Environmental and taxonomic drivers of bacterial extracellular vesicle production in marine ecosystems

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Abstract

Extracellular vesicles are small (~50-250 nm diameter) membrane-bound structures released by cells into their surrounding environment. Vesicles are abundant in the global oceans and likely play a number of ecological roles in these microbially dominated ecosystems, yet we know nothing about what influences their production and distributions. Here we examine how vesicle production varies among different strains of cultivated marine microbes and explore the degree to which this is influenced by some key environmental variables. We show that vesicle production rates – the number of vesicles produced per cell per generation – vary across an order of magnitude in cultures of marine Proteobacteria, Cyanobacteria, and Bacteroidetes. Vesicle production rates further differ among strains of the cyanobacterium *Prochlorococcus,* and vary across temperature and light gradients. These data suggest that both community composition and local environmental conditions modulate the production and standing stock of vesicles in the oceans. Examining samples from the oligotrophic North Pacific Gyre, we show depth-dependent changes in the abundance of vesicle-like particles in the upper water column in a manner broadly consistent with culture observations: highest vesicle abundances are found near the surface, where light irradiances and temperatures are greatest, and then decrease with depth. This work represents the beginnings of a quantitative framework for describing extracellular vesicle dynamics in the oceans – essential as we begin to incorporate vesicles into our ecological and biogeochemical understanding of marine ecosystems.

Importance

Bacteria secrete extracellular vesicles containing a wide variety of cellular compounds, including lipids, proteins, nucleic acids, and small molecules, into their surrounding environment. These structures are found in diverse microbial habitats, including the oceans, where their distributions vary throughout the water column. Differences in vesicle abundances likely affect their functional impacts within microbial ecosystems, but the factors influencing vesicle distributions in the environment remain poorly understood. Using quantitative analysis of marine microbial cultures, we show that bacterial vesicle production in the oceans is shaped by a combination of biotic and abiotic factors. Our data indicate that different marine taxa release vesicles at rates varying across an order of magnitude, and that vesicle production can change dynamically as a function of environmental conditions. Taken together with direct measurements of vesicle concentrations in the oceans, these culture-based measurements further provide a window into estimating vesicle loss rates. These findings represent a step forward in our understanding of marine vesicle distributions and provide a basis for quantitatively exploring vesicle dynamics in natural ecosystems.

Keywords

Extracellular vesicles, *Prochlorococcus*, *Pelagibacter*, *Alteromonas, Thalassospira, Alcanivorax, Marinobacter, Dokdonia, Polaribacter,* Station ALOHA

Introduction

Cells from all domains of life produce membrane-bound structures known as extracellular vesicles. Most of what we know about vesicle release by bacteria is derived from studies of model microbes relevant to advances in molecular biology or biomedical research. Recently, however, the theater has been expanded to examine the microorganisms that serve as the biogeochemical engines of natural ecosystems. In previous work, we demonstrated that the globally abundant marine cyanobacterium *Prochlorococcus* releases vesicles containing a wide range of biomolecules including lipids, nucleic acids, proteins, and metabolites (1–3). Extracellular vesicles are abundant in both coastal and oligotrophic waters, and they are produced by a diverse suite of marine bacteria, archaea, and eukaryotes from both temperate and polar regions (1, 2, 4–7). Though the precise functional roles of vesicles in marine ecosystems remain unknown, lab and field studies suggest that these structures contribute to many biological processes, including horizontal gene transfer, host-phage interactions, and nutrient exchange (1, 8–10). Vesicles represent an essentially unknown feature of the dissolved organic matter in ocean ecosystems (1). Still, we know nothing about what factors influence their production and, in turn, distributions along ocean environmental gradients.

While the production of extracellular vesicles appears to be essentially ubiquitous among bacteria, the cellular mechanisms of vesicle production remain unclear. In Gram-negative bacteria, vesicles released by intact, viable cells are largely thought to arise via 'blebbing' of the outer membrane, where small regions of the lipid bilayer protrude away from the cell and eventually separate in the form of a spherical vesicle (11, 12). These membrane protrusions could originate from numerous sources, such as localized membrane changes, uneven membrane synthesis, membrane turgor pressure, or the influence of small molecules on local regions of the membrane (13–17). Extracellular vesicles can arise through other mechanisms as well, such as through the pinching off of both outer and inner membrane to yield dual-membrane vesicles (18, 19), or via processes associated with the rotation of sheathed flagella (20). Self-annealing of membrane fragments released by cellular lysis can also lead to vesicle formation (21, 22).

Given the underlying complexity and diversity of vesicle biogenesis and release, understanding how this might be regulated – and even the degree to which it is directly regulated in the first place – represents a challenge. In some Gram-negative pathogens and human commensals, vesicle formation appears to involve some degree of genetic control, though it is

not known whether these impacts arise through direct or indirect effects (23–28). Further, stress induced by changes in temperature (6, 29), nutrient availability, reactive oxygen species, and UV exposure has also been correlated with increased vesicle production (29–31). These examples show the potential for vesicle production to vary in response to intracellular and extracellular conditions, but the magnitude to which such variables affect vesicle release by different microbes remains an open question.

