microLife, 2023, **4**, 1–11
DOI: 10.1093/femsml/uqac025
Advance access publication date: 20 December 2022
Research Article

Characterization of membrane vesicles in *Alteromonas macleodii* indicates potential roles in their copiotrophic lifestyle

Eduard Fadeev [1,*], Cécile Carpaneto Bastos[1], Jennifer H. Hennenfeind[1], Steven J. Biller[2], Daniel Sher[3], Matthias Wietz [4,5], Gerhard J. Herndl[1,6,7]

[1]Bio-Oceanography and Marine Biology Unit, Department of Functional and Evolutionary Ecology, University of Vienna, Djerassiplatz 1, 1030 Vienna, Austria
[2]Department of Biological Sciences, Wellesley College, Central Street 106, MA 02481, Wellesley, United States
[3]Department of Marine Biology, Leon H. Charney School of Marine Sciences, University of Haifa, Aba Khoushy Ave. 199, 3498838 Haifa, Israel
[4]Deep-Sea Ecology and Technology, Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research, Am Handelshafen 12, 27570 Bremerhaven, Germany
[5]Max Planck Institute for Marine Microbiology, Celsiusstrasse 1, 28359 Bremen, Germany
[6]Department of Marine Microbiology and Biogeochemistry, Royal Netherlands Institute for Sea Research (NIOZ), Utrecht University,1790 AB Den Burg, The Netherlands
[7]Vienna Metabolomics & Proteomics Center, Department of Functional and Evolutionary Ecology, University of Vienna, Djerassiplatz 1, 1030 Vienna, Austria
*__Corresponding author:__ Bio-Oceanography and Marine Biology Unit, Department of Functional and Evolutionary Ecology, University of Vienna, Djerassiplatz 1, 1030 Vienna, Austria. E-mail: eduard.fadeev@univie.ac.at
Editor: Oded Beja

Abstract

Bacterial membrane vesicles (MVs) are abundant in the oceans, but their potential functional roles remain unclear. In this study we characterized MV production and protein content of six strains of *Alteromonas macleodii*, a cosmopolitan marine bacterium. *Alteromonas macleodii* strains varied in their MV production rates, with some releasing up to 30 MVs per cell per generation. Microscopy imaging revealed heterogenous MV morphologies, including some MVs aggregated within larger membrane structures. Proteomic characterization revealed that *A. macleodii* MVs are rich in membrane proteins related to iron and phosphate uptake, as well as proteins with potential functions in biofilm formation. Furthermore, MVs harbored ectoenzymes, such as aminopeptidases and alkaline phosphatases, which comprised up to 20% of the total extracellular enzymatic activity. Our results suggest that *A. macleodii* MVs may support its growth through generation of extracellular 'hotspots' that facilitate access to essential substrates. This study provides an important basis to decipher the ecological relevance of MVs in heterotrophic marine bacteria.

Keywords: marine bacteria, EVs, membrane transporters, extracellular enzymes, iron uptake, moonlighting proteins

Introduction

Production of extracellular vesicles occurs in all three domains of life (Deatherage and Cookson 2012). Once considered as cell debris, it is now realized that vesicles play an important role in microbial physiology and ecology. Both gram-negative and -positive bacteria produce membrane vesicles (MVs; Nagakubo et al. 2019). The MVs are lipid structures, ranging between 20–400 nm in diameter, which can carry various biomolecules including proteins, enzymes, and nucleic acids (Nagakubo et al. 2019). The most common mechanism of MV release in Gram-negative bacteria is through blebbing of the outer membrane, releasing so-called outer-membrane vesicles (Schwechheimer et al. 2013). These MVs are typically spherical, with an external lipopolysaccharide and an inner phospholipid layer that encloses fragments of the periplasmic matrix (Deatherage et al. 2009). Several other types of MVs were recently characterized in Gram-negative bacteria, such as outer-inner membrane vesicles that contain both layers of the cellular membrane and have cytoplasmic content (Pérez-Cruz et al. 2015). Furthermore, tube-shaped membranous structures can form chains of MVs or enclosed extensions of cellular outer membranes (Fischer et al. 2019). The exact mechanisms behind the production of different MV types are still not fully understood, but different types of MVs might mediate different biological functions (Toyofuku et al. 2019).

Most knowledge of MV functional roles originates from research on bacteria-host interactions (Nagakubo et al. 2019). For example, human pathogenesis studies revealed that MVs can deliver virulence and toxin cargoes and may contribute to a range of serious illnesses, ranging from lung infection to cancer (Caruana and Walper 2020, Chronopoulos and Kalluri 2020). However, MVs also play key roles in bacteria-bacteria interactions, such as quorum sensing and cellular 'warfare' in *Pseudomonas aeruginosa* (Tashiro et al. 2013, Lin et al. 2018), as well as the distribution of antibiotic resistance genes between bacterial lineages (Yaron et al. 2000, Lee et al. 2013). The MV production can also be stimulated by environmental triggers (Orench-Rivera and Kuehn 2016) such as oxygen availability (Toyofuku et al. 2014) or iron starvation (Roier et al. 2016). This body of evidence suggests that MVs likely also have important functional roles in marine microbial communities (Schatz and Vardi 2018).

Sequencing of vesicle-associated DNA suggests that diverse groups of marine bacteria produce MVs (Biller et al. 2014,

Received: September 27, 2022. **Revised:** December 5, 2022. **Accepted:** December 16, 2022

Linney et al. 2022). However, to date, MV production has only been shown for few marine bacterial isolates (Biller et al. 2022). Two isolates of *Prochlorococcus*, the most abundant marine cyanobacterium, featured a continuous production of MVs harboring a diverse array of cellular compounds (Biller et al. 2014, 2017). Some *Prochlorococcus* MVs contain heterogeneous fragments of DNA, which might be associated with horizontal gene transfer (recently termed 'vesiduction'; Soler and Forterre 2020). Other *Prochlorococcus* MVs are characterized by a highly heterogeneous composition of nucleic acids, proteins and secondary metabolites, whose functional roles remain to be identified (Biller et al. 2021). Among heterotrophic marine bacteria, MV production has been observed in *Shewanella* (Gammaproteobacteria; Frias et al. 2010) and *Formosa* spp. (Bacteroidia; Fischer et al. 2019). In both taxa, the produced MVs had a high content of membrane and periplasmic proteins associated with organic matter and nutrient processing, with potential ecological importance.

To further elucidate the functional roles of MVs in heterotrophic marine bacteria, we investigated MV production, morphology and protein content in the marine bacterium *Alteromonas macleodii*. This gammaproteobacterium is a prominent member of pelagic bacterial communities in the ocean (García-Martínez et al. 2002) and can be associated with various phytoplankton groups, such as *Prochlorococcus* (Biller et al. 2016, Kearney et al. 2021). *A. macleodii* occurs both free-living and surface-attached (i.e. in biofilms) (Ivars-Martinez et al. 2008), and has been extensively studied through multiple genome-sequenced isolates from various oceanic regions (López-Pérez et al. 2012, 2013, Ivanova et al. 2015). *A. macleodii* strains harbor a high degree of genomic flexibility thought to arise through intra- and inter-species exchange of genomic islands and plasmids (López-Pérez et al. 2013, Fadeev et al. 2016, López-Pérez et al. 2017), facilitating ecological differentiations and niche specialization (López-Pérez and Rodriguez-Valera 2016, Koch et al. 2020). Nevertheless, all *A. macleodii* strains known to date are characterized as opportunistic copiotrophs, for instance specialized in polysaccharide degradation (Neumann et al. 2015, Koch et al. 2019), rapid uptake of organic carbon (Mitulla et al. 2016), as well as scavenging of iron through the production of siderophores (Manck et al. 2022). We hypothesize that some of these physiological and ecological traits of *A. macleodii* may involve MVs.

In this study we investigated the production and functional potential of MVs in *A. macleodii*. We determined MV production rates of six different *A. macleodii* strains from diverse global habitats, and compared the morphologically variable MVs populations. Supported by protein characterization of isolated MVs, we propose various potential roles of MVs in biological and biogeochemical traits of *A. macleodii*, which might contribute to the cosmopolitan occurrence and copiotrophic lifestyle of this marine bacterium.

Material and methods

Culturing *A. macleodii* Strains

Six *A. macleodii* strains (Table 1) were grown in artificial seawater AMP1 medium (Moore et al. 2007) amended with defined organic compounds (0.05% w/v of each lactate, pyruvate, acetate and glycerol) and a vitamin mix (Sher et al. 2011, Fadeev et al. 2016). Cultures were incubated at 20°C in the dark. To minimize sampling of cell debris, cultures were not mixed during the incubation and before sampling.

