Genetic classification of two *Vibrio* species isolated from Florida Gulf Coast using multi-locus sequence analysis

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Abstract

Prokaryotic species classification has lagged dramatically behind eukaryotic species due to the dependence on the difficult DNA-DNA hybridization technique required for species designation. This is disadvantageous for the general understanding of the diversity of microbial life and unique microorganisms, along with their metabolic abilities, like bioremediation of hazardous waste. This applies to the secondary metabolites microorganisms produce that can be beneficial for humans and other living organisms, such as antibiotics or other industrially important compounds. In an effort to isolate and identify new microbial species, environmental samples were collected from marine sediment environments as marine habitats are under-sampled compared to non-marine habitats. Two marine isolates designated MI-1 and MI-2 were isolated from marine sediment environments off the Gulf Coast of Florida and chosen for further study because of their antibacterial production and unique colony pigmentation. They were genetically analyzed through sequencing of the *16s rRNA* gene, shotgun cloning, and an eight-gene multi-locus sequence analysis comparison to 66 other species of the same genus. From these, MI-1 and MI-2 can be classified as members of the *Vibrio* genus most closely related to *Vibrio ruber* and a distinct strain from *V. ruber* type strain VR-1.

Introduction

The goal of biologists and taxonomists for over a century has been to classify organisms into species and taxonomic groups, thus bringing order to the great biodiversity seen in the world. Due to the lack of a broadly applicable species concept (56) and the historical reliance on the time-consuming and labor-intensive DNA-DNA hybridization (DDH) test required for species delineation (6, 28, 41), prokaryotic species classification has lagged far behind that of eukaryotes in achieving this goal. Compared to the number of known eukaryotic species (>1.2 million) (48), the ~12,000 recognized prokaryotic species (48, 67) are lacking, particularly when the number of prokaryotic species on the planet is estimated to be as high as 10^{11}-10^{12} (39, 44). Given the dominance of bacterial diversification as depicted in the recently expanded new tree of life (34), there is a need for increased attention to prokaryotic taxonomy to work towards classifying the remaining 99.999% of unclassified prokaryotic species (44).

Marine environments are a source of microorganisms with important roles including global nutrient cycling where they are responsible for half of the primary production on the planet (5, 14), production of industrially and medically important compounds (3, 10, 15, 19), and essential symbiotic relationships with larger organisms (14, 20, 32). As it is estimated that 91% of species in and around the ocean still await classification and the majority of these are microbial (48), marine environments represent a likely source for the isolation of novel microbes that may have medical or industrial importance. Because the challenges of growth in a marine environment are different from that of terrestrial organisms, marine microorganisms have acquired unique metabolic capabilities that allow for production of compounds not present in terrestrial organisms (14, 24). Antimicrobials are one type of compound that has generated considerable interest for production by marine microorganisms

due to both the increased prevalence of pathogens resistant to currently available antibiotics (33, 43) derived from terrestrial organisms and the comparative lack of sampling for antibiotic-producing organisms from marine environments (35, 38). Antibiotic compounds have been successfully purified from marine microorganisms (8, 21, 50), suggesting the marine environment offers an untapped source of antimicrobials that could help combat the impending antibiotic crisis.

One of the most abundant groups of microorganisms in marine environments is the *Vibrio* genus (64). Organisms classified as *Vibrios* are generally small Gram-negative, facultatively anaerobic rods with a single polar flagella that are primarily aquatic and require Na^+ for growth (2, 17, 61, 64). More than 100 *Vibrio* species have been validly described (22). Of these, several have been found to have close relationships with higher organisms, with effects ranging from beneficial symbiosis (32, 47) to harmful pathogenesis (27, 37, 68). *Vibrios* are believed to take part in nutrient cycling (62) and are particularly important for their production of polyunsaturated fatty acids that are vital for the aquatic food web but can only be produced by a handful of organisms (52) and their breakdown of chitin (60). Some *Vibrio* species have also been found to produce antimicrobial compounds (13, 45).

In addition to the *Vibrio* species already characterized, other new candidate *Vibrio* species have been observed but not yet described (22). Species determination in *vibrios* can be complicated as many different species have virtually identical biochemical phenotypes, and some species can have very similar genomes yet still represent evolutionary units in nature (2, 56). Multi-locus sequence analysis (MLSA) can serve as a substitute for the cumbersome DDH test (23, 26). This technique compares multiple housekeeping gene

sequences, at least four (22), concantenated together end to end into one larger sequence for phylogenetic analysis. MLSA has been used extensively for species determination in *Vibrio* (55, 61). The genetic classification of two antibiotic-producing *Vibrio* species isolated from the gulf coast of Florida through sequencing of the **16s rRNA** gene, shotgun cloning analysis, and MLSA is described here.

