

# CHARACTERIZATION OF PRODIGININE COMPOUNDS PRODUCED BY A *VIBRIO* SPECIES ISOLATED FROM SALT FLAT SEDIMENT ALONG THE FLORIDA GULF COAST

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- Prodigiosin
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## ABSTRACT

Prodiginines are secondary metabolites produced by several known species of bacteria. These metabolites are known for their bright pigmentation and their potential medicinal uses. Biosynthesis of prodiginine compounds, including the well-studied prodigiosin, has been well characterized in *Serratia marcescens* and other bacterial species, including several marine bacteria. In an effort to isolate and identify natural products from marine organisms, an environmental sample was taken from a salt flat along the Florida Gulf Coast and cultured for bacterial growth. A bacterial species that produces a vibrant pink pigment was isolated and identified as a member of the *Vibrio* genus and was named MI-2. Whole genome sequencing identified a 13-gene operon with homology to the *S. marcescens* prodigiosin biosynthetic operon. The pigment produced by MI-2 was hypothesized to be composed of prodigiosin or related prodiginine compounds and was purified by flash column chromatography and identified by mass spectrometry.

## INTRODUCTION

The prodiginine family of bacterial alkaloids includes many vibrantly pigmented compounds, most of which are red, produced as secondary metabolites by a variety of bacterial species. The most notable of these is *Serratia marcescens* from which the best studied prodiginine, prodigiosin, was first isolated in pure

form and structurally characterized (14, 19). Interest in the prodiginines comes not only from their strong red pigment but also from their potential medicinal uses. In addition to the antimalarial activity of prodigiosin itself (4), prodigiosin and other prodiginine derivatives have been shown to have immunosuppressive functions

with novel mechanisms of action (7, 21, 30, 38, 42) and apoptotic effects in human cancer cells (11, 27, 33, 42).

Since the discovery of prodigiosin in *S. marcescens*, prodiginine compounds have been discovered in other bacterial organisms, including *Streptomyces coelicor* A3(2), *Alteromonas rubra*, *Hahella chejuensis*, and *Vibrio gazogenes* (9, 19). Biosynthesis of prodigiosin has been best studied in *S. marcescens*, *S. coelicor*, and *H. chejuensis*. In *S. marcescens* ATCC 274, the prodigiosin biosynthesis (*pig*) cluster consists of 14 genes abbreviated as *pigA* through *pigN* arranged as an operon that are transcribed as a single 14-gene polycistronic mRNA (17). *H. chejuensis* KCTC 2396 also contains 14 prodigiosin biosynthetic genes, *hapA* through *hapN*, that are similar in their gene layout to that of *S. marcescens* (22). In *S. coelicor* A3(2), the *red* cluster is responsible for prodiginine biosynthesis (5). The genetic arrangement of the *red* cluster is significantly different from *S. marcescens* and *H. chejuensis*, however the presence of 12 homologous genes between it and the

*pig* operon suggests the two biosynthetic pathways are similar (17).

Several of the known prodiginine producers, including *H. chejuensis*, *V. gazogenes*, and *A. rubra* are marine microorganisms. The isolation of natural and potential medicinal products from diverse marine microorganisms has been described recently, with several new products originating from microorganisms isolated from marine environments (2, 3, 45). We report here the isolation of a prodiginine-producing *Vibrio* species known as Marine Isolate-2 (MI-2) from a marine salt flat environment along the central Gulf Coast of Florida. Originally isolated as a marine antibiotic-producing bacterium, MI-2 was unique in its ability to produce a distinctly pink prodiginine product under appropriate media conditions. Whole genome sequencing of MI-2 identified a 13-gene prodiginine biosynthetic gene cluster. The analysis of this prodiginine biosynthetic pathway and structural identification of the purified prodiginine compounds are discussed.

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## MATERIALS AND METHODS

### ISOLATION AND GENUS IDENTIFICATION OF MI-2

MI-2 was isolated from a salt flat sediment sample aseptically obtained from Leffis Key in Bradenton, FL. The sediment sample was diluted in 0.5 M NaCl to  $10^{-5}$  grams soil/ml and grown on 0.5 M NaCl potato dextrose agar (PDA) modified with the following contents: tryptone (2

g/l), NaCl (0.5 M), glucose (5 mM), and 10X Neidhardt MOPS salts (0.1X final concentration). The Neidhardt MOPS salt components were prepared as described (32). To inhibit fungal growth, 100  $\mu$ g/l cycloheximide was added to the initial isolation plates. After initial isolation of MI-2, the organism was maintained on 0.5 M NaCl modified PDA plates and 0.5 M NaCl LB agar plates and stored at 25°C.

Identification of the genus of MI-2 was completed by whole colony PCR amplification of the 16S rDNA. 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 using the MyTaq™ mix obtained from Biorline (Taunton, MA). The whole colony PCR reaction conditions were: 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 on a 1% agarose gel in 1X TAE buffer and purified using the Isolate II PCR and Gel Kit from Biorline. Purified PCR products were sequenced at the DNA Analysis Facility on Science Hill at Yale University.

## MEDIA AND GROWTH EFFECT ON PIGMENT PRODUCTION

The effect of media composition and NaCl concentration on the pigment production of MI-2 was determined qualitatively by quadrant streaking MI-2 onto modified PDA plates prepared as described previously with supplementation of 2%, 4%, or 6% NaCl and LB plates supplemented with 2%, 4%, or 6% NaCl. The plates were incubated at 30°C for two days before being photographed. The effect was determined quantitatively by inoculating a colony into modified Potato Dextrose Broth (PDB) and LB liquid cultures at 2%, 4%, and 6% NaCl and incubating at 30°C for 48 hours. Absorbance of the sample was taken by wavelength scan from 400 to 700 nm at 5 nm intervals.

The effect of time on pigment production of MI-2 was determined qualitatively by quadrant streaking MI-2 onto modified PDA or LB plates with supplementation of 2% or 4%. The plates were incubated at 30°C for nine days, and photographs were taken after two and nine days. The effect was determined quantitatively by inoculating a colony into PDB and LB liquid cultures at 2% and 4% NaCl and incubating at 30°C for 48 hours. Absorbance of the sample was taken by wavelength scan from 400 to 700 nm at 5 nm intervals.