Here we examine factors that may influence vesicle production within marine microbial ecosystems, focusing on potential roles for community composition and environmental variables. To this end, we measured vesicle production rates along a spectrum of environmentally relevant conditions for a suite of taxonomically diverse, and numerically abundant, marine bacteria. We further explored the degree to which vesicle production varies among closely related strains of *Prochlorococcus*, and how vesicle production rates change across gradients of light and temperature. Finally, we examined the depth-distribution of vesicles at a site in the oligotrophic North Pacific Gyre to explore how natural vesicle abundances correlate with environmental parameters.

Results and Discussion

Vesicle production rates vary among marine microbial taxa

We first wondered about the degree to which marine bacteria vary in their production of extracellular vesicles. Focusing solely on vesicles released by intact, growing cells, we measured production rates by several diverse marine microbes during exponential growth in terms of vesicles produced per cell per generation, a standardized metric that enables comparisons across differences in both cell abundances and growth rates (Methods and (1)). We surveyed representatives from several of the most abundant bacterial phyla in marine systems – Proteobacteria, Cyanobacteria, and Bacteroidetes – and found that their vesicle release rates, when growing under similar conditions, spanned across an order of magnitude (Fig. 1a). Median vesicle production rates during exponential growth ranged from ~4 vesicles cell^{-1} generation^{-1} among strains of *Prochlorococcus* to ~58 vesicles cell^{-1} generation^{-1} in the heterotroph *Marinobacter*, with other taxa producing vesicles at intermediate values.

Though the total amount of cellular surface area available to form membrane vesicles

117 might be expected to play some role in bounding the number of vesicles that can be formed at
118 any time, vesicle production was not significantly associated with cell surface area (Fig. S1a).
119 Cellular growth rates, on the other hand, were positively correlated with vesicle release rates
120 (Pearson's correlation $p < 0.05$), but growth rates only explained a portion of the variation in
121 vesicle production (see below). Together, our results indicate that vesicle production rates differ
122 markedly among marine taxa, and are due at least in part to physiological differences between
123 strains.
124
125 *Growth conditions impact vesicle production by marine heterotrophs*
126 To explore how environmental conditions might influence vesicle production, we first
127 measured vesicle production rates of selected strains of marine heterotrophic bacteria under
128 different culture conditions. Focusing first on media composition, we compared vesicle
129 production rates by *Pelagibacter* (SAR11) grown in either a defined artificial seawater medium
130 (AMS1) or a variant based on natural seawater but with otherwise equivalent nutrient
131 amendments (ProMS*; see Methods). *Pelagibacter* grew 3.2x faster in ProMS* than AMS1, and
132 vesicle release rates increased by ~15x (from ~0.4 vesicles cell^{-1} generation^{-1} in AMS1 to ~6
133 vesicles cell^{-1} generation^{-1} in ProMS*; Fig. 1b). While we do not know which component(s) of
134 the natural seawater background are responsible for the difference, this indicates that nutrient
135 availability can affect vesicle production. We next asked whether changes in temperature
136 influenced vesiculation by comparing production rates at two temperatures in *Marinobacter* and
137 *Thalassospira.* An increase in growth temperature from 21 °C to 24 °C increased the rate of
138 vesicle production by a factor of ~4-8x in both strains (Fig. 1c), broadly consistent with trends
139 observed in host-associated and terrestrial microbes such as *Pseudomonas aeruginosa* (29) and
140 *Acinetobacter baylyi* (32).
141 Looking across all heterotrophs and growth conditions examined, cells tended to produce
142 more vesicles per generation when they grew faster (Fig. S2). On average, each growth rate
143 increase of 1 day^{-1} resulted in ~2.6 additional vesicles released per cell per generation. However,
144 changes in growth rate only accounted for ~26% of the observed variance (linear regression $p <$
145 0.01; Fig. S2), indicating that other factors are at play. One possible explanation for the influence
146 of growth rate on vesicle production is that increasing growth rate requires increased cell
147 wall/membrane biosynthesis activity, which could in turn transiently weaken the connections

148 between the outer membrane and the rest of the cell to create more opportunities for vesicle
149 release. Environmental conditions might also modulate vesicle production via changes in
150 membrane composition and/or fluidity (17).
151
152 *Combinatorial impacts of light and temperature on* Prochlorococcus *vesicle production*
153 For abundant marine photosynthetic organisms such as *Prochlorococcus*, light and
154 temperature are key environmental variables impacting cellular growth and physiology (33–35),
155 and we suspected that they also influence vesicle production. To explore this, we measured
156 production rates under different combinations of light irradiance and temperature using five
157 different strains of *Prochlorococcus* that encompass phylogenetically and ecologically distinct
158 high-light and low-light adapted clades (Table 1). Median production rates varied significantly
159 among strains, and ranged from 1.2 – 7.7 vesicles cell^{-1} generation^{-1} across all conditions tested
160 (two-way ANOVA, $p < 0.01$; Table 2; Fig. 2a). Vesicle production by individual
161 *Prochlorococcus* strains was remarkably dynamic, varying by an average factor of ~27 between
162 the lowest and highest production rates observed (Table 2). On average, high light-adapted
163 *Prochlorococcus* produced fewer vesicles per cell per generation than low light-adapted strains;
164 the high-light strains also had a lower dynamic range of production rates (Table 2). What might
165 account for this? High light-adapted *Prochlorococcus* are smaller than low light-adapted cells
166 but, as with the heterotrophs, differences in cell size had little explanatory value for differences
167 in vesicle production rates (Fig. S1b). Unlike the heterotrophs, however, differences in
168 *Prochlorococcus* growth rate were not significantly correlated with variation in vesicle
169 production (linear model $R^2 = 0.065$; Figs. 2a and S4), suggesting that other physiological factors
170 must play key roles.
171 Looking at the impact of light and temperature, we found that individual strains
172 responded differently. Vesicle production by the high-light adapted strains did not significantly
173 change as a function of irradiance (Table 2; Fig. 2b), as consistent trends were only observed at
174 some, but not all, temperatures (Figs. 2a, S3). By contrast, the low-light adapted cells exhibited
175 significant variation in production rates with both light and temperature, again with higher light
176 or increased temperature leading to increased vesicle production rates (Figs. 2a, 2b, S3; Table 2).
177 Differences in these production patterns thus join the properties that contribute to the ecological
178 differentiation between high-light and low-light adapted *Prochlorococcus*.

