Ectohydrolytic enzyme activities of bacteria associated with *Orbicella annularis* coral

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Abstract Ectohydrolytic enzyme activity (EEA) potential of 37 bacterial isolates derived from *Orbicella annularis* coral and 2 coral pathogens (*Vibrio shilonii* and *V. coralliilyticus*) was measured as model to infer the role of bacteria in organic matter processing within coral reef ecosystems. Bacterial cell-specific activities of eight enzyme types were measured after incubation in organic matter enriched and unenriched filtered seawater. Max value of activities of alkaline phosphatase, oleate-lipase, stearate-lipase and proteinase were 769.3, 327.6, 82.9 and 36.7 amol cell^{-1} h^{-1}, respectively. Chitinase, α-mannosidase, α-glucosidase and β-glucosidase were generally lower by comparison (max 4.7–20.7 amol cell^{-1} h^{-1}). No "super" isolates (bacteria expressing high levels of all ectohydrolases) were found suggesting a "specialization" among individual bacterial strains. Cumulatively, the 39 isolates tested displayed a broad range of cell-specific enzyme activities in both organic matter conditions. Culture-independent measurement of coral mucus layer EEA in *O. annularis* off a Panama reef showed comparable EEA patterns and diversity as the isolates. Volume-specific EEAs of all enzymes except alkaline phosphatase were 8–48 times higher in mucus than in surrounding seawater (SSW) samples. However, cell-specific EEAs in mucus were generally lower than in the SSW partly due to more abundant cells in the mucus than in SSW. For field samples, ≥ 85% of proteinase was cell-bound, while lipase was preferentially dissolved (40–96%). In general, the production of dissolved EEAs varied among measurements depending on sample source and enzyme types, suggesting a potential role of ectoenzyme size distribution in linking the whole reef ecosystem. Our findings support that the cumulative ectoenzyme expression ("ectoenzymome") of the coral microbiome has the potential to maintain the functional resilience of the coral holobiont and response to stress through its contribution to organic matter processing within coral reef ecosystems.

Keywords Coral-associated bacteria · Ectohydrolytic enzyme activity · Organic matter degradation · Reef nutrient cycling · Coral holobiont

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Introduction

Marine heterotrophic bacteria transform and remineralize a substantial fraction of marine organic matter (Hedges 1992; Azam 1998; D'Ambrosio et al. 2014). Bacterial ectohydrolases (measured as ectohydrolytic enzyme activities "EEAs") play crucial roles in the processing of polymeric and particulate organic matter and consequently in the rates and regulation of elemental fluxes in the sea (Martinez et al. 1996; Arnosti et al. 2009). Due to their central importance in the ecology of marine bacteria and in bacteria-mediated organic matter cycling, ectohydrolases have been studied in diverse marine environments and some marine isolates (Hoppe et al. 1988; Smith et al. 1992; Martinez et al. 1996; D'Hondt et al. 2004; Hoarfrost and Arnosti 2017). However, less is currently known regarding the role and biogeochemical significance of EEAs of bacteria occurring within the coral holobiont and their roles in the functioning of coral reef ecosystems.

Coral reefs are highly productive and biodiverse ecosystems that, intriguingly, occur in oligotrophic waters (Kohn 2002) yet display levels of primary productivity that are comparable to tropical rain forests (Hatcher 1988; Rougerie and Wauthy 1993). This could be due in part to efficient nutrient cycling and tightly coupled nutrient and energy flows among various members of the coral holobiont, the coral host, its endosymbiotic Symbiodiniaceae (LaJeunesse et al. 2018) and microbiome (Ceh et al. 2013). Corals as meta-organisms have been considered to host three distinct microbiomes: a ubiquitous core microbiome, a microbiome filling functional niches and a highly variable bacterial community (Hernandez-Agreda et al. 2016). Still, the coral holobiont was conceptualized as a diverse transient microbial community responsive to the surrounding environment (Hernandez-Agreda et al. 2018) and bacterial communities of the surface mucus layer was found to be particularly sensitive to environmental change (Osman et al. 2020). Ectohydrolases of bacteria may reflect the range of biochemical capabilities for organic matter transformation and nutrient cycling by the microbiome within the context of the coral holobiont.

Understanding the variability and regulation of the in situ activities of ectohydrolases of microbes (herein "ectoenzymome") and microbiomes is an important goal in marine biogeochemistry and specifically in the study for the functioning of coral reef ecosystems. The ectoenzymome expression is expected to be responsive to the composition and concentration of oligomeric, polymeric and particulate organic matter encountered by the bacterial communities. On the other hand, the community composition of bacteria itself may shift depending on various factors including the enzyme expressions of bacterial community, the nature of the organic matter as well as various other physicochemical and trophic parameters (Rosenberg et al. 2007; Tout et al. 2015). The enzyme expression may also respond to the nutrient status and demand of the Symbiodiniaceae. For instance, phosphorus limitation or high inorganic N/P ratio has been shown to promote coral bleaching in a warming ocean (Ezzat et al. 2016; Rosset et al. 2017) and patterns of EEAs may influence these parameters within the holobiont.

Since it is currently not technically feasible to relate the natural holobiont community composition with in situ EEA profiles of various bacterial individuals isolated from coral, we considered that a suite of bacterial isolates (Rypien et al. 2010) from a well-studied coral *Orbicella annularis* (Edmunds 2015) could tentatively serve as a model system to begin to constrain the in situ ectohydrolases activities of the coral microbiomes. Extrapolating from the current knowledge of EEAs of pelagic marine isolates, including free and particle-associated bacteria (Martinez et al. 1996; Arnosti 2008; Arnosti et al. 2009; D'Ambrosio et al. 2014), we hypothesized that ectohydrolase profiles of individual taxa display substrate specializations and that community diversity reflects the potential of the coral microbiome for effective transformation of organic matter and response to variations in nutrient status of the environment. We recognize, however, that isolates do not necessarily accurately represent the natural microbiome due to culture bias (Rohwer et al. 2001), as the majority of bacteria are uncultivable using present technology. Still, interrogating the ectoenzymomes of a large number of isolates was likely to uncover a broad range of EEA profiles as in previous studies of free and particle-associated pelagic marine isolates (Martinez et al. 1996; Arnosti 2008; Arnosti et al. 2009; D'Ambrosio et al. 2014). Furthermore, the cumulative EEA of sets of isolates (all or select) within those tested here might be viewed as "ectoenzymomes" of the culturable communities. A culture-independent study of the ectoenzymome of *O. annularis* field-collected coral mucus and surrounding seawater (SSW) further provided a context for the culture-dependent measurements.