## GENOMIC SEQUENCING AND IDENTIFICATION OF A PUTATIVE PRODIGIOSIN BIOSYNTHESIS GENE CLUSTER

Genomic DNA from MI-2 was extracted from an overnight culture grown in 4% NaCl LB liquid media using the phenol chloroform method (44). The extracted DNA 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 the sequence viewer and annotator tool Artemis (35). The genes with homology to the *pig* gene cluster of *S. marcescens* were identified manually using the National Center for Biological Information (NCBI) protein basic local alignment tool (blastp) (15).

## COMPARISON OF PUTATIVE PRODIGIOSIN BIOSYNTHESIS GENE CLUSTER FROM MI-2 AND OTHER ORGANISMS

Comparison of prodigiosin biosynthesis genes from MI-2 and other known prodiginine compound producing organisms *Serratia marcescens* ATCC 274, *Hahella chejuensis* KCTC 2396, and *Streptomyces coelicor* A3(2) was done by aligning amino acid sequences using Clustal Omega for each gene within the cluster (37). Gene sequences were acquired using the NCBI GenBank database and the accession numbers as follows: *S. marcescens* – AJB33002, *H. chejuensis* – DQ266254, and *S. coelicor* – AL645882.

## PURIFICATION OF PRODIGIOSIN

An overnight culture of MI-2 in 4% NaCl LB liquid media was centrifuged at 6000 Xg for 10 minutes; the supernatant was discarded and the pellet was resuspended in approximately 2 ml of a methanol and 2 N HCl mixture (24:1). The addition of acid was necessary to break down a suspected prodigiosin-associated protein that may sequester the pigment molecule (14, 24). The resuspended pellets were combined in 35 ml scintillation vials wrapped in aluminum foil and left for 12-18 hours on an orbital rotator. Extracts were then centrifuged at 6000 Xg for 10 minutes to remove any cellular residue. The supernatant was collected, and the solvent was evaporated using a rotary evaporator. Hydrophilic impurities were removed

by a series of chloroform-water liquid-liquid extractions that were repeated on the organic layer until the water layer no longer appeared cloudy (1). The chloroform was evaporated via rotary evaporation, and pigment was redissolved in acetonitrile. Flash column chromatography was performed using silica gel as the stationary phase and acetonitrile as the mobile phase. Fractions from flash chromatography that corresponded to a pigmented smear at 0.82 to 0.63 retardation factor (Rf) regions on silica thin layer chromatography (TLC) plates with acetonitrile as the mobile phase were combined and concentrated by rotary evaporation.

## UV-VIS PH ASSAY

The pH of methanol solvents was adjusted to 1.8, 5.0, 7.0, 8.0, 11.1 and 12.0 using solutions of 1 M HCl and 1 M NaOH. One hundred  $\mu$ l of purified prodigiosin pigment extract was suspended in each methanol solvent, and absorbance spectra were measured from 350 nm to 700 nm at 0.25 nm intervals. A baseline correction was performed for all samples.

## MASS SPECTROMETRY

Electrospray Ionization (ESI) tandem mass spectrometry (MS/MS or MS<sup>2</sup>) was performed using Fourier transform mass spectrometry (FTMS) mode on an LTQ-Orbitrap with helium used as the collision gas. Relative collision energy (rCE) ranged from 30-35 in the ion trap component of the instrument. High-resolution mass spectra were obtained with full width half maximum resolving power of 100,000 at 400 m/z in profile mode.

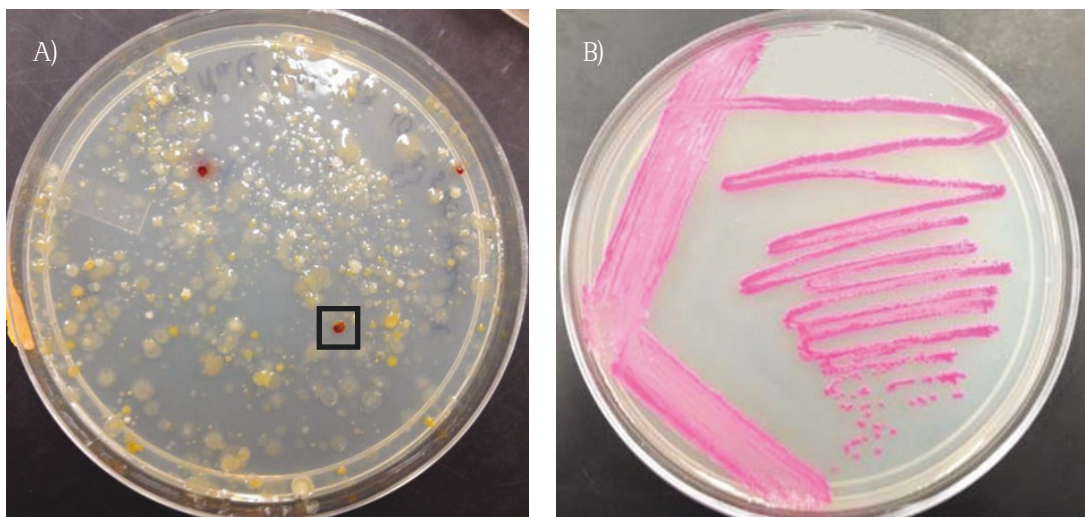


Figure 1. Isolation and Colony Appearance of MI-2  
Panel A shows the original isolation of MI-2 from Leffis Key in Bradenton, FL on modified PDA media. The red/pink colony of MI-2 selected for pure culture isolation is shown in the black square. Panel B shows the pure culture and pink pigment production of MI-2 on modified PDA media.