179

180 *Vesicle release as a response to light stress in* Prochlorococcus

181 Our experiments indicate that light and temperature impact *Prochlorococcus* vesicle
182 production in complex ways. Given that most strains showed an increase in production at the
183 highest light intensities tested (Fig. 2b), we wondered whether differences in production might
184 be associated with light stress. Irradiance levels higher than that which can be utilized by
185 photosystems can disrupt intracellular redox balance, inhibit photosynthesis, or generate reactive
186 oxygen species in cyanobacteria. Cells must then activate one or more stress response pathways
187 to repair the damage and/or help to manage the excess energy input (36–38). Across all
188 *Prochlorococcus* strains examined, production rates were not influenced by irradiance when cells
189 were grown at or below the levels which maximized growth rates (Figs 2c, S4). Above this light
190 optimum, when cells are assumed to experience some degree of light stress, production was
191 significantly and positively correlated with relative increases in light (R^2=0.23, linear model $p <$
192 0.01; Fig. 2c). Changepoint threshold regression analysis corroborated this difference, indicating
193 a significant step increase in vesiculation when light levels were increased above the growth
194 optima at each particular temperature (threshold range: 1-2x optimal light level, $p < 0.001$). This
195 suggests that chronic light stress may directly or indirectly lead to increases in vesicle release.

196 Might vesicle production directly contribute to mitigating light stress, or does it instead
197 reflect a side effect of other physiological changes occurring within the cell? Though we can
198 only speculate at this point, cyanobacteria can utilize several strategies to dissipate excess light
199 energy during light stress, including modifying their light-harvesting apparatus,
200 nonphotochemical quenching, and increased production of enzymes with antioxidant functions
201 (37). In addition, *Prochlorococcus* can utilize excess light energy to power increased levels of
202 carbon fixation and then 'dump' the unneeded organic molecules into the extracellular space to
203 maintain redox balance (36). While perhaps an indirect metabolic route, increased synthesis of
204 lipids and secretion of fixed organic compounds via vesicles might conceivably provide yet
205 another route for releasing the products of overflow metabolism. Intracellular oxidative stress,
206 such as might arise from high light conditions, increases the number of vesicles produced by
207 *Pseudomonas aeruginosa* (29) and a similar mechanism could be at play here. *Prochlorococcus*
208 vesicles can contain oxidized metabolites, raising the possibility that they could aid the cell by
209 removing cellular material damaged by oxidative stress (3, 12). While there remains much to

210 uncover about this association, differences in light stress responses may explain, in part, the
211 different degree to which light impacts vesicle release by high-light vs low-light adapted
212 *Prochlorococcus*.
213
214 *Vesicle size varies across microbial taxa and environmental conditions*
215 Bacteria release extracellular vesicles of varying sizes, often ranging between ~50 - 250
216 nm diameter (12). Vesicle size distributions can differ among strains of the same organism (39),
217 as a function of growth phase (40), and in response to changes in membrane composition (41).
218 As vesicle size is associated with differences in both proteomic (42) and DNA content (43),
219 shifts in size distributions may have functional consequences. Among the marine microbes we
220 studied, vesicle sizes differed significantly by taxonomic group (one-way ANOVA $p < 0.001$;
221 Fig. 3), with members of the Bacteroidetes releasing the largest (~170 nm mode diameter) and
222 *Prochlorococcus* releasing the smallest (~70 nm). Vesicle size was not significantly correlated
223 with cellular growth rate or production rate across the heterotrophs or *Prochlorococcus* strains.
224 However, within *Prochlorococcus*, vesicle size did vary with light intensity and, to a lesser
225 extent, growth temperature (Fig. S5; two-way ANOVA $p < 1e\text{-}6$ and $p < 0.01$, respectively).
226 Strains grown at lower light intensities tended to release larger vesicles than cells experiencing
227 higher light fluxes, even at levels below the optimum (Fig. S5a). The largest vesicles also tended
228 to come from cultures grown in the middle of the temperature range tested (Fig. S5b).
229 Do taxa differ in the relative investment of resources they put into vesicles? As an initial
230 approach to addressing this question, we calculated how much membrane material is required to
231 produce the median number of vesicles released per generation compared to each cell's total
232 surface area. Despite differences in cell size, vesicle diameter, and vesicle production rates, we
233 estimate that most strains, including *Prochlorococcus, Pelagibacter*, and *Alteromonas*, release
234 vesicles equivalent to a median of ~3 – 6% of their cell surface lipid membranes per generation
235 (Fig. S6). *Alcanivorax* and *Marinobacter* were notably distinct from the others and released
236 vesicles equivalent to ~40% of their surface area per generation (Fig. S6). Forming a given size
237 vesicle requires proportionately less membrane material from larger cells than from smaller cells,
238 but it appears that increased production rates can balance these lower proportional investments in
239 individual vesicles. Given the dynamic range of vesicle production rates, and thus relative
240 cellular investment, cells must substantially increase membrane synthesis under certain growth

