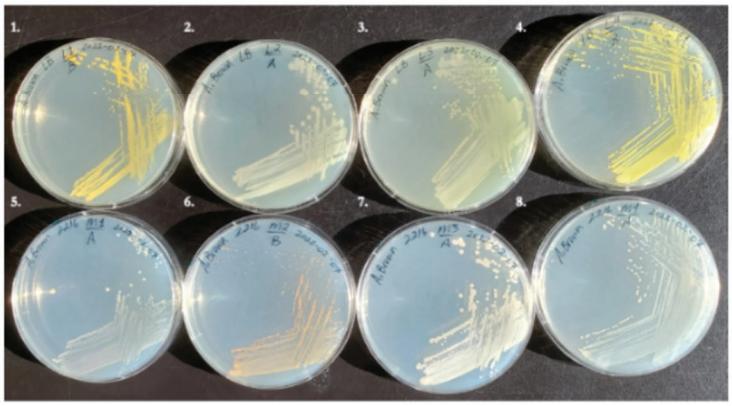

1C.

No.	Bacterial Species	Percent Abundance
1	*Sphingomonas dokdonensis*	0.000
2	*Pseudomonas guineae*	0.001
3	*Pseudomonas alcaligenes*	0.000
4	*Microbacterium oleivorans*	0.001
5	*Pseudomonas benzenivorans*	0.008
6	*Erythrobacter citreus*	0.001
7	*Rheinheimera aquimaris*	0.005
8	*Pseudomonas benzenivorans*	0.008

Figure 1. Determining the bacterial composition of bacterial strains isolated at Pensacola Beach. (1A) This graph shows the bacterial genera that made up the majority of the sand sample analysis as calculated using total metagenomic reads, which were generated via Nanopore sequencing and EPI2ME analysis. **(1B)** The eight bacterial isolates streaked on media; Luria-Bertani agar in the top row of plates (1-4), and Marine 2216 agar in the bottom row (5-8). **(1C)** The eight bacterial isolates, which correspond to **1C** by number, were identified employing 16S rRNA Sanger sequencing and BLAST analysis and the bacterial abundance was determined referencing the Epi2Me report.

Analysis of barcoded 16S metagenomic samples extracted from Pensacola Beach sand revealed that Thioalkalivibrio was the most abundant genus relative to the sample (**Figure 1C**). Geobacter, Nitrospira, and Halangium followed close behind. Most genera described in the EPI2ME report, including the four mentioned previously, were gram-negative. While the metagenomic data provided valuable information about the types of bacteria that thrive in our site of interest, culturing bias prevents us from growing the most present bacteria due to non-reproducible environmental conditions. Despite this, serially diluted spread plates were made using a portion of the same Pensacola Beach sand sample to isolate bacterial strains for phage hunting. Single colonies appeared on LB and Marine 2216 plates with a dilution factor of 10^{-2} to 10^{-5}, and morphology was analyzed under a light microscope. Eight unique single colonies were picked, four from LB plates and four from Marine 2216 plates. The strains were

purified twice and streaked on LB and Marine 2216 nutrient agar plates (**Figure 1B**). Subsequently, 16S rRNA genes from each purified bacterial sample were amplified and sequenced by North Carolina State University's Genomic Sciences Laboratory. The resulting sequence data were then compared to the NCBI database using the nucleotide BLAST tool [19]; the identified bacterial species are summarized in the table of (**Figure 1A)**. All isolated strains were found to be present in the metagenomic data. The eight sequenced isolates matched seven different species from the NCBI database. Two isolates were named *M. oleivorans* Pensacola AB and *E. citreus* Pensacola AB based on their matches with *M. oleivorans* and *E. citreus*, respectively; the former is gram-positive, and the latter is gram-negative. These strains were selected as representative local hosts for marine phage hunting due to their differing gram stains to study the potential phage diversity.

Choosing growth media for phage hunting

The medium preferences of *M. oleivorans* and *E. citreus*, *M. foliorum,* and *M.* sp. Casco Bay) were evaluated to optimize growth conditions for phage hunting. All four bacterial hosts were tested for growth on agar plates of the three medium types: PYCa, Marine 2216, and mock seawater medium. All plates were incubated at 28 °C for 48 hours (see **Table 1** and **Figure 2**, and data not shown).

