

FINAL PROGRESS REPORT

Project Title	Testing and Application of Novel Probiotic Bacteria for Use in Marine Aquaculture
Reporting Period	
Author (Project Coordinator)	Name of person submitting this report. <i>Dennis McIntosh</i>
Key Word	Probiotic, Fundulus, finfish, trout, tilapia, hybrid striped bass
Funding Level	Total funds allocated for this project to date. <i>Year One: FY 2012, \$ amount \$97,716</i> <i>Year Two: FY 2013, \$ amount \$98,708</i>
Participants	Name(s)/Role(s): Harold J. Schreier*, Co-PI; Eric J. Schott*, Co-PI Institution/Agency/Business: UMCES; IMET Address(s): 701 E. Pratt St., Baltimore, MD 21202 Phone(s): (410) 234-8874; (410) 234-8881 Email(s): schreier@umbc.edu ; schott@umces.edu
Project Objectives	This project was designed with six objectives: <ol style="list-style-type: none"> 1. Test the effect of probiotic candidates on larval performance of four (two freshwater and two marine) commercially important aquaculture species. 2. Measure the protective effect of probiotic candidates in the presence of pathogens. 3. Select strains having significant probiotic activity and introduce a molecular tag to aid in identification and develop a quantitative assay to facilitate year two objectives. 4. Host special session on probiotics in aquaculture at Aquaculture 2016 in Las Vegas, NV. 5. Determine dosing conditions (concentrations, dosing intervals, routes of administration) and track probiotic in intestine and culture water over time. 6. Characterize novel probiotics with respect to inhibition of pathogen growth and biofilm formation/retention.

<p>Anticipated Benefits</p>	<p>State briefly how the project will benefit the aquaculture industry – directly or indirectly.</p> <p>One strategy for controlling disease has been to utilize probiotics, which are live microbial supplements that beneficially affect the host by modifying the host-associated microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response to disease, or by improving water quality of its ambient environment. The probiotic acts by either competing with other bacteria for essential resources or nutrients, antagonism, or by producing their own broad-spectrum antibiotics. The application appears to be useful in a wide range of life-history stages, from larvae to adults. In the aquaculture industry, the application of probiotics is not systematically used and little is known about the specific mechanisms used by individual probiotic bacteria for protection. To optimize their effectiveness, probiotics should be selected from (adapted to) the environment in which they will be eventually used. <i>Fundulus heteroclitus</i> lives in habitats with a wide range of salinities, and can be cultivated in a similarly wide range in aquaculture. Therefore, the potential probiotics that we derived from this species as part of our 2012 NRAC Mini-grant have the potential to be applied to commercially important species from a range of salinities. Impacts from this research will be applicable throughout the Northeast region and beyond for both marine and freshwater aquaculture. This work will expand our knowledge base with respect to probiotic bacteria, and thereby allow us to directly address NRAC goal TRA-13-5 Improving shellfish and finfish health maintenance and disease control.</p>
<p>Project Progress</p>	<p>Summarize concisely for each objective the progress toward accomplishment to date. This has an 8,000 character limit.</p> <p>DSU</p> <ol style="list-style-type: none"> 1. Probiotic Trial 1 <ol style="list-style-type: none"> 1. Larval Nile Tilapia were chosen for the first growth and development trials given their importance and equity in the Northeast. 2. Trial parameters included daily water quality (ammonia, nitrites, nitrates, temperature), weekly weights and mortality count. 400 tilapia (1-2 days post hatch) were fed Zeigler “starter diet” three times daily. Culture was done in 16 separate 1-L containers with a 50% daily water exchange. Each container was assigned and dosed with one of the three probiotics (two <i>Bacillus</i> spp. and one <i>Shewanella</i> spp.) previously used in trials carried out at IMET. Dosing was done at 1 microliter to maintain 10⁶ concentration in culture units. Trial was terminated after 30 days due to high mortality throughout all tanks, most likely due to poor water quality due to a static system. 3. Statistical analysis showed that there was no statistically significant differences in the weights of the probiotic dosed animals versus the

controls, or among the probiotic treatment groups. This has several possible reasons, one of which being tilapia's resistance to stressors (environmental as well as bacterial).