Materials and Methods

Isolation of MI-1 and MI-2

MI-1 was isolated from a marine sediment sample aseptically collected from the intertidal zone near a nature park in Pinellas County, FL. The GPS coordinates of the MI-1 collection site were 27°53'19"N, 82°50'22"W. MI-2 was isolated from a salt flat sediment sample aseptically collected from Leffis Key in Bradenton, FL, Manatee County. The GPS coordinates of the MI-2 collection site were 27°27'07"N, 82°41'20"W. One sediment sample was taken from each location. Permission to collect samples was obtained from local authorities in both counties. Both sediment samples were serially diluted in 0.5 M NaCl to 10^{-5} grams soil/ml and grown on starch agar plated supplemented with 0.5 M NaCl (MI-1) or modified potato dextrose agar (PDA) with the following contents (MI-2): tryptone (2 g/l), potato starch (4 g/l), NaCl (0.5 M), glucose (5 mM) filter sterilized and added to the media after autoclaving, and agar (20 g/l). The Neidhardt MOPS salt components were prepared as described (51). To control for fungal growth from environmental samples, 100 μg/1 cycloheximide was added on the initial isolation plates. After four days of growth at 25°C, colonies were isolated and selected for their ability to inhibit the growth of **Staphylococcus epidermidis**, **Pseudomonas putida**, and **Chromohalobacter salexigens**, a marine microorganism, by spread-plating **S. epidermidis**, **P. putida**, or **C. salexigens** onto the separate starch

agar or modified PDA plates supplemented with 0.5 M NaCl and then patch-plating the environmental isolates on top. After initial isolation, MI-1 and MI-2 were maintained on 0.5 M NaCl modified PDA plates and 0.5 M NaCl LB agar plates and stored at 25°C.

Chemical Extraction of Antimicrobial Compound from MI-2

The antimicrobial compound from MI-2 was crudely extracted and tested for activity against five different bacterial species. Isolates of MI-2 were continuously streaked onto 0.5 M NaCl modified PDA plates and incubated at room temperature for seven days. Agar from the plates was cut into cubes and placed into 50 ml media bottles. The bottles were placed in -80°C freezer for 30 minutes. After freezing, 20 ml of ethyl acetate was added to the bottle and shaken on an orbital shaker for 24 hours. The organic ethyl acetate layer was decanted into 20 ml scintillation vials where the ethyl acetate was allowed to evaporate, leaving the extract behind. Extracts were resuspended in 1 ml of ethyl acetate. Four 20 μl portions of the resuspended extract were plated onto a single region on the side of an LB plate and allowed to dry. The same procedure was repeated on the other side of the plate with an ethyl acetate control. A tester strain culture was plated on top of the extract and ethyl acetate control be mixing 100 μl of an overnight culture of **S. epidermidis**, **Bacillus subtilis**, **Citrobacter freundii**, **C. salexigens**, or **Enterobacter aerogenes** in with 10 ml of top agar and poured evenly over the plate. The plates were incubated for 24 hours at 30°C. The zones of inhibition surrounding the chemical extract were measured.

Genus Identification of MI-1 and MI-2

Identification of the genus of MI-1 and MI-2 was completed by PCR amplification of the **16s rRNA** gene sequence. The 16S primers 63F (5'- CAG GCC

TAA CAC ATG CAA GTC – 3') and 1387R (5'-GGG CGG WGT GTA CAA GGC – 3') obtained from Integrated DNA Technologies (IDT) were used to amplify the gene along with the MyTaq™ mix from Bioline. The whole colony PCR reaction conditions were run as follows: 1 cycle of 95°C for 10 minutes; 30 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute; and 1 cycle of 72°C for 5 minutes. Successful PCR amplification was confirmed by agarose gel electrophoresis using a 1% agarose gel in 1X TAE buffer and purified using the Isolate II PCR and Gel Kit from Bioline. Purified PCR products were sequenced at the DNA Analysis Facility on Science Hill at Yale University. Sequencing results were analyzed by BLAST against the **16s rRNA** database of EZtaxon (11).