M. foliorum, *M.* sp. Casco Bay, and *M. oleivorans* grew best on PYCa media and grew moderately on both Marine 2216 and mock seawater media despite a slower generation time. For *M. foliorum* and *M.* sp. Casco Bay, there was rapid growth in the PYCa medium. *M. oleivorans* grows well in the PYCa medium but at a slower pace than *M. foliorum* and *M.* sp. Casco Bay. All three *Microbacterium* strains grew consistently but slowly in Marine 2216 and had the most minor growth on mock seawater media. *E. citreus* grew best on the Marine 2216 medium and had significantly reduced colony growth on the mock seawater medium. *E. citreus* had no growth onPYCa media. Ultimately, PYCa was chosen to grow, assay, and amplify *M. foliorum*, *M. oleivorans*, *M.* sp. Casco Bay, and respective phages. Likewise, Marine 2216 was chosen for *E. citreus* and its isolated phages.

Bacteria Growth			
Bacterial Strain	**PYCa**	**Mock Seawater**	**Marine 2216**
M. foliorum	++	+	+
***M.* sp. Casco Bay**	++	+	+
M. oleivorans	++	+	+
E. citreus	–	+	+

Table 1. Testing three different growth media on the four bacterial hosts for optimal growth. Each bacterial host was tested on three different growth media. A single plus (+) sign indicates decent growth, a double plus (++) indicates rapid and significant growth, and a dash (-) symbol indicates no growth. The responses in red indicate the chosen media used in the subsequent phage research.

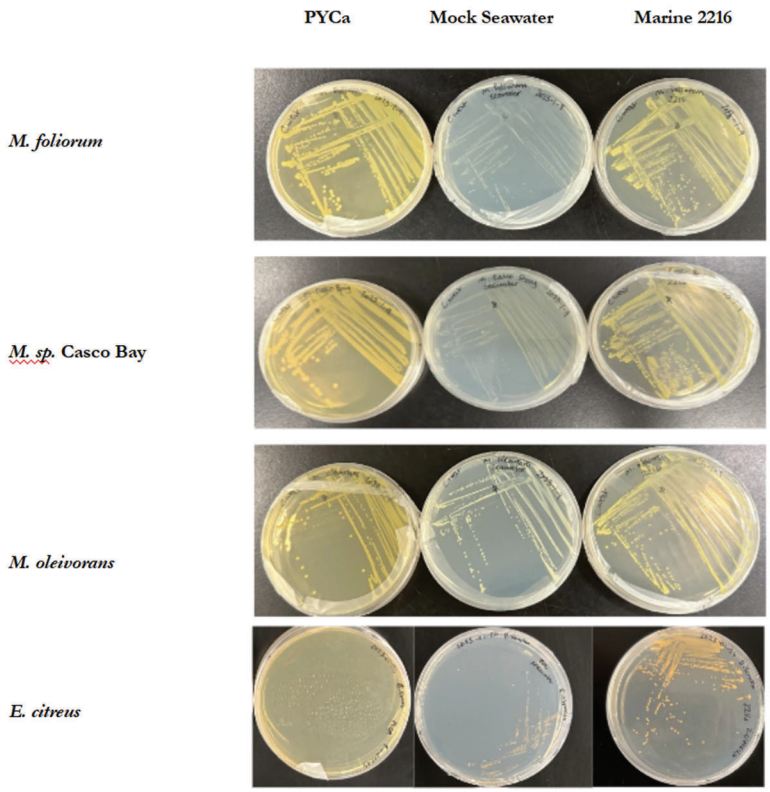

Figure 2. Bacterial growth on three different media. Hdfk#edfwhulddkrw#z dv#hwwhg#rq#wkh#kuhh#gliihuhqw#jurz wk#p hgld#S\Fd/#P rfn#Vhdz dwu/#bqg# P dulq#5549,#diwu#7;#krxu#ri#ltxlg#fxowuh#qfxedwrq#dw#5;°C.

Phage hunting trials

This project used plaque assay to identify phages' presence and measure the efficiency of phage hunting using the locally isolated marine bacterial strains and non-marine bacterial strains. **Figure 3** shows two examples of plaque morphology observed during phage hunting trials described below.

