Exploring Antimicrobial Activity of Crab Bacterial Isolates Against Potential Marine Pathogens

THE UNIVERSITY of NORTH CAROLINA at CHAPEL HILL

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Introduction

Globally, an increasing number of pathogens are evolving resistance to our current antibiotics, signaling the need for new antibiotics. While 70% of our current antibiotics were discovered from terrestrial microbes, marine bacteria are an understudied source of potential new antibiotics.

Previous research has shown that bacteria isolated from shrimp[1], lobster[2], and squid eggs[3] have antimicrobial activity that protects the eggs against infections. This study investigates similar activity in mud crab eggs collected from the North Carolina coast. Twenty-one bacteria were isolated from the crabs *Eurypanopeus depressus* and *Petrolisthes armatus.* These crab isolates were tested against marine bacteria isolated from seawater collected off the coast of North Carolina which may be potential pathogens of marine organisms.

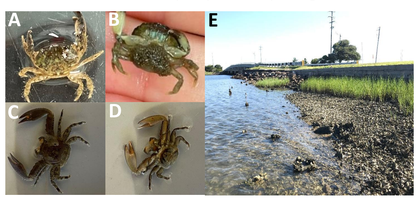


Figure 1. Mud Crabs and Sampling Site
Eggs were sampled from different mud crabs, with the sample IDs C14 **(A & B)** and C17 **(C & D)**. C14 was identified as the mud crab, *Eurypanopeus depressus,* and C17 is a porcelin crab, *Petrolisthes armatus*. They were collected from intertidal areas near the UNC Institute of Marine Sciences at Morehead City, North Carolina **(E)**.

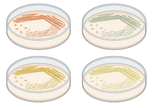
Objectives

- Use zone of inhibition assay to test for antimicrobial activity of crab strains against the environmental bacterial targets *Alteromonas*, *Pseudoalteromonas*, and *Ruegeria* species
- Sequence crab and crab isolate DNA using PCR to find their identities

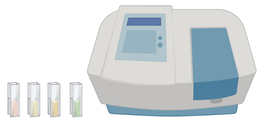
Methods

Creating and Measuring the Zone of Inhibitions

1. Plate crab isolates and chosen potential pathogens onto a SWT plate and incubate at 29°C for 24-72 hours

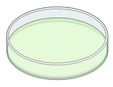


2. Measure cell density using a spectrophotometer at 600nm



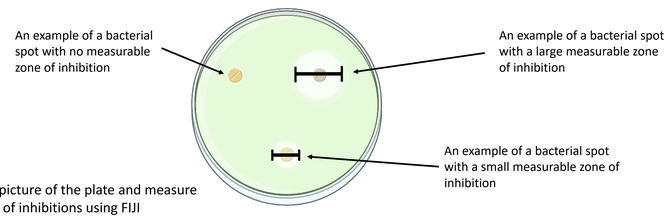
3. Normalize samples to an optical density (OD600nm) equal to 1

4. Spread a lawn of the potential pathogen and allow it to dry for 20 minutes



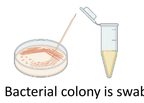
5. Spot the crab isolate bacteria onto the lawn and allow it to dry for 5-10 minutes

6. After 24-72 hours, measure the diatmeter of zone of inhibition (black bars below)



7. Take a picture of the plate and measure the zome of inhibitions using FIJI

PCR



1. Bacterial colony is swabbed from an agar plate and placed into Tris-HCl buffer

2. Heat samples in thermomixer to 95°C for 15 min

3. Centrifuge samples at 10,000 xg for 15 min

4. Collect the supernatant containing DNA

5. Mix 1µL of 16S rRNA forward primer, 1 µL of 16S rRNA reverse primer, and a ratio amount of MiliQ water and DNA supernatant with 12.5 µL Econotaq mastermix to perform PCR

6. Gel electrophoresis of the PCR product to confirm 16S rRNA gene was amplified

7. Clean PCR product with ZYMO DNA Clean and Concentrator Kit, then send to Eton Biosciences for Sanger DNA Sequencing

Antimicrobial Activity of Crab Isolates

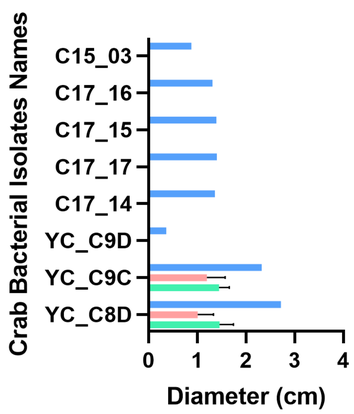


Figure 2. Zone of Inhibition Diameters of Crab Isolates Against Target Bacteria
The graph above represents the relationship between the crab bacterial isolates and their zone of inhibition diameters measured in centimeters. Each bar represents a zone of inhibition against a potential pathogen *Alteromonas* (green), *Pseudoalteromonas* (red), and/or *Ruegeria* (blue). Some bacterial isolates inhibited all potential pathogens, while others do not inhibit any. A larger zone of inhibition indicates stronger inhibition. Data is an average of three trials.

