Protective Bacteria in Algal Ponds: Inducible Protection to Maximize Response

DOE BIOENERGY TECHNOLOGIES OFFICE

2021 PROJECT PEER REVIEW

March 22, 2021

ADVANCED ALGAL SYSTEMS

PI: Rhona Stuart, LLNL

This work was performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344.

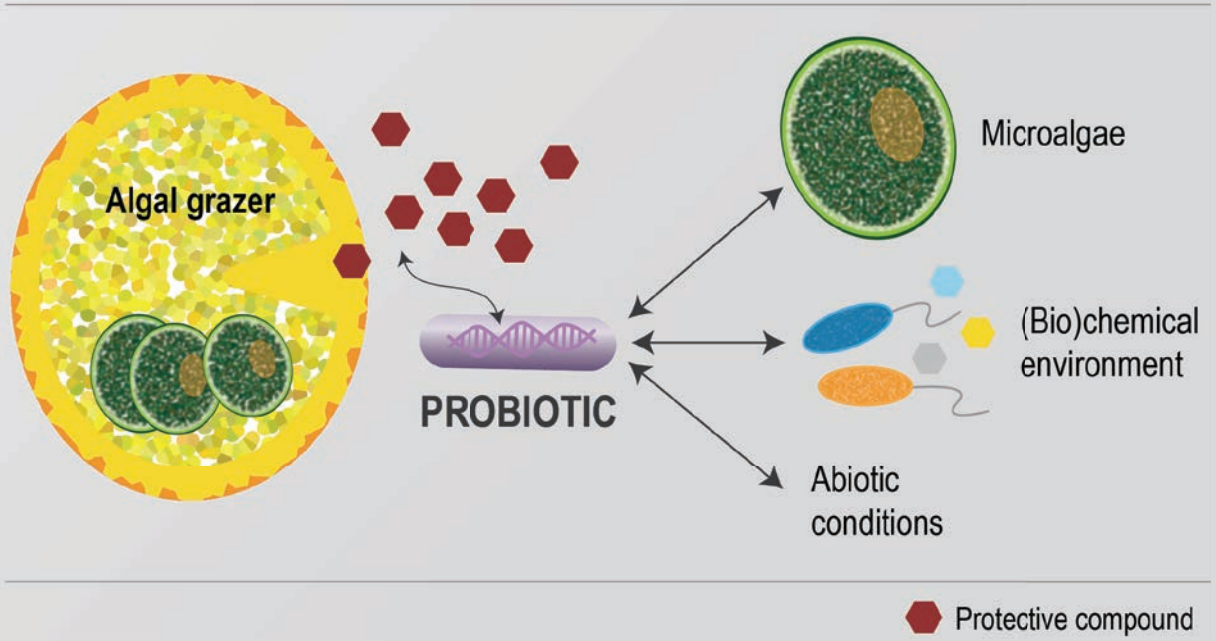
Protective Bacteria in Algal Ponds: *Inducible Protection to Maximize Response*

NEED:

- Novel tool for crop protection
  - Pest-driven loss decreases productivity by at least 10-30%
- *Janthinobacter lividum* is a **promising probiotic**
  - Our previous BETO-funded project (2015-2018) discovered that *J. lividum* protected algae from predation at laboratory and outdoor scales
- *BUT protection was inconsistent, especially in complex communities, and required dose was cost-prohibitive*

GOAL: *J. lividum* application regimes that improve the baseline protective effect found in our previous project:

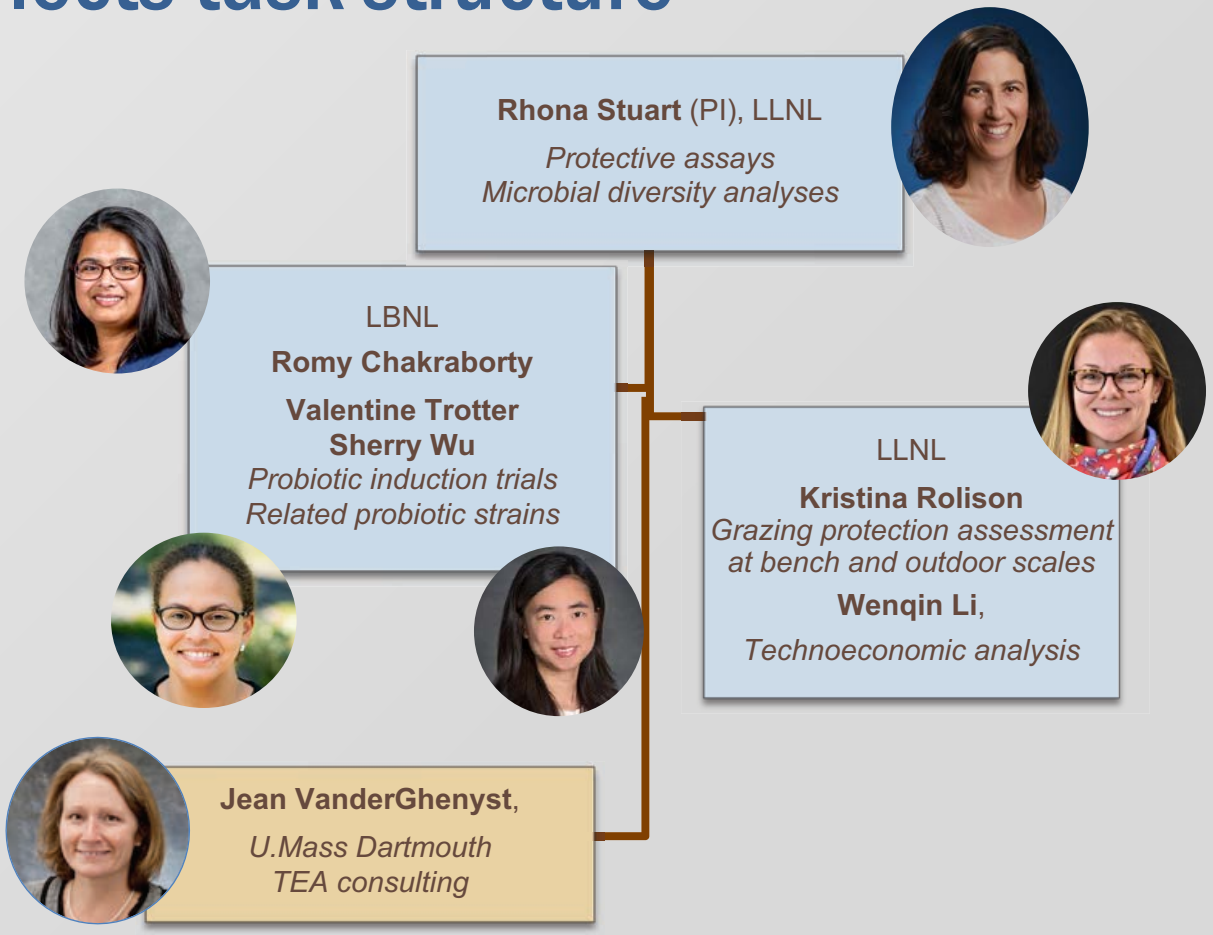
- Magnitude ↑ 25%
- Duration ↑ 25%



Back to basics: By identifying the biological mechanism and regulation of protection, we can induce and increase the protective response

Management structure reflects task structure

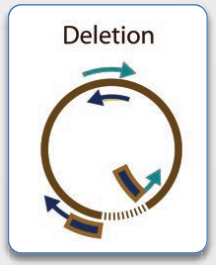
- LLNL: Expertise in complex microbial community analyses and algal ecophysiology
- LBNL: Our partners provide expertise on our probiotic of choice, *Janthinobacter*
- Management includes:
  - Decision making through consensus
  - Team leads responsible for achieving task milestones
  - PI retains ultimate decision-making authority
  - Monthly telecon with team members