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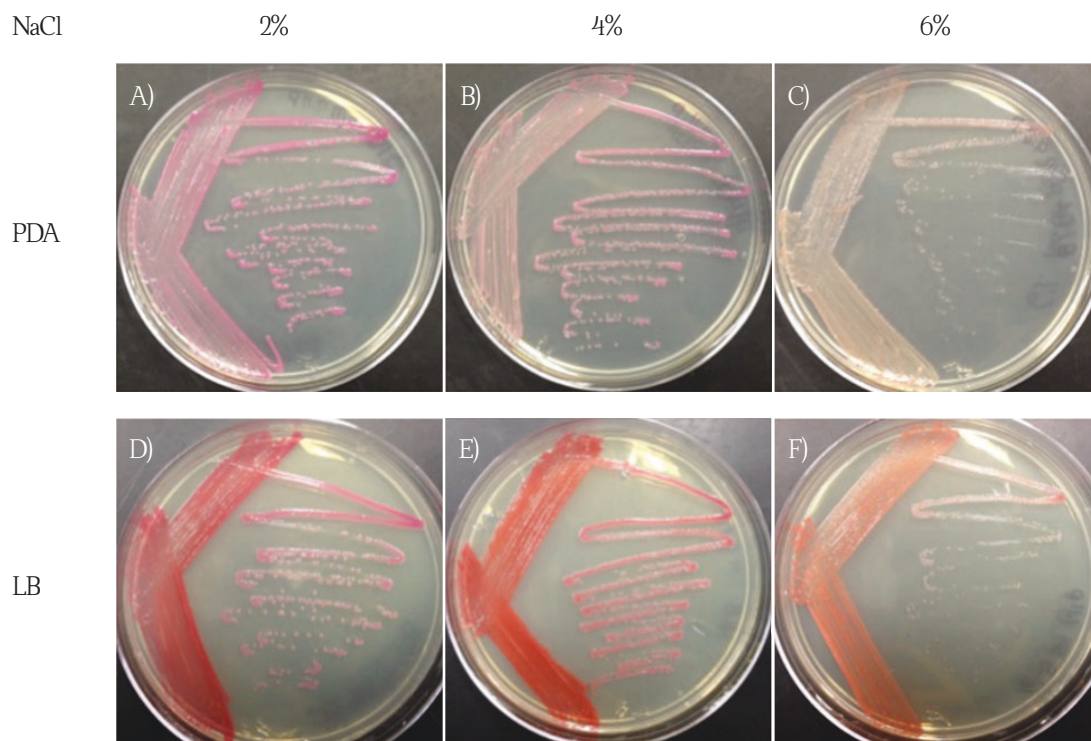
## RESULTS

### ISOLATION AND GENUS IDENTIFICATION OF MI-2

The marine bacterium MI-2 was originally cultured from salt flat sediment obtained at Leffis Key in Bradenton, FL on modified PDA media supplemented with 0.5 M NaCl for marine organisms and tryptone as described in the methods to support growth of fastidious organisms. Figure 1 shows the original marine sediment isolation plate in panel A and the pure culture growth of MI-2 in panel B.

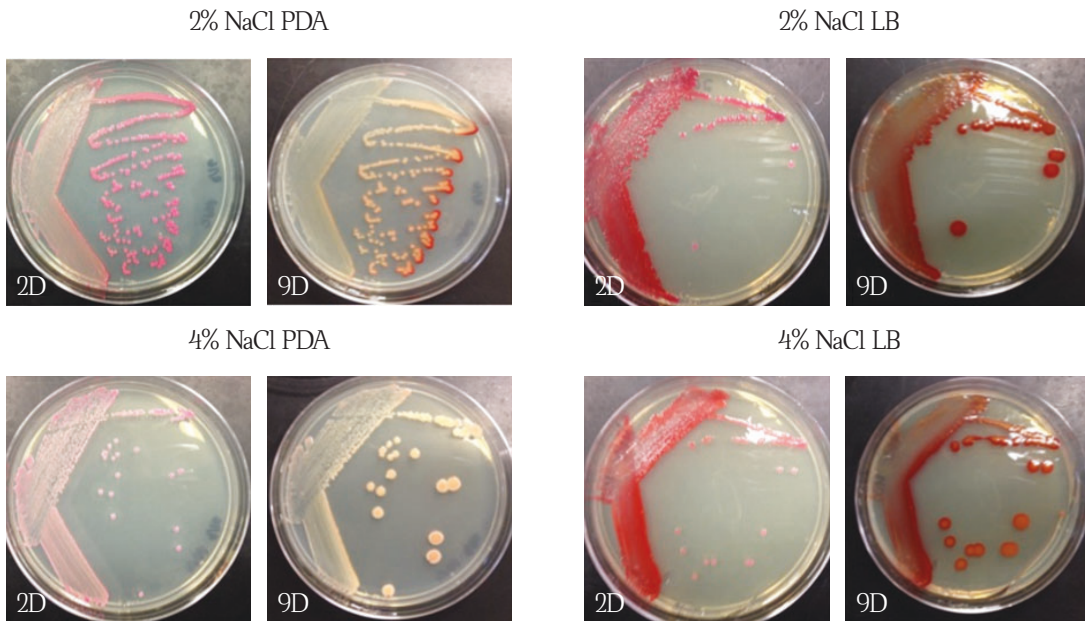
The purpose of this isolation was to identify antibiotic-producing microorganisms from diverse marine environments. MI-2 was identified as an antibiotic-producer (data not shown), but its ability to produce a bright pink pigment when grown on the modified PDA prompted further characterization of this microorganism. Sequencing of the 16S rRNA gene identified the organism as being a member of the *Vibrio* genus with *Vibrio ruber* being the closest related species (data not shown).





