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Bacterial Vesicles in Marine Ecosystems

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Many heterotrophic bacteria are known to release extracellular vesicles, facilitating interactions between cells and their environment from a distance. Vesicle production has not been described in photoautotrophs, however, and the prevalence and characteristics of vesicles in natural ecosystems is unknown. Here, we report that cultures of *Prochlorococcus*, a numerically dominant marine cyanobacterium, continuously release lipid vesicles containing proteins, DNA, and RNA. We also show that vesicles carrying DNA from diverse bacteria are abundant in coastal and open-ocean seawater samples. *Prochlorococcus* vesicles can support the growth of heterotrophic bacterial cultures, which implicates these structures in marine carbon flux. The ability of vesicles to deliver diverse compounds in discrete packages adds another layer of complexity to the flow of information, energy, and biomolecules in marine microbial communities.

Cells from all domains of life produce membrane vesicles (*1*). In Gram-negative bacteria, spherical extracellular vesicles (between ~50 and 250 nm in diameter) are thought to be formed when regions of the outer membrane "pinch off" from the cell and carry with them an assortment of proteins and other molecules (*2*, *3*). Vesicle release occurs during the normal growth of many species, and although growth conditions, stressors, and membrane structure can influence the number of vesicles produced (*4*), the regulation of vesicle production is still unclear (*2*). These structures have been shown to contribute to diverse processes in model bacteria, including virulence (*5*, *6*), quorum signaling (*7*), biofilm formation (*8*), redox reactions (*9*), cellular defense (*10*, *11*), and horizontal gene transfer (*12*, *13*). Despite the numerous ways in which vesicles may affect microbial communities, their abundance and functions in marine and other ecosystems remain unknown.

While examining scanning electron micrographs of an axenic culture of the marine cyanobacterium *Prochlorococcus*, we noticed the presence of numerous small spherical structures near the cell surface (Fig. 1A). This strain has no prophage or gene transfer agents in its genome; thus, we suspected the spheres to be membrane vesicles. Because *Prochlorococcus* is the numerically dominant marine phytoplankter—with a global population of ~10^{27} cells (*14*) accounting for 30 to 60% of the chlorophyll a in oligotrophic regions (*15*)—*Prochlorococcus*-derived vesicles could have a notable influence on the function of marine microbial systems. To study the phenomenon of vesicle release, we first isolated the <0.2-μm fraction from a *Prochlorococcus* culture and visualized these structures by transmission electron microscopy (TEM) (*16*). Negative stain and thin-section micrographs (Fig. 1, B and C) of samples from exponentially growing cultures of healthy, intact cells (fig. S1) confirmed the presence of membrane-bound vesicles ~70 to 100 nm in diameter (Fig. 1D). Their presence was independently observed using nanoparticle tracking analysis of unperturbed cultures, which showed that the particle size distributions were as would be predicted for *Prochlorococcus* cells and vesicles (Fig. 1E). Vesicles were produced under both constant light and diel light-dark cycling conditions and were found in cultures of all six axenic *Prochlorococcus* strains available (representing both high-light– and low-light–adapted ecotype groups) (table S1). A *Synechococcus* strain (WH8102) also produced vesicles (fig. S2), which demonstrated that vesicle release is a feature of the two most numerically abundant primary producers in the oligotrophic oceans.

Because membrane vesicles were the dominant small particle between 50 and 250 nm in our cultures as seen by TEM, we used the concentration of all particles in this size range, determined using nanoparticle tracking analysis, as a measure of vesicle concentration. Vesicles were at least as abundant as *Prochlorococcus* cells in growing cultures, and in some strains they could be 10 times as numerous as cells in exponential and/or stationary phase (Fig. 1F and fig. S3). Vesicles appear to be produced continually during exponential growth, consistent with observations in other bacteria (*17*), and by our estimates, the rate of vesicle production varied from about two to five vesicles per cell per generation among three different *Prochlorococcus* strains (table S1; supplementary text). Vesicles are stable under laboratory conditions, as their size and concentration remained essentially unchanged over the course of 2 weeks in sterile seawater at 21°C (fig. S4). Although many factors could influence vesicle production by *Prochlorococcus* in the wild, production estimates based on these initial data yield global release rates on the order of 10^{27} to 10^{28} per day (supplementary text).