Materials and methods

Culture-based study

We measured the activities of eight enzymes for 39 coral-associated bacterial isolates (Table 1). Thirty-seven of the isolates were isolated in a previous study from coral mucus, tissue and skeleton of healthy *O. annularis* colonies collected in the Florida Keys, USA (Rypien et al. 2010). In addition, two model coral pathogens maintained in the

Table 1 Taxonomy and source of bacterial isolates used in this study

Phylum, Class	Family	Genus, species[a]	Isolate ID#	Source/citation
α Proteobacteria	Rhodobacteraceae	*Pseudovibrio denitrificans*	1	*Orbicella annularis* coral, Florida Keys, FL, USA (Rypien et al. 2010)
			2	
			3	
			4	
			5	
		Ruegeria arenilitoris	6	
			7	
			8	
γ Proteobacteria	Pseudoalteromonadaceae	*Pseudoalteromonas phenolica*	9	
		Pseudoalteromonas elyakovii	10	
			11	
			12	
	Alteromonadaceae	*Aliagarivorans taiwanensis*	13	
	Ferrimonadaceae	*Ferrimonas marina*	14	
			15	
	Vibrionaceae	*Grimontia celer*	16	
		Photobacterium aquimaris	17	
		Vibrio campbellii	18	
		Vibrio coralliilyticus	19	
		Vibrio parahaemolyticus	20	
		Vibrio sinaloensis	21	
		Vibrio owensii	22	
			23	
			24	
			25	
			26	
			27	
		Vibrio harveyi	28	
			29	
			30	
			31	
			32	
			33	
		Vibrio fortis	34	
			35	
		Vibrio xuii	36	
			37	
		Vibrio coralliilyticus	38	Ben-Haim et al. (2003)
		Vibrio shilonii	39	Kushmaro et al. (1996)

[a]Taxonomy based on top-hit (97–100% strain similarity) blasted on EZBiocloud (Yoon et al. 2017)

Ben-Haim et al. (2003), Kushmaro et al. (1996), Rypien et al. (2010) and Yoon et al. (2017)

Azam Lab were included: *V. coralliilyticus*[#38] (Ben-Haim et al. 2003) and *V. shilonii*[#39] (Kushmaro et al. 1996).

Based on 16S rDNA similarity, these isolates were comprised of 16 species of Proteobacteria. Phylogenetic relationship among all isolates used in this study is shown as a phylogenetic tree of 16S rDNA for coral isolates and type strains with the highest similarity (Supplementary Fig. 1). Culture-independent study by Barott et al. (2011) reported that *O. annularis*-associated bacteria community in Curacao was dominated by Proteobacteria (75%) and

approximately 70% of 16S rDNA sequences from other culture-independent studies on healthy and diseased corals were of α and γ Proteobacteria (Rohwer et al. 2001, 2002; Rohwer and Kelley 2004). Thus, we believe our suite of tested isolates to be relatively representative at the class level for *O. annularis* and other corals.

We measured EEAs of bacteria isolates grown in ZoBell 2216E medium (ZoBell) and then nutritionally downshifted one culture aliquot by transfer to GF/F (0.7 μm nominal pore size) filtered-autoclaved seawater (FASW) for 72 h. The ZoBell medium consisted of the same batch of FASW plus 0.1% yeast extract and 0.5% bacteriological peptone (ZoBell 1941). The downshift from enriched to unenriched media was designed to test if enzyme activity levels were influenced by high and low organic matter concentrations that bacteria might experience within the holobiont or in the surrounding seawater, respectively.

Experimental conditions and treatments

Each bacteria isolate was grown overnight as one batch-culture in ZoBell medium at room temperature (RT, 24–25 °C). Cells were collected by centrifugation (3,200 × *g*) for 7 min. In order to avoid possible competition between the ZoBell organic components and fluorogenic substrates used in the EEA measurement for the same bacterial enzymes, the cells were washed by centrifugation and resuspended in FASW to final cell concentrations range of 10^6 to 10^7 cells mL^{-1}. We assume that the cells' enzyme profiles did not change by this treatment given the short time frame of the processing. These bacteria suspensions were divided into two equal parts. One aliquot was used directly for enzyme activity measurement of cultures grown in an organic rich condition, "T0 hr (Zobell)"; the other aliquot of the washed cells was incubated at RT for 72 h and then directly used for enzyme activity measurement of cultures acclimated to unenriched seawater, "T72 hr (FASW)". The seawater used for making the media in this experiment was collected off Ellen Browning Scripps Memorial Pier, La Jolla, California, USA.

EEA measurement

Hydrolytic enzyme activities were assayed using fluorogenic model substrates, 7-amino-4-methylcoumarin (AMC) or 4-methylumbelliferone (MUF) derivatives that undergo large fluorescence enhancement upon enzymatic hydrolysis (Hoppe 1993). Hydrolysis rates of leucine-AMC, MUF-phosphate, MUF-α-D-glucoside, MUF-β-D-glucoside, MUF-α-D-mannopyranoside, MUF-stearate, MUF-oleate and MUF-*N*-acetyl-β-D-glucosamine were used to assay the activities of proteinase, alkaline phosphatase, α-glucosidase, β-glucosidase, α-mannosidase, stearate-lipase, oleate-lipase and β-*N*-acetylglucosaminidase (chitinase), respectively. Substrates were purchased from Sigma. We tested a range of substrate concentrations (20, 50 and 100 μM) and duration of incubation (5, 15, 30 and 60 min) for several isolates to optimize the protocol. Near maximal activity (Ectoenzyme Activity Potential, EAP Böer et al. 2009) was achieved at 50 μM/15 min; therefore, these conditions were subsequently selected and used for each assayed isolate.

The fluorescence of three technical replicates for each isolate batch-culture was assayed at RT using a 96-well plate reader (SpectraMax 190, Molecular Devices) with an excitation/emission of 355 nm/460 nm. Hydrolysis rates were calculated using standard curves established with standard solutions of AMC and MUF. As negative control, aliquots of FASW were incubated with the same enzyme substrates and assayed in parallel. The hydrolysis rates were reported as "volume-specific EEAs" (nmol L^{-1} h^{-1}) and "cell-specific activities" (amol $cell^{-1}$ h^{-1}). Cell-specific activities were calculated by dividing the volume-specific EEAs by bacteria cell abundance in the corresponding sample (cell enumeration methodology described below). Dissolved EEAs were not measured for any isolates. Statistical analysis was run using MATLAB (R2018b).

Field study

Upon analysis of our isolate results, we found it desirable to acquire culture-independent field data that would allow us to contextualize our findings and further strengthen our hypotheses on the role of ectoenzyme activity within coral-reef ecosystems. Logistical constraints prohibited us from visiting the Florida Keys, USA, to sample the same *O. annularis* colonies that our isolates came from. However, our laboratory capitalized on an opportunity in September 2014 to sample ectoenzyme activity from *O. annularis*-associated mucus and seawater at a well-studied coral reef (Hospital Point; 9° 19′ 3″ N, 82° 12′ 14″ W; Levitan et al. 2004, 2014)) during a field trip to the Smithsonian Tropical Research Institute in Bocas del Toro, Panama.