Lawrence Livermore National Laboratory

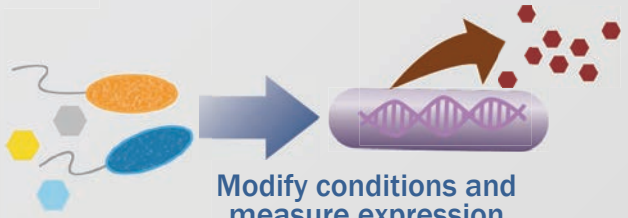
BERKELEY LAB

Technical Approach



Mutagenesis

Grazing assays

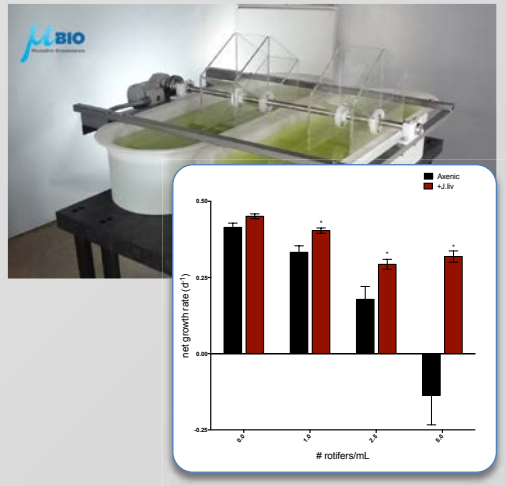


Modify conditions and measure expression

Screening at bench scale

G/NG

Open system testing



100 L scale tests and Technoeconomic Analysis

AIM 1: Identify protective genes

AIM 2: Test induction conditions with scale

AIM 3: Assess improvements with increasing complexity, and cost improvements

AIM 4: Methods Development:
Effect of protection on algal carbon loss and in situ pond failure frequency

Stepwise scale up and early testing outdoors to mitigate risks

Impact

This project has advanced the SOT through **Cultivation System** R&D targeting both **Enhanced Productivity** and **Robust Yields** to overcome barriers in **Biomass Availability** and **Sustainable Production**

- **2 Publications (3rd in prep)**
  - Provide a bacterium that industry could apply for one pest (rotifers)
  - Demonstrate a roadmap for how to discover and test probiotics for other pests
- Applications could increase the current annual productivity (15.9 g/m^2/day) by up to 30% and decrease mean time to failure in ponds

GOALS **directly address barriers to sustainable algae production through CROP PROTECTION research and development, and translating** LABORATORY SUCCESS **to** SCALABLE OUTDOOR CULTIVATION STRATEGIES.

Technical Accomplishments Outline (2.25 of 3 years)

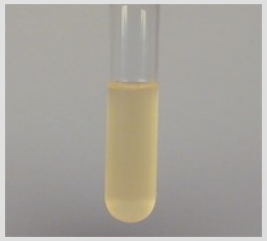
1. Identified a mechanism of protection: violacein production
2. Induced increased violacein production and improved protection at laboratory scale by 56-71% over our low violacein baseline, leading to reduced dose
3. Ongoing systematics to identify and optimize induction of violacein
4. Demonstrated improvements to baseline protection (at low violacein) with outdoor mesocosms (Outdoor testing ongoing)
5. Currently updating our TEA to include these cost improvements
6. Schedule: Met our Go/No-Go 2 months ahead of schedule, and have met our milestones*

*with some alterations due to pandemic shutdowns

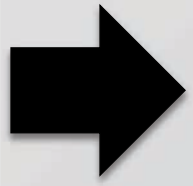
Intro: we designed a grazing assay to assess effects of treatments to reduce grazing

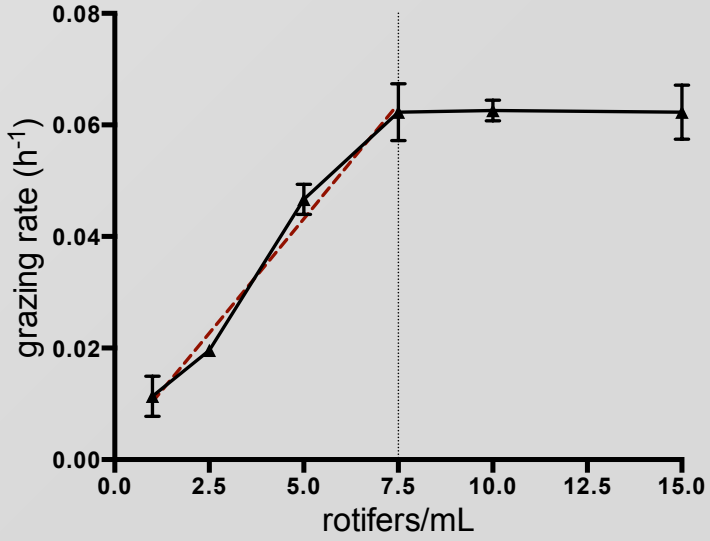
Axenic *M. salina* 2mL
~2 x 10^6 cells ml^{-1}