2. Probiotic Trial 2

1. Larval rainbow trout were chosen for the second growth and development trials given their importance in aquaculture as well as their commercial value.
2. Trial parameters included daily water quality (ammonia, nitrites, nitrates, temperature), weekly weights and mortality count. 120 rainbow trout (1-2 days post hatch) were fed Zeigler "starter diet" three times daily. Culture was done in 16 separate 1-L containers with a 50% daily water exchange. Each container was assigned and dosed with one of the three probiotics (two *Bacillus* spp. and one *Shewanella* spp.) previously used in trials carried out at IMET. Dosing was done at 1 microliter to maintain 10^6 concentration in culture units. Trial was terminated after the scheduled 45 days.
3. Statistical analysis showed that there was no statistically significant differences in the weights of the probiotic dosed animals versus the controls, or among the probiotic treatment groups.

3. Probiotic Trial 3

1. Larval Hybrid striped bass were chosen for the third growth and development trials given their availability and their use in aquaculture.
2. Trial parameters included daily water quality (ammonia, nitrites, nitrates, temperature), weekly weights and mortality count. 120 hybrid striped bass (1-2 days post hatch) were fed Zeigler "starter diet" three times daily. Culture was done in 16 separate 1-L containers with a 50% daily water exchange. Each container was assigned and dosed with one of the three probiotics (two *Bacillus* spp. and one *Shewanella* spp.) previously used in trials carried out at IMET. Dosing was done at 1 microliter to maintain 10^6 concentration in culture units. Trial was terminated after 38 days due to high mortality throughout all tanks, most likely due to poor water quality due to a static system.
3. Statistical analysis showed that there was no statistically significant differences in the weights of the probiotic dosed animals versus the controls, or among the probiotic treatment groups.

IMET

Summer 2018 Hybrid Striped Bass challenge experiment:

We tested the ability of the three potential probiotic bacterial strains, ISO5, ISO11 and ISO12 (each at a final concentration of 10^6 CFU/ml) to protect hybrid striped bass (HSB) larvae against *Vibrio anguillarum* strain NB10 (@ 1×10^5 CFU/ml), which our previous year's study showed to be pathogenic to HSB. After a four-day pre-treatment with probiotic, fish were treated with one dose of *V. anguillarum*. Probiotic was then added

	<p>daily, monitoring temperature, water quality (ammonia, nitrate/nitrite, hardness and pH) and fish death. Our results indicated that the addition of the probiotic strains had no effect on fish mortality over the course of the 15-day experiment. In the presence of pathogen, we found no significant difference in the % survival of fish treated with probiotic compared to those that were not. However, there appeared to be a correlation between mortality and temperature in the presence of pathogen, with fish survival decreasing with increasing temperature (from 73.7°F to 81°F), which appeared to be alleviated significantly in the presence of strain ISO12. The protection provided by ISO12 against <i>V. anguillarum</i> at elevated temperatures is interesting and requires additional study.</p> <p>Genome sequence for <i>Bacillus</i> sp. strain ISO11: The genome sequence for strain ISO11 was determined and examined (Schreier, 2018). Analysis revealed approximately 86 kb of DNA encoding polyketide synthases and related proteins having >98% similarity to the aminopolyol zwittermicin A cluster of several <i>B. cereus</i> strains. Zwittermicin A is active against Gram-negative and Gram-positive bacteria as well as fungi and protists. Genes for biosynthesis of a thiazole/oxazole-modified microcin (TOMM) antimicrobials that may play a role in probiotic activity along with an associated microcin C7 immunity-encoding <i>mccF</i> were also identified. The ISO11 genome was also found to encode motility, adhesion, and aggregation functions needed for probiotic activity including flagella, exopolysaccharide biosynthesis (<i>epsBCDE</i> genes), enolase and fibronectin, as well as arginine deiminase pathway enzymes for protection against the acidic environment of the host's stomach. Hemolysin and hemolysin-like proteins were identified including hemolysin BL (Hbl) and non-hemolytic enterotoxin (Nhe) as well as cytotoxin K (CytK), which are causative agents of gastrointestinal disease. While we have found that ISO11 will not cause disease in finfish the genome sequence will be advantageous for characterizing ISO11-host interactions.</p>
Accomplishments:	
Outreach Overview	<p>Describe in general how your results have been extended to the intended users. OR, if they haven't yet, explain when & how this will occur.</p> <p>Efforts to share this knowledge include formal presentations at Aquaculture America 2017, the 2017 ARD Research Symposium by DSU Graduate Students Jasmine Smalls and Jackie Myer (see below). In addition, this project is regularly shared with tour groups visiting the DSU Aquaculture Research and Demonstration Facility. Visitors include K-12 teachers and students from across Delaware, various local and state policy makers, members of the research community, as well as current and prospective aquaculture producers.</p>
Targeted Audiences	<p>Provide information on the target audience for efforts designed to cause a change in knowledge, actions, or conditions.</p>