Mucus was collected from three independent healthy *O. annularis* coral colonies using a 50-mL sterile syringe. Surrounding seawater (SSW) was also collected in triplicate from ∼ 3 m above the coral colonies using 5 L acid washed and Milli-Q rinsed Cubitainer® collapsible bottles. Aliquots from each of the three samples of mucus and SSW were briefly vortexed and then size fractionated through 0.2-μm polycarbonate-membrane filters (Millipore). Three technical replicates for each of the unfractionated "Whole sample" and "Dissolved fraction" samples were assayed for enzyme activities as stated above, except here we used 50 μM/60 min to achieve maximal activities. FASW was

also set as negative control. Volume-specific EEAs contributed by cell-bound section ("Whole sample" minus "Dissolved fraction") were used when calculating cell-specific activities.

Cell enumeration

Epifluorescence microscopy was used to determine the bacteria cell abundance for each sample that was measured for EEA. The same general protocol was used for the isolate samples and field samples. Within 15 min after starting the EEA measurements, one aliquot for each sample was fixed with 0.22 µm filtered formalin (2% v/v final) for 15 min at 4 °C. After brief vortex, fixed samples were filtered onto 0.2 µm pore-size polycarbonate filters and stained with 4, 6-diamidino-2-phenylindole (DAPI Vectashield; Vector Labs) (Porter 1980). The remaining fixed sample was archived at − 20 °C. At least fifteen microscopic fields of view were counted for each sample. When counting, we observed bacteria aggregates for a few isolate samples. These isolates were treated with 10 µg mL^{-1} (final) Tween-80 followed by sonication on ice for 30 s to disperse the cells. Field samples were only mixed thoroughly with vortex mixer before fixation and DAPI staining.

Results and discussion

EEA profiles of coral isolates

EEA overview of the different isolates

As a general result (Supplementary Table 1), all isolates at T0 hr (ZoBell) expressed at least five of the eight tested EEA types, while 15 of 39 isolates expressed all eight. After the nutrient downshift at T72 hr (FASW), each isolate expressed a maximum of only three of the eight EEA types and the proportion of isolates expressing all eight EEAs dropped to 9 of 39. At T72 hr (FASW), about half of the coral isolates showed positive bacterial yields while others the opposite (Fig. 1). The cell number varied per volume of sample, and then cell-specific EEAs were analyzed in detail to assess the EEA potential of each coral isolate.

As shown in Table 2, cell-specific alkaline phosphatase activity comprised among the highest values and also the widest range from 0 to 769.3 amol $cell^{-1}$ h^{-1}. Alkaline phosphatase and two lipases' activity were relatively high and widely distributed in all isolates. The other five EEAs were generally low (proteinase activity had a mean value of 5.3 and 2.6 amol $cell^{-1}$ h^{-1} at T0 hr (ZoBell) and T72 hr (FASW), respectively; three glycosidases were generally lower with narrow ranges (0–9.99 amol $cell^{-1}$ h^{-1}) except two values larger than 20).

All measured isolate EEA values in our study fell within the range of values measured in 21 other marine studies (see compiled summary in Supplementary Table 2). It is noteworthy that the variability of EEA types expressed

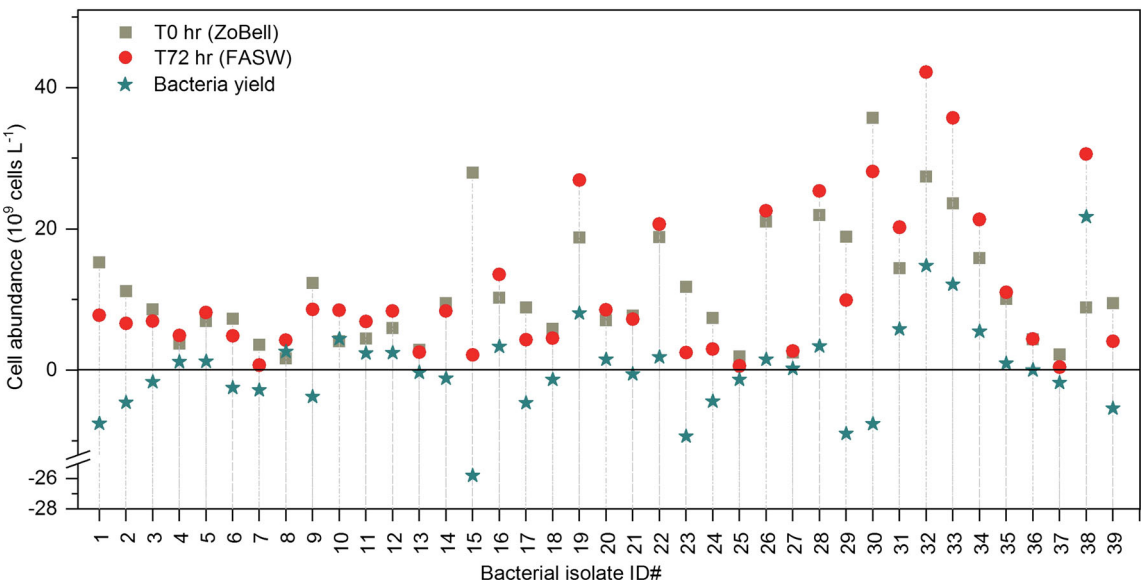


Fig. 1 Bacteria abundance for each isolate (n = 39) at T0 hr (ZoBell) and T72 hr (FASW) and their total yield after incubation (abundance at T72 hr − T0 hr = Bacteria yield)

among our isolates, along with our observation that none of the individual isolates exhibited high activity levels for all eight tested enzymes (i.e., no "super" isolates were found) was consistent with the results of Martinez et al. (1996) who measured EEA profiles for 44 diverse marine isolates. Taken together, these results further support the hypothesis that 'specialization' is required among individual bacterial strains to process complex organic matter within natural assemblages (Smith et al. 1992; Martinez et al. 1996) and suggests that such specialization may also be necessary within coral reef ecosystems.

Regulation of nutrient shift and taxonomic assignments on EEAs

Cell-specific EEAs of isolates varied enormously before and after nutrient shift (Fig. 2). Activities of all enzyme types decreased for more than half of the isolates (19–30 of 39) except chitinase which showed as activity increase for 29 of 39 isolates following the nutrient down-shift (Table 3). When organic status (ZoBell or FASW) was set as the only variable, one-way ANOVA found activities of proteinase ($p < 0.01$), oleate-lipase ($p < 0.05$) and stearate-lipase ($p < 0.001$) showed significant variance.