To estimate MV production rates, each strain was grown in 12 biological replicates of 50 mL each. After 12, 24, 72, and 144 h of incubation, three replicates from each strain were sampled. For protein analysis and morphological characterization of MVs, 2 L cultures of each *A. macleodii* strain were grown for 48 h. For EEA assays, *A. macleodii* ATCC27126 was grown in triplicates of 50 mL and sampled after 24 h. From each culture, 1 mL of cell suspension was preserved with 4% glutaraldehyde (final conc.) and stored at −80°C until flow cytometric analysis. The remaining culture was used for isolation of MVs.

Bacterial cell enumeration

Bacterial cells were stained with 1× SYBR Green I (Thermo Fisher Scientific, MA, USA) for 15 min and then quantified using a flow cytometer (Accuri™ C6, BD, NJ, USA). Counts were processed using R package 'flowWorkspace' v4.8.0 (Finak and Jiang 2022) with hierarchical gating of singletons using R package 'flowStats' v4.8.2 (Hahne et al. 2022) and removal of cell debris using R package 'openCyto' v4.2 (Finak et al. 2014).

Isolation of MVs

The MVs were isolated following Biller et al. (2022) for filtration size cut-offs for marine bacteria and according to the guidelines for definition of extracellular vesicles and their functions of the International Society for Extracellular Vesicles (Lötvall et al. 2014).

For production rates and EEA measurements, *A. macleodii* cultures were filtered manually using 50 mL syringes through 25 mm diameter, 0.22 μm polycarbonate filters (Merck Millipore, MA, USA) to remove bacterial cells. To remove small molecular weight solutes, the filtrate was concentrated ca. 10 times using Amicon centrifugal filter units (Merck Millipore) with a 100 kDa cut-off to a final volume of 5 mL. The 100 kDa–0.22 μm size fraction containing the MVs was then used for nanoparticle tracking analysis (NTA).

For protein analysis and morphological characterization, the 2 L cultures were filtered using a peristaltic pump through a 142 mm diameter, 0.22 μm polycarbonate filter (Merck Millipore, MA, USA) at a low pumping rate of 30 mL min^{-1} to minimize cell lysis. The filtrate was concentrated ca. 10 times using a tangential flow filtration with a 100 kDa cut-off (Vivaflow, Sartorius, Germany) to 200 mL, and further concentrated ca. 10 times using Amicon centrifugal filter units (Merck Millipore) with a 100 kDa cut-off to a final volume of 20 mL. A 5 mL subsample of the concentrated MVs was collected for NTA. The remaining 15 mL of the concentrated MVs were pelleted using ultracentrifugation at 100 000 × g at 4°C for 4 h, and resuspended in 10 mL of clean PRO99 medium. After an additional round of ultracentrifugation, pelleted MVs were resuspended in 250 μL of clean PRO99 medium. All isolated MV samples (excluding those for EEA assays) were stored at −20°C until further analysis.

Enumeration of MVs using nanoparticle tracking analysis

The abundance of MVs was measured by NTA using a NanoSight NS300 instrument (Malvern Panalytycal, UK) equipped with a 488 nm laser. Each MV sample was injected using a syringe pump with a flow speed of 100 μL min^{-1}, and five videos of 60 sec were recorded. Videos were analyzed using NanoSight NTA software v3.2. For MV production rate measurements, samples were analyzed with a consistent per-strain-threshold setting of 4–6. All particles in the size range of 20–450 nm were included in the final MV abundance estimates. To achieve high accuracy in MV size distribution estimates, concentrated MV samples contained 20–100 particles per frame following the manufacturer's standard protocols.

Table 1. *Alteromonas macleodii* strains used in this study.

Strain	Origin	NCBI Reference Sequence	Reference
ATCC 27126^T	North Pacific (surface)	NC_018632.1	Baumann et al. 1984
AD45	Adriatic Sea (surface)	NC_018679.1	Pujalte et al. 2003
BS11	Black Sea (surface)	NC_018692.1	López-Pérez et al. 2012
BGP6	Pacific Ocean (surface)	NZ_CABDWP000000000.1	Koch et al. 2020
HOT1A3	North Pacific (surface)	NZ_CP012202.1	Fadeev et al. 2016
MIT1002	North Atlantic (surface)	NZ_CABDXM010000001	Biller et al. 2015

The flow cell was thoroughly flushed with Milli-Q water (Merck Millipore) between samples, and visually examined to exclude carry over. The performance of the instrument was routinely checked by measuring the concentration and size distribution of standardized 100 nm silica beads.

MV production rate determination

The production rates of MVs were calculated following Biller et al. (2014) based on the assumption that each bacterial cell produces a constant number of MVs per generation (*r*). By estimating the number of generations based on changes in cell abundances and the change in MV concentrations, the MV production rate *r* can be calculated as follows:

$$r = \frac{V_n}{(2^{n-1}) \times N_0}$$

Where:

- *V*—number of produced MVs
- N_0–initial cell abundance
- *n*—number of generations

Production rates for early (0–24 h) and late (24–72 h) stages were calculated based on initial cell and MV abundances at 0 or 24 h, respectively.

Cryogenic electron microscopy (cryo-EM)

To verify the presence and size of MVs, 50 μL of the purified MVs was used for cryo-EM imaging. Plunge freezing and imaging was conducted at the Electron Microscopy Facility of the Vienna Bio-Center on a Glacios transmission electron microscope (Thermo Fisher Scientific).

Protein content characterization

For each *A. macleodii* strain, the protein content of cells retained on 0.22 μm filters and in 200 µL of purified MVs (total of 10^{10}–10^{11} MVs) were determined as described elsewhere (Bayer et al. 2019, Zhao et al. 2020). Extracted proteins were subjected to in-solution trypsin digestion, and the resulting peptides sequenced on a Q-Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific) at the Vienna Research Platform for Metabolomics & Proteomics. Using the Proteome Discoverer v3.0 (Thermo Fisher Scientific), tandem mass spectrometry spectra were searched using SEQUEST-HT against the corresponding genome of each strain. Search parameters were enzyme: trypsin, fragment mass tolerance: 0.6 Da, max. missed cleavages: 2, fixed modifications: carbamidomethyl (Cys), optional modifications: oxidation (Met). Percolator parameters were: max. delta Cn: 0.6, max. rank: 0, validation based on q-value, false discovery rate (calculated by automatic decoy searches) 0.05. Protein quantification was conducted using the chromatographic peak area-based label-free quantitative method.

The genomes of *A. macleodii* were investigated using Anvi'o v7.0 (Eren et al. 2015) and annotated against the Clusters of Orthologous Groups of proteins (COG) database (Tatusov et al. 2000, Galperin et al. 2015). The average nucleotide identity was calculated using PyANI v0.2.12 (Pritchard et al. 2015). Further analyses were done in R v4.2.1 (R Core Team 2022) using RStudio v2022.02.3 (RStudio Team 2019) and R packages 'phyloseq' v1.40 (McMurdie and Holmes 2013) and 'vegan' v2.6–2 (Oksanen et al. 2022). Subcellular localization of the identified proteins was predicted using the CELLO2GO online tool (Yu et al. 2014), and supported by model predictions in PSORTdb v4.0 (Lau et al. 2021).

Extracellular enzymatic activity (EEA) assays

The EEA assays base on the hydrolysis of fluorogenic substrate analogues to estimate the activity of leucine aminopeptidase (LAPase), alkaline phosphatase (APA), as well as alpha- and beta-glucosidase (AGase and BGase; Hoppe 1983). Substrate working solutions were prepared in 2-methaoxyethanol and kept at −20°C. Standard calibration curves were established with 7 to 15 different concentrations of the fluorophores methylumbelliferyl (MUF) and 4-methylcoumarinyl-7-amide (MCA) prepared in 0.22 μm filtered artificial seawater ranging from 1 nmol L^{-1} to 1 μmol L^{-1}.

Three technical replicates (300 µL each) from the different size fractions (bulk, 100 kDa-0.22 µm, < 100 kDa) were incubated with the fluorogenic substrates in Bio-One 96-well non-binding microplates (Greiner, Germany). Concentrations of each substrate were determined based on previously conducted enzyme kinetics (data not shown): 600 μmol L^{-1} of MCA-L-leucine-7-amido-4-methylcoumarin (LAPase), 100 μmol L^{-1} MUF-phosphate (APA), and 400 μmol L^{-1} for both MUF-α-D-glucopyranoside MUF-β-D-glucopyranoside (AGase and BGase, respectively). Incubations were carried out at room temperature in the dark. Fluorescence in each sample was measured after 1.5 h using a Fluorolog-3 fluorometer with a MicroMax 384 microwell plate reader (Horiba, Japan) at excitation/emission wavelengths of 365/445 nm and 380/440 nm for MUF and MCA, respectively. Culture media with substrates were used as blanks to determine background fluorescence, which was then subtracted from the sample fluorescence. Using the calibration curves for each fluorochromes, the linear increase in fluorescence over time was then transformed to enzymatic hydrolysis activity (nmol L^{-1} h^{-1}).