	<p>Direct end users of the knowledge generated by this project will be members of the research community that are exploring the potential of probiotics for use in aquaculture to enhance production, improve nutrient use efficiency and/or mitigate disease impacts.</p>
Outputs:	<p>Outputs are tangible, measurable products (website, events, workshops, products [AV, curricula, models, software, technology, methods, websites, patents, etc.], trainees, etc.). Do NOT include publications as they're listed separately.</p> <p>Graduate student Jackie Myer was sent to Virginia Tech Agricultural Research and Extension Center (June 2017) to participate in a larval rearing class. Class centered mostly around clown fish and live feed techniques required for many larval fish, but included other species including cobia and rainbow trout. Class was taught by VT's lab manager, Steve Urick, and was hands on in their lab.</p>
Outcomes/Impacts:	<p>Describe how findings, results, techniques, or other products that were developed or extended from the project generated or contributed to an outcome/impact. Outcomes/impacts are defined as changes in Knowledge, Action, or Condition.</p> <p>Following our presentation at Aquaculture 2016 in Las Vegas in February, we were approached by a representative from Epicore Bionetworks, Inc. (http://www.epicorebionetworks.com/). They are interested in licensing our probiotics for potential commercialization. Schreier and Schott are currently collaborating with Epicore on the effects of probiotics on shrimp challenged with Acute Hepatopancreatic Necrosis Disease (<i>V. parahaemolyticus</i>).</p>
Impacts Summary	<p>Provide short statements (2-3 sentences) about each of the following: (pre-established fields for Researchers to complete short statement answers)</p> <ol style="list-style-type: none"> Relevance: Issue – what was the problem? One strategy for controlling disease has been to utilize probiotics which are live microbial supplements that beneficially affect the host. The probiotic acts by either competing with other bacteria for essential resources or nutrients, antagonism, or by producing their own broad-spectrum antibiotics. While the application appears to be useful in a wide range of life-history stages, from larvae to adults, the application of probiotics is not systematically used in the aquaculture industry. Response: What was done? Growth and survival trials are underway at the DSU ARDF: having already completed our work with tilapia. Challenge trials with <i>Vibrio</i> pathogens were conducted at IMET with sea bass, tilapia and hybrid striped bass (collaborators Schott and Schreier). The project has increased the institutional capacity of IMET to conduct vertebrate pathogen challenges. Results: How did your work make a difference (change in knowledge, actions, or conditions) to the target audiences? Our work is not yet at a point where the technologies being developed could be put into practical use at the farm level, though we have begun discussions with multiple companies interested in commercializing our results. Recap: One- sentence summary Applying preventive measures may lead to less reliance on the use of chemicals - disinfectants, pesticides and antimicrobials - that treat the symptoms of the problem rather than affecting a cure.