Genotypic Characterization of MI-1 and MI-2

For all genotypic characterization, genomic DNA was obtained from overnight cultures of MI-1 and MI-2 grown in LB with 4% NaCl using the phenol-chloroform extraction method as described (70). Shotgun cloning of MI-1 and MI-2 was completed. Genomic DNA from MI-1 and MI-2 was digested with Eco-RI and HindIII (New England Biolabs). The resulting fragments were ligated into pGEM7 (Promega) after digestion with the same enzymes. Successful recombinant plasmids were transformed into Escherichia coli DH5α and isolated after overnight growth using the GenElute Plasmid Miniprep Kit (Sigma). Dideoxy-sequencing of the isolated plasmids was performed by Functional Biosciences using the T7 promoter primer. The generated sequences were analyzed using the BLAST (1) feature in the PATRIC, the Bacterial Bioinformatics Resource Center (69), comparing against the genomes of **V. ruber** VR1 (listed as DSM 16370 in PATRIC), **V. rhizosphaerae** DSM 18581, **V. gazogenes** DSM 21264, and V. mangrove CECT 7927. The shotgun clones from MI-2 were deposited in Genbank (9) with the accession numbers shown in Table S1 in the supplemental material.

Whole genome sequencing of MI-1 and MI-2 was completed. After ethanol precipitation of extracted genomic DNA, the sample was checked for integrity by agarose gel electrophoresis and quantified using a NanoDrop 2000 (Thermo Scientific). Whole genome sequencing of the extracted DNA was conducted using HiSeq 2500 technology at Purdue University. The resulting genomic sequence was analyzed using RAST (7).

MLSA of MI-1 and MI-2 along with 66 known *Vibrio* species type strains was completed using eight protein-coding housekeeping genes: **ftsZ**, **gapA**, **mreB**, **recA**, **rpoA**, **rpoD**, **topA**, and **toxR**. The gene sequences for all eight genes from MI-1 and MI-2 were obtained from the whole genome sequencing results. Gene sequences for the 66 known *Vibrio* species type strains were obtained from PATRIC (69). Table S2 in the supplemental material contains the locus tag numbers or reference sequence numbers as available for the genes sequences found through PATRIC. All sequences for each gene were aligned using Clustal Omega (29) and trimmed so each gene sequence covered the same aligned region. The aligned regions from each gene were concentrated to create an 8-gene chain. The concatenated sequences were aligned with Clustal Omega and analyzed phylogenetically with MEGA7 (42). Nucleotide percent identity values were acquired using a percent identity matric in Clustal Omega. Maximum likelihood trees with a phylogenetic test of 100 bootstrap replications was created using previously suggested parameters (22) in MEGA7.

Results

Isolation of MI-1 and MI-2

Marine Isolates 1 and 2 (MI-1 and MI-2) were isolated

from marine sediment samples collected in intertidal regions of the Gulf coast of Florida in Pinellas County and Manatee County, FL, respectively. These locations were selected because they are undersampled marine environments that do not receive a large amount of daily foot traffic. Each isolate was selected for further study based on its ability to produce antimicrobial compounds that completely inhibit the growth of *S. epidermidis*, *P. putida*, and *C. salexigens*. These organisms were chosen as *S. epidermidis* and *P. putida* represent safe relatives of *S. aureus* and *P. aeruginosa*, two of the ESKAPE pathogens whose increasing rates of antibiotic resistance represent a great threat to the healthcare field (54). *C. salexigens* is a halophilic marine microorganism with a broad range of salinity tolerance (4) that was chosen to see if MI-1 and MI-2 can inhibit halophilic organisms as well as halotolerant *S. aureus* and non-halophilic *P. aeruginosa*. MI-1 was initially isolated on NaCl-supplemented Starch agar where is appeared orange while MI-2 appeared bright pink on the modified-PDA. Different media were used for initial isolation to provide different environments and increase the chances of isolating unique microorganisms. Colonies

of MI-1 and MI-2 appear anywhere from pink, red, or orange in pigmentation depending on the type of media on which it is grown.

Fig. 1 shows the antibacterial inhibition and pigmentation of both MI-1 and MI-2. Figure 2 shows the results of chemical extraction of the antimicrobial compound produced by MI-2 against five different bacterial species. The crude chemically extracted antimicrobial compound was capable of inhibiting all organisms it was tested against: two Gram positive species (*S. epidermidis* and *B. subtilis*) and three Gram negative species (*C. freundii*, C. *salexigens*, and *E. aerogenes*). Its strongest inhibition was against Gram positive species. Similar zones of inhibition were seen for MI-1 (data not shown). A Gram stain of both isolates showed small, slightly curved Gram-negative rods.