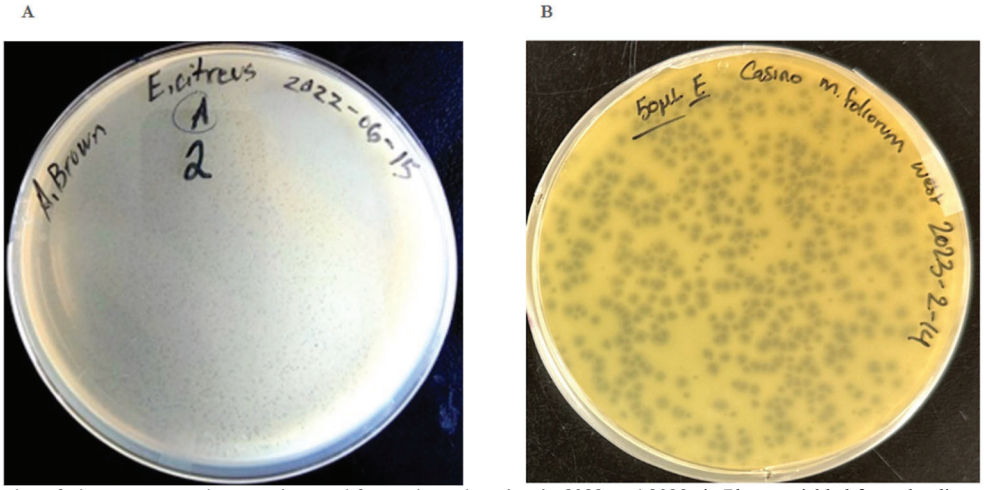

Figure 3. Two examples of plaque assay- plaques observed from phage hunting in 2022 and 2023. A. Plaques yielded from the direct isolation of a dry sand sample from Pensacola Beach, Florida, infecting *E. citreus*. Marine 2216 was used for the media preparations. **B.** Plaques yielded from enriched isolation of a mixed marine sample from Pensacola Beach, Florida, infecting *M. foliorum* in PYCa media preparations.

The first two marine phage hunting attempts were to isolate phages from dry, wet, and underwater sand samples collected from Pensacola Beach. These sample filtrates were tested using *M. oleivorans* and *E. citreus*. No phages from the three environmental

samples were found when using *M. oleivorans* as the host either through direct isolation or medium-enrichment methods. All three ecological samples showed the presence of phage for *E. citreus* for direct samples using the direct isolation method. Wet and underwater sand had few plaques, and dry sand had too many to count.

Determining the preferred method of phage lysate precipitation

Lysate precipitations with various agents, such as $ZnCl_2$ and PEG solutions, help concentrate the sample lysate before infection.[20,21] To determine which reagent was more effective, *M. foliorum* phage Zepp, previously isolated in the lab, was used to test for usage in environmental phage hunts. After the precipitation treatment of Zepp with either $ZnCl_2$ or PEG, plaque assay was used to determine the efficiency of its infection of *M. foliorum* PEG-precipitated lysates showed a 1.6-fold titer increase from $2.5*10^8$ PFU/mL to $4*10^8$ PFU/mL, while $ZnCl_2$ precipitated lysates saw a 0.23-fold decrease from $6*10^8$ PFU/mL to $1.4*10^8$ PFU/mL. The percentages of phage particles from the original lysate that remained in the precipitated lysates were calculated to be 80% and 0.2% for PEG and $ZnCl_2$ precipitation, respectively (**Figure 4A**).

DNAs from the PEG-precipitated and unprecipitated Zepp phage lysates were examined to verify that PEG-precipitation did not damage phage DNA. **Figure 4B** shows that the lane containing undiluted DNA extracts from PEG-precipitated samples had a band (12 kb and larger sized) with a higher concentration of phage DNA than unprecipitated, and the DNA was still intact for use in downstream sequencing applications. Note that the DNA extracts were not treated with nuclease before incubation with SDS (See Methods and Procedures), which indicates that the smear in Lane 3 might be due to either degraded phage or host DNA fragments that remained in the lysate buffer during the Zepp lysate preparation. Judged by the increased titer, high percentage of infectious particle yield, and maintained DNA quality of PEG-precipitated Zepp lysates, it was found that precipitation of phage lysates with a PEG/NaCl solution was highly effective and can be used in various lysate concentration methods. These results showed that for our purposes, PEG-precipitation, but not $ZnCl_2$, is an effective method to concentrate a sample lysate and can be used throughout the phage-hunting process to increase the isolation yield. The PEG-precipitation method was adopted in the phage hunting trial in 2023.