241 conditions, and this burden may not fall just on the smallest cells. The costs and benefits of
242 changing vesicle production rates under a given environmental context, especially when
243 considering other resources invested besides just the membranes, are clearly complex (44).
244
245 *Extracellular vesicle abundances across environmental gradients in the North Pacific*
246 We have shown that extracellular vesicle concentrations released by the entire microbial
247 community decreases with depth at an oligotrophic site in the Atlantic ocean (1). To explore
248 whether those results may be generalizable to other regions of the ocean, we examined vesicle
249 abundances at Station ALOHA in the North Pacific subtropical gyre (Fig. 4a). As at the Atlantic
250 site, vesicle-like particles were most abundant near the surface, with their concentration
251 decreasing by approximately an order of magnitude over the upper 500 m. Vesicle-like particle
252 concentrations in this set of Pacific ocean samples were of the same magnitude as those
253 previously seen in the Atlantic and declined with depth to a similar degree. This overall
254 congruence of results across different oceans, seasons, and years, provides an early indication
255 that this general pattern of vesicle distributions in the water column might be generally
256 representative for mid-latitude oligotrophic waters.
257 Assuming loss rates are constant with depth, the data from our culture studies would
258 broadly predict that, all else being equal, vesicle abundances should be greatest where light
259 irradiance and temperature are highest, and this is what we observed (Fig. 4b-c). Not only were
260 overall vesicle-like particle concentrations greatest at the surface, but there were proportionally
261 more vesicles per cell at the surface than in deeper samples, where environmental conditions
262 would be expected to yield lower vesicle production rates (Fig. 4). These data are consistent with
263 local environmental factors, such as those explored above, influencing *in situ* vesicle distribution
264 patterns.
265 Given production rates in pure cultures, measurements of microbial standing stocks in the
266 wild, and estimates of *in situ* cellular growth rates, we can also begin to estimate vesicle loss
267 rates – an aspect of vesicle dynamics in the environment that is completely unknown. Assuming
268 that marine phytoplankton grow at a rate of ~0.5 per day (45, 46), with heterotrophs growing at
269 ~0.1 per day (45), the vesicle production rates measured here suggest that the surface community
270 at Station ALOHA might produce in excess of 1 vesicle cell^{-1} day^{-1}, or >10^{5} vesicles mL^{-1} day^{-1}.
271 This estimated daily input vesicle flux is comparable to the measured standing stocks in the

oceans; if we further assume that standing vesicle concentrations are roughly constant, this would suggest that vesicle loss rates must be similar to production rates. Vesicle production rates in the ocean could certainly differ from those measured under laboratory conditions due to strain differences, nutrient availability, the influence of biotic or abiotic interactions, consideration of vesicle production through cell lysis mechanisms, or other factors. Regardless, loss rates must be significant, and could arise from processes including natural decay, consumption or degradation, fusion with other cells, or physical transport. More work is needed to arrive at a detailed estimate of losses and examine the relative contribution of these and other mechanisms.

Conclusions

This study shows that extracellular vesicle production rates differ among marine microbes and highlights the dynamic nature of vesicle production by individual bacterial cells. The composition of extracellular vesicle populations in a given location will be influenced by a complex set of factors involving differences in cellular community structure and the vesicle production rate of each taxa under local environmental conditions. As differences in cell and vesicle concentrations would be expected to impact their encounter rates in an aqueous, planktonic environment, vesicle-mediated interaction dynamics may also changes as a result of environmental shifts. Studies addressing the fate and turnover of vesicles will be essential for developing detailed models of vesicle distributions and functions in the oceans.

Materials and Methods

Culturing conditions

Axenic *Prochlorococcus* cells were grown in 0.2 µm filtered and autoclaved Sargasso Sea water amended with Pro99 nutrients (47). Triplicate cultures were grown on a 14:10 hr light:dark cycle at temperatures ranging from 15 °C to 26 °C and at four light levels: very high light (VHL), high light (HL), medium light (ML), and low light (LL), with specific values determined by each strain's light tolerance (Table 1). Culture axenicity was regularly verified using a suite of purity test broths (MPTB, ProAC, ProMM) (48–50) and by flow cytometry. Cultures were maintained at the indicated temperature and light intensity for at least 12 generations (approximately four complete transfers), and were not sampled until the culture