Further, distinct EEA patterns were found among isolates of the same genera or species. All *P. denitrificans* isolates $^{\#1–5}$ had high alkaline phosphatase and lipase activities and low activities of other enzymes, but for the same ectoenzyme, the activity value among isolates was highly variable. Moreover, EEAs following DOM down-shift showed an increase for some isolates but decrease for others, even when all isolates $^{\#6, 7, 8}$ belonged to *Ruegeria arenilitoris* (i.e., strain-specific variations). Highly variable EEA profiles were also found among isolates of *V. owensii*, *V. harveyi* and other Vibrios. Detailed analysis of each enzyme type was as below.

Fig. 2 Cell-specific enzyme activities for each tested bacterial isolate ▶ ($n = 39$). All activities were reported as quantity of MUF produced, except for proteinase activity (AMC produced). Error bars represent ± SD

Widespread existence of alkaline phosphatase

Alkaline phosphatase is produced by bacteria to catalyze phosphoester hydrolysis to supply their metabolism with phosphorus (Cotner et al. 1997; Wambeke et al. 2002; Sebastian and Ammerman 2009) as well as organic substrates such as glucoses (Chróst 1990). In our study, all 39 cultures expressed alkaline phosphatase activity at T0 hr (ZoBell) and one $^{\#8}$ ceased to express it at T72 hr (FASW). Change in alkaline phosphatase activity values covered a broad range from − 462 to 145 amol cell^{-1} h^{-1} (Fig. 3). Bacteria themselves are P rich (marine bacteria C/P ratio ~ 25 (Lee and Fuhrman 1987)), and coral animal feeding on bacteria could be another pathway for P introduction into the holobiont. Further, bacteria could take up (in addition to inorganic P) the organic carbon moieties released due to the action of alkaline phosphatase on its substrates.

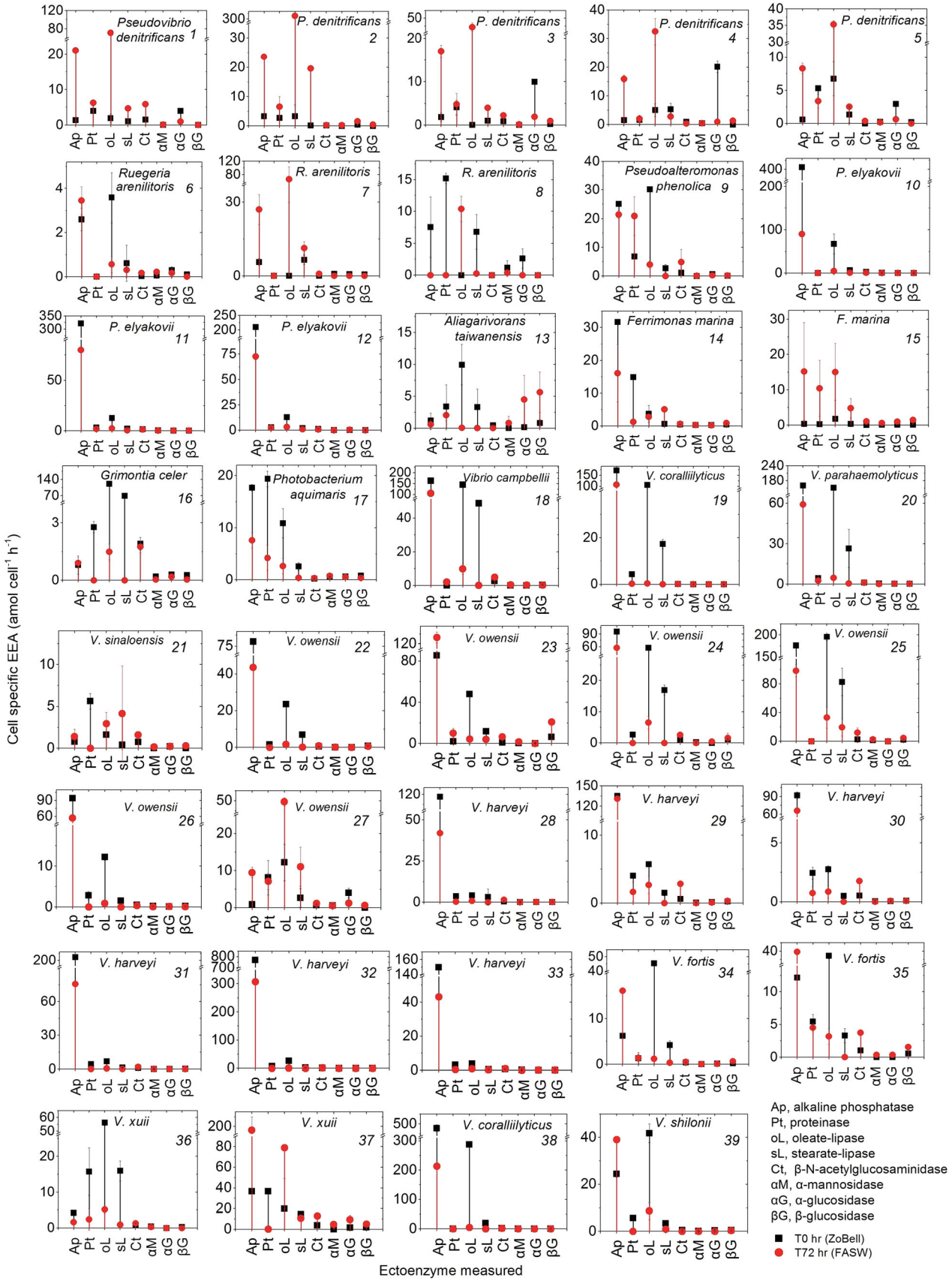
Proteinase activity and its downregulation with nutrient downshift

Nitrogen, one dominant element in protein, is a major limiting nutrient in sustaining highly productive reef ecosystems. It is required via bacterial regeneration of dead organic matter and animal catabolism, as well as external nitrogen inputs mainly from nitrogen fixation utilized by

Table 2 Summary of measured cell-specific EEAs for all tested bacterial isolates

	T0 hr (ZoBell)			T72 hr (FASW)			Isolates tested (n)
	Range	Mean	Median	Range	Mean	Median	
Alkaline phosphatase	0.4–769.3	105.3	31.7	0.0–306.8	57.5	39.5	39
Proteinase	0.0–36.7	5.3	3.4	0.0–20.9	2.6	1.3	39
Oleate-lipase	0.0–287.1	40.6	12.2	0.1–327.6	21.7	4.0	39
Stearate-lipase	0.2–82.9	10.2	3.2	0.0–19.6	2.8	0.3	39
β-*N*-acetylglucosaminidase	0.0–3.7	1.0	0.8	0.0–12.7	2.1	1.2	39
α-Mannosidase	0.0–1.4	0.3	0.1	0.0–4.7	0.4	0.1	39
α-Glucosidase	0.0–20.1	1.3	0.2	0.0–9.2	0.7	0.2	39
β-Glucosidase	0.0–6.3	0.5	0.2	0.0–20.7	1.3	0.2	39

