flow cytometry

Website: https://www.bco-dmo.org/dataset/994735
Version: 1
Version Date: 2026-03-17

Project
» Collaborative Research: Extracellular vesicles as vehicles for microbial interactions in marine Black Queen communities (Vesicle Interactions)

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Abstract
The cyanobacterium Prochlorococcus has a conspicuously reduced genome causing it to require help from co-existing organisms for survival under a variety of stressful conditions. In this work we used conditioned media experiments to demonstrate that exudates of the heterotrophic bacterium Alteromonas macleodii EZ55 facilitated the survival of Prochlorococcus MIT9312 batch co-cultures as they entered stationary phase. Microscopy revealed the presence of extracellular membrane vesicles and protein complexes in the exudates. When the exudates were stained with an amine-reactive dye, we were able to observe them interacting directly with Prochlorococcus cells by flow cytometry, where stained exudates (but not dye alone) added both green fluorescence and size (forward scatter) to Prochlorococcus cells.

Table of Contents

Coverage

Location: Laboratories at the University of Alabama at birmingham

Methods & Sampling

Strains and culture conditions:
All strains used in this study were taken from those used for a Long-Term Phytoplankton Evolution (LTPE) experiment (1). *Prochlorococcus* strains were streptomycin-resistant derivates of the high light-adapted strain MIT9312 obtained as described previously (2, 3), either before (Ancestor) or after 500 generations of evolution at either 400 ppm or 800 ppm pCO_2 conditions (i.e., modern day or projected year 2100 conditions (4)). *Alteromonas* strains were derivatives of strain EZ55, originally isolated from a *Prochlorococcus* MIT9215 culture (3). As with our *Prochlorococcus* strains, we used both ancestral and evolved varieties of EZ55 co-evolved with *Prochlorococcus* at the two pCO_2 treatments and subsequently isolated. *Prochlorococcus* cultures were revived from cultures cryopreserved with 7.5% DMSO in liquid nitrogen vapor, and *Alteromonas* cultures were revived from cultures preserved with 20% glycerol stored at -80^{o} C. Prior to use in experiments, all *Prochlorococcus* cultures were grown in co-culture with *Alteromonas* EZ55 helpers (3) and were acclimated to culture conditions for at least 4 generations prior to data collection.

Alteromonas cultures were grown in YTSS medium (5) and *Prochlorococcus* cultures were grown in Pro99 medium (6) or PEv medium (1), both made in an artificial seawater base (ASW) (1). Prior to addition to co-cultures *Alteromonas* strains were pelleted at 2000 g for 2 minutes and washed twice in sterile ASW, then added to cultures at approximately 10^6 cells ml^{-1}. *Alteromonas* was grown at 30^o C with 120 rpm shaking. Unless otherwise noted, *Prochlorococcus* and co-cultures were grown in static 13 mL conical bottom acid-washed glass tubes under approximately 75 mmol photons m^{-2} s^{-1} cool white light in a Percival incubator set to 23^o C. When medium additions were employed, all solutions were filter sterilized with a 0.2 mm filter. Cell densities of *Prochlorococcus* cultures to standardize inoculations between experiments were determined using a Guava HT1 flow cytometer (Luminex Corporation, Austin, TX) by the distinctive signature of these cells on plots of forward light scatter vs. red fluorescence (Fig. S1A). Day-to-day culture growth was tracked using the *in vivo* chlorophyll *a* module for the Trilogy fluorometer (Turner Designs, San Jose, CA) with a custom 3D-printed adapter designed for conical bottom tubes. Fluorometer measurements and cell counts were linearly related across the range of cells examined in this study (Pearson correlation coefficient 0.835, $p = 1.38 \times 10^{-6}$, Fig. S1B).

Concentration of *Alteromonas* exudates:
EZ55 was grown in Pro99 media supplemented with 0.1% glucose to sustain growth in the absence of *Prochlorococcus* exudates. We scaled cultures up progressively from 12 mL to 2 L. The 2L culture was grown in a vented bottle with an outlet connected to a filter with 0.22 µm pore size. After removing most of the cells by centrifugation, we produced size-fractionated, concentrated exudates using tangential flow filtration using Sartorius Vivaflow 200 cassettes. The 2L culture supernatant was passed first through a 0.22 µm cassette using a Masterflex L/S peristaltic pump (Cole-Parmer) to remove bacterial cells, then through a 50 kDa module and a 5 kDa module in succession to produce >50 kDa and <50 kDa fractions that were each concentrated approximately 100-fold. A portion of the >50 kDa fraction was placed in boiling water for 5 minutes to denature proteins. When these concentrated extracellular products were added to culture media for growth experiments they were diluted 100-fold, returning them to approximately their original concentration prior to filtration.