To explore the potential roles of vesicles from marine cyanobacteria, we analyzed the content of *Prochlorococcus* vesicles. As one might expect for membrane-bound vesicles, they contained both lipopolysaccharides, a common component of the Gram-negative outer membrane, and a number of typical cyanobacterial lipids (*18*). The most abundant lipid species in the vesicles were monoglycosyldiacylglycerol, sulfoquinovosyldiacylglycerol, and some unidentified glycolipids (fig. S5A). The lipid fatty acids from vesicles of *Prochlorococcus* MED4 were dominated by saturated $C_{14:0}$, $C_{16:0}$, and $C_{18:0}$ species, which suggested that these vesicles' membranes are relatively rigid (fig. S5B).

We examined the proteome associated with vesicles produced by two ecologically distinct *Prochlorococcus* strains (the high-light–adapted MED4 and low-light–adapted MIT9313) and found these vesicles to contain a diverse set of proteins, including nutrient transporters, proteases, porins, hydrolases, and many proteins of unknown function (tables S2 and S3). Although it is presently unclear whether these enzymes are bioactive, vesicles released by other bacteria can contain active enzymes (*2*), and proteins enclosed within related liposomal structures are known to be protected from degradation by marine bacteria (*19*). Bacterial membrane vesicle release may account, in part, for the relative abundance of membrane proteins among all dissolved proteins in seawater (*20*).

Prochlorococcus MED4 vesicles also contained DNA (fig S6A). To be certain that the detected DNA was associated with vesicles, purified vesicles were treated with deoxyribonuclease (DNase) before lysis and DNA purification. DNA fragments associated with vesicles were heterogeneous in size, with some measuring at least 3 kilobase pairs (kbp) (fig. S7)—enough to encode multiple genes. We amplified and sequenced DNA from ~10^{11} MED4 vesicles and found that it

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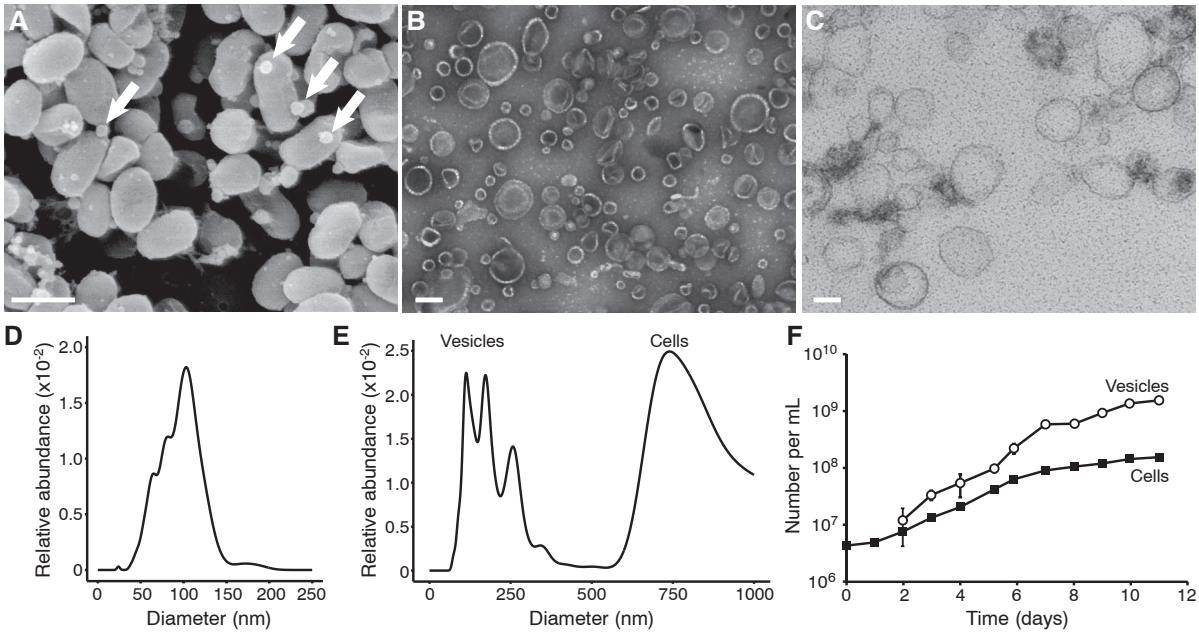