Brachionus plicatilis
added at 4 concentrations including zero



J. lividum ~6x10^7 cells
(baseline dose)

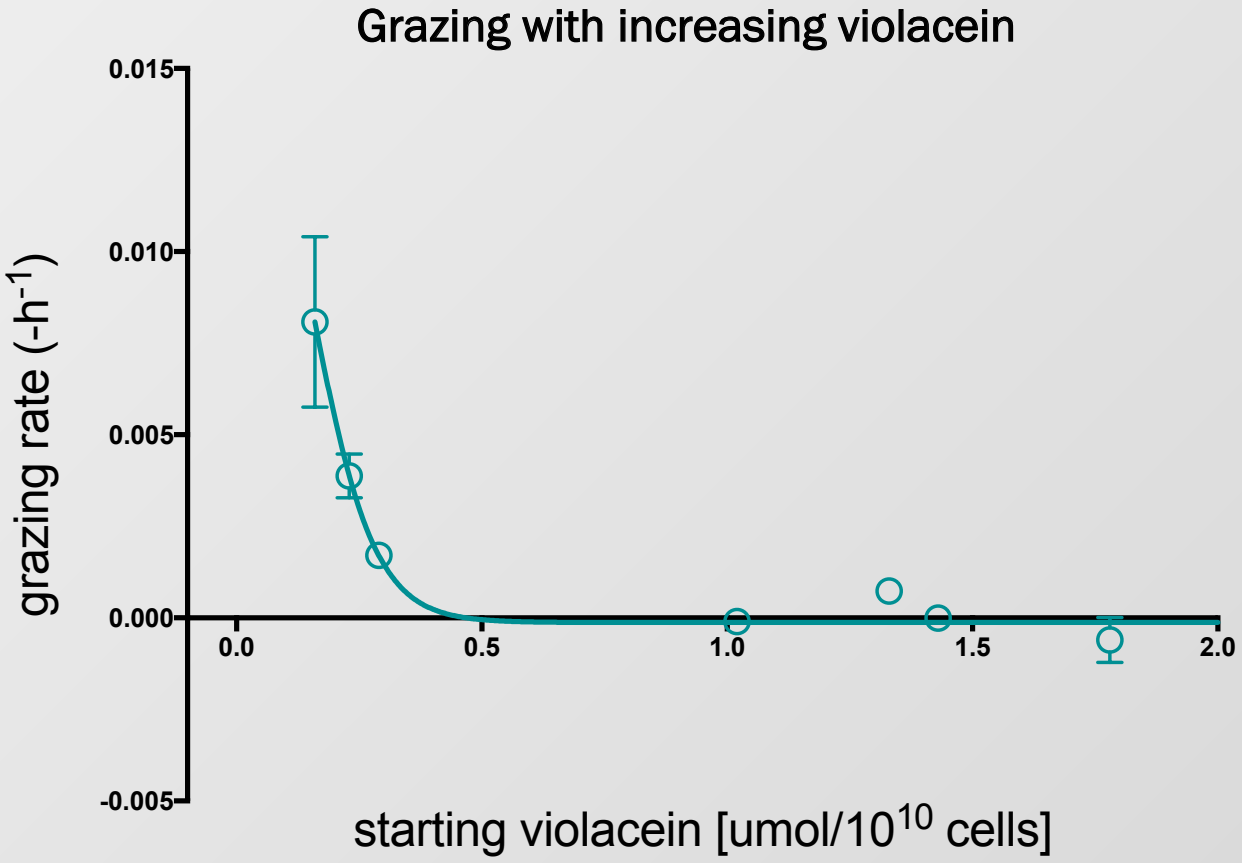


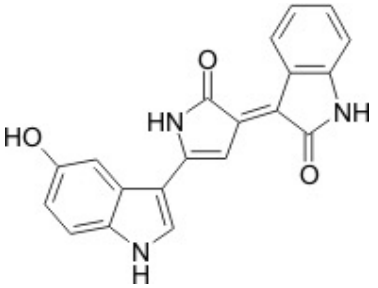


- Laboratory: 24-well plates, 2 mL per well
- Bacteria and rotifers added to axenic algal culture on Day 1
- Fluorescence and ODs measured at least daily for ~1wk

Increase concentration of rotifers—increase grazing rate.

1 Identified mechanism of protection: Violacein production

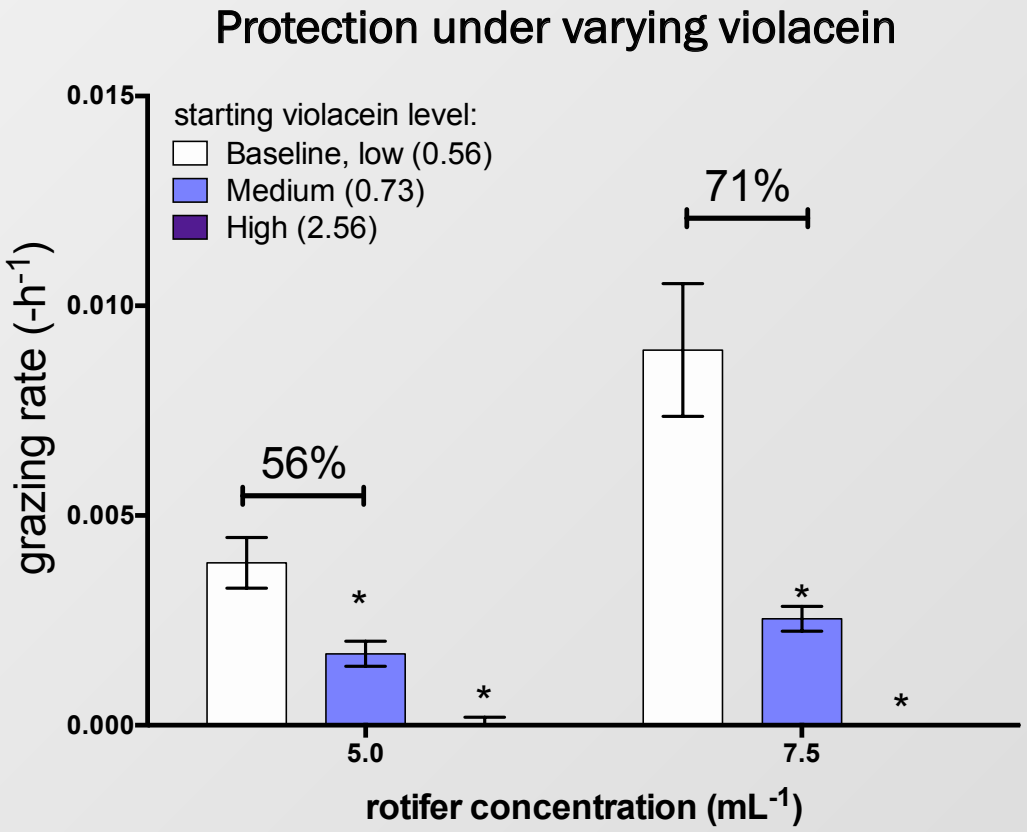




violacein

Starting violacein concentration in *J. lividum* correlated with declines in grazing rate.

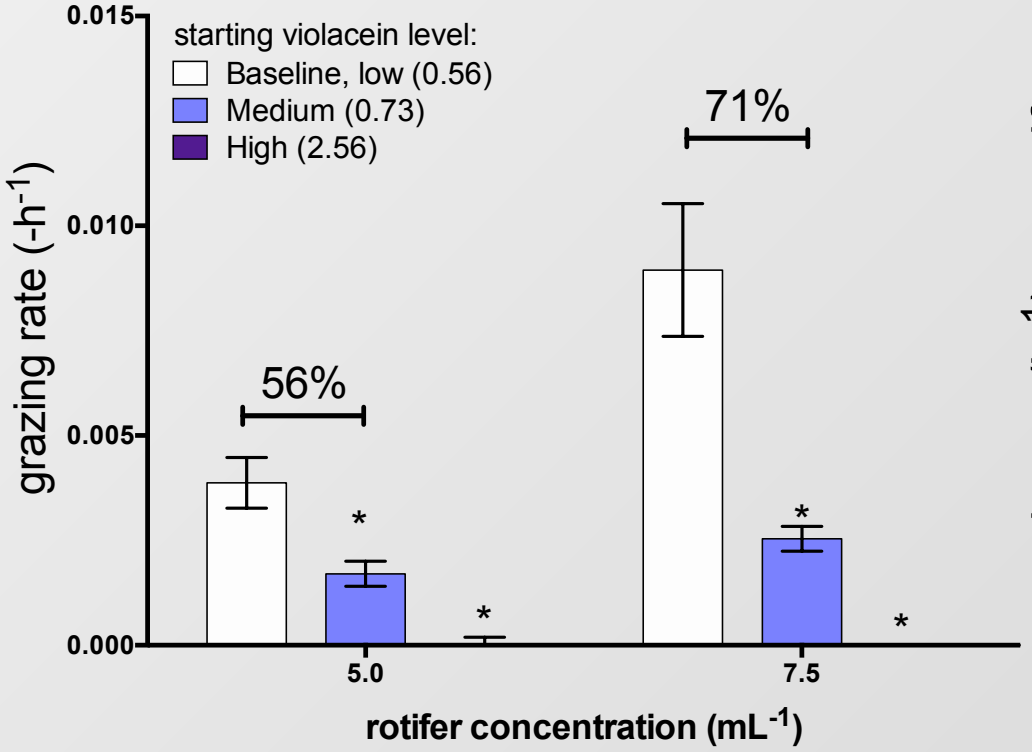
2 Improvements to baseline protection at lab scale