Exudate interaction with Prochlorococcus: In order to visualize interactions between exudates and *Prochlorococcus* cells, we covalently labelled the >50 kDa concentrates with an amine-reactive Alexa Fluor 488 5-SDP ester dye (Molecular Probes/ThermoScientific) (7). First, 100 µL of each sample was concentrated with 100 kDa centrifugal ultrafilters (Pall) at 14,000 rpm for 15 minutes and then resuspended with 0.1 M sodium bicarbonate buffer (pH 8.3). Then, 2.5 µL of Alexa Fluor 488 5-SDP ester dye was added and incubated for 1 hour in the dark at room temperature with gentle mixing. The mixture was washed by centrifugation at 14,000 rpm for 15 minutes, removal of the supernatant, and resuspending with 1x PBS three times, with a final resuspension in 100 µL ASW. The labeled and washed exudate was added to 100 µL axenic *Prochlorococcus* culture and incubated for 2h at 28 ℃ with 120 rpm orbital shaking. Control samples were prepared with *Prochlorococcus* cells only or with *Prochlorococcus* stained with Alexa Fluor 488 5-SDP ester dye but without added exudate concentrate. Cells were analyzed with a Guava HT1 flow cytometer tracking cells by size (forward scatter), chlorophyll (red) fluorescence, and dye (green) fluorescence. Flow cytometry data was visualized using the online Floreada platform (https://floreada.io). Cells were also examined by using a Nikon 80i epifluorescence compound microscope using the GFP and Texas Red filter cubes. Images were collected and processed using Nikon NIS-Elements imaging software.

Data Processing Description

All statistical analyses were performed in R v. 4.4.1. Most analyses used linear models followed by post hoc extended marginal means testing of pairwise differences between treatment groups using the *emmeans* package (Searle et al., 1980). Assumptions of linear regression were checked for models by Shapiro-Wilk tests of the normality of residuals and plots of residuals vs. fitted values for homoscedasticity; where these assumptions were violated we used the Box-Cox procedure to find an optimal power transformation (Sokal & Rohlf, 2012). Statistical differences between lysate and exudate protein localization counts were determined using Fisher's exact test implemented in R.

[table of contents | back to top]

Parameters

Parameters for this dataset have not yet been identified

[table of contents | back to top]

Instruments

Dataset-specific Instrument Name	Percival
Generic Instrument Name	Algal Growth Chamber
Generic Instrument Description	A chamber specifically designed for the growth of algae in flasks. The chamber typically provides controlled temperature, humidity, and light conditions.

Dataset-specific Instrument Name	Guava HT1
Generic Instrument Name	Flow Cytometer
Generic Instrument Description	Flow cytometers (FC or FCM) are automated instruments that quantitate properties of single cells, one cell at a time. They can measure cell size, cell granularity, the amounts of cell components such as total DNA, newly synthesized DNA, gene expression as the amount messenger RNA for a particular gene, amounts of specific surface receptors, amounts of intracellular proteins, or transient signalling events in living cells. (from: http://www.bio.umass.edu/micro/immunology/facs542/facswhat.htm)

[table of contents | back to top]

Project Information

Collaborative Research: Extracellular vesicles as vehicles for microbial interactions in marine Black Queen communities (Vesicle Interactions)

Coverage: Laboratory cultures

NSF Award Abstract:
The function and stability of microbial communities in the ocean depends on exchanges of biological products and services between individual cells. Marine microbes are typically far apart from one another, so some of these exchanges occur through the release of products or services into the surrounding water, where they travel to other cells via simple diffusion. Understanding the degree to which such valuable products made by one organism are targeted to a specific partner, and how, has important implications for our understanding of the ecology and evolution of the marine microbiome. This project examines the role played by a poorly understood type of very small particle - extracellular membrane vesicles - in mediating functional interactions within the oceans. Extracellular vesicles are released by most marine microbes and are abundant in ocean waters, but our understanding of their functions remains in its infancy. As vesicles can contain diverse molecules, including active enzymes, and transport them between cells, they may work as a packaging and delivery system for goods and services traded between ecologically important microorganisms. Broader impacts of the project include providing hands-on research experiences for undergraduate and graduate students - including those from groups historically underrepresented in STEM fields - and the development of new active learning exercises to help increase knowledge about the roles microbes play in students' lives.

This project explores vesicle functions across multiple scales, combining -omics analyses, field experiments, and functional studies in cultures of diverse and ecologically important microbes to arrive at new

understandings of vesicle contributions to cellular exchanges. These experiments incorporate an evolutionary perspective for exploring the range of vesicle functions and genetic mechanisms affecting their production, examining how their contents have changed in co-cultures of phytoplankton and heterotrophic bacteria following hundreds of generations of experimental laboratory evolution. Fundamental ecological questions are addressed concerning whether vesicles, and their associated functions, act as truly 'public goods' in the oceans or can instead be targeted to a subset of cells, possibly yielding 'club goods' that define interacting, cooperative networks. Collectively, this effort will generate new insights into the mechanisms marine microbes use to interact with one another, and experimentally define the functional potential and ecological impact of EV-mediated trafficking networks in the oceans.

This project is jointly funded by the Biological Oceanography Program and the Established Program to Stimulate Competitive Research (EPSCoR). This award reflects NSF's statutory mission and has been deemed worthy of support through evaluation using the Foundation's intellectual merit and broader impacts review criteria.

[table of contents | back to top]

Funding

Funding Source	Award
NSF Division of Ocean Sciences (NSF OCE)	OCE-2304066
NSF Division of Ocean Sciences (NSF OCE)	OCE-2304067

[table of contents | back to top]