Genus Identification of MI-1 and MI-2

PCR amplification and sequencing of the *16s rRNA* gene of MI-1 and MI-2 showed both organisms are in the *Vibrio* genus. This confirmed what was previously
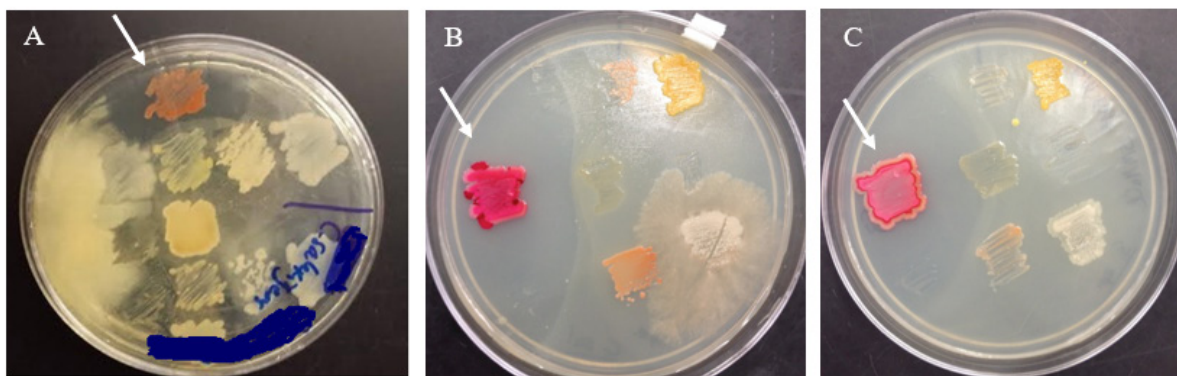

Figure 1. The figure shows the antibiotic production of patch plated MI-1 against C. salexigens (panel A), and patch plated MI-2 against S. epidermidis (panel B) and C. salexigens (panel C). White arrows indicate the location of MI-1 and MI-2 on each plate. Antibiotic production can be seen by the zones of inhibition surrounding the patch plated MI-1 and MI-2. The orange pigment production of MI-1 and pink pigment production of MI-2 can be seen.
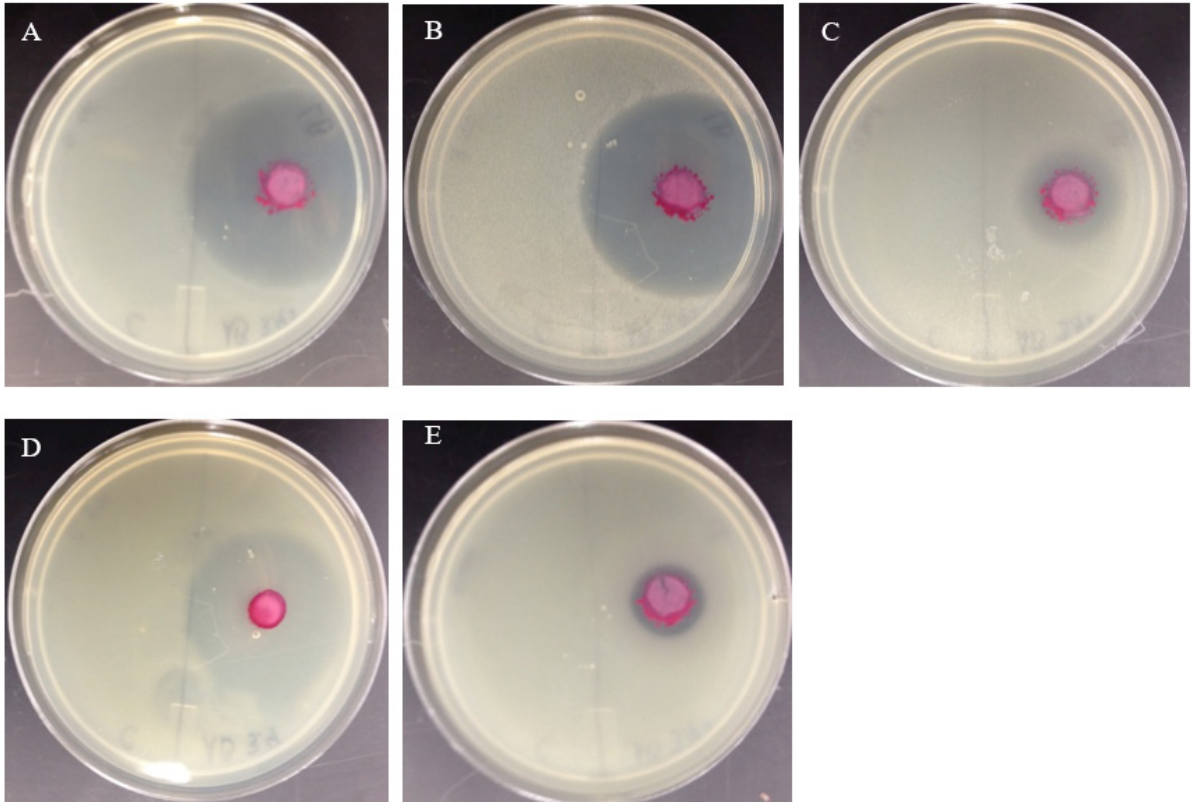
Bacterial Species	S. epidermidis	B. subtilis	C. freundii	C. salexigens	E. aerogenes
Radius of Zone of Inhibition	24 mm	23 mm	7 mm	19 mm	5 mm