Values are amol MUF produced cell^{-1} h^{-1} except for proteinase (amol AMC produced cell^{-1} h^{-1}). FASW, filtered autoclaved seawater; Zobell, Zobell 2216E medium


Pseudovibrio denitrificans 1
P. denitrificans 2
P. denitrificans 3
P. denitrificans 4
P. denitrificans 5
Ruegeria arenilitoris 6
R. arenilitoris 7
R. arenilitoris 8
Pseudoalteromonas phenolica 9
P. elyakovii 10
P. elyakovii 11
P. elyakovii 12
Aliagarivorans taiwanensis 13
Ferrimonas marina 14
F. marina 15
Grimontia celer 16
Photobacterium aquimaris 17
Vibrio campbellii 18
V. coralliilyticus 19
V. parahaemolyticus 20
V. sinaloensis 21
V. owensii 22
V. owensii 23
V. owensii 24
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V. harveyi 28
V. harveyi 29
V. harveyi 30
V. harveyi 31
V. harveyi 32
V. harveyi 33
V. fortis 34
V. fortis 35
V. xuii 36
V. xuii 37
V. coralliilyticus 38
V. shilonii 39
Ap Pt oL sL Ct αM αG βG
Cell specific EEA (amol cell^{-1} h^{-1})
Ectoenzyme measured
Ap, alkaline phosphatase
Pt, proteinase
oL, oleate-lipase
sL, stearate-lipase
Ct, β-N-acetylglucosaminidase
αM, α-mannosidase
αG, α-glucosidase
βG, β-glucosidase
T0 hr (ZoBell)
T72 hr (FASW)

Table 3 Proportion of isolates expressing ectoenzyme activities in each treatment

Enzyme	Isolates expressing activity at			Isolates w/ EEA profiles that changed after nutrient downshift	
	T0 hr (ZoBell)	T72 hr (FASW)	Neither at T0 hr nor at T72 hr	Activity increased	Activity decreased
Alkaline phosphatase	39/39	38/39	0/39	16/39	23/39
Proteinase	33/39	25/39	5/39	9/39	25/39
Oleate-lipase	36/39	39/39	0/39	11/39	28/39
Stearate-lipase	39/39	28/39	0/39	9/39	30/39
β-*N*-acetylglucosaminidase	36/39	36/39	1/39	29/39	9/39
α-Mannosidase	28/39	26/39	2/39	16/39	21/39
α-Glucosidase	30/39	29/39	6/39	12[a]/39	21/39
β-Glucosidase	32/39	29/39	2/39	18/39	19/39

[a]The absolute change values of EEAs (decreased/increased) were larger than 0.01 amol cell^{-1} h^{-1} except one of the 12 isolates here

coral symbionts either in dissolved or particulate organic form (Benavides et al. 2017). Six isolates at T0 hr (ZoBell) expressed no proteinase activity and 14 isolates failed to express it following the down-shift to FASW including five isolates #6, 7, 10, 25, 38 which had undetectable proteinase activity in either timepoints. The maximum cell-specific proteinase activity decreased from 36.7 to 20.9 amol cell^{-1} h^{-1} following the shift. The change values ranged from − 37 to 14 amol cell^{-1} h^{-1}. It was unexpected that 25 of 39 isolates' proteinase activity was lower at T72 hr (FASW) than at T0 hr (ZoBell).

Taking these results on face value, they are inconsistent with the literature findings that corals can satisfy their N demand through bacterial nitrogen fixation and heterotrophic feeding of the animals on particles including bacteria (Rädecker et al. 2015; Bednarz et al. 2017; Benavides et al. 2017). We did not test for the release of proteinase into the ZoBell culture medium, so we cannot rule out that our isolates (unlike most pelagic isolates) released any proteinase into the medium.

Wide distribution of lipase

The substrates we used for lipase measurement are substrates for acid and alkaline lipases instead of phospholipase used in past studies. All 39 cultures expressed stearate-lipase and 36 isolates expressed oleate-lipase at T0 hr (ZoBell), but at T72 hr (FASW), all isolates produced oleate-lipase but 11 isolates #9, 13, 16, 18, 24, 26, 28–31, 35 lacked detectable stearate-lipase activity. The maximum oleate-lipase and stearate-lipase activity at T72 hr (FASW) were both from isolate #2 with the absolute change value of 324 and 19 amol cell^{-1} h^{-1} separately (Fig. 3).

Lipase is involved in pathogen establishment on hosts including human (Bender and Flieger 2010) and plants (Subramoni et al. 2010). It is interesting that a number of isolates and particularly one coral pathogen #38 in our study displayed very high oleate-lipase activity (the highest) and stearate-lipase activity (5th highest) at T0 hr (ZoBell) but decreased a lot at T72 hr (FASW). It is worth investigating whether the very high MUF-oleate/stearate hydrolysis activity from our study is involved in pathogen invasion. Further, we found a very large increase in oleate-lipase activity of α Proteobacteria when shifted to unenriched condition (Fig. 2). Indeed, the lipase activity of coral-associated bacteria may generally be involved in nutrient acquisition. This is evidenced by a field study that found significant bacterial growth and elevated lipase activity in seawater samples that contained degraded, lipid-rich, coral gametes (Guillemette et al. 2018). In our experiment, the observed upregulation of lipase would be consistent with a nutritional role for lipase activity in these isolates.

Chitinase and its upregulation for 29 of 39 isolates following the nutrient down-shift

Chitin, which is resistant to degradation (Akaki and Duke 1999), is among the most abundant polymers in the marine environments (Pruzzo et al. 2008). Chitinase activity was detectable in 36 coral isolates with a mean value of 1.0 and 2.1 amol cell^{-1} h^{-1} at T0 hr (ZoBell) and T72 hr (FASW), respectively. ZoBell and FASW used in this experiment were from the same seawater collection; there would not be any increase of chitin during the nutrient down-shift. On the contrary, compared to other enzymes, chitinase activity showed the biggest upregulation (29 of 39 isolates) following the nutrient down-shift. Enhanced rate of chitin