Results and discussion

Alteromonas macleodii produces morphologically diverse MVs

To expand our understanding of MV production in marine bacteria, we characterized MVs in six strains of the cosmopolitan marine bacterium *A. macleodii*. Cryogenic electron microscopy (cryo-EM) analysis of these cultures revealed heterogeneous MVs with high morphological diversity and sizes of 40–300 nm (Fig. 1).

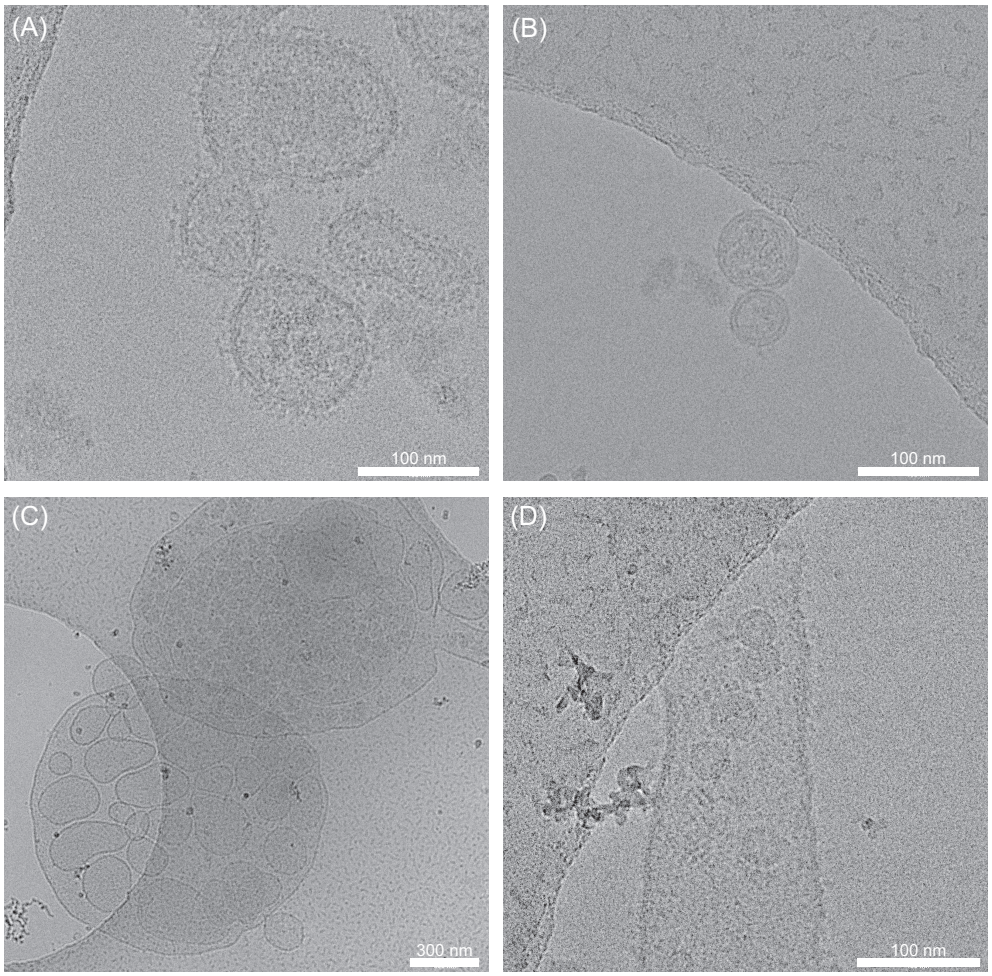

Figure 1. Distinct morphological shapes in *A. macleodii* MVs, visualized using cryo-EM. **(A)** strain BS11–protrusions of potentially lipopolysaccharide or proteins surrounding the MVs. **(B)** strain HOT1A3–outer inner-MVs. **(C)** strain BGP6–micron-large membrane-bound structures. **(D)** strain BS11–tube-shaped membranous structures. Note the larger scale bars in panels C and D.

Further investigation using nanoparticle tracking analysis (NTA) revealed a bi-modal size distribution, with a pronounced main population of MVs (diameter of 80–120 nm) and a secondary population (diameter of 200–240 nm; Fig. 2). This size range overall agrees with observations in other marine bacteria (Biller et al. 2014, 2017, 2022). Although the size of MVs may affect their functionality (Turner et al. 2018), it is largely unknown whether there is a direct link between size and function (Nagakubo et al. 2019). However, morphologically different MVs likely mediate different biological functions (Toyofuku et al. 2019).

Due to the limited number of MVs that could be visualized by cryo-EM and size filtrations applied to the NTA, we are unable to fully link morphologies and quantitative size distributions. Nonetheless, in addition to outer- and outer inner-MVs with diameter of <300 nm (Fig. 1A and B), some *A. macleodii* strains produced larger membrane-bound spherical and tube-shaped structures that enclosed large numbers of smaller MVs (Fig. 1C and D). These potentially represent self-assembled structures, for example from lysed cells (Walker et al. 1997, Turnbull et al. 2016). However, microscopic observations in other bacteria suggest that such structures are actually produced by the bacteria, and have specific functional roles (Kaplan et al. 2021). The origin of these structures in *A. macleodii* is unknown, however, morphologically similar structures in other marine bacteria were recently linked to sensing and utilization of polysaccharides (Fischer et al. 2019, Dürwald et al. 2021). This suggests that future investigations into larger size classes of MVs may be warranted in *Alteromonas*.

Previous estimates of MV production in pelagic marine bacteria range from few to several dozen MVs per bacterial cell (Biller et al. 2014, Kwon et al. 2019, 2022). Based on temporal changes in cell and MV abundances, the six strains of *A. macleodii* showed overall net MV production rates within the same order of magnitude to previous observations (Fig. 3A; Biller et al. 2014, 2022). Despite the slightly different growth dynamics of the strains, we observed differences in net MV production rates between the early stage (0–24 hours) that was mostly characterized by exponential bacterial growth, and the late stage (24–72 hours) that represented the transition to the stationary phase of the bacterial culture (Fig. 3B). During the early stage the MV production rates were 1–4 MVs cell^{-1} generation^{-1} in all strains except BS11, that exhibited a production rate of 64±5 MVs cell^{-1} generation^{-1}. The high production rate by BS11 is potentially related to its slower growth, and thus lower cell abundances compared to other strains, in the first 12 hours of incubation. During late stage four strains exhibited several-fold higher MV production rates (Fig. 3B), whereas BS11 showed a strong decrease and HOT1A3 no changes, respectively. We cannot exclude that higher abundances of MVs in 'older' bacterial cultures correspond to MVs that were formed from

Table 2. Overview of proteins detected in cellular and MV fractions of each *A. macleodii* strain.

Strain	Total protein encoding genes	Proteins detected in cells	Proteins detected in MVs	Shared proteins*	Proteins detected only in cells*	Proteins detected only in MVs*
AD45	3817	1239	704	16	45	19
ATCC27126	3770	1512	1218	37	38	23
BGP6	3869	1356	1076	35	32	18
BS11	3677	1554	1317	45	32	20
HOT1A3	3839	1169	556	10	48	17
MIT1002	3845	1455	1279	31	41	32

