Proteomic analysis of Alteromonas macleodii exudates

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

Contributors	Affiliation	Role
Morris, James	University of Alabama at Birmingham (UA/Birmingham)	Co-Principal Investigator

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. Based on mass spectrometry analysis, Alteromonas exudates contained a wide variety of proteins. These proteins were significantly different between exudates and whole-cell lysates, suggesting they represented programmatic release instead of simple lysis. Moreover, the composition of exudates changed after 500 generations of adaptation to co-culture with Prochlorococcus, suggesting genetic regulation of protein release.

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Coverage

Location: Laboratories at the University of Alabama at birmingham
Spatial Extent: **Lat**:0 **Lon**:0

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 30º 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 23º 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.

Proteomics: The >50 kDa fraction described above was further concentrated using a 30 kDa centrifugal filter (MilliporeSigma™ Amicon™ Ultra-15, Darmstadt, Germany) to ~1.5 ml by centrifugation at 7000 g. Then, 13.5 mL sterile milli-Q water was added to the filtrate and was concentrated to ~1.5 mL again. The above wash step was repeated, and the final ~1.5 mL sample was transferred to a sterile 2 mL tube for storage at 4º C. We also isolated proteins from whole EZ55 cells from the same cultures used to produce the >50 kDa fraction using a Bacterial Cell Lysis kit (GoldBio). The total protein concentration for each sample was measured using a DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA). The samples were then diluted with 4X Laemmli Sample Buffer (Bio-Rad, Hercules, CA, USA) containing 2-mercaptoethanol (Bio-Rad, Hercules, CA, USA) at the rate of 3 parts sample to 1 part buffer. The diluted sample was heated at 95°C for 5 min, and 20 µL was loaded onto a 4-20% Mini-PROTEAN TGX precast polyacrylamide gel (Bio-Rad, Hercules, CA, USA). Gel electrophoresis was performed in a vertical direction in a Mini-PROTEAN Tetra cell (Bio-Rad, Hercules, CA, USA) at ~200V for 20-40 min until the blue band in the marker line reached the bottom of the gel. After electrophoresis was complete, the gel was gently removed from the cassette and was rinsed in a shallow staining tray with milli-Q water. The rinsed gel was soaked in fixing solution (40% ethanol, 10% acetic acid) for 15 min with gentle agitation, rinsed with milli-Q water again, and stained with colloidal Coomassie blue for 14 h with gentle agitation at room temperature. The stained gel was destained in three changes of milli-Q water over 3 h with gentle agitation.

For protein identification, the portion of the destained gel containing target bands of interest was cut into 8 slices with equal length (Figure S2), and each slice was digested following the In-Gel Digestion Protocol described by (7). Each digest was analyzed as previously described (8). An aliquot (5 µL) of each digest was loaded onto a Nano cHiPLC 200 µm ID x 0.5 mm ChromXP C_{18} -CL 3-µm 120-Å reverse-phase trap cartridge (Eksigent, Dublin, CA) at 2 µL/min using an Eksigent 415 LC pump and autosampler. After the cartridge was washed for 10 min with 0.1% formic acid in ddH_2O, the bound peptides were flushed onto a Nano cHiPLC 200-µm ID x 15-cm ChromXP C -CL 3-µm 120-Å reverse-phase column (Eksigent) with a 100-min linear (5 to 50%) acetonitrile gradient in 0.1% formic acid at 1,000 nL/min. The column was then washed with 90% acetonitrile + 0.1% formic acid for 5 min and re-equilibrated with 5% acetonitrile + 0.1% formic acid for 15 min. A Sciex 5600 Triple-TOF mass spectrometer (Sciex, Toronto, Canada) was used to analyze the protein digest. The IonSpray voltage was 2,300 V, and the declustering potential was 80 V. Ion spray and curtain gases were set at 10 and 25 lb/in^2, respectively. The interface heater temperature was 120°C. Eluted peptides were subjected to a time-of-flight survey scan from m/z 400 to 1250 to determine the top 20 most intense ions for tandem mass spectrometry (MS/MS) analysis. Product ion time-of-flight scans (50 ms) were carried out to obtain the MS/MS spectra of the selected parent ions over the range from m/z 400 to 1,000. The spectra were centroided and

deisotoped by Analyst software (v1.7 TF; Sciex). A β-galactosidase trypsin digest was used to establish and confirm the mass accuracy of the mass spectrometer.

The MS/MS data were processed to provide protein identifications using an in-house Protein Pilot 4.5 search engine (Sciex) using the NCBI *Alteromonas* EZ55 protein database and a trypsin digestion parameter and carbamidomethylation for alkylated cysteines as a fixed modification. Proteins of significance were accepted based on the criteria of having at least two peptides detected with a confidence score of >95% using the Paradigm method embedded in the Protein Pilot software. Complete amino acid sequences of predicted proteins were downloaded using the Bio.Entrez package from BioPython (9). Subcellular localization of proteins was predicted using PSORTb v 3.0 (10). KEGG orthology group codes were obtained for proteins using BlastKOALA (11) and were binned into pathways using KEGGREST (12) in R (13). Estimated molecular weights for EZ55 proteins were calculated using the CusaBio molecular weight calculator (https://ww.cusabio.com/m-299.html). Data were statistically analyzed and visualized within R.

References

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Statistical Computing, Vienna, Austria. https://www.R-project.org/.

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 (1). 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 (2). Statistical differences between lysate and exudate protein localization counts were determined using Fisher's exact test implemented in R.

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Parameters

Parameters for this dataset have not yet been identified

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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.

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