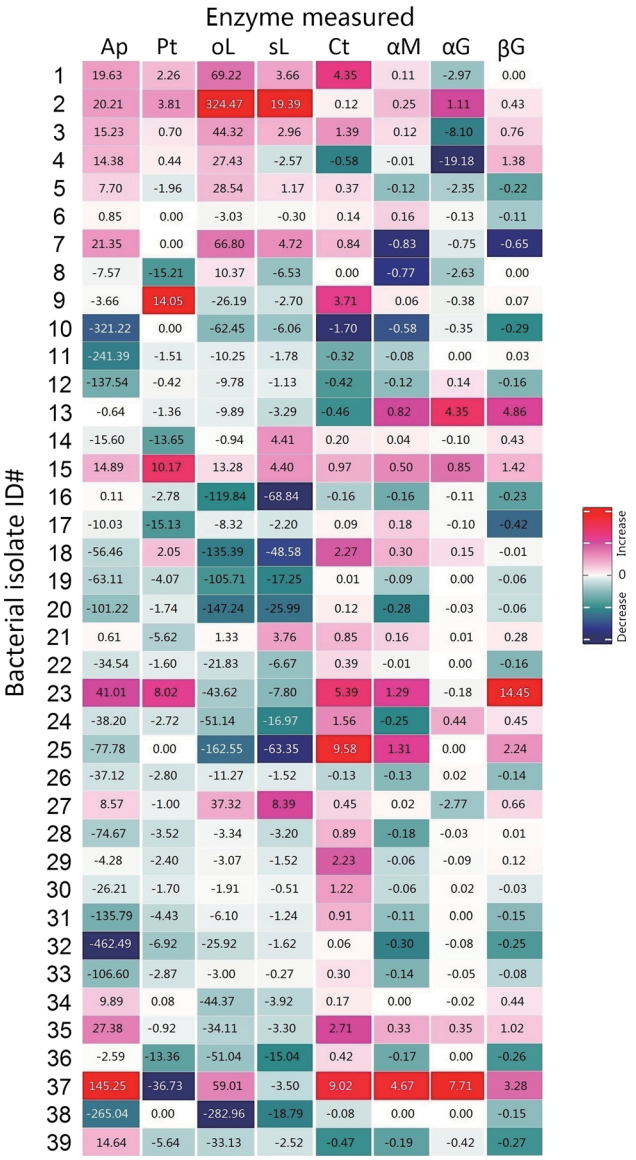

Fig. 3 Heatmap of cell-specific EEA changing value after nutrient downshift (n = 39)

hydrolysis in oligotrophic FASW than eutrophic ZoBell suggests the potential role of coral-associated microorganisms as chitin degraders, especially in oligotrophic conditions. However, the activity of both coral pathogens decreased following nutrient down-shift (Fig. 2).

It has been demonstrated that chitinase is critical for successful fungus pathogen infection on insects (Peng et al. 2009) and are a defense strategy for plants against pathogen infection (Herrera-Estrella and Chet 1999; Naher et al. 2012). It would then be interesting to test whether high chitinase activity expressed by the two pathogens in organic rich condition plays a role in pathogen infection or that high chitinase expressed by coral microbiome isolates (not known to be pathogens) act against pathogen invasions.

Low glycosidases' activities

Percentage of isolates expressing α-mannosidase, α-glucosidase and β-glucosidase in ZoBell was 72%, 77% and 82%, respectively. Compared to other enzymes, glycosidases were less commonly distributed and the activities when detectable were low, similar with past studies of pelagic isolates (Martinez et al. 1996). Activities less than 0.25 amol cell^{-1} h^{-1} occupied more than a half for all the three glycosidases detected (Supplementary Fig. 2). Activity of α-glucosidase at T0 hr (ZoBell) was relatively high for isolates $^{\#1,\ 3,\ 4,\ 8,\ 27}$ (2.6–20 amol cell^{-1} h^{-1}) but decreased greatly following nutrient down-shift. Isolates $^{\#13,\ 23,37}$ had relatively high β-glucosidase activity (5–21 amol cell^{-1} h^{-1}) after the shift to FASW. For α-mannosidase, it was low for all isolates at both timepoints with the top three values ranging from 1.7 to 4.7 amol cell^{-1} h^{-1}.

EEAs of field-collected *O. annularis* mucus and surrounding seawater

To gain further assessment on in situ ectohydrolase activities, we measured EEAs from a natural *O. annularis*-associated microbial community that included mucus and surrounding seawater (SSW) field samples. Ectohydrolytic enzyme activities were measured for unfractionated (whole sample) and 0.2-μm-filtrate (Dissolved-fraction) samples to evaluate whether enzymes were free dissolved or cell-bound.

Volume-specific EEAs

The predominant EEAs measured in the field samples were proteinase, alkaline phosphatase and lipases (Fig. 4 left Y axis); a similar trend to what we observed in the isolate experiments. Activities of all enzymes except alkaline phosphatase were 8–48 times higher in mucus than in SSW samples.

Alkaline phosphatase activity was nearly identical between mucus (322.0 ± 154.9 nmol L^{-1} h^{-1}) and SSW samples (334.3 ± 228.0 nmol L^{-1} h^{-1}); each with about one-third coming from the dissolved fraction (Supplementary Table 3). They were strikingly higher than non-reef seawater samples (20 nmol L^{-1} h^{-1} by Martinez et al. (1996); 0.01–13.7 nmol L^{-1} h^{-1} by Mahaffey et al. (2014); 0–120 nmol L^{-1} h^{-1} by Malfatti et al. (2019)). While corals in pristine waters face the challenge of P extraction from low-nM Pi, the corals exposed to coastal over nutrition and thermal stress paradoxically may