Media	2% NaCl		4% NaCl		6% NaCl	
	Max Abs. $\lambda$ (nm)	Avg. Abs. ( $\pm$ SE)	Max Abs. $\lambda$ (nm)	Avg. Abs. ( $\pm$ SE)	Max Abs. $\lambda$ (nm)	Avg. Abs. ( $\pm$ SE)
PDB	545	1.70 $\pm$ 0.14	545	1.87 $\pm$ 0.08	540	1.73 $\pm$ 0.08
LB	500	0.72 $\pm$ 0.02	500	1.12 $\pm$ 0.03	500	1.24 $\pm$ 0.05

Figure 2. Effect of Media Composition and NaCl on MI-2 Pigment Production  
MI-2 was quadrant streaked onto modified PDA and LB plates at the indicated NaCl concentrations and incubated at 30°C for 48 hours. Panels A–C show growth on PDA at 2%, 4%, and 6% NaCl concentrations. Panels D–F show growth on LB at 2%, 4%, and 6% NaCl concentrations. The shade of the pigment is different when grown on PDA compared to LB. This is confirmed by the corresponding table that shows the maximum absorbance wavelength (Max Abs.  $\lambda$ ) and the average absorbance value (Avg. Abs.)  $\pm$  standard error (SE) for three replicates grown in PDB and LB at 30°C for 48 hours.



NaCl Concentration	PDB				LB			
	Two Days		Five Days		Two Days		Five Days	
	Max Abs. $\lambda$ (nm)	Avg. Abs. $\pm$ SE	Max Abs. $\lambda$ (nm)	Avg. Abs. $\pm$ SE	Max Abs. $\lambda$ (nm)	Avg. Abs. $\pm$ SE	Max Abs. $\lambda$ (nm)	Avg. Abs. $\pm$ SE
2%	545	1.70 $\pm$ 0.14	545	2.02 $\pm$ 0.30	500	0.72 $\pm$ 0.02	450	0.781 $\pm$ 0.10
4%	545	1.87 $\pm$ 0.08	540	2.30 $\pm$ 0.01	500	1.12 $\pm$ 0.03	450	1.39 $\pm$ 0.20

Figure 3. Effect of Time on MI-2 Pigment Production

MI-2 was quadrant streaked onto LB and modified PDA with 2% and 4% NaCl and incubated at 30°C for nine days. Photographs were taken after two days of growth (2D) and after nine days of growth (9D). The corresponding table shows the Max Abs.  $\lambda$  and Avg. Abs.  $\pm$  SE for three replicates grown in PDB and LB at 30°C for two days and five days. The Max Abs.  $\lambda$  changed dramatically from two days (500 nm) to five days (450 nm) when grown in LB with 2% and 4% NaCl but not in modified PDB at the same salt concentrations. The pigment intensity appeared to be notably decreased after nine days of growth, but this decrease was not reflected quantitatively in the absorbance values.



## MEDIA AND GROWTH EFFECTS ON PIGMENT PRODUCTION

The effects of media (LB vs. PDA) and NaCl concentration (2%, 4%, or 6%) on pigment production of MI-2 are shown in Figure 2. While the pigment produced by MI-2 appeared bright pink on the 2% NaCl PDA plate, it was more red on the 2% NaCl LB plate. This difference was quantitatively confirmed by measuring the absorbance after growth in liquid samples (tabular data in Figure 2). The maximum absorbance wavelength (Max Abs.  $\lambda$ ) was 540–545 for PDB and 500 for LB. There was a slight change in the Max Abs.  $\lambda$  for PDB at 4% (545 nm) to 6% (540 nm) which may explain the slight change seen in the pigment color from panels B to C. Pigment production on both media types visually appeared to show a decrease in intensity as the concentration of NaCl in the media increased as seen by comparing panels A to C and D to F, however this decrease was not replicated quantitatively as there is no decreased absorbance seen with higher NaCl concentrations.

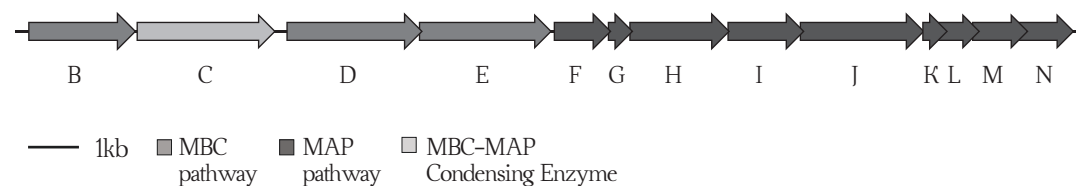
The effect of time on pigment production of MI-2 is shown in Figure 3. After nine days of growth, pigment appearance of MI-2 appeared notably altered from where it was after two days of growth. Extensive growth on PDA appeared to result in decreased pigment content, and altered pigment color from red–pink to red–orange was seen after growth on LB. The change in pigment appearance was more dramatic on both media types at 4% NaCl than at 2% NaCl. Quantitative analysis of this (tabular data in Figure 3) confirmed a change in the max. abs.  $\lambda$  from 500 nm to 450 nm when grown in LB while the max. abs.  $\lambda$  of PDB remained relatively unchanged

by prolonged growth. Average absorbance values do not show the decreased pigment production that is visible in the figure as values for all samples actually increased from two days to five days.

## IDENTIFICATION OF PUTATIVE PRODIGIOSIN BIOSYNTHESIS GENE CLUSTER

In order to better characterize and identify MI-2, its genome was sequenced. After whole genome sequencing and annotation, the putative prodigiosine biosynthesis gene cluster was identified based on homology to the well characterized 14 gene *pig* operon found in *S. marcescens* (17). The MI-2 prodigiosine biosynthesis gene cluster is approximately 20.3 kb in length and consists of 13 genes (Figure 4). The overall structure of the MI-2 cluster is highly similar to prodigiosine biosynthesis clusters in both the arrangement of protein homologs as well as in the relative sizes of proteins and intergenic spacing. Six of the open reading frames (ORF) overlap with one another, but a significant gap of 177 base pairs was found between the putative *pigC* and *pigD*. This is comparable with the structure of the *pig* cluster in *S. marcescens* as well as the *hap* cluster in *H. chejuensis* (17, 22).

The major difference between the structure of the putative *pig* cluster in MI-2 and *S. marcescens* is the absence of *pigA* in MI-2. A nucleotide alignment of bases preceding *pigB* with the coding nucleotide sequence for *pigA* in *S. marcescens* ATC 2744 showed short regions of homology (data not shown). This suggests that a *pigA* homolog may have been present at one time but has since been lost.