*Comparison of the top 5% of proportionally most abundant proteins

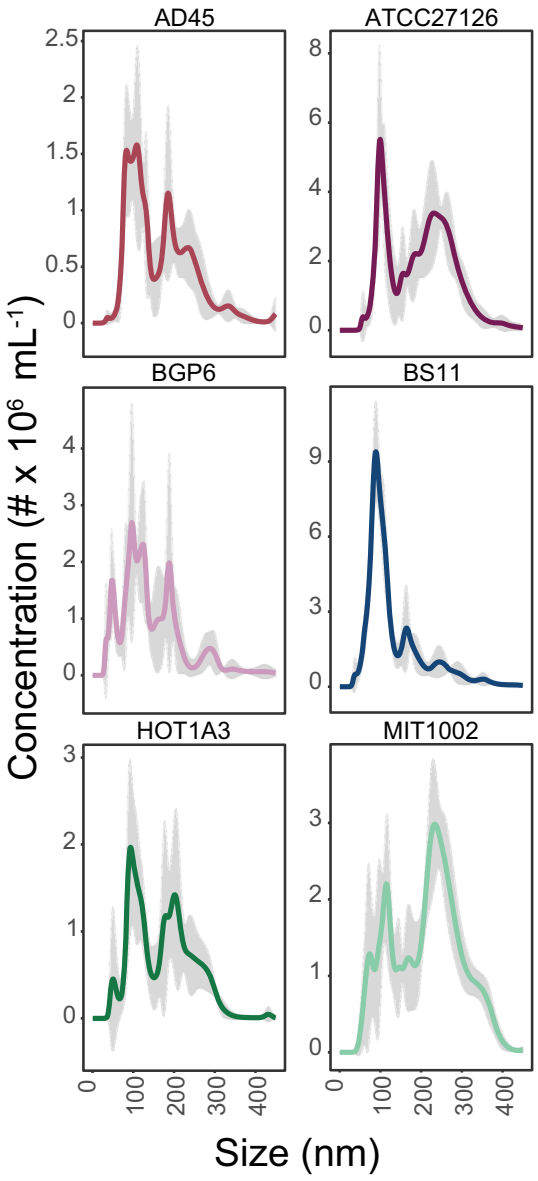

Figure 2. Size characterization of MVs in *A. macleodii* strains. Each plot represents the NTA size distribution of MVs; the shaded area representing the standard deviation between the technical replicates of the NTA runs.

increasing cell lysis (Toyofuku et al. 2019). Nonetheless, higher MV production during stationary phase could result from changing growth conditions in the culture, such as nutrient limitation or oxidative stress (Orench-Rivera and Kuehn 2016).

Alteromonas macleodii MVs likely play a role in its copiotrophic lifestyle

To further elucidate the functional potential of MVs in *A. macleodii*, we characterized the protein content of the cellular (>0.22 μm) and the MV-associated fractions (100 kDa–0.22 μm) in each strain. Overall, no significant differences in the total detected proteome were found between the *A. macleodii* strains (PERMANOVA, $p > 0.05$), with >85% of all detected proteins shared among the six strains (Table 2). This consistency probably corresponds to the high genomic similarity of the strains (average nucleotide identity >70% and 2900 shared genes; Fig. S1) and the similar non-limiting growth conditions applied here. Furthermore, up to two thirds of MV-associated proteins in each strain were shared with all other *A. macleodii* strains, suggesting high intraspecific conservation of the produced MVs. Therefore, we focus on MV-associated proteins that were observed across all strains.

The MVs are generally fragments of the producing cell and thus, their associated proteins represent a subset of the cellular proteome (Schwechheimer and Kuehn 2015). From all proteins encoded by each strain, 36±5% were detected in the cellular and 27±9% in the MV-associated fraction respectively (Table 2). From the 5% most abundant proteins in each strain, more than half were fraction-specific (32–48 proteins in the cellular and 17–32 proteins in the MV-associated fraction, respectively; Table S1). Additional proteins, found in both cellular and MV-associated fractions, had significantly different proportional abundance between the fractions (PERMANOVA, $P < 0.05$). In all six strains, predicted localization of these most abundant proteins indicated that the MV-associated fraction comprised a significantly higher portion of proteins with membrane- and periplasm-related origins (Fig. 4). The localization profiles of MV-associated proteins overall agreed with previous studies (Kulkarni et al. 2014, Veith et al. 2015, Biller et al. 2021). Proteins abundant in the MV-associated fraction matched those found enriched in other bacterial MVs (Deatherage et al. 2009, Schwechheimer and Kuehn 2015). However, we also detected that a notable portion of the MV-associated fraction had a cytoplasmic origin, consistent with outer inner-MVs encapsulating the cytoplasmic matrix fraction in *A. macleodii* cultures (Fig. 2). This MV type could contribute to the presence of cytoplasmic proteins in the MV-associated fraction (Pérez-Cruz et al. 2015). Nonetheless, based on the relatively high portion of proteins of cytoplasmic origins, we cannot exclude some level of cellular contamination in the MV-associated fraction.

In all six *A. macleodii* strains, the MV-associated fraction contained high proportions of various cell membrane-related proteins (Fig. 5), with a more than two-fold higher proportional abundance of outer-membrane proteins (*OmpA* and *OmpW*), inner-membrane type 1 secretion system transporters (*TolC*), and efflux channels (both alpha and beta subunits of F0F1-type ATP synthase as well as *MotA/TolQ/ExbB* proton channels; Table S1).

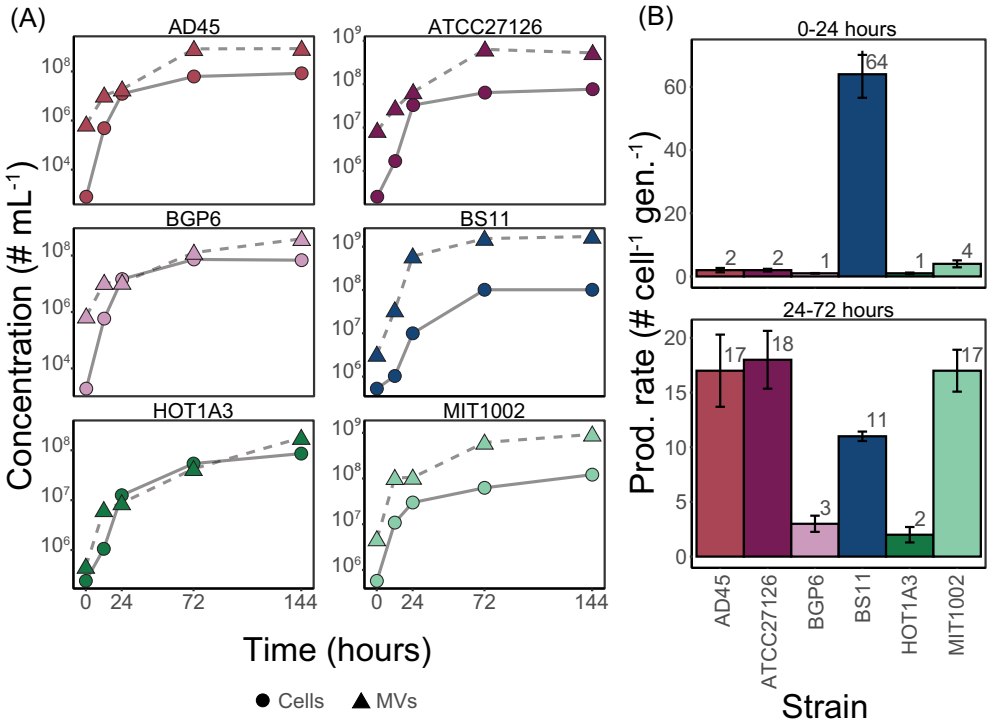

Figure 3. Production of MVs by *A. macleodii*. **(A)** Total cell (circles) and MV (triangles) abundances in six different strains of *A. macleodii*. The standard errors between the biological replicates are smaller than the symbols. **(B)** Calculated production rates of MVs during the early (0–24 h) and late (24–72 h) growth stages of each strain. The color coding of the individual strains is the same for panels A and B.

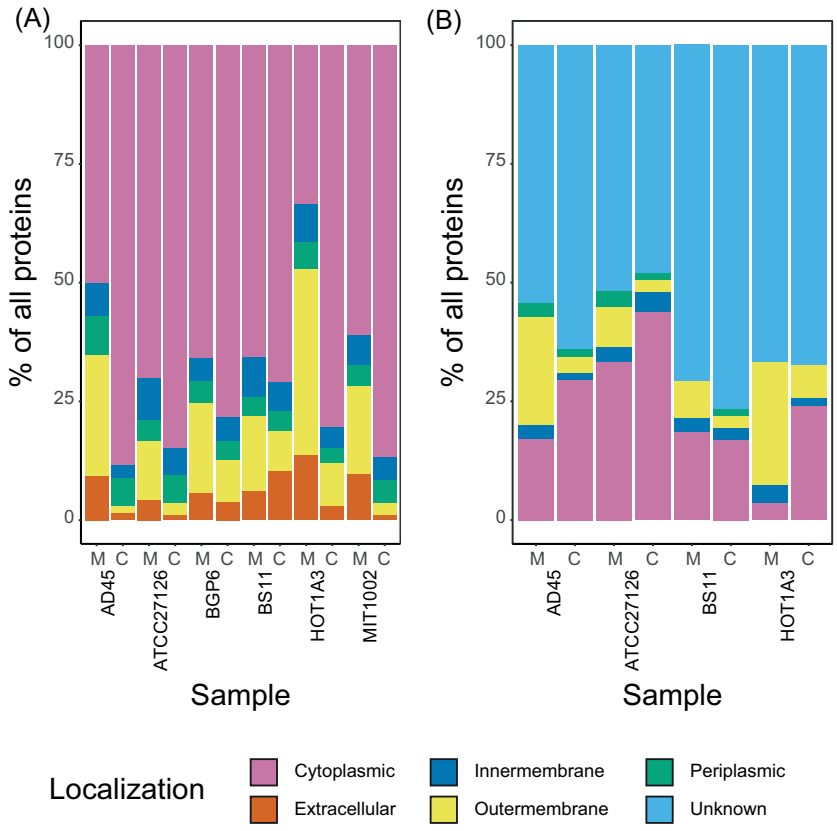

Figure 4. Proportions of subcellular localizations among the most abundant proteins in the cellular ('C') and MV ('M') fractions in each *A. macleodii* strain. The color code represents the different subcellular origins, predicted by CELLO2GO **(A)** and psortdb **(B)**.