Figure 2. The figure shows the inhibition of the crude chemically extracted antimicrobial compound from MI-2 against *S. epidermidis* (Panel A), *B. subtilis* (Panel B), *C. freundii* (Panel C), *C. salexigens* (Panel D), and *E. aerogenes* (Panel E) which has been recently renamed as *Klebsiella aerogenes*. Zones of inhibition can be seen surrounding the pink-pigmented chemical extract of MI-2. The radius of the zones of inhibition is indicated in the table accompanying the figure. The zones were measured 24 hours after plating.

Isolate	Top 3 Matches	Accession #	% Identity
	Vibrio gazogenes ATCC 29988	X74705	97.58%
MI-1	*Vibrio ruber* VR1	AF462458	97.40%
	Vibrio rhizosphaerae MSSRF3	DQ847123	97.03%
	Vibrio ruber VR1	AF462458	98.64%
MI-2	*Vibrio rhizosphaerae* MSSRF3	DQ847123	98.30%
	Vibrio mangrovi MSSRF38	EU144014	98.19%

Table 1 – *16s rRNA* Analysis for MI-1 and MI-2. The table shows the three top matches for the ***16s rRNA*** gene sequence of MI-1 and MI-2 after BLASTing the sequence against the ***16s rRNA*** sequence database of EZtaxon. The matches generated by EZtaxon represent the type strains for each of the species.

MI-1 Clone	Best Match in PATRIC	% Identity
1	*V. ruber pulD*	93%
	V. ruber TPR- repeat-containing protein	95%
2*	*V. ruber proV_1*	93%
3	*V. ruber bglB_1*	97%
	V. ruber bglX_1	99%
4*	*V. ruber glpE_1*	91%

MI-2 Clone	Best Match in PATRIC	% Identity
	V. ruber murR	97%
1*	*V. ruber* Sodium/bile acid symporter family	97%
	V. ruber recN	91%
2*	*V. ruber* hypothetical protein	95%
	V. ruber cheV_1	95%
3*	*V. ruber* Potential *queD*	100%
4	*V. ruber lysU*	97%
5	*V. ruber rps1*	94%
6	*V. ruber pgk*	94%
7	*V. ruber celC*	94%
8	*V. ruber purB*	97%

Table 2 – Shotgun Cloning Results for MI-1, MI-2, and *V. ruber*. The table shows the shotgun cloning results for MI-1 and MI-2 when sequences were on a BLAST in the PATRIC database against the type strain genomes of *V. ruber, V. rhizosphaerae, V. gazogenes,* and *V. mangrovi*. The top organism match for each gene present on the shotgun clone is shown along with the information about the gene provided and the percentage identity of the match. * indicates the shotgun clone produced a sequence that contained two genes, each having a separate match.

	MI-1	MI-2	V. aerogenes	V. gazogenes	V. mangrovi	V. quintilis	V. rhizosphaerae	V. ruber
MI-1	100	99.6	80.1	85.9	86.3	80.4	90.8	97.9
MI-2	99.6	100	80.1	85.9	86.3	80.3	90.8	97.8
V. aerogenes	80.1	80.1	100	75.4	80.2	88.3	80.0	80.1
V. gazogenes	85.9	85.9	75.4	100	81.0	75.6	85.3	85.6
V. mangrovi	86.3	86.3	80.2	81.0	100	81.0	86.2	86.2
V. quintilis	80.4	80.3	88.3	75.6	81.0	100	80.0	80.3
V. rhizosphaerae	90.8	90.8	80.0	85.3	86.2	80.0	100	90.6
V. ruber	97.9	97.8	80.1	85.6	86.2	80.3	90.6	100