Fig. 1. *Prochlorococcus* releases membrane vesicles. (**A**) Scanning electron micrograph of *Prochlorococcus* strain MIT9313 shows the presence of numerous small spherical features (vesicles, indicated by arrows) near the cells. Scale bar, 1 μm. (**B**) Purified *Prochlorococcus* vesicles as seen by negative-stain TEM. Scale bar, 100 nm. (**C**) Thin-section electron micrographs confirm that *Prochlorococcus* vesicles are circular, membrane-enclosed features lacking notable internal structure. Scale bar, 100 nm. (**D**) Particle size distribution of purified *Prochlorococcus* vesicles as determined by nanoparticle tracking analysis. (**E**) Particle size analysis of an unperturbed culture confirms the presence of particles of two broad size classes consistent with whole cells and vesicles. (**F**) Vesicle production during growth of MIT9313, comparing the number of vesicles (open circles) with cells (squares) in the culture. Values represent means ± SD of three biological replicates. Vesicle limit of detection was ~10^7 ml^{-1}.

encoded broadly distributed regions of the MED4 genome, representing over 50% of the entire chromosomal sequence (Fig. 2 and table S4). We noted a reproducible overabundance of reads from a region of the chromosome roughly centered on the terminus region, which suggested a possible link to the cell cycle; however, the mechanisms through which these DNA fragments are generated and packaged into vesicles are unclear. The vesicles also contained RNA (fig. S6B), and sequences from 95% of open reading frames in the genome were recovered (fig. S8). Membrane vesicles have been shown to facilitate horizontal gene transfer among a number of bacteria including *Escherichia coli* (*12*) and *Acinetobacter* (*13*). Given the abundance of *Prochlorococcus* in the oceans, our finding that it releases DNA within vesicles could have important implications for mechanisms of horizontal gene transfer in marine ecosystems.

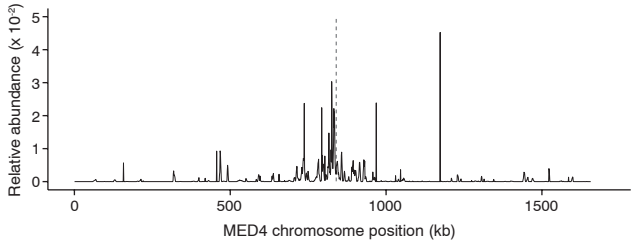

Fig. 2. *Prochlorococcus* membrane vesicles contain genomic DNA sequences. Distribution of DNA associated with vesicles from *Prochlorococcus* MED4 cultures. Sequence reads were mapped against the MED4 chromosome; mean relative abundance from two replicate samples is plotted over a 1-kb window. In total, 948,424 bp of the 1,657,990 bp chromosome (56%) was sequenced at least once from the vesicle populations. Dashed vertical line indicates the predicted chromosome terminus location.

To explore the prevalence of vesicles in the oceans, we examined surface samples from both nutrient-rich coastal seawater (Vineyard Sound, MA) and from the oligotrophic Sargasso Sea [at the Bermuda Atlantic Time-series Study (BATS) station]. Using density-gradient purification to separate vesicles from other small particles in seawater, we isolated numerous structures from both sites with a morphology and size distribution similar to those of the vesicles found in *Prochlorococcus* cultures (Fig. 3, A and B, and fig. S9A). Although some particles resembling filamentous phage copurified with the vesicles (thin white lines in Fig. 3B), we observed negligible numbers of apparent tailed or tailless phage, gene transfer agents, viruslike particles (*21*), and inorganic colloids in our enriched fractions. Examination of the putative vesicles by thin-section electron microscopy showed numerous examples of circular, membrane-bound features lacking internal structure or electron density (Fig. 3C), consistent with vesicle morphology and not that of bacteria. These samples contained protein as well as diverse lipid species, also consistent with membrane vesicles (fig. S10 and supplementary text). Although it is possible that some of these structures could have been formed by other mechanisms, the combination of culture and field data support the interpretation that these are membrane vesicles.