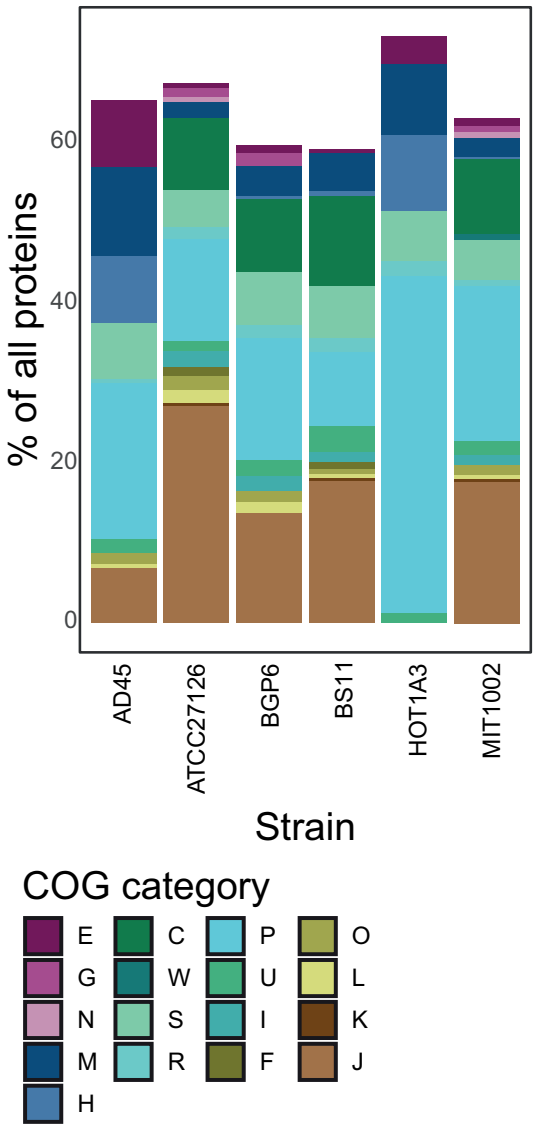

Figure 5. Proportion of selected COG categories among the most abundant MV-associated proteins in each *A. macleodii* strain. Included COG categories are: E—Amino acid transport and metabolism; G—Carbohydrate transport and metabolism; N—Cell motility; M—Cell wall/membrane/envelope biogenesis; H—Coenzyme transport and metabolism; C—Energy production and conversion; W—Extracellular structures; S—Function unknown; R—General function prediction only; P—Inorganic ion transport and metabolism; U—Intracellular trafficking, secretion, and vesicular transport; I—Lipid transport and metabolism; F—Nucleotide transport and metabolism; O—Posttranslational modification, protein turnover, chaperones; L—Replication, recombination and repair; K—Transcription; J—Translation, ribosomal structure and biogenesis.

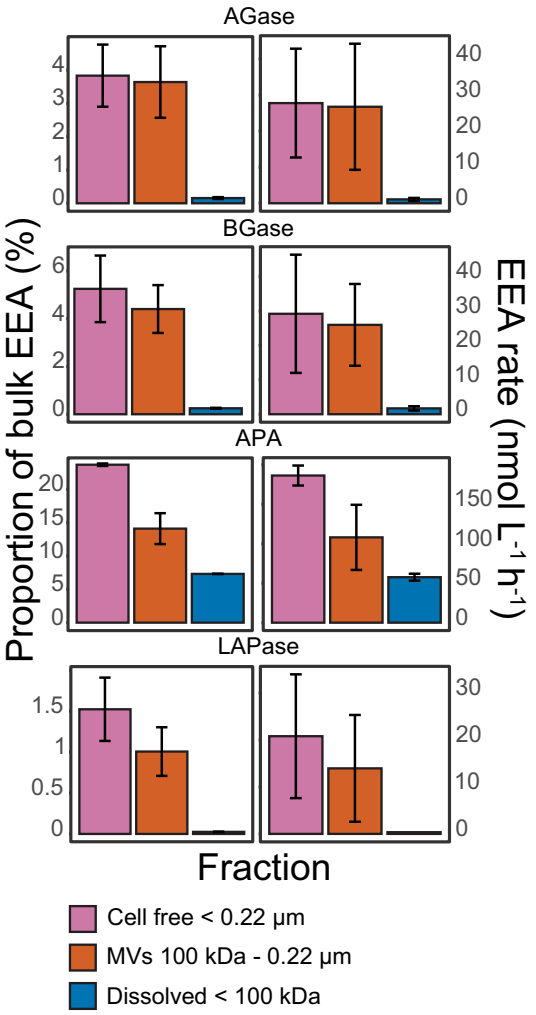

Figure 6. Extracellular enzymatic activity (EEA) of different size fractions in culture of *A. macleodii* type strain ATCC27126. The bulk represents EEA of non-filtered samples (i.e. bacterial cells + ectoenzymes). The color coding of the individual fractions is the same for all plots. AGase—alpha-glucosidase, BGase—beta-glucosidase, APA—alkaline phosphatase, LAPase—L-aminopeptidase.

The abundance of outer-membrane *TonB*-dependent siderophore receptors constituted 7–33% of all MV proteins, making them the most prominent MV-associated protein group (Table S1). The TonB-dependent receptors mediate the transfer of various solutes, such as organic molecules and siderophores, via the outer membrane (Braun 1995). In *A. macleodii*, distinct sets of TonB-dependent receptors are expressed during carbon- and iron-limiting conditions (Manck et al. 2020) and during polysaccharide utilization (Neumann et al. 2015). Several non-marine Gram-negative bacteria also produce MVs enriched in TonB-dependent receptors (Veith et al. 2009, Zakharzhevskaya et al. 2017, Dhurve et al. 2022). Although MV-associated receptors cannot transport solutes due to the absence of an energy source inside the MVs, they could still bind the solutes (Veith et al. 2015). Then, through fusion, bacterial cells could incorporate MV-bound solutes such as siderophores (Prados-Rosales et al. 2014). In the ocean, such mechanisms of solute enrichment on MVs could potentially serve as community public goods, for instance supporting bacterial lineages without iron-scavenging capabilities (Hogle et al. 2022, Manck et al. 2022).

Interestingly, the MV-associated fraction of all six *A. macleodii* strains comprised cytoplasmic proteins linked to cellular metabolic pathways, such as glutamine synthetase, superoxide dismutase, ketol-acid reductoisomerase, and riboflavin synthase (Fig. 5). The MV-associated fraction also contained various proteins associated with post-translational modifications including chaperones (e.g. chaperonin *GroEL*), thioredoxins (*cnoX*), peptidylprolyl isomerase (*surA*), and elongation factors (*tufA* and *FusA*; Table S1). Some of these proteins might be associated with the cellular matrix encapsulated in outer inner-MVs (Kulkarni et al. 2014, Park et al. 2015, Pérez-Cruz et al. 2015, Biller et al. 2021). However, others were moonlighting proteins that have independent and different intra- vs. extracellular functionalities (Huberts

and van der Klei 2010, Singh and Bhalla 2020). Such moonlighting proteins are present on membranes of Gram-negative bacteria and their MVs (Park et al. 2015, Turnbull et al. 2016, Ebner and Götz 2019), with potential specific functional roles. For example, in *P. aeruginosa*, MVs enriched in various moonlighting proteins mediate antibiotic resistance (Park et al. 2015). Among the proteins observed in this study, the chaperonin *GroEL*, responsible for proper protein folding inside the cytoplasm, can turn into a toxin outside of the cell (Yoshida et al. 2001). Additionally, glutamine synthetase, which was highly abundant in the MVs of all *A. macleodii* strains, can function as adhesion factor when exposed on the membrane (Candela et al. 2007). Similar adhesive functions have also been described for other proteins, such as the superoxide dismutase (Reddy and Suleman 2004) and the ketol-acid reductoisomerase (Castaldo et al. 2009). Based on observations on *Helicobacter pylori*, where MVs can enhance biofilm formation (Yonezawa et al. 2009), we hypothesize that the potentially adhesive nature of MVs facilitates surface colonization of *A. macleodii* (i.e. in biofilms; Ivars-Martinez et al. 2008).