303 reached balanced growth. Growth was routinely monitored by culture fluorescence with a 10AU
304 or TD700 Fluorometer (Turner Designs), and growth rates were calculated by exponential
305 regression from the log-linear portion of the growth curve.
306 *Alcanivorax* MIT1350 was isolated from a water sample collected at 150m depth from
307 Station ALOHA (22.75 °N 158 °W) in the North Pacific in 2013. This strain was flow-sorted
308 from a 'small cell, low nucleic acid' population derived from a *Prochlorococcus* enrichment
309 culture. Following isolation, *Alcanivorax* MIT1350, along with *Alteromonas macleodii* strain
310 MIT1002 (51), *Thalassospira* strain MIT1004 (2), and *Marinobacter* MIT1353 (52) were
311 maintained in ProMM medium. The genome sequence of *Alcanivorax* MIT1350 is available at
312 the IMG database (53) under Genome ID 2681813576.
313 *Pelagibacterales sp.* HTCC7211, a member of the abundant warm-water surface-
314 dwelling Ia.3 ecotype of the SAR11 bacterial clade (54), was cultured in either a defined
315 artificial medium (AMS1; (55)) or Sargasso seawater-based ProMS* (a modified version of
316 ProMS media (52) with pyruvate, glycine, methionine, and vitamin mixes added to
317 concentrations equal to those in AMS1). *Dokdonia* MED134 (56) and *Polaribacter* MED152
318 (57) were grown in in Pro99 media supplemented with 5 g L^{-1} peptone and 1 g L^{-1} yeast extract
319 (BD Difco). All heterotrophs were grown at 24 °C, except for HTCC7211, which was
320 maintained at 22 °C.
321 We manually examined the genome sequence of each strain listed above and found no
322 evidence for the presence of lysogenic phage and/or gene transfer agents in these organisms
323 (based on BLASTp searches using the *Rhodobacter capsulatus* gene transfer agent sequences
324 RCAP_rcc01682 - RCAP_rcc01698), and the cultures contained no evidence of lytic phage
325 activity. Given this and prior electron microscopy data (2), our data assume that all particles
326 between ~50-250 nm diameter in these culture supernatants are extracellular vesicles.
327
328 *Cell and vesicle enumeration*
329 At each time point sampled, cells were preserved for flow cytometry by fixation in
330 0.125% glutaraldehyde, flash frozen in liquid nitrogen, and stored at -80 ºC until analysis.
331 Vesicles were sampled by collecting 1 mL of culture and filtering it through a 0.22 µm Supor
332 syringe filter (Pall). The <0.22 µm fraction was stored at -20 ºC until ready for analysis.
333 *Prochlorococcus* cells were enumerated using a Guava easyCyte 12HT flow cytometer

334 (Luminex). Heterotrophs were stained with 1x SYBR Green I (Molecular Probes) for 1 hr and
335 then counted using either an easyCyte 12HT or a ZE5 flow cytometer (Bio-Rad).
336 *Prochlorococcus* cell sizes were normalized to 2 µm beads (Polysciences). Cells were excited
337 with a blue 488 nm laser and analyzed for red fluorescence (692/40 nm), green fluorescence
338 (530/40 nm), and size (forward scatter). Cell lengths were determined by imaging >500 cells on
339 a Zeiss Axioskop microscope equipped with a 100x objective lens and a Nikon D90 digital
340 camera. Cell lengths were determined using ImageJ, and calibrated using a 1mm / 0.01mm scale
341 stage micrometer (Meiji Techno).
342 Vesicles were measured from samples collected at two or more timepoints during
343 exponential growth phase. Vesicles were enumerated by nanoparticle tracking analysis using a
344 NanoSight LM10HS instrument (Malvern/NanoSight), equipped with a LM14 blue laser module
345 and NTA software V3.1. Three technical replicate videos (60s each) were collected from each
346 sample using a camera level setting of 11. Samples containing >80 particles per frame were
347 diluted with clean seawater media to a final concentration between 20-80 particles per frame.
348 The sample chamber was thoroughly flushed with 18.2 MΩ cm^{-1} water (Milli-Q; Millipore)
349 between samples, and visually examined to ensure that no particles were carried over between
350 samples. Videos were analyzed using NTA V3.1 software with a threshold of 1. Vesicle
351 concentrations in each sample are based only on particles between 50 - 250 nm diameter. Media
352 blank controls were routinely run for each batch of media used, and any background
353 concentration was subtracted. A small number of samples containing abnormally high particle
354 counts (defined as containing >10x higher concentrations than other replicates of that timepoint
355 and/or the surrounding timepoints) or in which large, highly refractive particles were present
356 (which mask the underlying particle counts) were removed from the final dataset. The source of
357 these contaminants is unknown and their occurrence in samples did not follow any obvious
358 systematic patterns; we hypothesize that they could have arisen from manufacturing variation or
359 defects in individual filters, plastic tubes used to store samples, or plastic syringes used in fluid
360 handling.
361
362 *Vesicle production and surface area calculations*
363 We measured vesicle production rates as the average number of vesicles produced on a
364 per cell, per generation basis based on averaged cell and vesicle measurements from all