Table 3 – Percent Identity Matrix from MLSA MI-1 and MI-2. The table shows the percent identity matrix for MI-1 and MI-2 and the six other *Vibrio* species from the eight gene MLSA that showed greater than 80.0% identity to MI-1 and MI-2. Percent identity was determined using Clustal Omega.
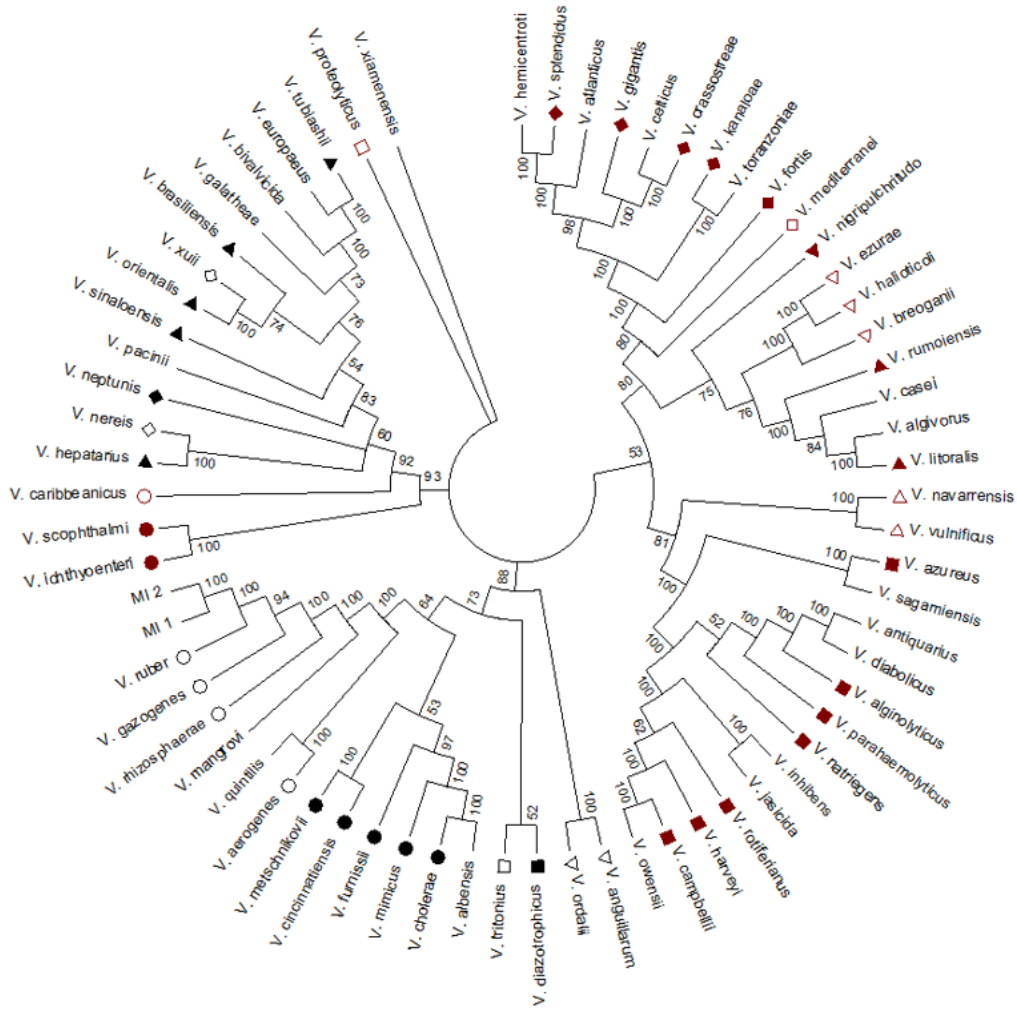
Figure 3 – Molecular Phylogenetic Analysis from MLSA MI-1 and MI-2. The figure shows the phylogenetic tree resulting from the eight gene MLSA of MI-1 and MI-2 with 66 known *Vibrio* species. Symbols indicate the clade identity of each organism as assigned from the eight gene MLSA of Sawabe, 2013; organisms without symbols were not included in this MLSA (61). Evolutionary analyses were conducted in MEGA7 (42). The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (40). The bootstrap consensus tree inferred from 100 replicates is taken to represent the evolutionary history of the taxa analyzed (18). All positions containing gaps and missing data were eliminated. There were a total of 9807 positions in the final dataset.

Legend of *Vibrio* Clade Symbols

○ Gazogenes	● Cholerae	▢ Porteresiae	■ Diazotrophicus
▽ Anguillarum	▼ Orientalis	◇ Nereis	◆ Coralliilyticus
○ Pectinicida	● Scophthalmi	▢ Proteolyticus	■ Harveyi
△ Vulnificus	▲ Rumoiensis	▽ Halioticoli	▼ Nigripulchritudo
◇ Mediterranei	◆ Splendidus	No Symbol – Not included in MLSA of Sawabe, 2013 (61)	

shown for MI-2 (49). The PCR sequencing was completed from three unique colonies with the same results obtained each time. The sequencing results were analyzed using the curated *16s rRNA* EZtaxon database. Sequencing results are shown in Table 1. The match producing the highest similarity was *Vibrio ruber* VR1 for MI-2 at 98.64% while the best match for MI-1 was *Vibrio gazogenes* ATCC 29988 at 97.58%. Both of these values are below the suggested 98.65% similarity cutoff for species delineation with the *16s rRNA* gene sequences (39). As *V. ruber* VR1 was the best match for MI-2 and the second match for MI-1, it was obtained from the BCCCM/LMG bacterial culture collection and used as a comparison for genetic characterization for MI-1 and MI-2.