Publications	<p>Follow the format to list publications in the following categories:</p> <ul style="list-style-type: none"> • Oral Presentations: <ul style="list-style-type: none"> ○ Smalls, J. and D. McIntosh. The Use of Probiotics in Shrimp Aquaculture. Aquaculture America 2017 Book of Abstracts, San Antonio, TX, USA. ○ Myer, J. L., and D. McIntosh. Probiotics and Fish Growth. 2017 ARD Research Symposium, Atlanta, GA. ○ Smalls, J. and D. McIntosh. The Use of Probiotics in Shrimp Aquaculture. 2017 ARD Research Symposium, Atlanta, GA. • Poster Presentations <ul style="list-style-type: none"> ○ Myer, J. L., E. Schott, H. J. Schreier, and D. McIntosh. Probiotics and Fish Growth. Aquaculture America 2017 Book of Abstracts, San Antonio, TX, USA • Peer-reviewed: <ul style="list-style-type: none"> ○ Schreier, H. J. (2018) Draft genome sequence of marine <i>Bacillus</i> sp. ISO11, a candidate finfish and shellfish probiotic. Microbiology Resource Announcement, http://doi.org/10.1128/MRA.01227-18. ○ Myer, J., H. J. Schreier, E. J. Schott, G. Blank and D. McIntosh. Examination of the probiotic potential of bacteria isolated from <i>Fundulus heteroclitus</i> in the aquaculture of commercially important fish larvae. J. World Aquaculture Soc., in preparation. • Non-Peer-reviewed: <ul style="list-style-type: none"> ○ Extension factsheets - none to report at this time ○ Popular articles - none to report at this time
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Students/Participants:	<p>Provide the following information for every student that worked with you during the reporting period:</p> <p style="text-align: center;">Delaware State University</p> <table border="1" style="width: 100%;"> <thead> <tr> <th>Name</th> <th>Level</th> <th>Completed</th> <th>New/Continuing</th> <th>Thesis</th> </tr> </thead> <tbody> <tr> <td>Jasmine Smalls</td> <td>MS</td> <td>No</td> <td>Continuing</td> <td>The Use of Probiotics in White Shrimp (<i>Litopenaeus vannamei</i>) Aquaculture</td> </tr> <tr> <td>Jackie Myer</td> <td>MS</td> <td>No</td> <td>Continuing</td> <td>The effect of probiotics in commercially important fish in the Northeast</td> </tr> <tr> <td>Charles Wallace</td> <td>BS</td> <td>Yes</td> <td>Continuing</td> <td>NA</td> </tr> <tr> <td>Diondre Oliver</td> <td>BS</td> <td>Yes</td> <td>Continuing</td> <td>NA</td> </tr> <tr> <td>Dean Johnson</td> <td>BS</td> <td>Yes</td> <td>Continuing</td> <td>NA</td> </tr> </tbody> </table> <p style="text-align: center;">UMD Summer Interns</p> <table border="1" style="width: 100%;"> <thead> <tr> <th>Name</th> <th>Level</th> <th>Completed</th> <th>New/Continuing</th> <th>Thesis</th> </tr> </thead> <tbody> <tr> <td>Sarah Avery</td> <td>MS</td> <td>No</td> <td>Continuing</td> <td></td> </tr> <tr> <td>Jennifer Kuhlman</td> <td>BS</td> <td>N</td> <td>New</td> <td>NA</td> </tr> <tr> <td>Susannah Ruzbarsky</td> <td>BS</td> <td>No</td> <td>Continuing</td> <td>NA</td> </tr> <tr> <td>Amanda Hise</td> <td>BS</td> <td>No</td> <td>New</td> <td>NA</td> </tr> </tbody> </table>	Name	Level	Completed	New/Continuing	Thesis	Jasmine Smalls	MS	No	Continuing	The Use of Probiotics in White Shrimp (<i>Litopenaeus vannamei</i>) Aquaculture	Jackie Myer	MS	No	Continuing	The effect of probiotics in commercially important fish in the Northeast	Charles Wallace	BS	Yes	Continuing	NA	Diondre Oliver	BS	Yes	Continuing	NA	Dean Johnson	BS	Yes	Continuing	NA	Name	Level	Completed	New/Continuing	Thesis	Sarah Avery	MS	No	Continuing		Jennifer Kuhlman	BS	N	New	NA	Susannah Ruzbarsky	BS	No	Continuing	NA	Amanda Hise	BS	No	New	NA
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Partnerships	<p>List any partners that you worked with on your project. Provide the following information for each Partner:</p> <p>Fish Suppliers Tilapia, trout Steve Hughes, Cheyney University</p>
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	Tilapia	Jeff Reeser, Baltimore Polytechnic High School
	Clownfish	Mid-Atlantic Aquatic Technology
	Hybrid Striped Bass	Skip Bason, Delmarva Aquatics
	Hybrid Striped Bass	Mike Freeze, Keo Fish Farms