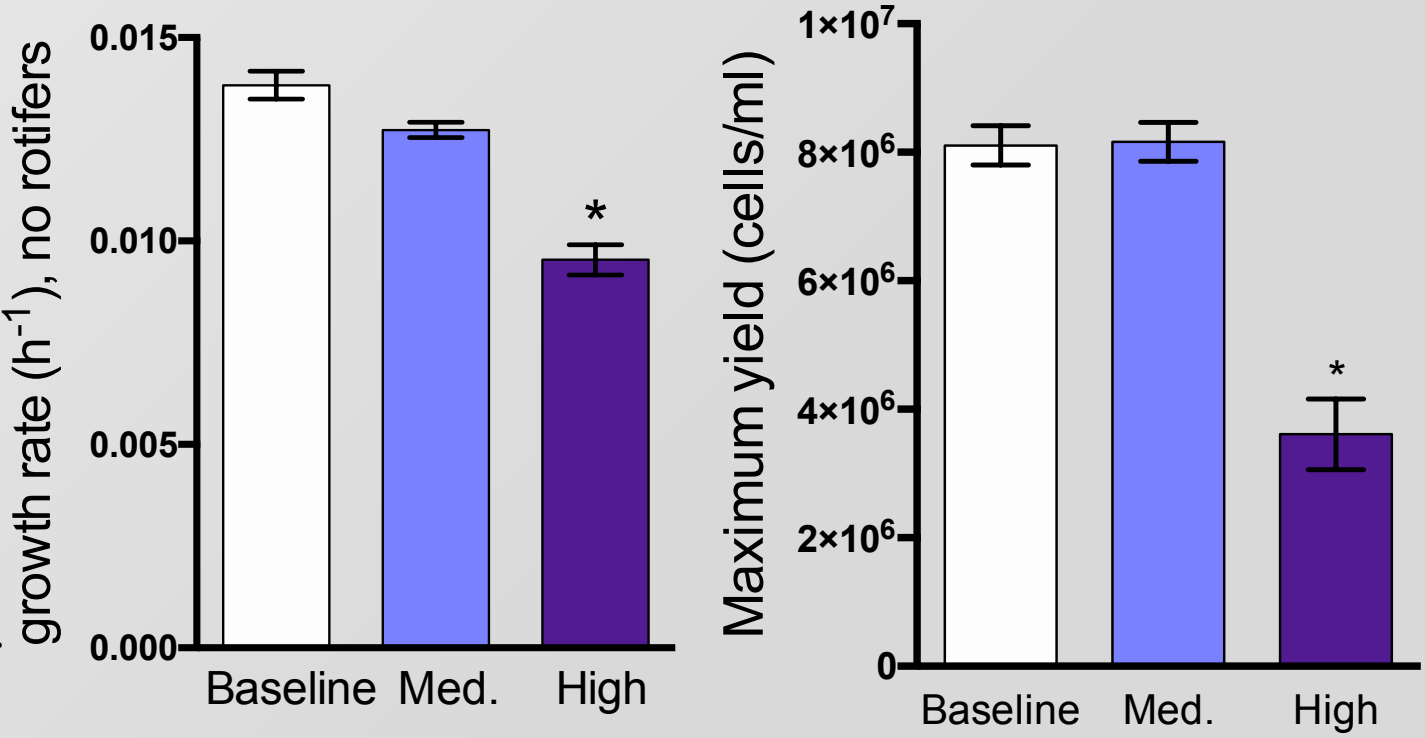
Inducing violacein production before adding to algal cultures ("priming") **ABOLISHED** grazing.

2 Improvements to baseline at lab scale

Protection under varying violacein

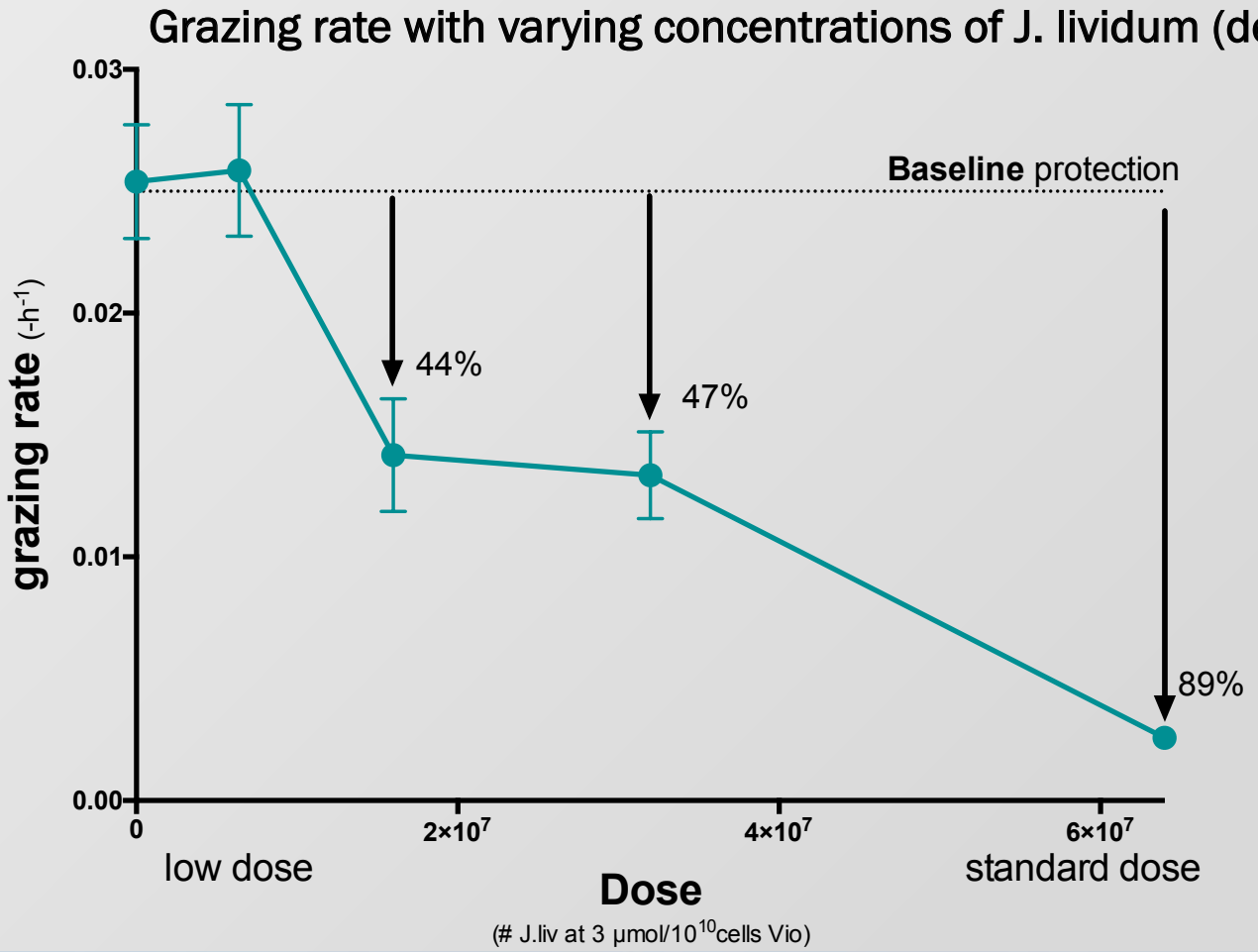


Algal growth and yield under varying violacein (no rotifers)