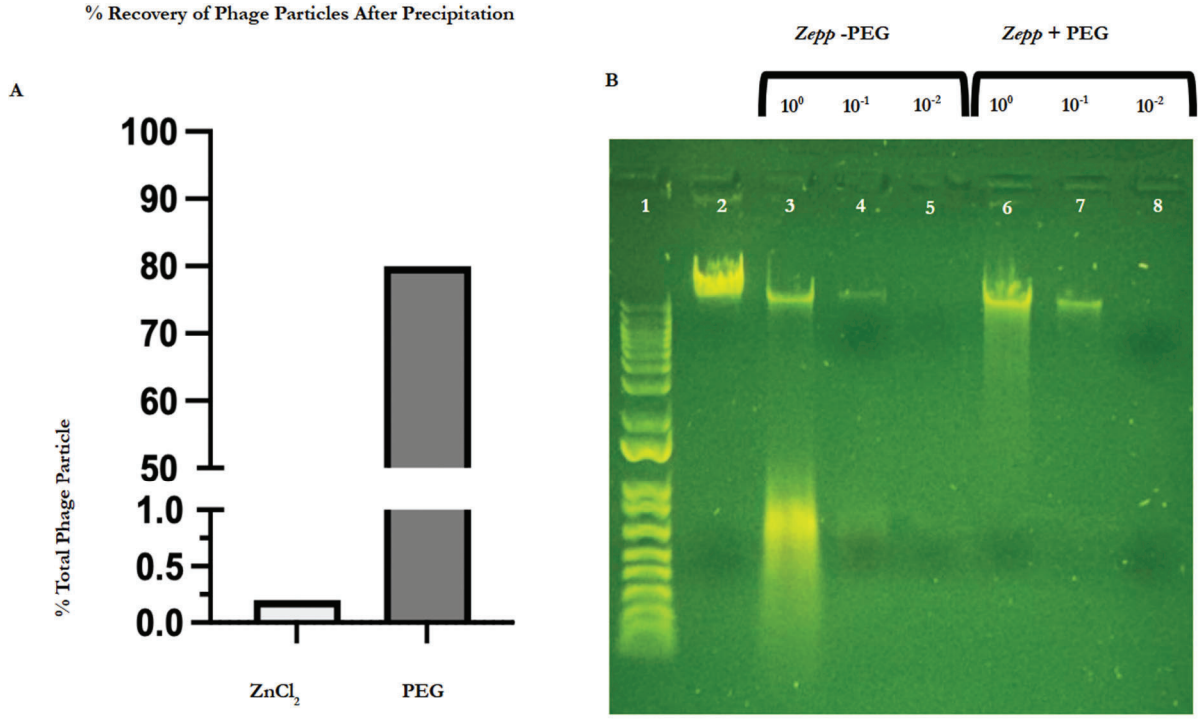

Figure 4. Comparison of Phage Lysate Precipitation Methods. The *M. foliorum* phage Zepp was precipitated with 2M $ZnCl_2$ or 20% PEG/2.5M NaCl. Titers were calculated through plaque assays with Zepp's host, *M. foliorum*. **Figure 4A** shows the percentage of phage particles remaining in lysate after precipitation. (**B**) Gel electrophoresis of isolated DNA from above PEG-precipitated samples. **Lane 2** shows DNA from the *E. coli* phage *Lambda* (λ). Lanes 3-5 are 10-fold dilutions of DNA from PEG-precipitated Zepp lysates and Lanes 6-8 are 10-fold dilutions of DNA from unprecipitated Zepp lysates.

Table 2 summarizes the phage-hunting results of all the trials in 2022-2023, with and without PEG precipitation treatments. Overall, six environmental samples collected from areas along Santa Rosa Island (Pensacola Beach and Navarre Beach) were used, including dry sand, underwater sand, ocean water, marine animals, seagrass, and mixed samples. Three findings were made: a) when using environmental sample lysates to infect *E. citreus*, the PEG-precipitation method was not helpful as plaque assays

identified no indication of phages. b) The PEG-precipitation method was successful when *M. foliorum* and *M.* sp. Casco Bay were used as bacterial hosts. c) Numerous *E. citreus* phages were isolated from sand samples. Phages isolated from the seagrass sample infected *M.* sp. Casco Bay, and the mixed sample infected *M. foliorum*. These results suggest that, without concentrating sample lysates, bacterial isolate *E. citreus* was more efficient in marine phage hunting than the non-local bacterial strains *M. foliorum* and *M.* sp. Casco Bay, which is consistent with our hypothesis.

	Phage Hunting Results							
	M. oleivorans		***M. foliorum***		***M. sp* Casco Bay**		***E. citreus***	
Samples	**-PEG**	**+PEG**	**-PEG**	**+PEG**	**-PEG**	**+PEG**	**-PEG**	**+PEG**
Dry /Wet Sands	–	N/A	–	–	–	–	+	–
Seagrass	N/A	N/A	–	–	–	+	–	–
Mixed sample**	N/A	N/A	–	+	–	N/A	N/A	N/A

Table 2. Summary of phage hunting results using either no PEG ("-PEG") or with PEG ("+PEG") treatment in 2022-2023. The results are denoted with (-) for no phage presence and (+) for the presence of phages. Phage hunting trials occurred in January, June, and August of 2022, February and March of 2023. Due to the culture maintenance issues with *M. oleivorans,* this host was not used in 2023 for phage hunting. ** Mixed sample included coquina, beach sand at the waterline and dry beach sand.