<i>pig</i> Homolog	ORF Size (bp)	Start*	End*
B	2,070	1	2,070
C	2,288	2,440	4,728
D	2,628	4,905	7,533
E	2,556	7,530	10,086
F	1,010	10,170	11,186
G	204	11,197	11,461
H	1,944	11,464	13,408
I	1,467	13,410	14,877
J	2,471	14,821	17,292
K	320	17,302	17,622
L	814	17,563	18,377
M	1,220	18,210	19,430
N	1,094	19,119	20,213

\*Nucleotide start and end positions are given relative to the start position of ORF B.  
Table rows highlighted in gray indicate an ORF that overlaps with the ORF preceding it.

Figure 4. Schematic Representation of Putative Prodiginosin Biosynthesis Gene Cluster in MI-2. The cluster is approximately 20.3 kb in length and contains 13 genes. The arrows show the directionality of ORFs. Letters for ORFs correspond to the *pig*B–N homologs. Different arrow patterns indicate the putative role of the enzyme in the bifurcated prodiginine biosynthesis pathway in which *Pig*C condenses the compound 4-methoxy-2,2-bipyrrole-5-carboxyaldehyde resulting from synthesis of the MBC pathway with the compound 2-methyl-3-n-amylyl-pyrrole resulting from the synthesis of the MAP pathway). The corresponding table shows the size of each ORF, the nucleotide start position for each ORF and the nucleotide end position for each ORF.

Table 1. AAI of Prodiginine Biosynthetic Gene Cluster Homologs

The table shows the AAI of MI-2 putative prodigiosin biosynthetic gene cluster homologs compared to known prodiginine biosynthetic proteins from *S. marcescens* ATCC 274, *H. chejuensis* KCTC 2396, and *S. coelicor* A3(2). AAI with MI-2 homologs were calculated using Clustal Omega.

MI-2	<i>S. marcescens</i>		<i>H. chejuensis</i>		<i>S. coelicor</i>	
	Homolog	% AAI	Homolog	% AAI	Homolog	% AAI
A*	PigA	55	HapA	54	RedW	43
B	PigB	57	HapB	38	RedS	35
C	PigC	69	HapC	54	RedH	40
D	PigD	73	HapD	51	-	-
E	PigE	80	HapE	61	-	-
F	PigF	73	HapF	58	RedI	19
G	PigG	64	HapG	45	RedO	21
H	PigH	72	HapH	58	RedN	59
I	PigI	58	HapI	43	RedM	40
J	PigJ	61	HapJ	33	RedX	
K	PigK	68	HapK	47	RedY	45
L	PigL	17	HapL	19	RedU	19
M	PigM	48	HapM	33	RedV	26
N	PigN	17	HapN	14	RedF	15

PigD and PigE homologs in *S. coelicor* have not been found.

\*PigA homolog is not found within the MI-2 cluster.

## PROTEIN SEQUENCE COMPARISON OF THE PUTATIVE PRODIGIOSIN BIOSYNTHESIS CLUSTER

The amino acid identities (AAI) of the proteins coded for by the putative prodigiosin biosynthesis genes were compared to their homologs of *S. marcescens*, *H. chejuensis*, and *S. coelicor* (Table 1). In addition to the structural homology of the gene cluster, there was also

consistent homology between genes in the MI-2 cluster and corresponding genes in the prodiginine biosynthesis operon of the other species. The AAI between MI-2 and Pig homologs typically ranged from 50% to 80%, a comparable range compared with other studies that have shown an AAI of only 23% or more between *Vibrio* and *Serratia* species (16, 20, 39). Exceptions to this can be seen in PigL and PigN homologs, which had very low AAI; however, neither of these two proteins are required for prodigiosin production (43).

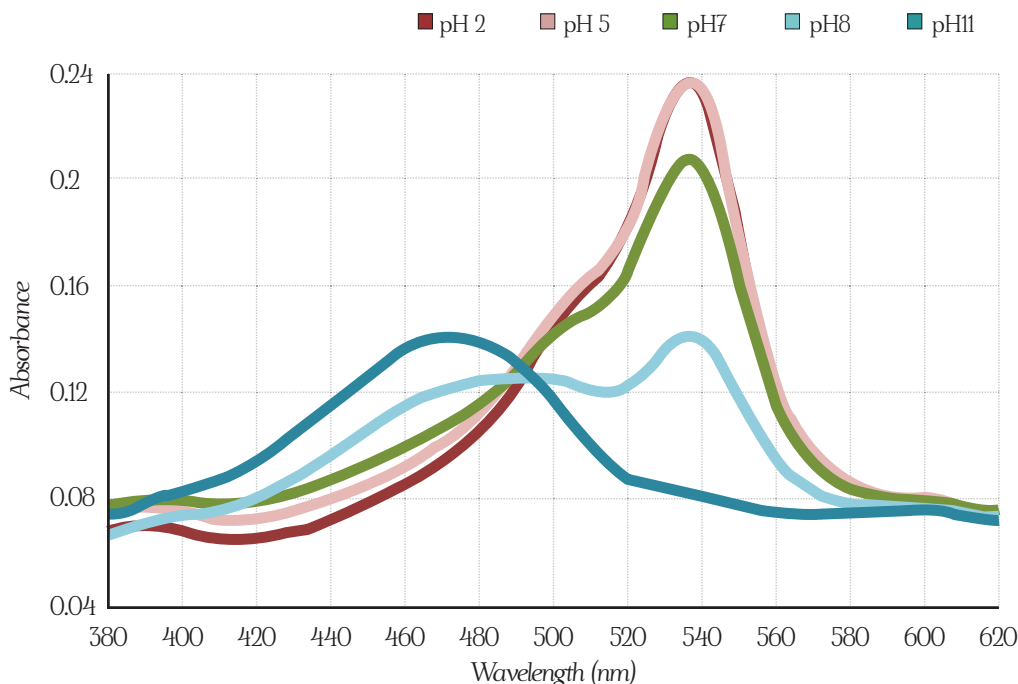


Figure 5. UV-Vis Spectra of MI-2 Pigment Extract at Various pHs

The graph shows the absorbance from wavelengths ranging from 380 nm to 620 nm of MI-2 pigment extract in aqueous methanol solvents at varying pHs. Two maxima can be observed at 470 nm and 535 nm. Absorbance at 535 nm is greater at acidic pH while absorbance at 470 nm is greater at basic pH. An isosbestic point can be observed at 490 nm.