Genotypic Characterization of MI-1 and MI-2

Genotypic characterization began with shotgun cloning to ascertain if MI-1 and MI-2 might be novel enough to warrant a larger investment through whole genome sequencing. Results of the shotgun cloning are showing in Table 2. There were four successful clones for MI-1 that matched up to six unique genes and eight successful clones for MI-2 that matched up to eleven unique genes. The top match for all clones when analyzed by BLAST through PATRIC (69) was *V. ruber*. The percentage identity ranged from 91-99% with a median of 94% for MI-1 and 86-100% with a median of 95% for MI-2. These values are right around and below the suggested speciation cutoff of 95-96% for average nucleotide identity (ANI) (28, 39, 59), suggesting MI-1 and MI-2 may be novel species and should undergo a more thorough genotypic characterization through MLSA.

An eight gene MLSA of MI-1 and MI-2 was completed with 66 known *Vibrio* type strains with genomes uploaded to the PATRIC database. The eight genes used were *ftsZ*, *gapA*, *mreB*, *recA*, *rpoA*, *rpoD*, *topA*, and *toxR*. With the exception of *rpoD* and *toxR*, these genes were previously used

for an extensive eight-gene MLSA analysis of the *Vibrio* clades (61). A percent identity matrix for all organisms showing greater than 80% identity to MI-1 and MI-2 from the MLSA is shown in Table 3. MI-1 and MI-2 share 99.6% identity from the MLSA. The closest percent identity match from known *Vibrio* species to MI-1 and MI-2 was *V. ruber* at 97.93 and 97.82% identical, respectively. The complete percent identity matrix for all 68 organisms is in Table S3 in the supplemental information. The phylogenetic tree generated from the MLSA data is shown in Figure 3. Organisms with symbols on the figure were included in the eight gene MLSA of Sawabe et al., 2013 (61), and the symbols indicate the *Vibrio* clade to which they were assigned. MI-1 and MI-2 appear most closely related to *V. ruber* and belong in the *Gazogenes* clade. *V. mangrovi* and *V. quintilis* were not included in the previous MLSA, but the results from this MLSA suggest they also belong in the *Gazogenes* clade along with *V. ruber*, *V. gazogenes*, and *V. rhizosphaerae*.

Discussion

Marine bacterial isolates MI-1 and MI-2 cultured from two locations on the Gulf Coast of Florida showed unique colony pigmentation and production of antibacterial compounds capable of inhibiting both Gram positive and Gram negative organisms. Results of the *16s rRNA* gene sequencing indicated MI-1 and MI-2 are in the *Vibrio* genus. *V. ruber*, *V. rhizosphaerae*, *V. mangrovi*, and *V. gazogenes* are the four most closely rated species by 16s rRNA sequence homology, however none were above the 98.65% cut-off for species delineation (39). Morphologically, MI-1 and MI-2 are small Gram-negative curved rods. This along with their isolation from marine environments is consistent with all other *Vibrio* species. The four most closely related *Vibrio* species are known to produce red pigments similar to that seen from MI-1 and MI-2 (31, 57, 58, 63). *V. ruber* and *V. gazogenes* have been

experimentally shown to produce the antibacterial compound prodigiosin (31, 46, 63), a chemical compound with red pigmentation responsible for the color produced by the colonies. It is best known for producing the characteristic red pigmentation of *Serratia marcescens* (16). *V. gazogenes* is also capable of producing cycloprodigiosin, a cyclicized analogue of prodigiosin and fellow member of the prodiginine family of chemical compounds (25) capable of antimicrobial activities. Prodiginine compounds have also been purified from cultures of MI-2 (49). It remains to be experimentally determined if MI-1, *V. rhizosphaerae* or *V. mangrovi* produce antimicrobial prodiginine compounds, but it is likely they do based on the color of the colony. *V. gazogenes* has also been shown to produce the antimicrobial compound magnesidin A (36). In an effort to contribute to the knowledge of marine bacterial species, these organisms were classified genetically through shotgun cloning and an extensive eight-gene MLSA.