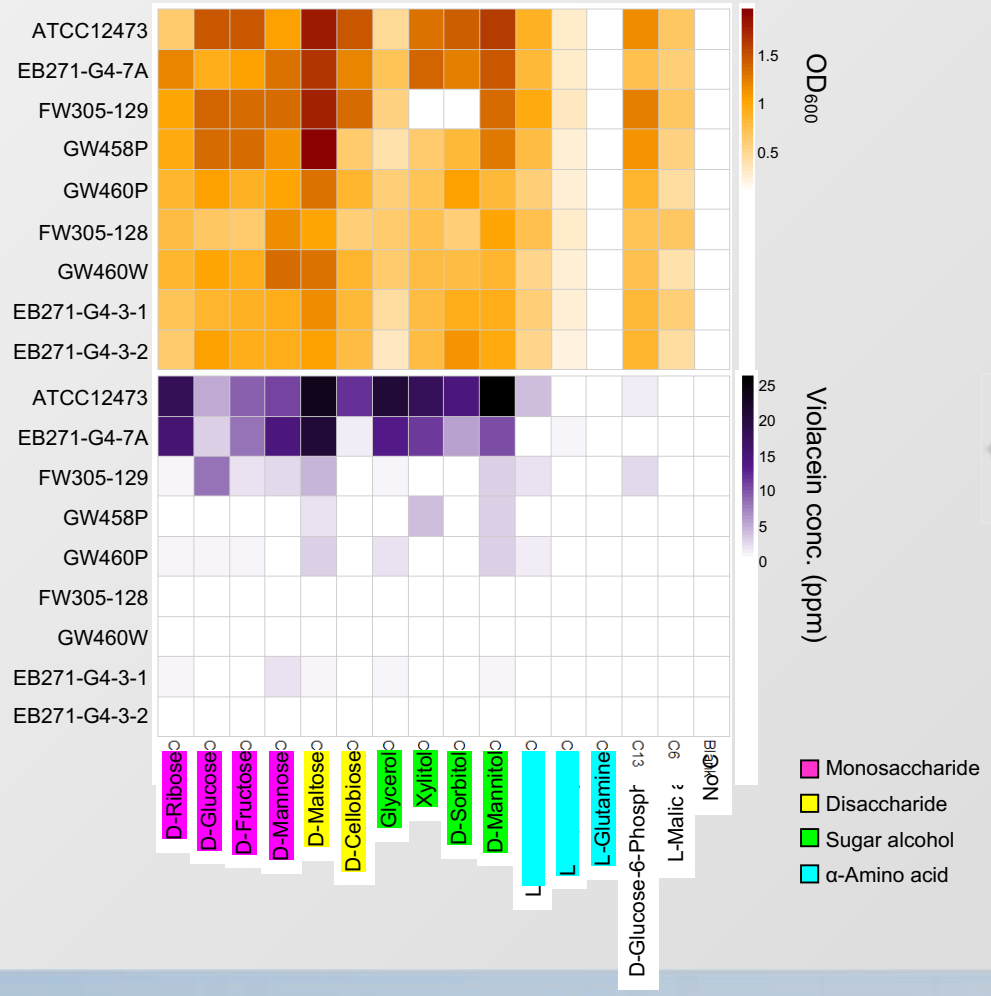
Inducing violacein production before adding to algal cultures ("priming") **ABOLISHED** grazing.

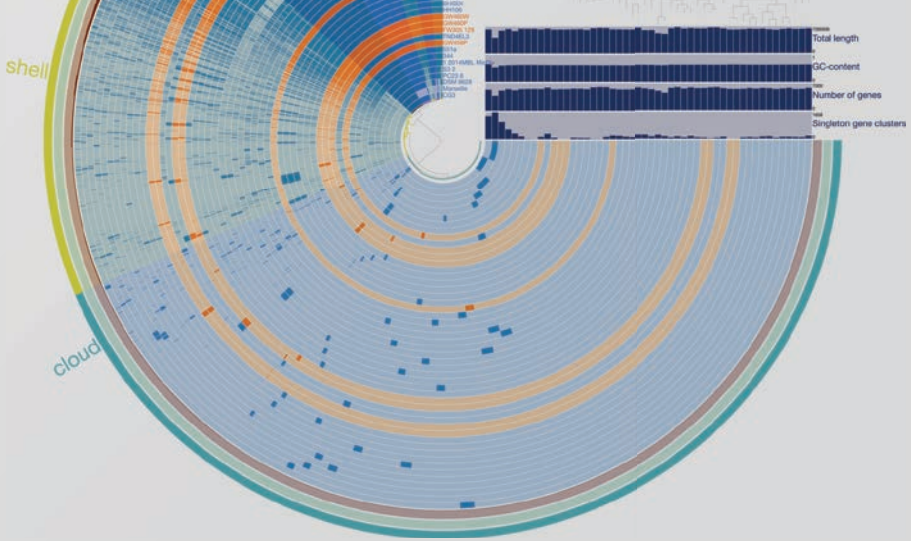
2 Decreasing dose to cut costs



Can improve 44% over baseline with a dose reduction of 75% (dependent on violacein content per cell).

3 Regulation and induction of violacein production: Systematics





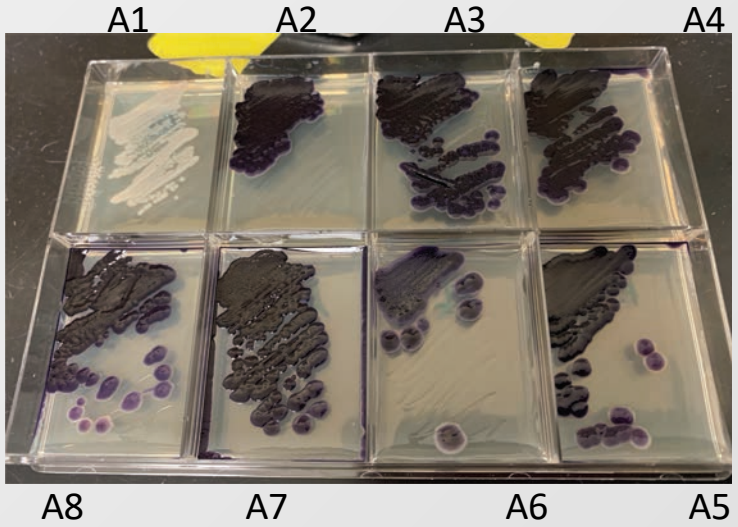
Sherry Wu

Romy Chakraborty

Carbon sources can induce violacein; comparative genomics shows quorum sensing genes link high violacein producers

Accomplishments

3 Regulation and induction of violacein production: Mechanisms and Ecology



- Traditional insertional mutagenesis of the violacein gene cluster proved challenging
- Multi-pronged mitigation has led to several new discoveries:
  - Successfully applied random transposon mutagenesis, and screened for white phenotypes
    - Several are conditionally white, and genes disrupted give hints to regulation of violacein
    - Grazing assay tests on these strains is underway
  - Obtained violacein-producing *E. coli* strain; no protection detected
    - This suggests that protection involves more than simply making violacein, export or delivery also may be important