Morphological and Genomic characterization of four phages

Four phages were successfully isolated and subjected to further purification and amplification. Among the four, WestPM, derived from a mixed sample is an *M. foliorum* phage; PortlandC27 and PensacolaC28, both derived from the sea grass sample, are *M.* sp. Casco Bay phages; Horizon is an *E. citreus* phage. All four phages were characterized by observing plaque morphology and through transmission electron microscopy (TEM). Regarding the plaque morphology, Horizon is distinctly different from the other three. While the phages from Microbacterium species had plaques roughly 1.5-2 mm in diameter, Horizon's plaques are extremely tiny and barely visible to the naked eye (about 0.25 mm). **Table 3** and **Figure 5** provide a further description and images of the plaque characteristics of all four phages. The TEM analysis showed that Horizon has a prolate polyhedral capsid shape with a tail, and the other three phages have isometric polyhedral capsids with a tail. **Figure 5** shows the morphology of WestPM, PensacolaC28, PortlandC27, and Horizon (Detailed analysis of WestPM [22] and PensacolaC28 [23] has been published separately). Each phage's average capsid transverse diameter and tail length were calculated based on measurements taken from two TEM photographs using ImageJ software. WestPM had an average capsid transverse diameter of 53.5 nm and an average tail length of 114.0 nm.[22] PortlandC27 had an average capsid transverse diameter of 39.0 nm and an average tail length of 94.4 nm. Similarly, PensacolaC28 had an average capsid transverse diameter of 39.2 nm and tail length of 91.4 nm.[23] Lastly, Horizon was determined to have an average transverse diameter width of 52.8 nm and an average tail length of 222.9 nm.

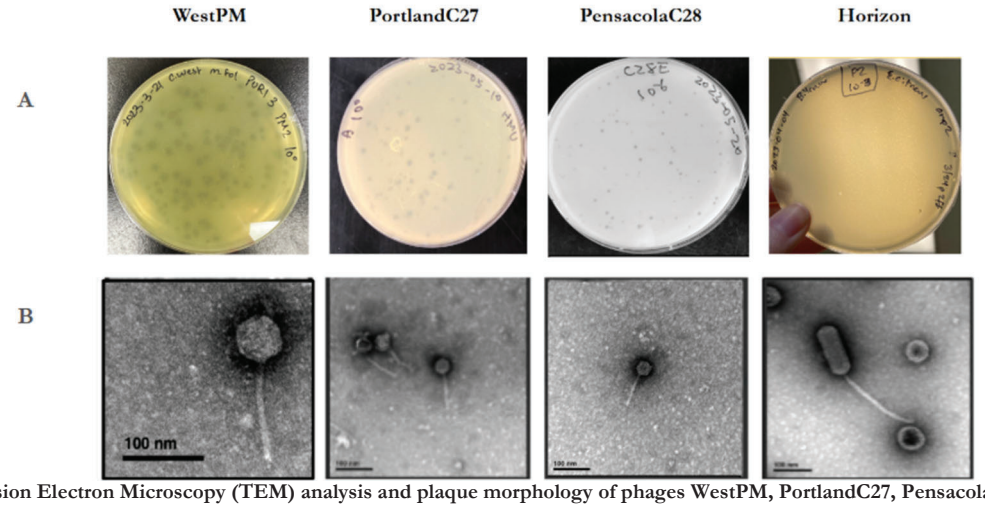

Figure 5. Transmission Electron Microscopy (TEM) analysis and plaque morphology of phages WestPM, PortlandC27, PensacolaC28, and Horizon. **A.** Plaque morphology of four phages (WestPM, PortlandC27, and PensacolaC28 on the PYCa medium, and Horizon on the Marine 2216 medium. **B.** Negative staining TEM of the four bacteriophages as seen in **A.** The average capsid width and tail length were measured using ImageJ software (n=2). Images for WestPM and PensacolaC28 were adapted from West *et al*, 2025 and Girard *et al*, 2025 respectively.