As mentioned previously, there is no homolog for PigA found in the MI-2 putative prodigiosin biosynthesis gene cluster. However, a different protein found elsewhere in the MI-2 genome did show homology to PigA. It is the sequence of this putative protein that is used for PigA comparison from MI-2 in Table 1.

## PURIFICATION AND UV-VIS SPECTRA OF MI-2 PURIFIED PIGMENT EXTRACT AT DIFFERENT PH

Extraction and purification of the MI-2 pigment was completed by flash column chromatography. The UV-Vis spectra of the purified MI-2 pigment extract resuspended in

different pH solutions is shown in Figure 5. The spectra showed two main peaks that depended on the pH of the solvent. This suggests that the compound exists in either a protonated or non-protonated form (34). The peak absorbance for what was presumed to be the protonated form occurred at a wavelength of approximately 535 nm and the non-protonated peak absorbance occurred at approximately 470 nm. These results were consistent with the UV-Vis spectra for prodigiosin (18).

The spectra for the MI-2 pigment extract revealed an isosbestic point at a wavelength of approximately 490 nm. This is the wavelength in which the absorbance remains constant as a conformational change occurs due to the changes in pH of the aqueous methanol. Additionally, the data suggested that the pH at

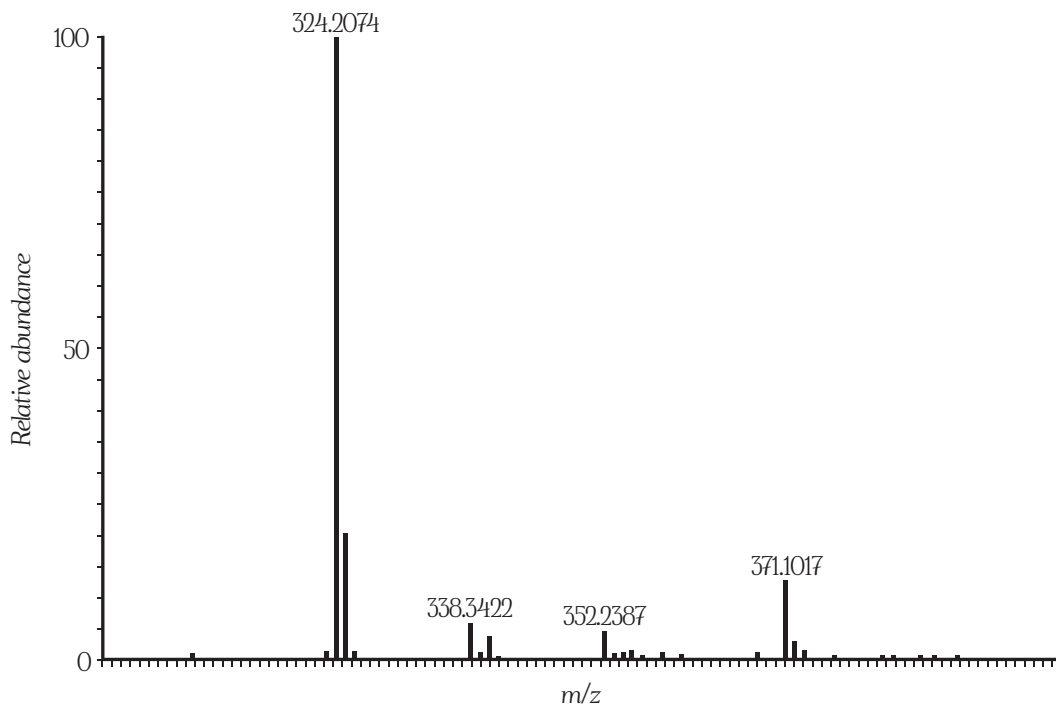


Figure 6. Electrospray Ionization Mass Spectra of MI-2 Pigment Extract  
The full mass spectra of the purified MI-2 pigment extract is shown. Peaks represent ions of a particular mass (x-axis) with a relative abundance (y-axis). The peak at 324 is the most prominent while peaks at 338, 352 and 371 appear as minor components.

which the absorbance between the two peaks would be equal was just above pH 8, which is consistent with the known pKa value of 8.25 for prodigiosin in acidified ethanol (18).

## MASS SPECTRA OF MI-2 PURIFIED PIGMENT EXTRACT

The full mass spectrum of purified pigment extract from MI-2 is shown in Figure 6. This spectrum revealed a mixture of four different compounds in different abundances. A compound with a mass of 324 g/mol was the main component of the mixture, and compounds with masses of 338 g/mol, 352 g/mol and 371 g/mol were present in the extract as minor components.