Romy Chakraborty

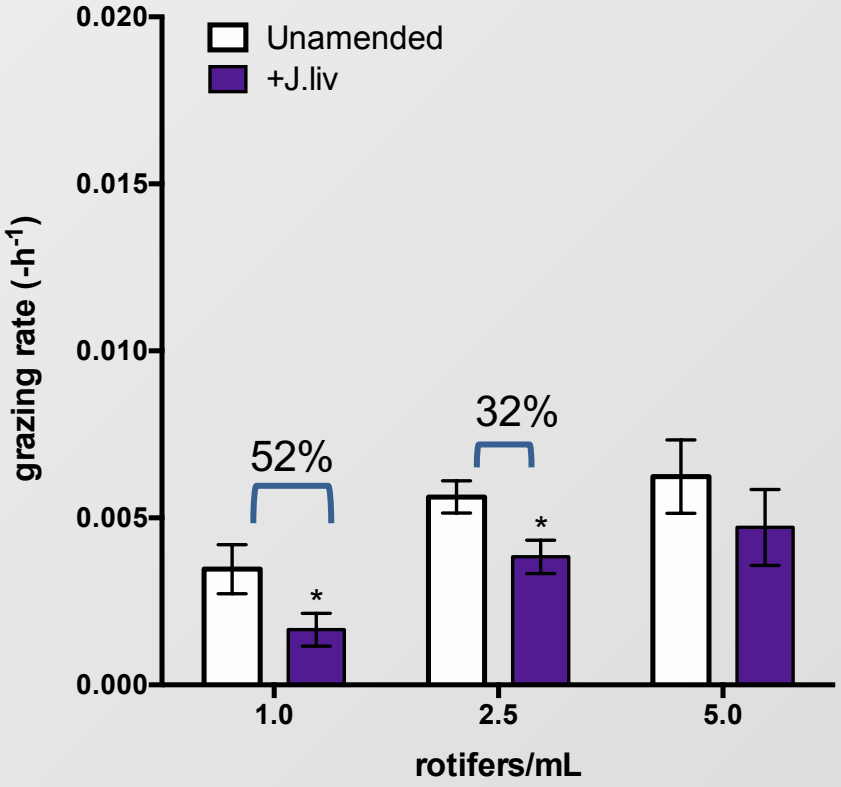
Valentine Trotter

Risk Mitigation: transposon libraries and *E. coli* knock-ins reveal putative mechanisms

4 Scale up: *Janthinobacter* in complex microbiomes

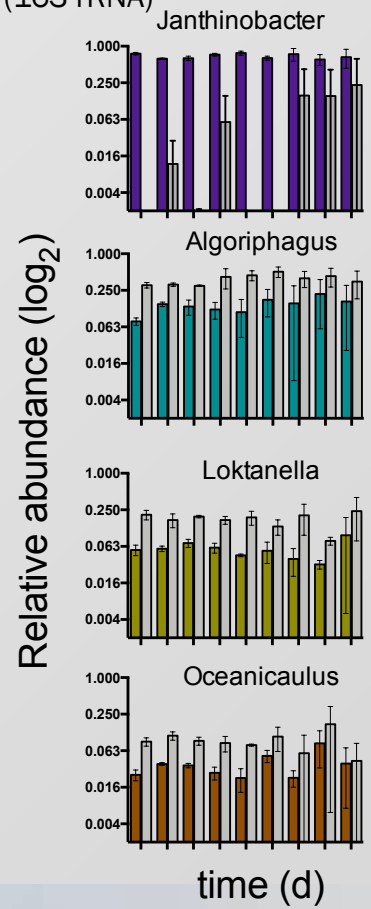
Chris Ward

Grazing in complex community with or without probiotic addition



Algal microbiome over time with and without probiotic

(16S rRNA)

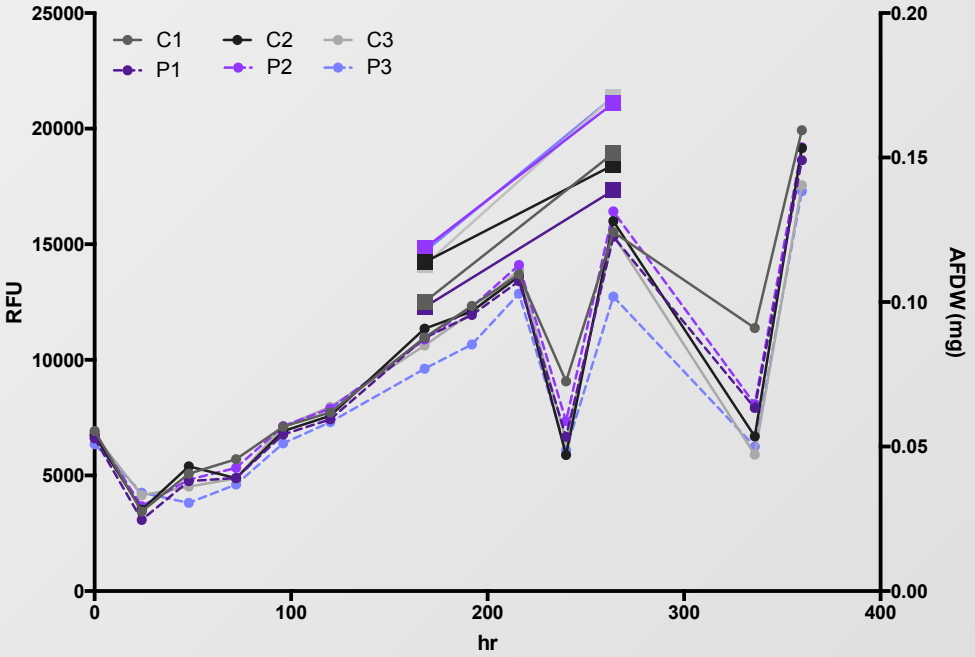


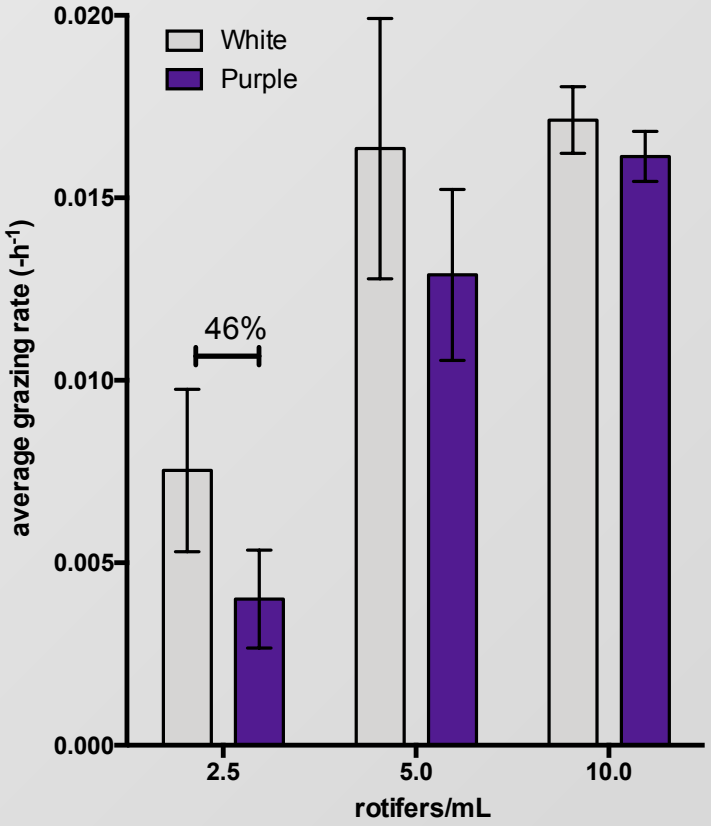
Protects in a complex microbiome and does not displace the native microbiome

Grazing assays conducted on diluted subsamples from outdoor triplicate mesocosms which were amended with *Janthinobacter* or unamended controls