All four phages mentioned maintained a high enough titer throughout the amplification process for sequencing. The titer of WestPM was approximately 1 x 10^9 PFU/mL. PensacolaC28 and PortlandC27 had the highest titers at 4.0 x 10^{10} PFU/mL. The titer of Horizon was hard to determine because the plaque size was tiny, and the bacterial lawns were faintly colored (**Figure 5.**), however, an estimate was 2.3 x 10^8 PFU/mL. Gel electrophoresis was used to test the quality of the extracted phage DNA. **Figure 6** shows that each of the phage genomic DNA was larger than 12 kb. A total of 2 μg of each extracted DNA was then sequenced using Illumina sequencing by North Carolina State University's Genomic Sciences Laboratory. Over a million reads were obtained for each phage DNA to assemble a single contig, i.e., a phage genome. Sequencing of WestPM and PensacolaC28 resulted in 6541-fold and 19692-fold coverage respectively. It was determined that PortlandC27 and PensacolaC28 had the shortest genome lengths at around 17 kb among the four phage genomes. WestPM had a genome length of 39.7 kb, and Horizon had the largest genome length of 151.4 kb. Recent genomic analysis of WestPM [22] and PensacolaC28 [23] showed that WestPM is in the subcluster of EA11 and PensacolaC28 is a singleton of the Actinobacteriophage database [15] and Horizon is in progress. Additionally, for PortlandC27 and PensacolaC28, the genome of the two phages only differed by 39 base pairs, suggesting that PortlandC27 and Pensacola C28 are duplicates of the same phage isolated from independent plaques on the sample plate.

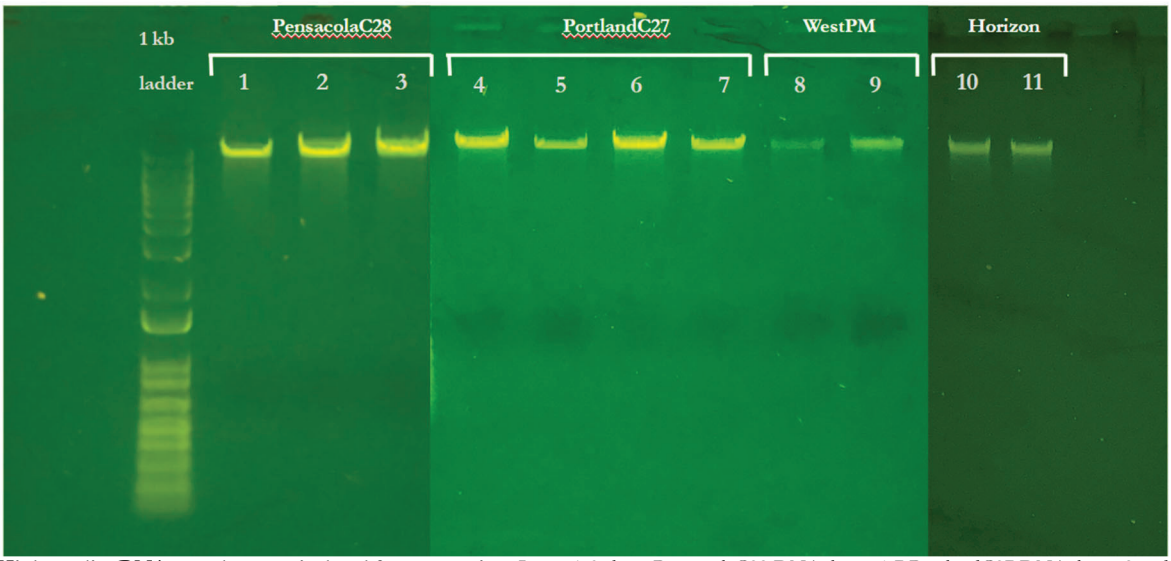

Figure 6. High-quality DNA samples were isolated for sequencing. Lanes 1-3 show PensacolaC28 DNA, lanes 4-7 PortlandC27 DNA, lanes 8 and 9 WestPM DNA, lanes 10 and 11 show Horizon DNA, 0.5 μg DNA per lane. DNA was extracted from the phage lysates using the Norgen Biotek Phage DNA Isolation Kit. A total of 2 μg of DNA per phage was sent off for sequencing.

Characterization of isolated phages					
Host	**Bacteriophage**	**Genome Length (kb)**	**GenBank Accession Number**	**Plaque Morphology**	**Phage Morphology**
Microbacterium foliorum	WestPM	39.693	PP978895	Turbid, with halo and irregular border ~1.5mm diameter	Siphoviral Capsid: 53.5nm Tail: 114.0nm
***Microbacterium* sp. Casco Bay**	PortlandC27	16.73	Duplicate of Pensacola C28	Translucent, with clear and consistent borders ~2mm diameter	Siphoviral Capsid: 39.0nm Tail: 94.4nm
***Microbacterium* sp. Casco Bay**	PensacolaC28	16.769	PP978844	Translucent, with clear and consistent borders ~2mm diameter	Siphoviral Capsid: 39.2nm Tail: 91.4nm
Erythrobacter citreus	Horizon	151.365	TBD	Tiny, pin prick-like plaques ~0.25mm diameter	Prolate Capsid: 52.8nm Tail: 222.9nm

Table 3. Summary of phage genome sizes. Four bacteriophage DNA samples were sent off for genome sequencing at the North Carolina State Genomic Sciences Laboratory. PortlandC27 and PensacolaC28 were determined to be identical. WestPM and PensacolaC28 genomes have been annotated and published in the GenBank database.