Genotypic investigation of MI-1 and MI-2 was completed by performing an eight gene MLSA with 66 known *Vibrio* species whose genomes have been sequenced. *V. ruber* showed the closest percent nucleotide sequence identity to MI-1 and MI-2, 97.93 and 97.82%, respectively. Both of these values are below the 98% cutoff suggested by Sawabe et al. as a species boundary (61), suggesting MI-1 and MI-2 may be novel species based on *Vibrio* guidelines, though these values are higher than the accepted 95-96% ANI cutoff value (12, 28, 39, 59). The 99.61% identity between MI-1 and MI-2 indicate they are the same species. The phylogenetic tree generated from the MLSA (Figure 3) showed MI-1 and MI-2 belong in the Gazogenes clad along with *V. ruber*, *V. gazogenes*, *V. rhizosphaerae*, and *V. aerogenes*, *V. magrovi*, and *V. quintilis* were not included in the original MLSA of Sawabe et al., 2013 (61), but based on their position in the phylogenetic tree, we suggest

they be included in the Gazogenes clade as well.

There was general agreement between the clustering of organisms in this MLSA and the clade designations from Sawabe et al., 2013 with the exception of the **Nereis** and **Orientalis** clades. The **Nereis** clade was reported to contain *V. nereis* and *V. xuii* (61), however this MLSA shows *V. xuii* to be more closely aligned with *V. orientalis* of the **Orientalis** clade and *V. nereis* to be more closely aligned with *V. hepatarius*, also of the Orientalis clade. Reasons for the differences could be explained by the use of *rpoD* and *toxR* genes in this MLSA in place of *gyrB* and *pyrH* in Sawabe et al., 2013. The substitution of *gyrB* and *pyrH* with *rpoD* and *toxR* occurred based off data from Pascual, 2010 that suggested *rpoD* and *toxR* are better for reliable species identification of *Vibrio* (53). This study also utilized sequences of the entire gene as opposed to smaller PCR fragments used in Sawabe et al., 2013. *V. neptunis* of the **Coralliilyticus** clade also aligned closely with *V. nereis* and *V. heptarius*; however the other member of its clade, *V. coralliilyticus*, was not included in this MLSA, so no conclusions regarding its appropriate clade can be made. Other *Vibrio* species not included in this MLSA of Sawabe et al., 2013 that are closely aligned with members of the **Orientalis** clade in this study include *V. pacinii*, *V. galatheae*, *V. bivalvicida*, and *V. europaeus*.

More clade designation suggestions regarding the *Vibrio* organisms not included in the MLSA of Sawabe et al., 2013 can be made. It appears *V. albensis* belongs in the **Cholerae** clade, not surprisingly considering it is a Non-O1 serovar of *V. cholerae* (30). *V. owensii*, *V. jasicida* and closely related *V. inhibens* (66), *V. diabolicus* and the closely related *V. antiquaries* (65), and *V. sagamiensis* closely align with members of the **Harveyi** clade; while *V. algivorus* and *V. casei*

appear most closely related to the **Rumoiensis** clade. Additionally, *V. toranzoniae*, *V. celticus*, *V. atlanticus*, and *V. hemicentroti* appear closely related to members of the **Splendidus** clade. *V. xiamenensis* appears to be in an orphan clade much like *V. proteolyticus* (61).

The genetic analysis has demonstrated that MI-1 and MI-2 are *Vibrio* isolates that can be classified as a strain of *V. ruber* distinct from the type strain *V. ruber* VR1 used in this study. Genotypically, they are on the threshold of being classified as distinct species. The refinement of the whole genome sequences of MI-1 and MI-2 are currently being completed to fill in the gaps and produce a complete genome sequence that can be confidently compared to *V. ruber* by whole genome ANI or digital DDH, an electronic version of the classic DDH mechanism (12). This complete genotypic characterization will be included as a part of a polyphasic approach along with phenotypic and chemotaxonomic characterization (67). Phenotypic characterization will consist of thorough biochemical and growth phenotype testing of MI-1 and MI-2 and comparison with *V. ruber*. Chemotaxonomic characterization will be performed through analysis of the fatty acid methyl ester (FAME) composition of membrane lipids in MI-1, MI-2, and *V. ruber*.

Additionally, future research will characterize the antibacterial secondary metabolites produced by MI-1 and MI-2. This will include confirming prodiginine compounds are responsible for pigment production in MI-1 as in MI-2 (49) and determining whether the antibacterial properties of these organisms is solely due to prodigiosin or if they produce additional antibacterial compounds, like *V. gazogenes* and its production of magnesidin A (36).

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