Ward et al *in revision*

4 Scale up: testing priming in complex microbiomes

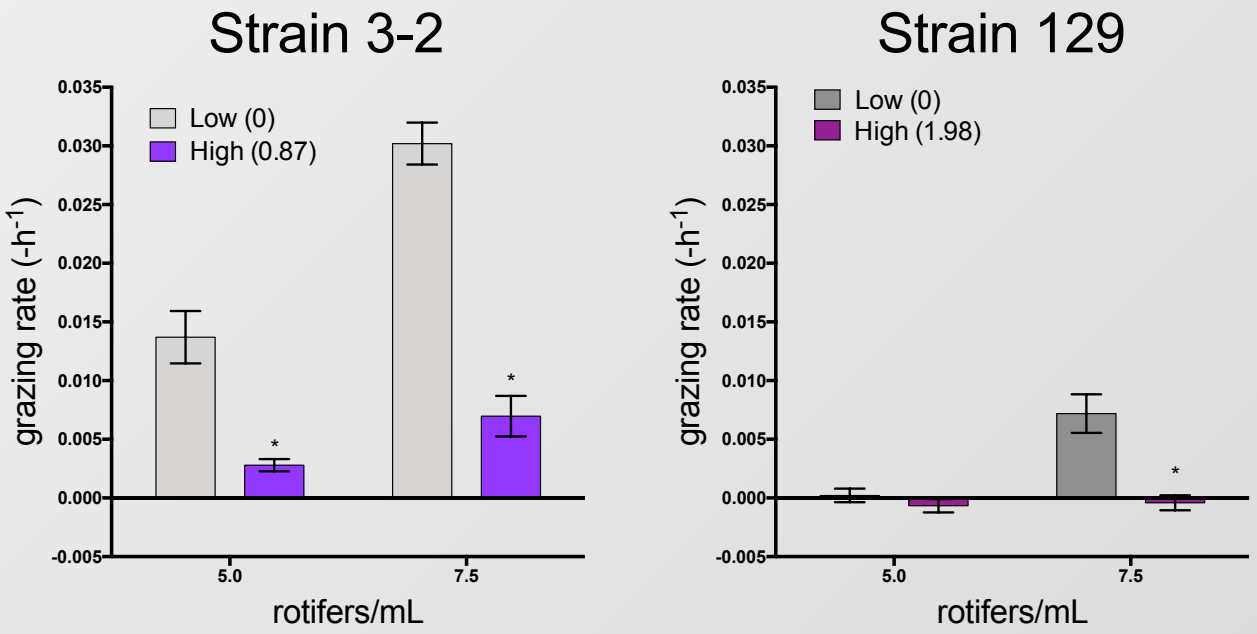


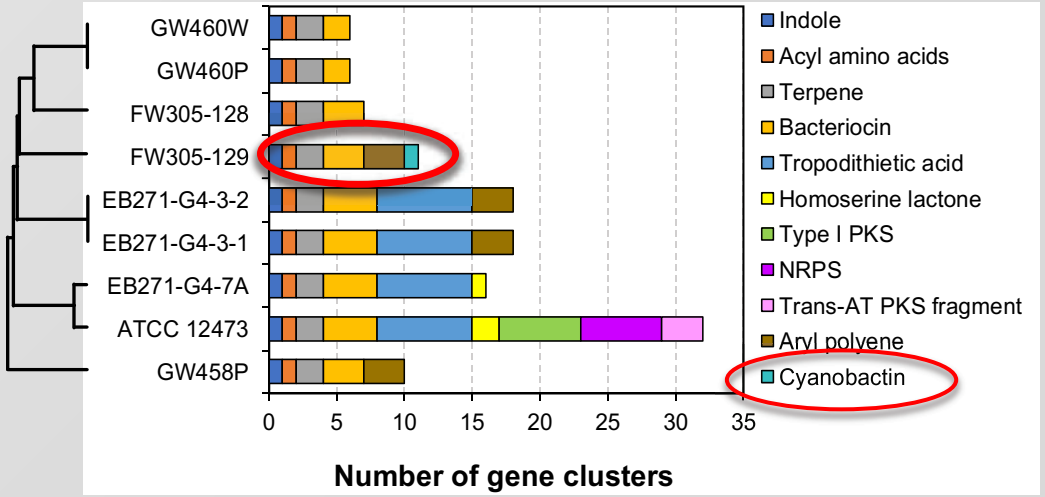


Grazing assays conducted on diluted subsamples from outdoor triplicate mesocosms which were either amended with white *Janthinobacter* (control/baseline) or purple *Janthinobacter*

In outdoor communities, priming does not negatively impact yields and improves protection

Different *Janthinobacter* strains also protect: Other toxins we can leverage?

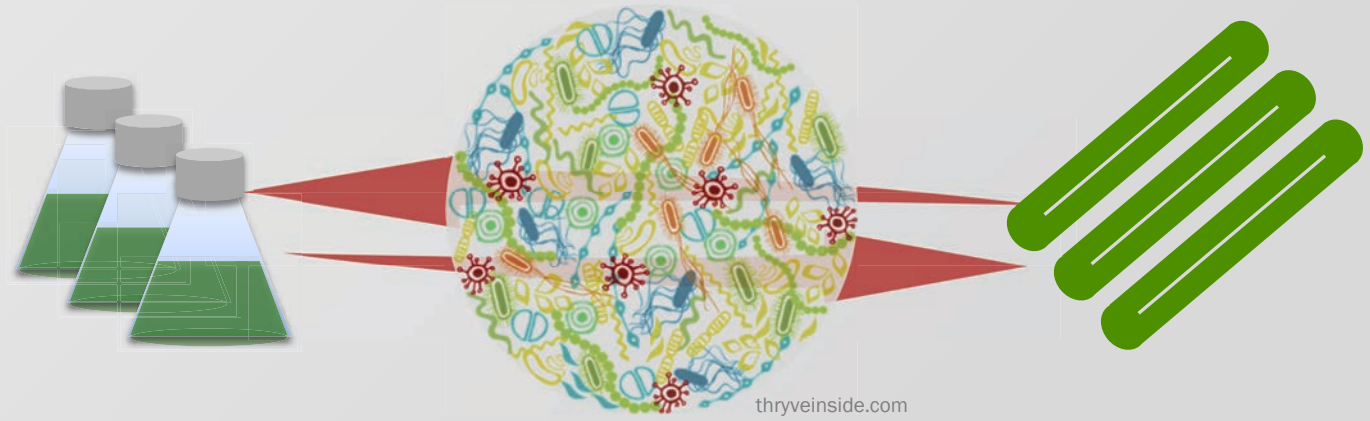




Differences between strains indicate additional toxins may also be grazing deterrents—expand to other pests?

Summary

1. Identified a mechanism of protection: violacein production
2. With higher violacein, improved our baseline protection at laboratory scale by 56-71%.
3. With a 75% lower dose (and high violacein), improved our baseline protection by 44%
4. Ongoing identification of regulation and induction of protection
5. One iteration of outdoor testing complete, with improvements to baseline
6. Currently updating our TEA to include these improvements



AOP Quad Chart Overview

- Project start: 10/1/18
- Project end: 9/30/21
- ~75% complete

	FY 20 Costs	Total Planned Funding (FY 19-project end date)
DOE Funding		

Project Partners	%
LLNL	85
LBNL	15

Barriers addressed:
- Aft-B, Sustainable Algae Production
- Aft-A, Biomass Availability and Cost

Project Goal: Improve application of protective probiotics to decrease algal grazing crop loss through induction of probiotic protective mechanism

Project Objectives

1. Identify genes/toxins involved in bacterial protective mechanisms ✓
2. Test and identify induction conditions for increased protection ✓
3. Apply promising induction conditions at increasing scales ✓
4. Determine effects of protection on in situ pond failure frequency (ongoing)

End of project goal:

Probiotic application regimes that:
- Increase probiotic effect over baseline
  - Magnitude ↑ 25% ✓ (>50%)
  - Duration ↑ 25%
- Decrease in situ algal carbon loss

Funding Mechanism: AOP

Credits

LLNL

- Kristina Rolison
- Chris Ward (now at BGSU)
- Michael Thelen (retired Jan. 2020)
- Courtney Swink