DISCUSSION/CONCLUSION

As the most abundant organisms in the ocean, phages play an essential ecological role in microbial balance.[25] The sheer abundance of phage particles in the ocean has been realized using traditional culture-based and metagenomic approaches.[3] Although there has been more research on marine microbial environments in recent years, it is essential to continue to better understand our local bacterial species and their interactions with associated phages. This undergraduate research project tested the hypotheses that the local beach bacterial composition could be determined through eDNA analysis, confirm that local bacterial strains could be used to isolate bacteriophage from local environmental samples, and that locally isolated bacterial strains are more efficient in isolating marine phages than non-local bacterial strains. By the end of this project in summer 2023, the authors were able to conclude that determining the bacterial composition is difficult due to low culturability of present bacteria in environmental samples, and that it is possible to use local marine bacterial isolates to perform phage hunting, however, which bacterial isolate is more suitable to use might require extensive characterization not within the limitations of this research.

Regarding choosing bacterial hosts for phage hunting

Not all bacteria in the environment can be readily cultured in a laboratory setting. The eight bacterial strains isolated from the Pensacola beaches have low abundance relative to the entire metagenomic sample, demonstrating a widely understood culturing bias. *M. oleivorans* and *E.citreus* were selected as representative local hosts for marine phage hunting due to the ubiquitous distribution of the genera and the absence of health risks. These two bacterial strains comprise a very small portion of the metagenomic bacteria composition profile, which might indicate a low number of marine phages capable of infecting these bacterial strains. However, this factor could not be solely responsible, as revealed by the drastic difference in phage hunting success between using *Microbacterium* and *Erythrobacter* species as hosts (**Table 2**). *Microbacterium* strains fall under the Actinobacteria phylum and are gram-positive, whereas *E. citreus* belongs to the phylum Pseudomonadota and is gram-negative; it is possible that these differences could have contributed to the varied results of our phage hunting. Some phage possess specialized endolysin production genes to combat the peptidoglycan layers in bacterial cell walls, such as Vibriophage VPp1, a double-stranded DNA phage specific to *Vibrio parahaemolyticus*.[25] Gram-negative bacteria are much more prevalent in the marine environment,[27] suggesting that marine phages are likely better suited to infect gram-negative hosts; this could cause the discrepancy in phage infection between *M. oleivorans* and *E. citreus*.

Another example of how the complex interactions between phages and bacteria in the marine world regulate the ocean's ecological balance is that some marine bacterial strains exist as lysogens, which contain prophage regions that inhibit infection by lytic phages.[28] While it is possible to identify prophage via sequencing the genome of newly isolated bacterial strains,[29] this poses an additional financial burden. The observed difficulties in using *M. oleivorans* as a host for phage hunting, might suggest the presence of a lysogen in this bacterial strain. Alternatively, it's possible that the inability of *M. oleivorans* to produce plaques from our environmental samples was due to the host's low abundance in the surrounding environment, as determined through bacterial profiling. Environments without a significant abundance of the chosen host bacteria present often yield lower phage hunting success due to decreased pressure for the development of associated phage. Because of this, increased volumes of environmental samples are necessary to increase phage hunting yield and it can be challenging to detect phages from a large volume of environmental samples without concentrating filtrates. While testing if the *M. oleivorans* isolate is lysogenic was beyond the scope of this research, concentrating the sample lysates to increase the success of phage hunting was worth trying.

When comparing another two *Microbacterium* strains, *M. foliorum* NRRL B-24224 [30] isolated from the phyllosphere of grasses and *M.* sp. Casco Bay, only five phages were isolated using *M.* sp. Casco Bay as the host.[24] In contrast, more than 5000 phages have been discovered using *M. foliorum* NRRL B-24224.[30] It is possible that originating from a close-to-marine environment, *M.* sp. Casco Bay might be a better *Microbacterium* strain host for marine phage hunting to be compared with *Erythrobacter* strains. Further research could shed light on this aspect.