Due to the considerable biomass required for the applied methodologies, we isolated *A. macleodii* MVs by targeting nanoparticles between 100 kDa–0.22 μm, an isolation strategy with intermediate recovery and intermediate specificity as compared with other isolation approaches (Théry et al. 2018). While the selected size fraction may have contained some other non-MV colloidal material (e.g. protein aggregates; López-Pérez et al. 2012), the cryo-EM and NTA data, the high abundance of membrane-associated proteins and the correspondence of our proteome data with that from other bacteria, together indicate that the targeted size fraction was highly enriched in MVs.

The MVs harbor active hydrolytic ectoenzymes

The physiological benefit from releasing MV-associated ectoenzymes was previously proposed as 'public goods' in human intestinal microbiome (Rakoff-Nahoum et al. 2014). It has been also experimentally demonstrated in *Escherichia coli* that MV-associated ectoenzymes strongly enhance cellulose hydrolysis (Park et al. 2014). The production of hydrolytic ectoenzymes is a prominent functional trait in marine heterotrophic bacteria, with an important role in organic matter turnover (Arnosti 2011, Arnosti et al. 2021). Among marine bacteria, active MV-associated hydrolytic enzymes were observed in *A. macleodii* KS62 isolated from seaweed (Naval and Chandra 2019), the coral pathogen *Vibrio shilonii* (Li et al. 2016), and *Prochlorococcus* (Biller et al. 2021). We found that also MVs of *A. macleodii* harbor a considerable amount and diversity of enzymes that are linked to bacterial extracellular enzymatic activity (EEA). Among these were leucyl aminopeptidases (EC 3.4.11.1), inorganic pyrophosphatases (EC 3.6.1.1), and putative alkaline phosphatases (EC 3.1.3.1). Other hydrolytic enzymes such as beta-glucosidase (EC 3.2.1.21) were also observed among MV-associated proteins, although with low proportional abundance. The molecular weights of these ectoenzymes in *A. macleodii*, as characterized by mass spectrometry, were all below 100 kDa. Thus, unless found in high molecular weight aggregates (Barrett et al. 2012), our purification processes indicate that *A. macleodii* also releases hydrolytic enzymes associated with MVs.

Ectoenzymes in marine bacteria can be both outer-membrane bound and periplasmic, with higher activity measured in the latter (Martinez and Azam 1993). This is especially relevant for MVs that, as a result of their formation process, could function as enzymatic hotspots. To test whether the observed MV-associated ectoenzymes were active, we conducted biochemical assays on isolated MVs of *A. macleodii* ATCC27126^T. We targeted three different enzymatic groups commonly used to estimate marine EEA, including peptidases, glucosidases and phosphatases. The total cell-free EEA of ATCC27126 ^{T} represented up to 20% of the bulk enzymatic activity in the culture (Fig. 6). Across all enzymatic groups tested, 60–90% of the cell-free EEA was associated with the MV fraction, with only a small portion of EEA measured in the <100 kDa fraction (Fig. 6). Previous size-fractionated EEA measurements in natural seawater demonstrated up to 30% to the cell-free EEA being contributed by the MV-containing size fraction (0.02–0.2 μm; Baltar et al. 2019). Based on our results, we speculate that a notable fraction of *A. macleodii* EEA is associated with MVs. These results encourage further targeted in-depth investigations of MV-associated enzymes in marine bacterial isolates, as well as in natural seawater, to estimate their contribution to enzymatic turnover of organic matter.

Conclusions

We showed that *A. macleodii*, a widespread heterotrophic marine bacterium, produces diverse MVs. The production and release of MVs represent an investment of cellular energy and resources, suggesting they likely have physiological and/or ecological benefits. While some of the observed characteristics correspond to findings in confined host-bacteria environments, the roles of MVs in marine bacterial assemblages remain largely unknown. In the oligotrophic marine environment, extended scavenging capabilities are highly beneficial. The herein demonstrated enrichment of receptors and adhesion proteins suggest that bacterial MVs can function as passive collectors of various solutes (e.g. iron and carbohydrates). The ability to produce and utilize such 'hotspots' of biologically relevant resources might provide an ecological advantage. Furthermore, in an environment where heterotrophic cleavage of organic matter mostly relies on extracellular enzymes, the ability to secrete vehicles for hydrolytic enzymes might increase the efficiency of EEA. Taken together, our study lays the groundwork for thorough, mechanistic investigations on bacterial MV diversity and function in the ocean, with potentially important implications for the understanding of the oceanic microbiome and biogeochemistry.

Author contributions

EF—designed and conducted MV characterization experiments, analyzed the data, and wrote the manuscript under the guidance of GJH. CCB—conducted enzymatic activity experiments and analyzed the results. JHH—conducted NTA measurements. SJB, DS and MW—provided *A. macleodii* cultures, and contributed to interpretation and writing.

Acknowledgments

The authors thank Mirjam Aubert for her assistance with the experimental work, as well as Dikla Aharonovich and Marlene Brandstetter for their assistance in producing and interpreting electron microscopy images. The authors also thank Susanne Erdmann for critically reviewing the manuscript. This project was funded by the Austrian Science Fund (FWF) grant numbers M2797-B to EF, and I4978-B to GJH.

Supplementary data

Supplementary data are available at *FEMSML* online.

Conflict of interest statement. The authors declare no competing interests.

Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD037008. Accession numbers of the analyzed *A. macleodii* strains are shown in Table 1.

All analytical workflows are publicly available on GitHub (https://github.com/edfadeev/Alteromonas_MVs/).

References

Arnosti C, Wietz M, Brinkhoff T *et al.* The biogeochemistry of marine polysaccharides: sources, inventories, and bacterial drivers of the carbohydrate cycle. *Ann Rev Marine Sci* 2021;**13**:81–108.

Arnosti C. Microbial extracellular enzymes and the marine carbon cycle. *Ann Rev Marine Sci* 2011;**3**:401–25.

Baltar F, De Corte D, Thomson B *et al.* Teasing apart the different size pools of extracellular enzymatic activity in the ocean. *Sci Total Environ* 2019;**660**:690–6.

Barrett AJ, Rawlings ND, Fred Woessner J. *Handbook of Proteolytic Enzymes*. Academic Press, 2012.

Baumann P, Baumann L, Bowditch RD *et al.* Taxonomy of Alteromonas: A. nigrifaciens sp. nov., nom. rev.; A. macleodii; and A. haloplanktis. *Int J Syst Evol Microbiol* 1984;**34**:145–9.

Bayer B, Pelikan C, Bittner MJ *et al.* Proteomic response of three marine ammonia-Oxidizing archaea to hydrogen peroxide and their metabolic interactions with a heterotrophic Alphaproteobacterium. *Msystems* 2019;**4**. DOI: 10.1128/mSystems.00181-19.

Biller SJ, Coe A, Chisholm SW. Torn apart and reunited: impact of a heterotroph on the transcriptome of *Prochlorococcus*. *ISME J* 2016;**10**:2831–43.

Biller SJ, Coe A, Martin-Cuadrado A-B *et al.* Draft Genome Sequence of Alteromonas macleodii Strain MIT1002, Isolated from an Enrichment Culture of the Marine Cyanobacterium Prochlorococcus. *Genome Announc* 2015. DOI: 10.1128/genomeA.00967-15.

Biller SJ, Lundeen RA, Hmelo LR *et al. Prochlorococcus* extracellular vesicles: molecular composition and adsorption to diverse microbes. *Environ Microbiol* 2022;**24**:420–35. DOI: 10.1111/1462-2920.15834.

Biller SJ, McDaniel LD, Breitbart M *et al.* Membrane vesicles in sea water: heterogeneous DNA content and implications for viral abundance estimates. *ISME J* 2017;**11**:394–404.