LBNL

- Romy Chakraborty
- Xiaoqin “Sherry” Wu
- Valentine Trotter

BETO project management support

Devinn Lambert, Jamie Meadows, Philip Lee (and previously Liz Burrows)

Additional Slides

Response to 2019 Peer Review Comments

- This project review is a combination assessment of the completed work and the new plan that is a continuation of this project. There were many key accomplishments exploring the use of probiotic bacteria in the cultivation of *Myrmica salina* as a protective approach to rotifer predation, including the development of a TEA around this strategy. The team is now building on this work to understand the potential mode of action and develop the process to scale. For the upcoming work, the project team will benefit from clarity on objectives in the outdoor trials to ensure project success.
  - Response: Our outdoor trial objectives are 1: to show no negative impact in yield from our induced protective response, and 2: to show significant improvement with induction as compared to baseline protective response (no induction).
- To reach the overall project goals of BETO, minimizing pond crashes is essential. This project has the potential to establish new and innovative solutions for pond pest management, but they seem to be focused only on mitigating saltwater rotifer. Rotifers are only one of many pests that hinder algal productivity. The project would make the most impact if the team focused on expanding their work of yearly sampling and characterizing the microbiome community to more sites while simultaneously using their techniques to find a solution for the different pests that arise during the different seasons.
  - Response: While we agree that rotifers are only one of many pests, we hope that by determining the mechanisms of protection by the probiotic, we can learn whether that mechanism could be applicable to other pests. Violacein, for example, has been found to protect against chytrid infection in amphibians. If the mechanism involves ingestion of the bacteria, then perhaps a wide range of grazers would be susceptible. Additionally, isolating and characterizing novel pests, and then setting up infection assays to test protection, is by no means trivial. We have set up a pipeline, but this would require many additional personnel and funding to replicate this approach to screen for probiotics for each individual pest.
- This project is focused on identifying, understanding, and scaling probiotic microorganisms for large-scale outdoor production, which has clear relevance to the BETO mission and MYP goals. The project included an appropriate proportion of laboratory, mesocosm, and field-testing, with experimental design appropriate for each scale. The group has completed most objectives and will be building on these accomplishments in a future AOP merit review cycle. The team is encouraged to incorporate outdoor testing early in the project to ensure bench-level successes will have relevance in an industrial environment in the presence of more challenging ecological pressures.
  - Response: We agree and plan to incorporate and iterate with outdoor experiments as soon as we have a strategy to test.
- The aim of the project is to improve the resilience of algal crops to predators and pathogens by using probiotic bacteria that will increase annual algal biomass yields above the 2015 SOT baseline. The team plans to improve the protective effect of probiotic bacteria by demonstrating probiotic application regimes that significantly increase the magnitude and duration of the probiotic protective effect by 25% each, above the current baseline, and significantly decrease *in situ* algal carbon loss compared to untreated, ultimately contributing to improved algal cultivation yields. The approach involves studying single cultivation relationships between algae and bacteria and identifying microbial communities that enhance algal cultures and scaling these up in a stepwise fashion. In the new AOP, the team will take an approach to identify protective genes because they hypothesize that violacein is the compound produced by bacteria that infers protection. The approach is deemed reasonable. Progress in the previous AOP is deemed reasonable. Success in developing probiotic mecocosms for algal cultures could potentially enhance robustness of cultures and overall productivity, helping to meet productivity goals for the BETO program.
- The team should provide a vision on the use of the identification of the gene(s) responsible for violacein production.
  - Response: Genes for violacein production are well described in the literature.

*These comments were not included in the 2019 report, we did not get them submitted in time because PI Stuart was on maternity leave.

Publications

1. Christopher Ward, Kristina Rolison, Max Li, Samuel Rozen, Carolyn Fisher, Todd Lane, Michael P. Thelen, and Rhona K. Stuart (in revision *Algal Research*). ***Janthinobacter* additions reduce rotifer grazing of microalga *Microchloropsis salina* in biotically complex communities**

2. Xiaoqin Wu, Alexey E. Kazakov, Sara Gushgari-Doyle, Xingli Yu, Valentine Trotter, Rhona Kayra Stuart, Romy Chakraborty (in review) **Comparative Genomics Reveals Insights into Regulation of Violacein Biosynthesis in *Janthinobacterium* Inhabiting Terrestrial Subsurface Sediment and Groundwater**

Tasks and Milestones (FY19-FY21)

Task	Task or Subtask Title	Milestone Description	End Date	Status
1.1	Aim 1: Genomic analyses to Identify target toxin genes for mutagenesis	To narrow down genetic mechanism candidates identify target list of 5 genes with genomic and literature support (LLNL/LBL)	FY19 Q1	Completed on time
1.1'	Aim 1:Global gene expression comparison to identify target genes for mutagenesis	Gene expression - Determine at least 10 target genes that are significantly differentially expressed by protective isolates	FY19 Q2	Mitigated and completed with modifications (transposon library)
1.2	Aim 1: Insertional mutagenesis of target genes	Demonstrate knockout of J. lividum target gene (full length gene not present in the genome)	FY19 Q2	Mitigated and ongoing
2.1	Aim 2: Compare isolate library protective induction under baseline	Characterize novel LBL isolates protective capabilities relative to TABB-established baseline (LLNL)	FY19 Q3	Completed on time
1.3	Aim 1: Determine whether toxin gene targets are required for protective effect	Measure net growth of algae in grazing assays of knockout J. lividum versus wildtype to thereby identify a genetic mechanism of protection by demonstrating gene product that is required for protective effect	FY19 Q4	Completed on time (mitigated)
2.2	Aim 2:Determine isolate library protective effects correlation with gene expression and toxin production	Quantify genetic mechanism expression and product production for all probiotic strains under protective conditions to establish baseline protection expression (LLNL/LBL)	FY20 Q1	Completed on time
2.3	Aim 2: Compare isolate library protective induction under a range of different cultivation conditions	Identify at least 2 cultivation conditions that induce protective expression over baseline (LBL/LLNL)	FY20 Q2	Completed on time
	Go/No-Go:	Identify at least one parameter that induces protective effect, determined through (a) significant ($p<0.05$) increase in toxin gene expression compared to specified baseline probiotic application condition or (b) significant ($p<0.05$) increase in protection in a grazing assay.	FY20 Q3	Completed early
2.4	Aim 2: Test promising conditions at mesocosm scale	Submitted publication for peer review on mechanism and induction of protective expression (LLNL/LBL)	FY20 Q4	Completed
3.1	Aim 3: Apply alternative strategies of probiotic application and compare protection with baseline probiotic application	Demonstrate significant ($p<0.05$) improvement in protection expression over baseline probiotic application for at least one time point (LLNL)	FY21 Q1	Completed on time
3.2	Aim 3: Modify TEA model to include alternative application strategies	Show feasibility and enhanced annual productivity of improved probiotic application as compared to baseline probiotic application (LLNL)	FY21 Q2	Ongoing
4.2	Methods: Quantify mesocosm failure frequencies	Determine baseline failure frequencies in amended mesocosms as compared to unamended mesocosms (LLNL)	FY21 Q3	Ongoing
3.3	Aim 3: Test scale up of alternative application strategy	Demonstrate alternative application of probiotic at increased scale (100 L) (LLNL)	FY21 Q4	Ongoing
		Demonstrate probiotic application regimes that significantly increase the magnitude and duration of the probiotic protective effect by 25% each, over current baseline, and significantly decrease in situ algal carbon loss as compared to untreated (LLNL)	*project end, 9/30/2021	