Determining the preferred method of phage lysate precipitation

In nature, phages and their hosts compete with many other bacteria and viruses, typically leading to lower concentrations than in pure lab cultures.[31] Phage hunting in the harsh marine environment might require large volumes of environmental samples to be plated for adequate plaque formation. To avoid this, phage precipitation can be performed to concentrate the phage particles into a smaller volume for plaque assay and titering.[20] Practice of such has been used in phage isolation from water samples. [32,33] In this project, the first few phage hunting attempts used various environmental samples from Santa Rosa Island, including dry sand, wet intertidal sand, underwater sand, seagrass, marine animals, and mixed samples. Results indicated no phage presence for further purification when *M. oleivorans* was used as the bacterial host. However, using direct isolation, *E. citreus* exhibited numerous plaques from all sand samples.

Several of the standard phage precipitation methods work through similar mechanisms. A salt solution is added to the lysate, allowing for the separation of water and clustering of phage particles similar to the salting out of proteins.[21] $ZnCl_2$ and PEG solutions were tested to determine efficacy for the concentration of infectious particles for downstream application with environmentally isolated lysates (**Figure 4**). Although $ZnCl_2$ has been used for phage research,[21] it was observed that the PEG-precipitation method was well suited to our phage hunting project, which intended to isolate and preserve phages for long-term usage.

Choosing growth media for phage hunting

This project used the media PYCa and Marine 2216 to culture *M. oleivorans, M. foliorum*, *M.* sp. Casco Bay, and *E. citreus* for phage hunting. After several months of culturing *E. citreus*, it was observed that the bacteria cultures struggled, possibly due to *E. citreus* being an environmentally derived host. The mock seawater medium supports the growth of all four bacterial strains but at a much slower rate than PYCa (*M. oleivorans, M. foliorum,* and *M.* sp. Casco Bay*)* or Marine 2216 (*E. citreus*), and yields smaller colonies. Although mock seawater media was not advantageous for culturing these bacteria, the growth suppression may be a possible stressor for the bacteria cells, leading to a higher phage infection rate. Whether using a sub-optimal medium could increase the potential for phage infection could be further studied in experiments involving marine bacterial strains.

Morphological and Genomic characterization of four phages

The morphology of WestPM, PortlandC27, and PensacolaC28 showed an isometric polyhedral capsid shape with a tail. Horizon images showed a prolate polyhedral shape with a tail. Regarding capsid volume and tail length, Horizon has the most significant capsid volume and extended tail compared to the other three (**Figure 4**). The prolate capsid shape indicates increased capsid capacity or volume.[34] A study has suggested that capsid volume linearly correlates with genome length regarding DNA viruses, including phages,[35] which is consistent with what was observed in this research when comparing the genome sizes of PortlandC27, PensacolaC28, WestPM, and Horizon (**Table 3**). Interestingly, upon observing plaque sizes, Horizon gave rise to miniature pin-prick-like plaques that are difficult to see and photograph. The large genome size of Horizon is consistent with other phages isolated from the bacterial genus *Erythrobacter*[36]. *Erythrobacter* phage vB_EliS-L02 has a genome length similar to Horizon's — around 150,000 base pairs —and the shape of its capsid is similarly prolate.[36]

Summary

In conclusion, wrapped by the summer of 2023, this undergraduate research tested the hypothesis that marine bacterial strains are equally suitable or better for marine phage hunting. The authors explored the feasibility of isolating phages from marine environments using a "start from scratch" approach, comparing the efficiency of isolated *Erythrobacter* and *Microbacterium* species in marine phage hunting. The discovery of the four phages offered an excellent opportunity to examine the phage-host interaction and analyze the genomic information. Annotations of WestPM [22] and PensacolaC28 [23] genomes suggest that these newly isolated marine phages share similar genomic structures with non-marine phages through comparisons using NCBI Blast database [19] and HHPred [37]. The genome annotation of Horizon is in progress, which will open a window for molecular analysis of phage gene functions in the future. For instance, thus far, very few *Erythrobacter* phages have been identified; [36] Horizon may be a valuable model to better understand marine phage and *Erythrobacter* species as hosts. Considering the possibility of lysogeny leading to superinfection resistance, metagenomic analyses may uncover more information on phage diversity and abundance within the marine environment. The lessons learned from this project can help structure future undergraduate marine phage hunting projects.

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PRESS SUMMARY

Bacteriophages, or viruses that infect bacteria, are among the most prevalent biological entities in the world. Many of these phages work to hijack the bacterial host's replication machinery to replicate, leading to host cell death (lytic cycle) or co-existing with the host (lysogenic cycle). The diverse genes phages use to modulate their host's fitness may be relevant in developing antimicrobials, environmental mitigation tactics, and other crucial research spheres. In this article, resident bacteria of Pensacola Beach, Florida, were characterized and cultured, allowing the isolation of four marine phages from environmental samples such as sand, ocean water, and crustacean tissue. The genomes of all four phages were sequenced, and morphological and phylogenetic information has been revealed.