365 biological replicates at each timepoint. These calculations are based on a model that incorporates
366 the relative rates of change in cell and vesicle concentrations in pure cultures over time as
367 detailed in (1). To better account for variation in slope across the time course, we computed the
368 average number of vesicles produced per cell per generation between each successive pair of
369 timepoints obtained during the exponential growth phase.
370 The surface area of vesicles from an individual strain was calculated assuming that
371 vesicles are spherical structures with a diameter equivalent to the mode value measured. Cellular
372 surface area is based on microscopy measurements and literature values (58, 59).
373 *Prochlorococcus* was estimated to be spherical, and the surface area of other cells were
374 estimated as cylinders with a hemisphere on each end. Relative cellular investment in vesicles
375 per generation was defined as (individual vesicle surface area * vesicle production rate per cell
376 per generation) / cell surface area.
377
378 *Statistics*
379 All statistical analyses were performed in R (V 4.0.2) and plots were produced using the
380 *ggplot2* package (60). ANOVA analysis of vesicle production rate data was performed on log-
381 transformed values. Model selection for two-way ANOVAs was carried out using the Akaike
382 information criterion metric, as implemented in the 'AICcmodavg' R package (61). Changepoint
383 threshold regression analysis was carried out using the 'chngpt' R package (62). Contour plots of
384 growth rate data were generated using the 'metR' and 'interp' R packages (63, 64).
385
386 *Field sampling*
387 Seawater samples for vesicle analysis were collected from Station ALOHA on cruise
388 HOT263 (June 2014). At each depth, we collected 100 - 175 L using a Niskin bottle array and
389 concentrated the < 0.2 μm fraction by tangential flow filtration following previously described
390 protocols (1). Vesicles were enriched across an Optiprep density gradient (Iodixanol; Sigma-
391 Aldrich) as previously described (1). Enumeration of field concentrations were based on
392 Nanosight concentration measurements of gradient-purified fractions containing the highest
393 abundance of vesicles (between ~1.14 – 1.19 g/mL) and consideration of the original sampled
394 volume. We note that particle losses incurred during the sample processing and analysis pipeline,
395 thus these *in situ* measurements should be considered lower-bound estimates. Cellular

abundance, PAR and temperature values were provided by the Hawaii Ocean Time-series program (https://hahana.soest.hawaii.edu/hot/hot-dogs/interface.html). As vesicle-like particles could be produced by microbes from all domains of life, cell abundances represent the combined counts for cyanobacteria, heterotrophic bacteria, and eukaryotes from the indicated depths.

Acknowledgments

We thank the captain and crew of HOT263 for their help with obtaining field samples. Stephen Giovannoni (Oregon State U.) kindly supplied *Pelagibacteriales sp.* HTCC7211, and Jarone Pinhassi (Linnaeus U.) shared the *Polaribacter* and *Dokdonia* cultures. We are also grateful to Christina Bliem and Madeline Williams for providing laboratory assistance. HOT data were obtained via the Hawaii Ocean Time-series HOT-DOGS application; University of Hawai'i at Mānoa National Science Foundation Award # 1756517.

This work was supported by the National Science Foundation (OCE-2049004 and DBI-2018337 to SJB, OCE-1356460 to SWC) and the Simons Foundation (SCOPE Award ID 329108, SWC). This is a contribution of the Simons Collaboration on Ocean Processes and Ecology (SCOPE).

The authors declare no conflicts of interest.

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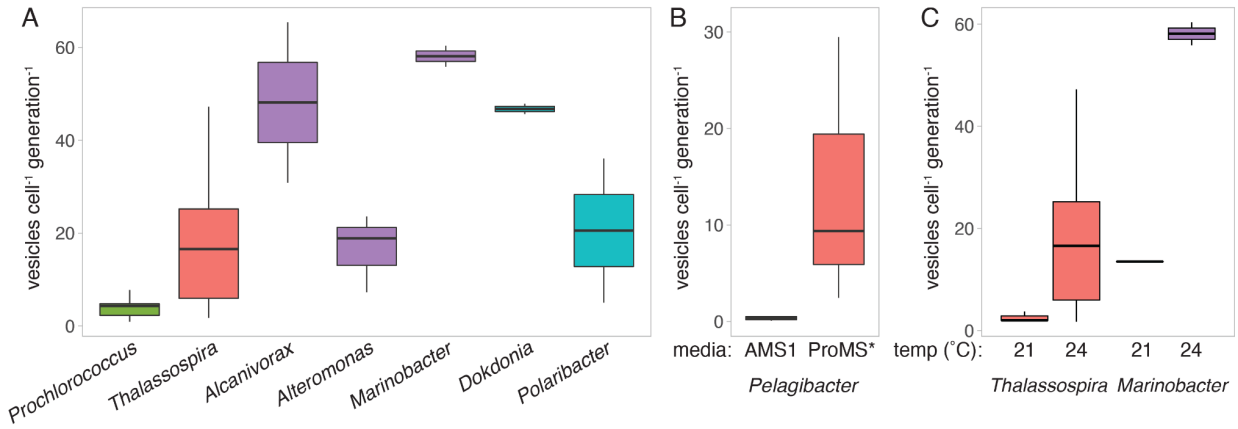


FIG 1 Extracellular vesicle production rates among marine microbes and as a function of growth environment. (A) Vesicle production rates for selected strains (indicated here by genus) of marine microbes at 24 °C. *Prochlorococcus* data represent median values from 5 strains grown at different light levels (see Fig. 2). (B) *Pelagibacter* (SAR11) vesicle release when grown at 22 °C on either a defined minimal medium (AMS1) or a natural seawater variant (ProMS*). (C) Impact of temperature on vesicle production by two heterotrophs. Colors represent taxonomic groupings of microbes at the Class level: Cyanophyceae (green), Alphaproteobacteria (red), Gammaproteobacteria (purple), Flavobacteriia (blue).