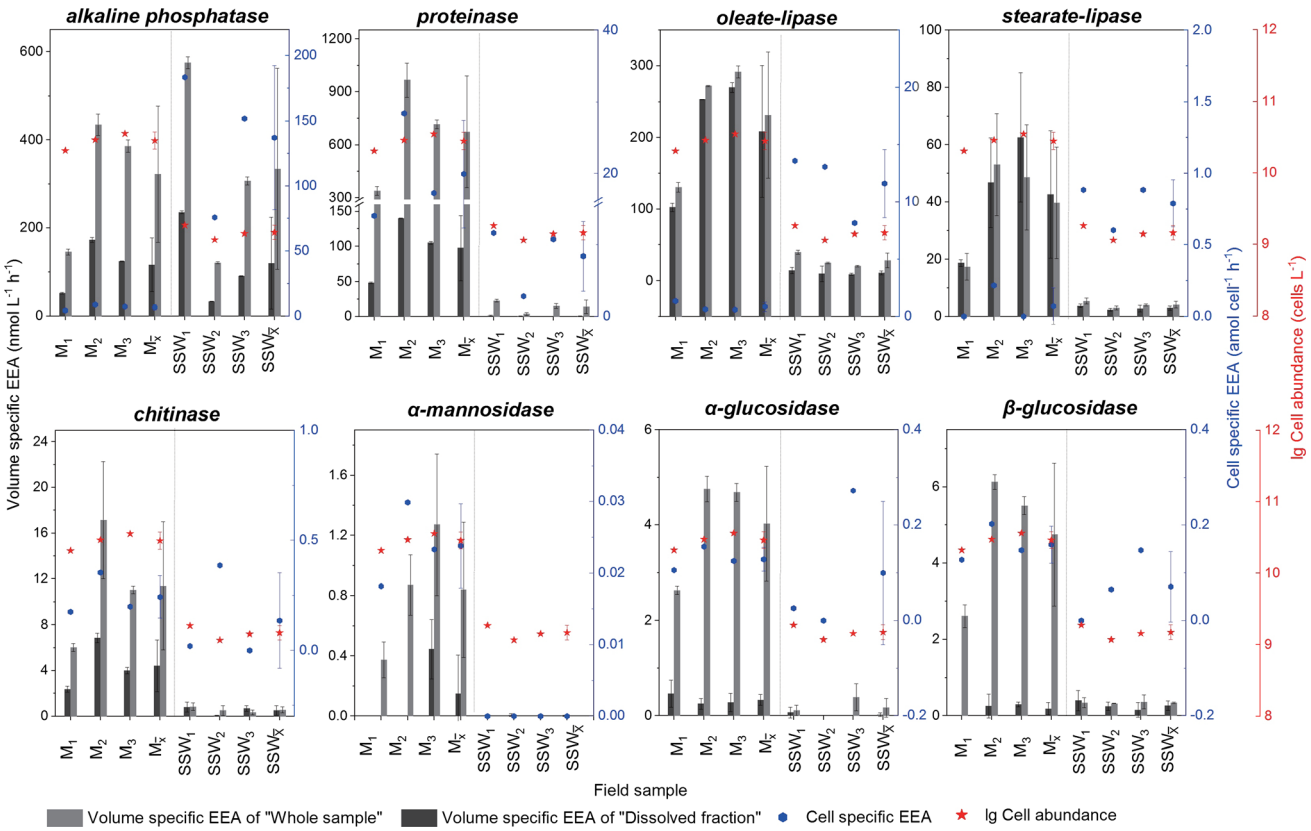

Fig. 4 Field sample EEAs and cell abundance for Mucus (M_1, M_2, M_3) and Surrounding seawater (SSW_1, SSW_2, SSW_3). M_X and SSW_X represent mean sample values, respectively. Error bars represent ± SD

become P-limited if the nutrient input has high N/P ratio (Lee and Fuhrman 1987). In nutrient replete waters subject to phytoplankton blooms, the rain of phytoplankton and detritus onto corals might be processed by bacterial hydrolases in the mucus layer including a critical role for alkaline phosphatase. Symbiodiniaceae either freshly isolated from corals or cultured showed extracellular alkaline phosphatase activity, which made it act as a sink of phosphate within the symbiosis (Godinot et al. 2009, 2013). Although we did not test the fate of the compounds targeted by alkaline phosphatase, our results agree with the hypothesis of persistence of high cell-associated alkaline phosphate supplying phosphorus to bacteria as well as to possibly contribute toward the P demand of Symbiodiniaceae in the holobiont environment. At a certain extent, our finding of high alkaline phosphatase as a common theme among the tested isolates and field samples suggests an important role for this enzyme activity in health and resilience of coral reefs.

In contrast, *proteinase* displayed the highest rates among mucus samples (673.8 ± 316.0 nmol L^{-1} h^{-1}) and was 48 times higher than the SSW (13.9 ± 9.6 nmol L^{-1} h^{-1}), which indicated much more active protein hydrolysis in mucus than in ambient seawater. This was a quite contrary scenario from proteinase activity of isolates. In addition, nearly all proteinase (85% in mucus and 95% in SSW) was cell associated versus dissolved in both sample types. Then, the speculation that hydrolysis of proteinaceous particle and polymers entering the mucus layer is the proteinase activity maintained through release by the coral animal is also excluded. Protein has been reported to be one of the richest mucus components of some corals (Meikle et al. 1988), and we deduce that mucus of *O. annularis* is also rich in proteins.

Activities of *oleate-lipase* and *stearate-lipase* in mucus were 231.4 ± 87.9 nmol L^{-1} h^{-1}, 39.7 ± 19.5 nmol L^{-1} h^{-1}, 8 and 20 times higher than SSW, respectively. Dissolved lipase in mucus samples reached 88–96%, while 40–72% in SSW. It has been concluded that dissolved ectoenzymes played a significant role in the hydrolysis of high-molecular weight substrates (Ziervogel and Arnosti 2008). Furthermore, a metabolome study (Ochsenkühn et al. 2018) has mapped along spatial transects from 0 to 50 cm from the coral a rich variety of lipids thus highlighting the holobiont as a hotspot in lipid production in oligotrophic environment.

Chitinase activity in mucus and SSW samples was 11.4 ± 5.6 and 0.6 ± 0.3 nmol L^{-1} h^{-1}, respectively. But

different from lipases, dissolved chitinase in mucus (39%) was less than in SSW samples (69%). Chitinase substrate *N*-acetyl-β-D-glucosamine we use is reported to be the main components of some coral mucus at lower temperature (Lee et al. 2016). In considering how the bacterial chitinase activity fits into the overall digestive system of the holobiont, we note that copepods and other chitin-rich food items are among the diet of the coral animal. Chitinase activity against the fungal pathogen, *Aspergillus sydowii*, has been detected from coral extracts (Douglas et al. 2007). Then, it would be interesting to study whether bacterial chitinase contributes to the holobiont processing and utilization of chitin, a C and N rich source for bacteria and for the coral animal.

Similar to isolate results, activities of both *α and β glucosidases* in mucus samples were at low level (4–4.7 nmol L^{-1} h^{-1}), but their activities in SSW samples were even lower (0.2–0.3 nmol L^{-1} h^{-1}). The *α-mannosidase* activity was even lower (0.8 nmol L^{-1} h^{-1} in mucus and not detectable in SSW). It is explainable as glucose was abundant while mannose was even undetected in *O. annularis* mucus (Klaus et al. 2007). In the context of coral holobiont, the mucus low glycosidase activity may lead to conservation of the structural integrity of mucus as bacterial environment. Also, another possibility of low glycosidase is that the assay substrates used here did not mimic major coral mucus carbohydrates such as fucose and arabinose (Klaus et al. 2007; Hadaidi et al. 2019).

Cell abundance and cell-specific EEAs

Similar to previous reports (Garren and Azam 2010), bacteria (Chl a negative cells under microscope) abundance was 20 times higher in mucus (2.8×10^{10} cells/L^{-1}) than in SSW (1.4×10^{9} cells/L^{-1}) (Supplementary Fig. 3). In addition, Chl a positive cells (Symbiodiniaceae and other smaller unknown autotrophs) were also detected in mucus (1.2×10^{8} cells/L^{-1}) and SSW (0.8×10^{8} cells/L^{-1}). Since no pretreatment was done to distinguish EEAs from Chl a negative or positive cells, here cell-specific EEAs were obtained by dividing volume-specific EEAs (excluding dissolved fraction) by summed cell abundance of both Chl a negative and positive cells.