Biller SJ, Muñoz-Marín MDC, Lima S *et al.* Isolation and characterization of cyanobacterial extracellular vesicles. *J Visual Experim* 2022. DOI: 10.3791/63481.

Biller SJ, Schubotz F, Roggensack SE *et al.* Bacterial vesicles in marine ecosystems. *Science* 2014;**343**:183–6.

Braun V. Energy-coupled transport and signal transduction through the gram-negative outer membrane via TonB-ExbB-ExbD-dependent receptor proteins. *FEMS Microbiol Rev* 1995;**16**:295–307.

Candela M, Bergmann S, Vici M *et al.* Binding of human plasminogen to *Bifidobacterium*. *J Bacteriol* 2007;**189**:5929–36.

Caruana JC, Walper SA. Bacterial membrane vesicles as mediators of microbe - microbe and microbe - host community interactions. *Front Microbiol* 2020;**11**:432.

Castaldo C, Vastano V, Siciliano RA *et al.* Surface displaced alfa-enolase of *Lactobacillus plantarum* is a fibronectin binding protein. *Microb Cell Fact* 2009;**8**:14.

Chronopoulos A, Kalluri R. Emerging role of bacterial extracellular vesicles in cancer. *Oncogene* 2020;**39**:6951–60.

Deatherage BL, Cookson BT. Membrane vesicle release in bacteria, eukaryotes, and archaea: a conserved yet underappreciated aspect of microbial life. *Infect Immun* 2012;**80**:1948–57.

Deatherage BL, Lara JC, Bergsbaken T *et al.* Biogenesis of bacterial membrane vesicles. *Mol Microbiol* 2009;**72**:1395–407.

Dhurve G, Madikonda AK, Jagannadham MV *et al.* Outer membrane vesicles of *Acinetobacter baumannii* DS002 are selectively enriched with TonB-Dependent transporters and play a key role in iron acquisition. *Microbiology Spectrum* 2022;**10**:e0029322.

Dürwald A, Zühlke M-K, Schlüter R *et al.* Reaching out in anticipation: bacterial membrane extensions represent a permanent investment in polysaccharide sensing and utilization. *Environ Microbiol* 2021;**23**:3149–63. DOI: 10.1111/1462-2920.15537.

Ebner P, Götz F. Bacterial excretion of cytoplasmic proteins (ECP): occurrence, mechanism, and function. *Trends Microbiol* 2019;**27**:176–87.

Eren AM, Esen ÖC, Quince C *et al.* Anvi'o: an advanced analysis and visualization platform for 'omics data. *PeerJ* 2015;**3**:e1319.

Fadeev E, De Pascale F, Vezzi A *et al.* Why close a bacterial genome? The plasmid of *Alteromonas macleodii* HOT1A3 is a vector for inter-Specific transfer of a flexible genomic island. *Front Microbiol* 2016;**7**:248.

Finak G, Frelinger J, Jiang W *et al.* OpenCyto: an open source infrastructure for scalable, robust, reproducible, and automated, end-to-end flow cytometry data analysis. *PLoS Comput Biol* 2014;**10**:e1003806.

Finak G, Jiang M. *flowWorkspace: Infrastructure for Representing and Interacting with Gated and Ungated Cytometry Data Sets*, 2022. https://www.bioconductor.org/packages/release/bioc/html/flowWorkspace.html.

Fischer T, Schorb M, Reintjes G *et al.* Biopearling of interconnected outer membrane vesicle chains by a marine Flavobacterium. *Appl Environ Microbiol* 2019;**85**. DOI: 10.1128/AEM.00829-19.

Frias A, Manresa A, de Oliveira E *et al.* Membrane vesicles: a common feature in the extracellular matter of cold-adapted Antarctic bacteria. *Microb Ecol* 2010;**59**:476–86.

Galperin MY, Makarova KS, Wolf YI *et al.* Expanded microbial genome coverage and improved protein family annotation in the COG database. *Nucleic Acids Res* 2015;**43**:D261–9.

García-Martínez J, Acinas SG, Massana R *et al.* Prevalence and microdiversity of *Alteromonas macleodii*-like microorganisms in different oceanic regions. *Environ Microbiol* 2002;**4**:42–50.

Hahne F, Gopalakrishnan N, Khodabakhshi AH *et al. flowStats: Statistical Methods for the Analysis of Flow Cytometry Data*, 2022. http://www.github.com/RGLab/flowStats.

Hogle SL, Hackl T, Bundy RM *et al.* Siderophores as an iron source for picocyanobacteria in deep chlorophyll maximum layers of the oligotrophic ocean. *ISME J* 2022;**16**:1636–46.

Hoppe H-G. Significance of exoenzymatic activities in the ecology of brackish water: measurements by means of methylumbelliferyl-substrates. *Marine Ecol Progr Ser* 1983;**11**:299–308.

Huberts DHEW, van der Klei IJ. Moonlighting proteins: an intriguing mode of multitasking. *Biochimica Et Biophysica Acta (BBA) - Molecular Cell Research* 2010;**1803**:520–5.

Ivanova EP, López-Pérez M, Zabalos M *et al.* Ecophysiological diversity of a novel member of the genus *Alteromonas*, and description of *Alteromonas mediterranea* sp. nov. *Antonie Van Leeuwenhoek* 2015;**107**:119–32.

Ivars-Martinez E, Martin-Cuadrado A-B, D'auria G *et al.* Comparative genomics of two ecotypes of the marine planktonic copiotroph *alteromonas macleodii* suggests alternative lifestyles associated with different kinds of particulate organic matter. *ISME J* 2008;**2**:1194–212.

Kaplan M, Chreifi G, Metskas LA *et al.* In situ imaging of bacterial outer membrane projections and associated protein complexes using electron cryo-tomography. *Elife* 2021;**10**. DOI: 10.7554/eLife.73099.

Kearney SM, Thomas E, Coe A *et al.* Microbial diversity of co-occurring heterotrophs in cultures of marine picocyanobacteria. *Environmental Microbiome* 2021;**16**:1.

Koch H, Dürwald A, Schweder T *et al.* Biphasic cellular adaptations and ecological implications of *Alteromonas macleodii* degrading a mixture of algal polysaccharides. *ISME J* 2019;**13**:92–103.

Koch H, Germscheid N, Freese HM *et al.* Genomic, metabolic and phenotypic variability shapes ecological differentiation and intraspecies interactions of *Alteromonas macleodii*. *Sci Rep* 2020;**10**:809.

Kulkarni HM, Swamy CVB, Jagannadham MV. Molecular characterization and functional analysis of outer membrane vesicles from the antarctic bacterium *Pseudomonas syringae* suggest a possible response to environmental conditions. *J Proteome Res* 2014;**13**:1345–58.

Kwon YM, Patra AK, Chiura HX *et al.* Production of extracellular vesicles with light-induced proton pump activity by proteorhodopsin-containing marine bacteria. *MicrobiologyOpen* 2019;**8**:e00808.

Lau WYV, Hoad GR, Jin V *et al.* PSORTdb 4.0: expanded and redesigned bacterial and archaeal protein subcellular localization database incorporating new secondary localizations. *Nucleic Acids Res* 2021;**49**:D803–8.

Lee J, Lee E-Y, Kim S-H *et al.* Staphylococcus aureus extracellular vesicles carry biologically active β-lactamase. *Antimicrob Agents Chemother* 2013;**57**:2589–95.

Li J, Azam F, Zhang S. Outer membrane vesicles containing signalling molecules and active hydrolytic enzymes released by a coral pathogen *Vibrio shilonii* AK1. *Environ Microbiol* 2016;**18**:3850–66.

Lin J, Cheng J, Wang Y *et al.* The Pseudomonas Quinolone signal (PQS): not just for quorum sensing anymore. *Front Cell Infect Microbiol* 2018;**8**:230.

Linney MD, Eppley JM, Romano AE *et al.* Microbial sources of exocellular DNA in the ocean. *Appl Environ Microbiol* 2022;**88**:e0209321.

López-Pérez M, Gonzaga A, Martin-Cuadrado A-B *et al.* Genomes of surface isolates of *Alteromonas macleodii*: the life of a widespread marine opportunistic copiotroph. *Sci Rep* 2012;**2**:696.

López-Pérez M, Gonzaga A, Rodriguez-Valera F. Genomic diversity of "deep ecotype" *Alteromonas macleodii* isolates: evidence for Pan-Mediterranean clonal frames. *Genome Biol Evol* 2013;**5**:1220–32.

López-Pérez M, Ramon-Marco N, Rodriguez-Valera F. Networking in microbes: conjugative elements and plasmids in the genus *Alteromonas*. *BMC Genomics* 2017;**18**:36.