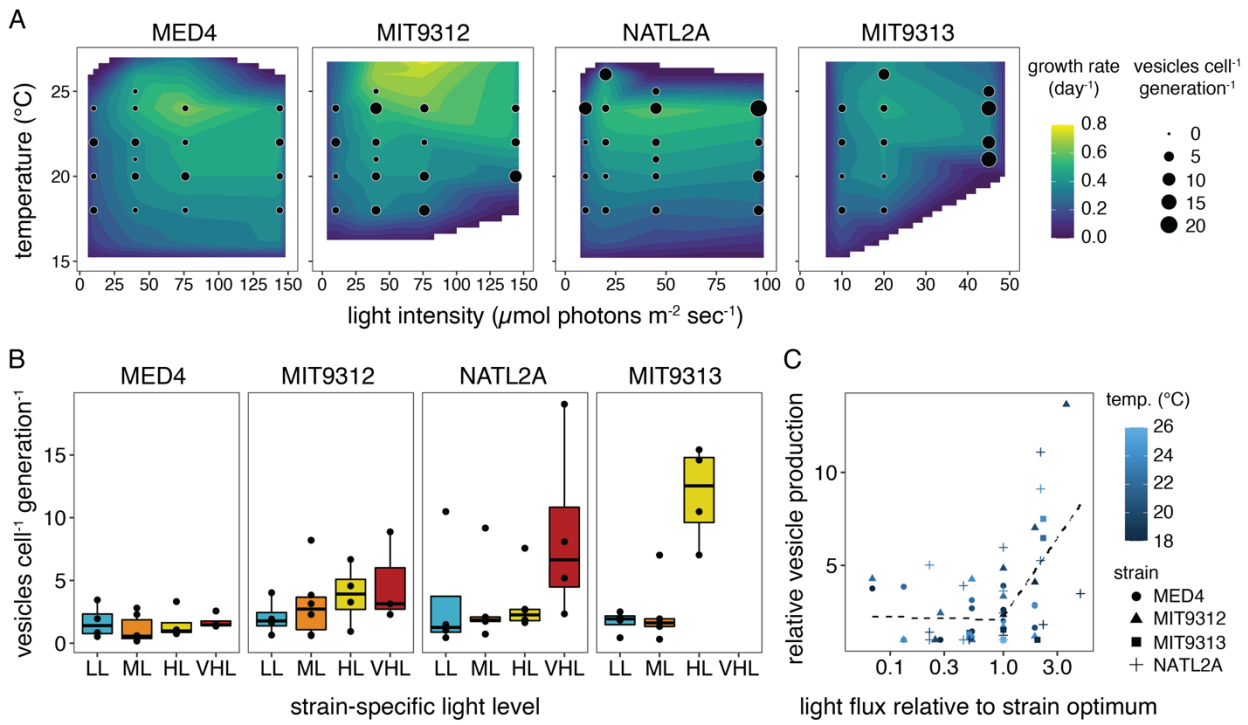
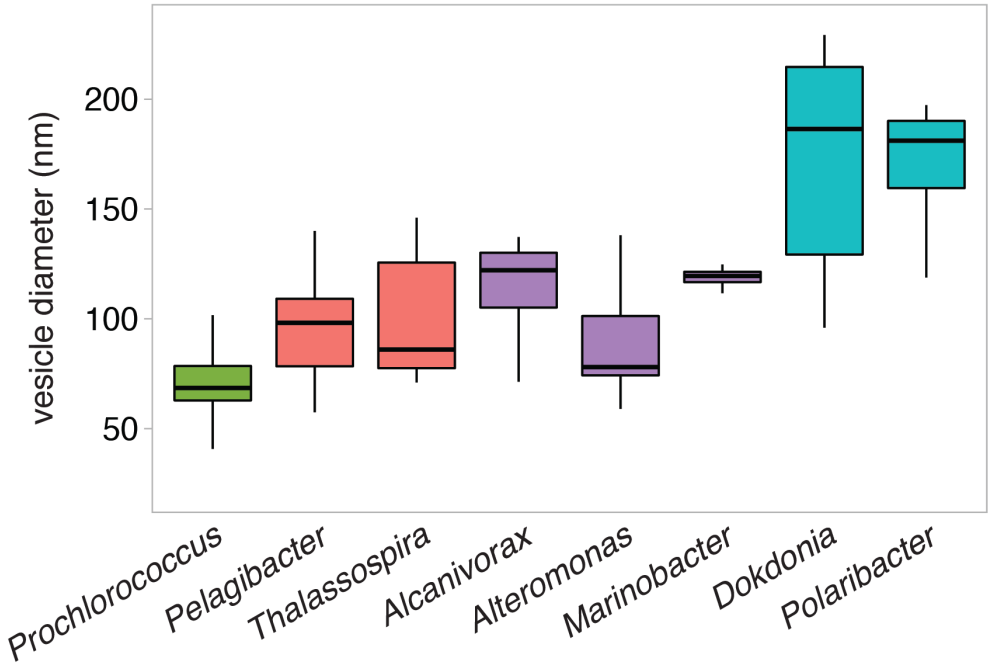


FIG 2 *Prochlorococcus* vesicle production rates as a function of light and temperature. (A) Vesicle production rates (black circles) for four *Prochlorococcus* strains grown at different combinations of light irradiance and temperature. Culture growth rate is indicated by background color. (B) Production rate data summarized across all growth temperatures measured. Light levels: LL= low light, ML= medium light, HL= high light, VHL=very high light (see Table 1). (C) Relative vesicle production rates as a function of relative light level. Vesicle release is normalized relative to the minimum measured value for each strain; light levels are compared to the irradiance that resulted in maximal growth rate for each strain (optimal = 1). Dashed lines represent the changepoint regression fit.