Vesicle abundances in the coastal surface water and Sargasso Sea samples were ~6×10^6 ml^{-1} and 3×10^5 ml^{-1}, respectively (Fig. 3D)—similar to the concentrations of bacteria at these sites. We analyzed a depth profile in the Sargasso Sea, and vesicles were observed both within and below the euphotic zone, declining with depth down to 500 m (Fig. 3D and fig. S9, B to D). The steady-state concentration of vesicles in the ocean is a function of rates of production, consumption, decay, and perhaps association with other ocean features such as marine microgels. Although we do not yet know enough about these processes to interpret the mechanisms that result in this distribution within the water column, the concordance between vesicle and bacterial abundance is consistent with the argument that the vesicles are of microbial origin.

It has been suggested that total *Prochlorococcus*-derived dissolved organic carbon (DOC) could support a substantial fraction of total bacterial production in oligotrophic regions (*22*). The release of vesicles by *Prochlorococcus*, with their diverse contents, implicates their secretion as a

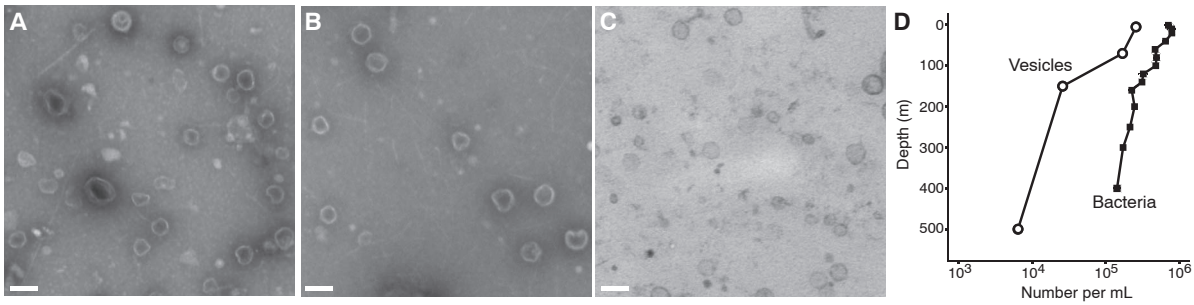

Fig. 3. Membrane vesicles are abundant in natural seawater samples. Negative-stain electron micrographs of membrane vesicles isolated from surface (**A**) coastal (Vineyard Sound, MA) and (**B**) oligotrophic waters (Sargasso Sea). (**C**) Thin-section electron micrograph of the sample from (B) shows that these structures are circular, enclosed by a membrane, and lack internal structure. Scale bars, 100 nm in (A) to (C). (**D**) Concentrations of vesicles (open circles) and total bacteria [squares; bacterial 4′,6′-diamidino-2-phenylindole (DAPI) count data from BATS] in the upper Sargasso Sea, December 2012. Vesicle concentrations are based on measurements of purified vesicle samples and represent lower-bound estimates of in situ abundance.