López-Pérez M, Rodriguez-Valera F. Pangenome evolution in the marine bacterium *alteromonas*. *Genome Biol Evol* 2016;**8**:1556–70.

Lötvall J, Hill AF, Hochberg F *et al.* Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles. *J Extracell Vesicles* 2014;**3**:26913.

Manck LE, Espinoza JL, Dupont CL *et al.* Transcriptomic study of substrate-Specific transport mechanisms for iron and carbon in the marine copiotroph *Alteromonas macleodii*. *Msystems* 2020;**5**. DOI: 10.1128/mSystems.00070-20.

Manck LE, Park J, Tully BJ *et al.* Petrobactin, a siderophore produced by *Alteromonas*, mediates community iron acquisition in the global ocean. *ISME J* 2022;**16**:358–69.

Martinez J, Azam F. Periplasmic aminopeptidase and alkaline phosphatase activities in a marine bacterium: implications for substrate processing in the sea. *Marine Ecol Progr Ser* 1993;**92**:89–97.

McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 2013;**8**:e61217.

Mitulla M, Dinasquet J, Guillemette R *et al.* Response of bacterial communities from California coastal waters to alginate particles and an alginolytic Alteromonas macleodii strain. *Environ Microbiol* 2016;**18**:4369–77.

Moore LR, Coe A, Zinser ER *et al.* Culturing the marine cyanobacterium *Prochlorococcus*: prochlorococcus culturing. *Limnol Oceanography: Methods* 2007;**5**:353–62.

Nagakubo T, Nomura N, Toyofuku M. Cracking open bacterial membrane vesicles. *Front Microbiol* 2019;**10**:3026.

Naval P, Chandra TS. Characterization of membrane vesicles secreted by seaweed associated bacterium *alteromonas macleodii* KS62. *Biochem Biophys Res Commun* 2019;**514**:422–7.

Neumann AM, Balmonte JP, Berger M *et al.* Different utilization of alginate and other algal polysaccharides by marine *Alteromonas macleodii* ecotypes. *Environ Microbiol* 2015;**17**:3857–68.

Oksanen J, Simpson GL, Blanchet FG *et al. Vegan: c ommunity ecology package*, 2022. https://CRAN.R-project.org/package=vegan.

Orench-Rivera N, Kuehn MJ. Environmentally controlled bacterial vesicle-mediated export. *Cell Microbiol* 2016;**18**:1525–36.

Park AJ, Murphy K, Surette MD *et al.* Tracking the dynamic relationship between cellular systems and extracellular subproteomes in *Pseudomonas aeruginosa* biofilms. *J Proteome Res* 2015;**14**:4524–37.

Park M, Sun Q, Liu F *et al.* Positional assembly of enzymes on bacterial outer membrane vesicles for cascade reactions. *PLoS One* 2014;**9**:e97103.

Pérez-Cruz C, Delgado L, López-Iglesias C *et al.* Outer-inner membrane vesicles naturally secreted by gram-negative pathogenic bacteria. *PLoS One* 2015;**10**:e0116896.

Prados-Rosales R, Weinrick BC, Piqué DG *et al.* Role for *mycobacterium tuberculosis* membrane vesicles in iron acquisition. *J Bacteriol* 2014;**196**:1250–6.

Pritchard L, Glover RH, Humphris S *et al.* Genomics and taxonomy in diagnostics for food security: soft-rotting enterobacterial plant pathogens. *Anal Methods* 2015;**8**:12–24.

Pujalte MJ, Sitjà-Bobadilla A, Alvarez-Pellitero P *et al.* Carriage of potentially fish-pathogenic bacteria in Sparus aurata cultured in Mediterranean fish farms. *Dis Aquat Organ* 2003;**54**:119–26.

R Core Team. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing, 2022.

Rakoff-Nahoum S, Coyne MJ, Comstock LE. An ecological network of polysaccharide utilization among human intestinal symbionts. *Curr Biol* 2014;**24**:40–49.

Reddy VM, Suleman FG. Mycobacterium avium-superoxide dismutase binds to epithelial cell aldolase, glyceraldehyde-3-phosphate dehydrogenase and cyclophilin A. *Microb Pathog* 2004;**36**:67–74.

Roier S, Zingl FG, Cakar F *et al.* A novel mechanism for the biogenesis of outer membrane vesicles in gram-negative bacteria. *Nat Commun* 2016;**7**:1–13.

RStudio Team. *RStudio: Integrated Development Environment for R*. Boston, MA: RStudio, Inc., 2019.

Schatz D, Vardi A. Extracellular vesicles - new players in cell-cell communication in aquatic environments. *Curr Opin Microbiol* 2018;**43**:148–54.

Schwechheimer C, Kuehn MJ. Outer-membrane vesicles from gram-negative bacteria: biogenesis and functions. *Nat Rev Microbiol* 2015;**13**:605–19.

Schwechheimer C, Sullivan CJ, Kuehn MJ. Envelope control of outer membrane vesicle production in gram-negative bacteria. *Biochemistry* 2013;**52**:3031–40.

Sher D, Thompson JW, Kashtan N *et al.* Response of *prochlorococcus* ecotypes to co-culture with diverse marine bacteria. *ISME J* 2011;**5**:1125–32.

Singh N, Bhalla N. Moonlighting proteins. *Annu Rev Genet* 2020;**54**:265–85.

Soler N, Forterre P. Vesiduction: the fourth way of HGT. *Environ Microbiol* 2020;**22**:2457–60.

Tashiro Y, Yawata Y, Toyofuku M *et al.* Interspecies interaction between *Pseudomonas aeruginosa* and other microorganisms. *Microb Environ* 2013;**28**:13–24.

Tatusov RL, Galperin MY, Natale DA *et al.* The COG database: a tool for genome-scale analysis of protein functions and evolution. *Nucleic Acids Res* 2000;**28**:33–36.

Théry C, Witwer KW, Aikawa E *et al.* Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J Extracell Vesicl* 2018;**7**: 1535750.

Toyofuku M, Nomura N, Eberl L. Types and origins of bacterial membrane vesicles. *Nat Rev Microbiol* 2019;**17**:13–24.

Toyofuku M, Zhou S, Sawada I *et al.* Membrane vesicle formation is associated with pyocin production under denitrifying conditions in *Pseudomonas aeruginosa* PAO1. *Environ Microbiol* 2014;**16**: 2927–38.

Turnbull L, Toyofuku M, Hynen AL *et al.* Explosive cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms. *Nat Commun* 2016;**7**:11220.

Turner L, Bitto NJ, Steer DL *et al.* Outer membrane vesicle size determines their mechanisms of host cell entry and protein content. *Front Immunol* 2018;**9**:1466.

Veith PD, Chen Y-Y, Chen D *et al.* Tannerella forsythia outer membrane vesicles are enriched with substrates of the type IX secretion system and TonB-Dependent receptors. *J Proteome Res* 2015;**14**:5355–66.

Veith PD, O'Brien-Simpson NM, Tan Y *et al.* Outer membrane proteome and antigens of *Tannerella forsythia*. *J Proteome Res* 2009;**8**:4279–92.

Walker SA, Kennedy MT, Zasadzinski JA. Encapsulation of bilayer vesicles by self-assembly. *Nature* 1997;**387**:61–64.

Yaron S, Kolling GL, Simon L *et al.* Vesicle-mediated transfer of virulence genes from *Escherichia coli* O157:H7 to other enteric bacteria. *Appl Environ Microbiol* 2000;**66**:4414–20.

Yonezawa H, Osaki T, Kurata S *et al.* Outer membrane vesicles of *Helicobacter pylori* TK1402 are involved in biofilm formation. *BMC Microbiol* 2009;**9**:197.

Yoshida N, Oeda K, Watanabe E *et al.* Chaperonin Turned Insect Toxin. *Nature* 2001;**411**:44–44.

Yu C-S, Cheng C-W, Su W-C *et al.* CELLO2GO: a web server for protein subCELlular localization prediction with functional gene ontology annotation. *PLoS One* 2014;**9**:e99368.

Zakharzhevskaya NB, Vanyushkina AA, Altukhov IA *et al.* Outer membrane vesicles secreted by pathogenic and nonpathogenic *Bacteroides fragilis* represent different metabolic activities. *Sci Rep* 2017;**7**:5008.

Zhao Z, Baltar F, Herndl GJ. Linking extracellular enzymes to phylogeny indicates a predominantly particle-associated lifestyle of deep-sea prokaryotes. *Sci Adv* 2020;**6**:eaaz4354.