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611

612 **FIG 3** Extracellular vesicle size variation among marine microbes. Vesicle diameters indicate
613 the mode size value from replicate vesicle populations. Colors represent taxonomic groupings of
614 microbes at the Class level: Cyanophyceae (green), Alphaproteobacteria (red),
615 Gammaproteobacteria (purple), Flavobacteriia (blue).

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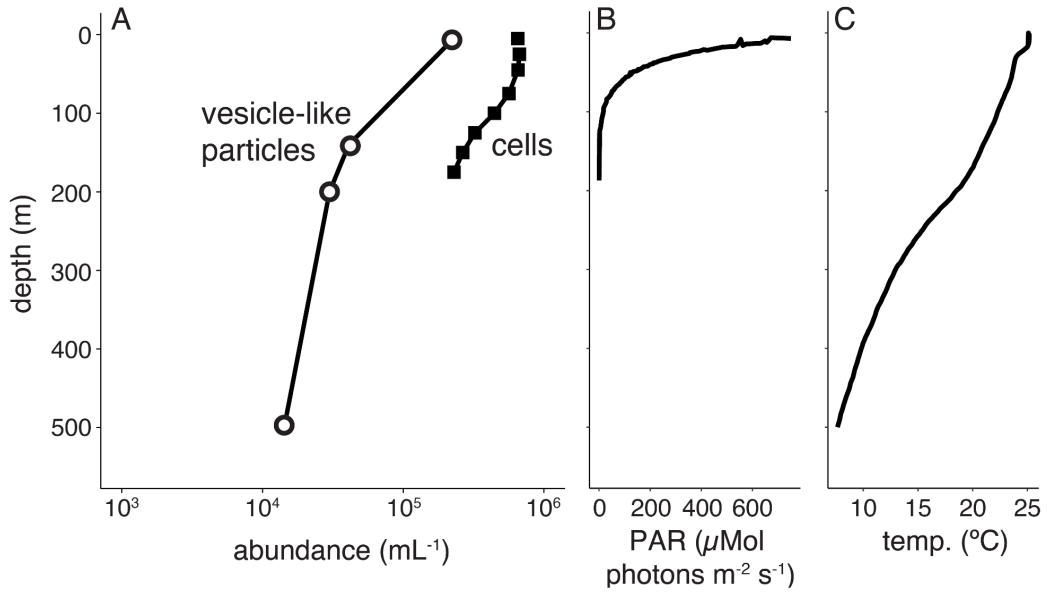

FIG 4 Distribution of vesicle-like particles in the North Pacific Subtropical Gyre. (A) Concentration of vesicle-like particles (open circles) and cells (filled squares) from Station ALOHA in June, 2014. Contextual environmental data are shown for (B) photosynthetically active radiation (PAR) levels and (C) temperature.

623 **TABLE 1** Light conditions examined for each strain. Light treatments are defined as very high
624 light (VHL), high light (HL), medium light (ML), and low light (LL), corresponding to intensity
625 values relative to the specific optima for that strain.

Strain	Ecotype/Clade	Light treatment	Light intensity (µmol photons m^{-2} sec^{-1})
MED4	high-light adapted I	VHL	144±4
MED4	high-light adapted I	HL	76±1
MED4	high-light adapted I	ML	40±1
MED4	high-light adapted I	LL	11±1[a]
MIT9312	high-light adapted II	VHL	144±4
MIT9312	high-light adapted II	HL	76±1
MIT9312	high-light adapted II	ML	40±1
MIT9312	high-light adapted II	LL	11±1
NATL2A	low-light adapted I	VHL	96±1
NATL2A	low-light adapted I	HL	45±1
NATL2A	low-light adapted I	ML	20±1
NATL2A	low-light adapted I	LL	10±0.5
MIT9313	low-light adapted IV	HL	45±1
MIT9313	low-light adapted IV	ML	20±1
MIT9313	low-light adapted IV	LL	10±0.5
MIT1223	low-light adapted VIII group	HL	20±1

626
627 [a]This strain was unable to grow at the indicated light level at 24 ˚C, and was instead grown at
628 17±0.5 µmol photons m^{-2} s^{-1}.
629

630 **TABLE 2** *Prochlorococcus* vesicle production rates. All rate values are in units of vesicles cell^{-1}

631 generation^{-1}.

632

Strain	Ecotype/Clade	Median vesicle production rate across all conditions tested (± SD)	Range of vesicle production rate (lowest-highest values in any combination of light/temp)	Significant variation with light (L) or temperature (T)[1]?
MED4	high-light adapted I	1.2 (±1.5)	0.2 – 3.5	T
MIT9312	high-light adapted II	2.2 (±9.1)	0.6 – 8.9	-
NATL2A	low-light adapted I	2.5 (±8.9)	0.4 – 19.0	T, L
MIT9313	low-light adapted IV	3.9 (±6.2)	0.3 – 15.4	T, L
MIT1223	low-light adapted VIII group	7.7 (±7.6)	7.7 – 11.1	*nd*[2]

633

634 [1]Two-way ANOVA, $p < 0.05$.

635 [2]Not determined due to limited number of conditions where this strain grew