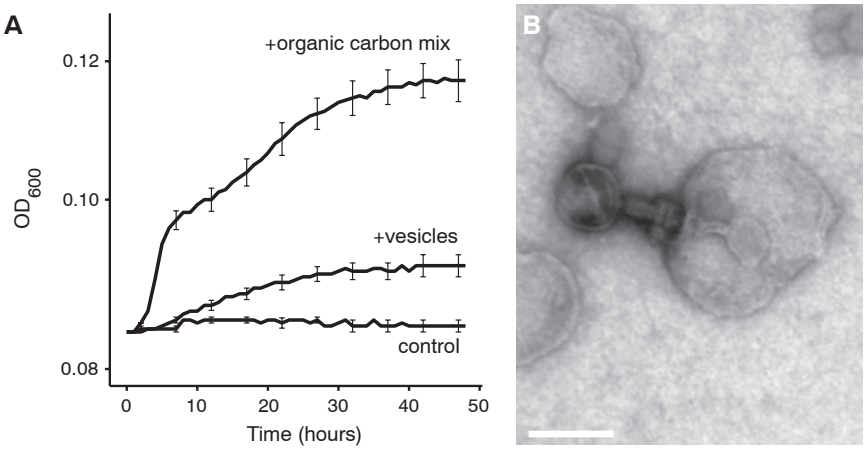

Fig. 4. Potential roles of bacterial vesicles in marine ecosystems. (**A**) Purified *Prochlorococcus* vesicles can support the growth of a heterotrophic marine *Alteromonas*. *Alteromonas* growth patterns are shown in a seawater-based minimal medium supplemented with media only (control), vesicles (+vesicles), or a defined mixture of organic carbon compounds (+organic carbon mix) as the only added carbon source. Growth curves represent the mean ± SEM of three replicates. The optical density at 600 nm (OD_{600}) increase of the "+vesicles" and "+organic carbon" trials both corresponded with a significant increase in *Alteromonas* cell concentration (measured by plate counts) as compared with the control after 48 hours (*t* test; $P < 0.05$). (**B**) Marine phage-vesicle interactions. TEM micrograph of cyanophage PHM-2 bound to a vesicle from *Prochlorococcus* MED4 (see also fig. S12). The shortened tail indicates that this phage has "infected" the vesicle and is therefore unable to infect a bacterial cell. Scale bar, 100 nm.

mechanism for production of some of this DOC. Given the data in hand, the lower bound of global *Prochlorococcus* vesicle production would represent roughly 10^4 to 10^5 tonnes of fixed C exported into the oceans per day (supplementary text). Lipids make up a notable component of total DOC in seawater (*23*), which is consistent with the presence of vesicle material among the high molecular mass fraction (>1000 daltons) of DOC. Although bacterial membrane vesicles have structural similarities to liposomes previously described as a component of DOC, they differ in two important respects: Vesicles are smaller [~50 to 150 nm diameter versus 400 to 1500 nm for liposomes (*24*)] and are not formed from the products of lysed or incompletely digested bacteria.

These observations implicate vesicle release by *Prochlorococcus* and other organisms in the flow of organic carbon in the oceans. To explore whether vesicles could serve as an organic carbon source for heterotrophs in marine food webs, we examined whether *Prochlorococcus* vesicles could support the growth of marine heterotrophic bacteria in culture. We found that marine isolates of both *Alteromonas* and *Halomonas* each grew in seawater media supplemented with purified *Prochlorococcus* vesicles as the only added carbon source (Fig. 4A and fig. S11), which illustrated that at least some of the vesicles' carbon is labile. Although vesicles may not constitute a large fraction of the total organic carbon consumed by heterotrophs in the oceans (each *Prochlorococcus* vesicle contains roughly 1/100th the carbon of an average bacterial cell in the open ocean) (supplementary text), they are clearly capable of moving fixed carbon from primary producers into the microbial food web. The presence of proteins and nucleic acids within vesicles implies that they could function as sources of N and P as well.

It is perhaps surprising that *Prochlorococcus*, or other microbes growing in the nutrient-poor oligotrophic oceans, would continually export nutrients in the form of membrane vesicles. *Prochlorococcus*, for example, has adaptations that reduce its phosphorus and nitrogen requirements, including the use of sulfolipids instead of phospholipids (*18*) and a proteome with relatively low nitrogen demand (*25*). Vesicle release seems inconsistent with the need to make efficient use of limited resources that presumably underlies these adaptations. What potential ecological roles, then, might vesicles play within marine microbial communities that could provide a fitness benefit sufficient to justify their costs? We can only speculate at this point, but what follows are some possibilities.

The growth of *Prochlorococcus* is often positively influenced by the presence of heterotrophs (*26*, *27*), so the release of DOC into the local environment could provide some fitness advantage by facilitating heterotroph growth. Vesicles might also play a role in defending cells against infection by phage—viruses that infect bacteria (*10*). Phage are abundant in the oceans, and cyanophage that infect *Prochlorococcus* can represent a substantial fraction of the total viral population (*28*). As vesicles contain bacterial outer membrane material, such as the external protein receptors phage use to identify their host, they represent a class of small particles that could influence marine phage infection dynamics. To explore this possibility, we mixed purified *Prochlorococcus* vesicles with a phage (PHM-2) known to infect the strain from which they originated and examined the population by electron microscopy. We observed numerous examples of phage bound to vesicles; in addition, many vesicle-attached phage had a shortened stalk and altered capsid staining density, which suggested that they had injected their DNA into the vesicle (Fig. 4B and fig. S12). Thus, the export of vesicles by marine bacteria could reduce the probability of a cell's becoming infected and killed by phage.