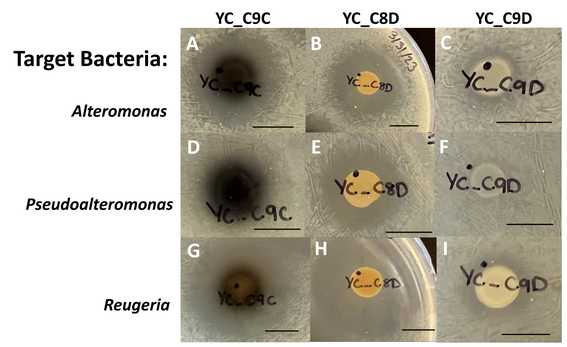
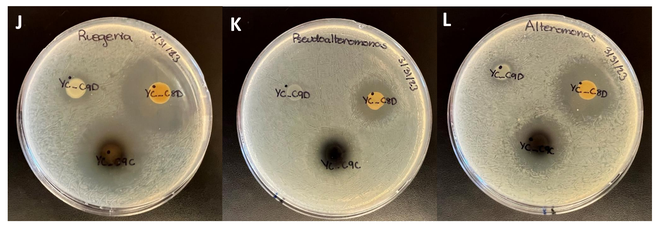
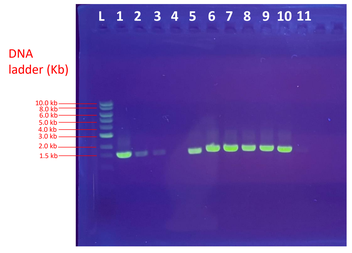


Figure 4. Zone of Inhibition Assay Images
Representative images of zone of inhibitions created by the isolate.
Images **A-C** show the isolates' activity against *Alteromonas*, **D-F** shows their responses to *Pseudoalteromonas*, and **G-I** shows their responses to *Reugeria.* Scale bars = 1cm.

Images **J-L** show how the spots were plated onto the lawns of the potential pathogens. Images were taken aproximately 24 hours after incubation.



PCR Results



Lane Number and Crab Isolate Identification
L. Ladder
1. YC_C9D
2. YC_C8D
3. YC_C9C
4. C14_29
5. C17_14
6. C17_15
7. C17_16
8. C15_03
9. C14_13
10. C17_17
11. Negative control

Figure 3. Gel Electrophoresis of Amplified 16S rRNA Genes from Crab Isolates
PCR products were run on a 1% agarose gel at 100 volts for 20 min. The bands of the crab isolates are between 1.5kb and 2.0 kb (expected product = 1,500 bp), with one lane (**sample 4**) that did not amplify. Crab isolate C14_13 was omitted from further sequencing as it did not show antimicrobial activity for any of the potential pathogens used in this experiment.

Identities of the Crab Bacterial Isolates

Using BLAST Results to Find the Possible Identitites of the Crab Bacterial Isolates

Crab ID	Isolate ID Name	Scientific Name	% ID	E-Value	Query Cover %	Genus
Eurypanopeus depressus	YC_C9D	*Vibrio mediterranei*	95.53	0	94	*Vibrio*
Petrolisthes armatus	YC_C8D	*Pseudoalteromonas piscicida*	97.34	0	92	*Pseudoalteromonas*
Eurypanopeus depressus	YC_C9C	*Pseudoalteromonas peptidolytica*	98.05	0	97	*Pseudoalteromonas*
Petrolisthes armatus	C17_14	*Leisingera aquaemixtae*	96.04	0	98	*Leisingera*
Petrolisthes armatus	C17_15	*Shewanella submarina*	95.22	0	95	*Shewanella*
Petrolisthes armatus	C17_16	*Shewanella submarina*	95.55	0	95	*Shewanella*
Petrolisthes armatus	C15_03	*Vibrio alginolyticus*	97.44	0	98	*Vibrio*
Petrolisthes armatus	C17_17	*Shewanella yunxiaonensis*	87.43	8.00E-119	49	*Shewanella*

Table 1. BLAST Results of Crab Isolate IDs
DNA samples were sent with the universal 16S rRNA primers to identify the taxonomy of the isolates. The table above shows the names and taxonomic lineages of the crab bacteria to find out which specific bacteria inhibit the potential pathogens. The percent identification shows how likely the bacteria named is similar to the one found through the BLAST database. E-value (expected value) is the probability that the ID is false, with zero and negative values indicating that it is unlikely to be false. Query Cover ID % shows the number of nucleotides in the DNA sample that aligned with the nucleotides in the BLAST database.

Conclusions

- Of the 21 strains (3 trials) tested, the results showed that 8 strains inhibited *Ruegeria*, 2 inhibited *Alteromonas*, and 2 inhibited *Pseudoalteromonas.*
- As the strains created zone of inhibitions, it is predicted that they are producing diffusible antimicrobials.
- The PCR results revealed that isolates YC_C9D and C15_03 are *Vibrios*; YC_C8D and YC_C9C are *Pseudoalteromonas*; C17_14 is *Leisingera*; and C17_15, C17_16, and C17_17 are *Shewanella*.
- In past studies, *Vibrio mediterranei*[4], *Pseudoalteromonas piscicida*[5], *Pseudoalteromonas peptidolytica*[6], and *Vibrio alginolyticus*[7] were shown to have antimicrobial activity against a variety of bacteria, including marine pathogens.

Future Directions

- Screen more of the crab egg isolates in the zone of inhibition assay. In this study, only 21 of the 104 crab isolates were screened.
- Test crab isolates against human pathogens to see if their antimicrobials have potential to be studied further for antibiotic development.
- Identify the antimicrobial chemicals being produced by the active crab isoaltes

Acknowledgements

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