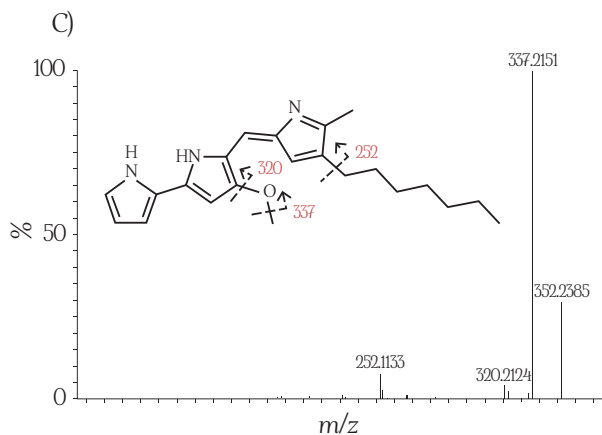
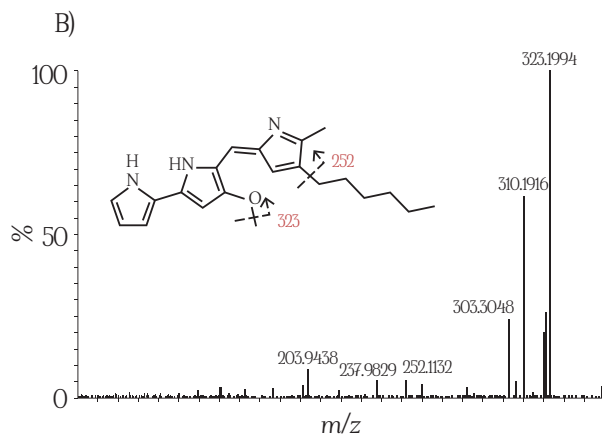
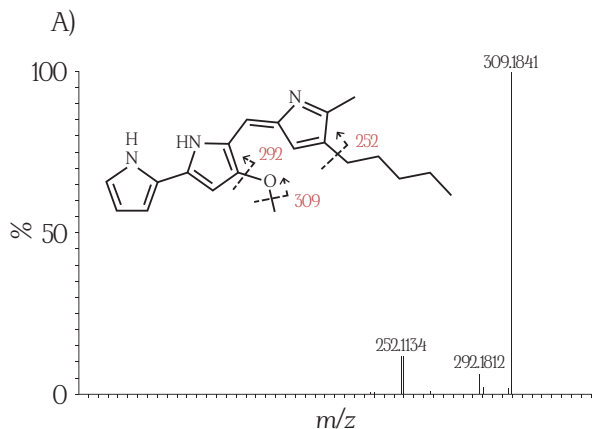
The tandem mass spectra of each of these four peaks are shown in Figure 7. Tandem mass

spectra of compounds with a mass of 324, 338 and 352 exhibited similar fragmentation patterns that were consistent with prodiginine compounds. Specifically, all three contained a peak at 252 m/z, which can be explained by the loss of the alkyl substituent group to yield the 2-methoxy prodiginine core. Additionally, all three showed an ion at a mass consistent with the loss of the methyl group from the methoxy. Compounds 324, 338 and 352 can be represented by compounds with varying lengths of alkyl chains, as there is a mass difference of 14 that can be represented by the addition of a CH<sub>2</sub>. The compound with a mass of 371 appeared to be unrelated as it did not contain the prodiginine core 252 peak; however, further analysis would be required to fully elucidate the structure of this compound. The spectrum from this peak is not shown in Figure 7.



Figure 7. Tandem Mass Spectra of Peaks from the Full Mass Spectra

The tandem mass spectra of the relevant prodiginine peaks from the full mass spectra in Figure 6 are shown. Peaks represent ions produced as a result of gas-induced dissociation (GID) of the parent molecule. Panel A shows the tandem mass spectra of a 324 molecular mass molecule with the proposed structure prodiginosin. The most stable ion produced appears at  $m/z$  309 and is the ion produced by cleavage of the methyl on the methoxy group. The peak at 252 represents an ion created as a result of the alkyl chain being cleaved from the molecule and 292 is the loss of the oxygen. These fragmentation patterns are shown with the dashed arrows. Panel B shows the tandem mass spectra of a 338 molecular mass molecule with the proposed structure 2-methyl-3-hexyl prodiginine. The most stable ion produced appears at  $m/z$  323 and is the ion produced by cleavage of the methyl on the methoxy group. The peak at 252 represents an ion created as a result of the alkyl chain being cleaved from the molecule, and the peak at 310 is likely loss of an ethene. Other peaks represented are not readily explainable given the proposed structure. It is possible this sample that is in low abundance is contaminated. Panel C shows the tandem mass spectra of a 352 molecular mass molecule with the proposed structure prodiginosin. The most stable ion produced appears at  $m/z$  337 and is the ion produced by cleavage of the methyl on the methoxy group. The peak at 252 represents an ion created as a result of the alkyl chain being cleaved from the molecule, and 320 is produced after the loss of the oxygen.



## DISCUSSION

The biosynthesis of prodigiosin is an interesting metabolic phenomenon as it is a secondary metabolite with no known direct benefit to cellular growth (43). Pure non-pigmented strains of *S. marcescens* show no significant difference in viability from pigmented strains (41). However, in the natural environment, prodigiosin and its derivatives may serve a purpose in bacterial defense as many have antibacterial properties (12, 25).

MI-2 is a prodigiosin-producing marine bacterium of the *Vibrio* genus. Its species designation appears to be closest to *Vibrio ruber*, a known prodigiosin producer (8), though its biochemical test profile differs from *V. ruber* in multiple ways, suggesting MI-2 is a strain of *V. ruber* unique from the wild type (manuscript in preparation). Although prodiginine compounds and the organisms that produce them have been studied for a long time, there is limited research on prodigiosin biosynthesis clusters in different genera. To date, prodigiosin biosynthesis clusters have only been examined in *S. marcescens* ATCC 274, *Serratia* spp. 39006, *H. chejuensis* KCTC 2396, and some *Streptomyces* species (5, 17, 22, 36, 43). The addition of another prodiginine biosynthesis cluster in *Vibrio* species will add more information on the potential catalytic mechanism for each enzyme by revealing key residues in conserved regions of each gene.

The appearance of the prodigiosin pigment production of MI-2 is affected by the media on which it grows and the length of incubation. The pigments produced by growth in LB and modified PDB showed different max absorbances, verifying the different colored appearance

seen on the plates in Figure 2. Pigment production on both LB and modified PDA media types appeared to show a decrease in intensity as the concentration of NaCl in the media increased, suggesting an inhibitory effect of excess NaCl on pigment production. This phenomenon could not be validated quantitatively in liquid broth (tabular data of Figure 2), however it is difficult to draw a comparison in intensities from the absorbance values as differences in the concentration of cells in each sample will affect the level of pigment production and therefore the absorbance values reached. These could be further complicated by the regulation of prodigiosin production by quorum sensing (43).