The finding that *Prochlorococcus* releases DNA within membrane vesicles suggests that they may also serve as a reservoir of genetic information and possible vector for horizontal gene transfer in marine systems. To characterize the nature of the vesicle-associated DNA pool in natural seawater, we sequenced the "metagenome" from purified and DNase-treated vesicles isolated from our two field sites. Based on the unique sequences recovered, these "wild" vesicles contained a diverse pool of DNA with significant homology to members of 33 phyla from all three domains, although bacterial sequences were dominant (table S5). The majority of unique bacterial sequences were most similar to members of the *Proteobacteria*, *Cyanobacteria*, and *Bacteroidetes*. Since *Prochlorococcus* and other bacteria (*29*) export fragments of their genome within vesicles, the taxonomic diversity of DNA we observed in the field samples implies that diverse marine microbes release vesicles. We also identified sequences with homology to tailed and other marine phage, despite the fact that these were not apparent in the fractions by TEM (table S5 and supplementary text). Although we cannot completely rule out the presence of phage in the samples, these sequences could reflect either the export of prophage sequences within vesicles or DNA arising from phage infection of vesicles in the field.

Although membrane vesicles constitute only a fraction of the $>10^9$ small (<200-nm) colloidal particles per ml observed in seawater (*30*), that they are known to move diverse compounds between organisms in other systems (*5*, *7*) suggests that they could serve specific functions in marine ecosystems. By transporting relatively high local concentrations of compounds, providing binding sites, or acting as reactive surfaces, vesicles may mediate interactions between microorganisms and their biotic and abiotic environment that would otherwise be impossible in the extremely dilute milieu of the oligotrophic oceans.

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Supplementary Materials

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Materials and Methods
Supplementary Text
Figs. S1 to S13
Tables S1 to S6
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Progenitor Outgrowth from the Niche in *Drosophila* Trachea Is Guided by FGF from Decaying Branches

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Although there has been progress identifying adult stem and progenitor cells and the signals that control their proliferation and differentiation, little is known about the substrates and signals that guide them out of their niche. By examining *Drosophila* tracheal outgrowth during metamorphosis, we show that progenitors follow a stereotyped path out of the niche, tracking along a subset of tracheal branches destined for destruction. The embryonic tracheal inducer *branchless* FGF (fibroblast growth factor) is expressed dynamically just ahead of progenitor outgrowth in decaying branches. Knockdown of *branchless* abrogates progenitor outgrowth, whereas misexpression redirects it. Thus, reactivation of an embryonic tracheal inducer in decaying branches directs outgrowth of progenitors that replace them. This explains how the structure of a newly generated tissue is coordinated with that of the old.

Many adult stem cells reside in specific anatomical locations, or niches, and are activated during tissue homeostasis and after injury (*1*–*4*). Although considerable effort has been made to identify factors that control stem cell proliferation and differentiation, how stem or progenitor cells move out of the niche and how they form new tissue are not well understood (*4*–*6*). Tissue formation in mature animals faces challenges not present in the embryo (*7*, *8*). The new cells migrate longer distances and navigate around and integrate into a complex milieu of differentiated tissues. In this work, we investigated the substratum and signals that guide *Drosophila* tracheal imaginal progenitor cells into the posterior during metamorphosis to form the pupal abdominal tracheae (PAT) that replace the posterior half of the larval tracheal system (tracheal metameres Tr6 to Tr10), which decays at this time (*9*, *10*) (Fig. 1A).

The PAT extend from the transverse connective (TC) branches in Tr4 and Tr5 (Fig. 1A). Each PAT consists of a multicellular stalk with many secondary branches, each of which has dozens of terminal cells that form numerous fine terminal branches (tracheoles) (*10*). There are two known tracheal progenitor populations at metamorphosis: dedifferentiated larval tracheal cells and spiracular branch (SB) imaginal tracheal cells set aside during embryonic tracheal

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