In contrast to volume-specific EEAs, cell-specific EEAs except proteinase and glycosidases were lower in mucus samples than in SSW due to the great cell abundance variance (Fig. 4 right blue and red Y axes). Cell-specific alkaline phosphatase and lipase activities in mucus were 20 and 10 times lower than in SSW, respectively. However, it must be noted that cell-specific proteinase of mucus samples was still slightly higher than SSW, as mucus proteinase activity was dramatically high with only 5% free dissolved. Mucus and SSW varied in nutrient composition, biomass and microbiome community. The paradox of volume/cell-specific EEAs between mucus and SSW samples triggered more questions to be solved in future.

Potential role of EEAs in coral holobiont functioning and future directions

Microbiomes influence the patterns and rates of particle and polymer hydrolysis, and therefore, ectoenzymes of bacteria and archaea are a fundamental mechanism of organic matter processing. However, the ecological role of bacterial EEAs in coral holobiont is currently not fully explored. In this study, we investigated hydrolytic EEAs relevant to C, N and P cycling in 39 coral derived bacterial isolates in both low and high organic matter conditions, concomitantly with freshly collected coral mucus and surrounding seawater samples. One caveat is that, due to reality, isolates are from corals collected in the Florida Keys, while field samples were collected in Panama, but fortunately, they were from the same coral species. FASW used is from a coastal environment, but the source is clean and safe for coral culturing, aquarium use in institutes and many other commercial uses. So, we assume that our experiment is reliable. First, we found that there were no "super" isolates expressing high levels of all ectoenzymes. Second, the specific EEA profiles of a large suite of coral derived isolates should enable progress toward constructing the "ectoenzymome" of coral derived microbiome and address the degree of functional redundancy among the isolates. Third, field-collected mucus and surrounding seawater comprised distinct ectoenzyme profiles and intensities possibly due to large differences in bacterial abundance, diversity and chemical composition.

As another caveat, the 39 isolates available from our study were cumulatively considered as model coral microbiome. However, we most probably missed a large proportion of the coral-associated bacterial community diversity due to culture bias (Staley and Konopka 1985; Rohwer et al. 2001). Additionally, the diversity represented by the suite of 39 isolates used in this study was not analyzed at the strain level, so we cannot test if different strains are capable of different EEA-based functions, as would be the case when exploring micro-diversity (Thompson et al. 2004). Whether the suite of tested isolates functionally comprised representative coral community is not known. Further, the bacterial ectohydrolases' spatial and biochemical relationship within the coral holobiont is likely complex and currently unconstrained. Despite these limitations, some valuable insights can be gained from our culture-dependent and culture-independent measurements.

Our working hypothesis is that the cumulative ectoenzyme activity ("ectoenzymome") of coral microbiome is optimized for the functioning and resilience of coral

holobiont. However, currently there are significant methodological and conceptual constrains on fully testing this hypothesis. Coral-associated microbes are believed to be an important source of coral's overall digestive system enzymes for coral feeding of particles or zooplankton (Raz-Bahat et al. 2017), similar to the role of the microbiome in animal including human gut (Sommer and Bäckhed 2013). Microbiome's contribution to the holobiont's digestive capability is likely critical for coral functioning, but the study of this contribution in situ is not feasible with current methods. In comparison, our study using current methods was largely exploratory and opportunistic.

But it is unexpected that extremely large cell-specific EEA variation were observed among isolates belonging to the same genus or species in our culture-dependent measurements (Fig. 2). Thus, phylogenetically closely related bacterial taxa and communities within coral holobionts could potentially express very different enzymomes, indicating that ectoenzymome was not necessarily constrained by microbiome phylogeny. Microbial community in mucus layers were revealed to be variable with response to environmental change (Osman et al. 2020). Dynamics of ectoenzymome is a reflection of ecological recycle and is far more than microbial community shift. Biotic interactions and environmental variances played a role in driving microbial community functioning (Louca et al. 2018).

Active or environment stresses-induced pathogen invasion (Bourne et al. 2008; Mao-Jones et al. 2010; Rypien et al. 2010) allows more pathogens, opportunistic or not to grow explosively (Rosenberg et al. 2009). Understanding the influence of such perturbation on the coral ectoenzymome will help uncover the underlying mechanisms. In the case of *V. coralliilyticus*, we note that Ben-Haim et al. (2003) found released proteinase which was found to kill the coral. Most hypotheses about the role enzymes may play in pathogen infection or resistance are based on plant or insect studies, while little is known about coral-associated bacteria.

Size distribution of EEAs is another term of microbial incorporation (Unanue et al. 1993). We failed to measure the dissolved enzymes of coral isolates, but we measured this section in field samples. It was found that percentage of dissolved section varied depending on sample sources and enzyme types (Supplementary Table 3). The linkage between pelagic microbial loop and benthic systems of coral reefs (Bak et al. 1998) may well be affected by EEAs through dissolved section. Since it is also possible that organisms other than bacteria (such as coral animals, zooplanktons, phytoplankton) could have contributed to the measured enzyme pool, it is necessary to figure out the detailed EEA size distribution as well as its transfer in the whole coral ecosystem.

Measurement of microbiome EEAs in situ remains an important yet challenging goal whether it concerns the coral or human microbiome. Methods used in this study employing fluorogenic substrate analogs are operationally defined and reproducible under ideal conditions at least as applied to pure cultures. As such, our culture-dependent findings offer a model coral-associated bacteria ectoenzymome displaying diverse ectoenzyme profiles of the constituent coral isolates in both rich and poor organic matter conditions. Direct measurements on the microbiomes of coral mucus and the surrounding water, using the fluorogenic substrates, enable comparisons of EEAs in the two environments.

As corals are declining worldwide, there is an urgent need and much research interest in understanding the role coral-associated microbial assemblages—the coral microbiomes—play in coral health, disease and resilience. Future research directions are summarized to further better understand the coral holobiont from the aspect of "ectoenzymome".

New methods to explore the "ectoenzymome" in coral reefs under complex near-natural environments

Exploring the biochemical activity at the individual cell level within complex communities could benefit from future microscopy-based approaches, e.g., a concerted application of quantitative phenotypic imaging and phylogenetic stains.

Influence of environmental stresses on the ectoenzymome of coral microbiome

It contributes to our mechanistic understanding of the role of coral microbiome in the functioning and resilience of coral holobiont in the natural environment.

Culturomics and ectoenzymome of coral microbiome

It aims to comprehensively establish the ectoenzymome of bacterial strains or species identified from corals.

In situ EEAs' size distribution and their relationship with chemical composition and microbial community

It aims to clarify their source and transfer from coral holobiont to reef water and then reveal the role EEAs play in coral ecosystem.

EEAs and coral disease

It aims to figure out the role EEAs may play in pathogen invasion or holobiont defense.

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Declarations

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest. The views expressed in this article are those of the author(s) and do not necessarily represent the views or policies of the U.S. Environmental Protection Agency. Any mention of trade names, products, or services does not imply an endorsement by the U.S. Government or the U.S. Environmental Protection Agency. The EPA does not endorse any commercial products, services, or enterprises.

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