Additionally, increasing the length of incubation from two days to nine days resulted in altered coloration, particularly in LB media, and the appearance of decreased pigment intensity in plates, though this occurrence could also not be reproduced quantitatively in liquid (Figure 3). The qualitative data suggest the pigment produced by MI-2 may not be stable for extended periods of time in the conditions it was grown (at room temperature and exposed to oxygen) as has been shown for other prodigiosin analogs (40). However further testing with a purified sample of the pigment would need to be performed to confirm this. Growth after nine days on PDA with 2% NaCl showed more red coloration on the right hand side of growth in the third quadrant compared to the rest of the plate. This difference is believed to be caused by the pattern of pigmentation fading in the colonies and not an effect of the media itself.

When comparing the structure of the putative prodigiosin biosynthesis cluster in MI-2 to *S. marcescens*, the most notable difference is the absence of *pigA* in MI-2. *PigA* functions as a flavoprotein desaturase whose catalytic role in the prodigiosin biosynthetic pathway occurs after *PigI* but before *PigJ*. An alternative reaction converting the substrate of *PigA* to the correct product without the use of *PigA* has been experimentally confirmed (13), perhaps negating the need for *PigA* in the prodigiosin biosynthetic pathway of MI-2. Additionally, as mentioned in the results, a different protein elsewhere in the genome did show homology to *PigA* and may be able to function in its place in prodigiosin biosynthesis.

As shown in Table 1, the AAI between proteins encoded by the putative prodigiosin biosynthetic gene cluster and known prodiginine biosynthetic proteins were typically between 50 to 80%. However, AAI comparison is likely not the best method for comparing protein function. Conservation of specific domains may be a better determinant as to whether a group of proteins will serve the same function. Although *H. chejuensis* and *S. marcescens* both produced prodigiosin, the amino acid identity between *Pig* and *Hap* homologs generally ranged from only 30% to 55% (23). Despite the seemingly lower amino acid identities between these homologs, these enzymes served the same function in prodiginine biosynthesis (23, 43). A better comparison of protein function can be done by comparing specific amino acid residues that are essential to the function of the protein.

According to the NCBI protein basic local alignment tool, the MI-2 homolog of *PigC* contained a domain belonging to the pyruvate phosphate dikinase (PPDK) superfamily. *PigC* performs the final reaction of the prodigiosin biosynthesis pathway, which combines the two precursor molecules 4-methoxy-2,2-

bipyrrole-5-carboxyaldehyde (MBC) and 2-methyl-3-n-amylyl-pyrrole (MAP) into the final prodigiosin structure (29, 43). The mechanism for *PigC* is similar to that of PPDK enzymes in that they phosphorylate a carbonyl by facilitating the transfer of a phosphoryl group from ATP using a histidine residue in a phosphoryl transfer domain (PTD) (6). *S. marcescens* strains with a *pigC* knockout completely lost the ability to produce the prodigiosin pigment (43). Selective mutagenesis experiments on *PigC* in *Serratia* spp. 39006 showed that replacement of His840 with an alanine residue completely eliminated activity. Similarly, residues predicted to be important for ATP binding, Glu281 and Arg295, showed significant reduction in activity when replaced with alanine residues (6). Closer analysis of the amino acid sequence in the MI-2 homolog also revealed residues His841, Glu282 and Arg296 that are analogous to key residues in *PigC* (data not shown). Given this similarity, it is likely that *PigC* and the MI-2 protein homolog function identically to one another. Investigations to experimentally confirm the function of the putative prodigiosin biosynthetic proteins in MI-2 are underway.

The prodiginine compounds isolated and identified in this study have been previously recognized and documented in several other species (1, 22, 26). Given the homology between *pig*, *hap*, and MI-2 prodiginine gene clusters it is plausible that MI-2 would produce similar prodiginine derivatives to those found in *Serratia* species and *H. chejuensis*. The mass spectrum of the purified MI-2 pigments contains multiple peaks from prodiginine compounds with different alkyl chain lengths. Prodiginine compounds varying in the length of the alkyl chain substituent group on a pyrrole of the structure are well documented and have been produced as minor byproducts in other species (1, 14, 26). The difference in MI-2 appearance on the modified PDA and the LB plates may be due to the amount of

overall prodiginine production and the ratio of different prodiginine compounds produced on each medium. This ratio has been previously correlated with color change in *V. gazogenes* (1).

Additional pigment spots on the TLC (data not shown) indicated that there may be additional prodiginine compounds produced by MI-2. In other studies, HPLC was used with a reversed-phase C-18 column to purify prodiginines (1, 28). Preparative TLC has also been used to purify different prodiginines (10, 31). Additionally, use of acetonitrile as a solvent for flash chromatography may have impeded separation by not stabilizing a single conformation during separation techniques. Prodigiosins prefer the  $\beta$  conformer in acetonitrile and would likely prefer the  $\alpha$  conformer in response to interactions with the silica, which is slightly acidic (34). A similar separation was achieved using ratios of chloroform, methanol and ethanol but was not extensively tested due to the convenience of using a single solvent; however, a better separation might be achieved by experimenting with the solvent ratios further.

Finally, prodiginine compounds have been well documented as possessing antibacterial capabilities against Gram negative and Gram positive organisms (9). The initial antibacterial properties observed in MI-2 (data not shown) may be due to the

sole production of prodiginine compounds, production of an unrelated antibacterial molecule, or the combined production of prodiginine and another antibiotic. It is not clear whether another antibiotic is produced by MI-2 or not. Further investigation using techniques such as bioautography on crude MI-2 extracts may show the production of more than one antibiotic.

In summary, a novel pigment and antibiotic-producing marine bacterium named MI-2 was isolated and identified as belonging to the *Vibrio* genus. The coloration and intensity of the pigment is effected by the media type, salt concentration, and time of incubation. After whole genome sequencing, a putative prodigiosin biosynthetic pathway was computationally identified MI-2 by sequence comparison to known prodigiosin producers, suggesting the pigment produced could be a prodigiosin derivative or a member of the prodiginine family. Purification and structural elucidation of the pigment produced by MI-2 demonstrated a mixture of four compounds, three of which were shown to be in the prodiginine chemical family and two of which were prodigiosin. Further research to experimentally verify the function of the 13 genes in the putative prodigiosin biosynthetic pathway and investigate the regulation of their expression is underway.

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