Chitinolytic activity of bacterial symbionts from the gorgonian coral *Eunicella labiata*

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Abstract

Corals are well known to live in association with complex microbial communities. These microbial symbionts are believed to produce many natural products and enzymes that are found in coral extracts and therefore are of great biotechnological interest. Chitin is the most abundant polymer in marine environments and its hydrolysis is mediated by chitinolytic enzymes, chitinases, which find wide industrial applications. Here, the chitinolytic capacity of 36 bacterial symbionts isolated from the octocoral *Eunicella labiata* (Thomson, 1927), is addressed using genomics and several *in-vitro* bioassays. Moreover, the relative abundances of chitinolytic genes within 20 microbial metagenomes of three octocoral species and their surrounding environments is investigated. Strong chitin-degradation on chitin agar plates was observed for 12 strains of the genera *Aquimarina (Bacteroidetes), Vibrio* and *Enterovibrio (Gammaproteobacteria),* with haloes >1cm. Extracellular endo-chitinase (EC 3.2.1.14) activity was detectable in all *Aquimarina* and *Vibrio* strains and quantified as 0.335 Units/L for *Aquimarina* (EL43) and 0.53 Units/L for *Vibrio* (EL36). Genome inspections revealed strong taxon-specific variations in the number of chitinase-encoding genes and, an unprecedented within- and across-taxon diversity of endo-chitinase (*chiA*) sequences. Metagenomic analysis showed that the frequency of chitinase-encoding genes in octocoral microbiomes was similar to that of seawater and sediments. Yet a significant enrichment of chitin-binding domains was found in octocoral microbiomes, likely enhancing the cells' binding capacity to chitin which could lead to more efficient chitin degradation. In conclusion, suspension-feeding octocorals are a valuable source of taxonomically diverse chitin-degrading bacteria and potentially novel biocatalyst enzymes.

Keywords: chitinase; chitin; *chiA*; microorganisms; octocorals; biotechnology

INTRODUCTION

Corals (phylum Cnidaria, class Anthozoa), the most important members of coral reefs, are often referred to as holobionts, a term that comprises the animal itself, its endosymbiotic dinoflagellates called "zooxanthellae" and its associated community of internal and external microorganisms (Rohwer *et al.,* 2002; Rosenberg *et al.*, 2007). In the coral holobiont we can find viruses and microorganisms like bacteria, archaea, fungi, alveolates and endolithic algae (Bourne *et al.,* 2009) present in several body parts of the coral, such as the mucus (Ritchie, 2006; Banin *et al*., 2001), the inner and superficial tissue (Williams *et al.,* 1987) and the calcium carbonate skeleton (Kushmaro *et al.,* 1996). Coral-microbiome interactions is a prevailing research topic, not only due to the current environmental concern of coral bleaching, but also due to the potential pharmaceutical, medical or biotechnological value of new natural products and enzymes synthesized by corals and their symbionts (Hernandez-Agreda *et al*., 2016). Microbial symbionts are likely the true producers of many

marine invertebrate-derived natural products (Davidson *et al.*, 2001; Piel *et al*., 2004).

Chitin is the polymer of (1→4)-β-linked N-acetylglucosamine (GlcNAc) and it is the most abundant polymer in the marine environment (Paulsen *et al.*, 2016; see **Figure 1**). It can be found in three crystalline forms: alpha- (α-), beta- (β-), and gamma- (γ-), differing in the orientation of chitin micro-fibrils. Chitin does not accumulate in marine habitats as it is hydrolyzed by marine microorganisms that can use chitin as a carbon, nitrogen and/or energy source (Beier & Bertilsson *et al.*, 2013). This process is often mediated by chitinolytic enzymes, the chitinases, that hydrolyze the β-1,4 glycosidic bonds between the GlcNAc residues, producing chito-oligosaccharides. There are two types of chitinases: endo-chitinases (EC 3.2.1.14), that cleave chitin randomly at internal sites, generating oligomers of GlcNAc, such as chitotetraose, chitotriose and the dimer chitobiose; and exo-chitinases (EC 3.2.1.52), that can be further divided into two subtypes: chitobiosidases, which catalyse the progressive release of chitobiose starting at the nonreducing end of the chitin microfibril; and N-acetyl-β-glucosaminidases, which cleave the oligomeric products of endo-chitinases and chitibiosidases generating monomers of GlcNAc (Cohen-Kupiec & Chet, 1998). Commonly, the endo-chitinase are extracellular enzymes which act outside the cell while the exo-chitinase N-acetyl-β-glucosaminidase frequently acts inside the bacterial cell (Beier & Bertilsson, 2013). In the biotechnological field, these enzymes present applications in the food, medical and agricultural sectors (Beier & Bertilsson *et al.*, 2013) since they have hypoallergenic, anti-cancer and antimicrobial properties and can enhance blood coagulation, lower cholesterol and be used in adhesives for wound healing (Fukamizo, 2000; Ngo *et al.*, 2008; Madhumathi *et al*., 2010; Yusof *et al*., 2003; Hudson, 1997; Rathke & Hudson, 1994; Hudson, 1998). A recent developing application for chitinases is their use to treat chitinous waste, mainly in the seafood industry (Wang *et al.*, 1995; Suginta *et al.*, 2000). Also, chitin and its derivatives are being studied for their use in novel drug delivery systems (Hata *et al*., 2000; Nsereko & Amiji, 2002; Xing *et al*., 2017; Komenek *et al.*, 2017; Shevtsov *et al*., 2018; Xu *et al.*, 2018).

Chitinolytic activities and genes have already been reported in some corals. A study on the gorgonian coral *Gorgonia ventalina* revealed that crude extracts from the coral contained detectable levels of exo-chitinase activity (Douglas *et al.*, 2007). Recently, Keller-Costa and colleagues reported 19 chitinase-encoding genes on the genome of *Aquimarina* sp. strain EL33, isolated from the gorgonian *Eunicella labiata* (Keller-Costa *et al*., 2016). Last year, Yoshioka and colleagues identified two chitinase-like genes in the genome of *Acropora digitifera.* Moreover, the authors demonstrated chitinolytic activity in seven coral species, which may indicate that chitinases are widely distributed in the coral-holobiont (Yoshioka *et al.*, 2017).

Enicella labiata (Thomson, 1927) is an octocoral (subclass Octocoralia) from the family Gorgoniidae (“gorgonians”) (Costello *et al.*, 2001) that can be found in the Atlantic Ocean and Mediterranean Sea (Coll *et al*., 2010), and is the most abundant gorgonian along the Southern and South-Eastern coast of Portugal (Curdia *et al*., 2013). It is a rich source of unique amino-diterpenoids (“labiatamids”) that possess cytotoxicity against human cancer cell lines (Berrue & Kerr, 2009; Roussis *et al.*, 2010). Apermanent culture collection of 175 bacteria (consisting of 36 unique phylotypes) associated with *E. labiata* has been established previously (Keller-Costa *et al*., 2017) and the authors demonstrated that many of these bacterial isolates are dominant members of the *E. labiata*

microbiome. This study now surveys the chitinolytic capabilities and chitin-degradation pathways of these 36 unique bacterial associates using *in-vitro* bioassays and genomics. Moreover, the relative abundances of chitinolytic genes within 20 microbial metagenomes of three octocoral species and their surrounding environments is investigated.

MATERIALS AND METHODS

2.1. Bacterial strains

The 36 bacterial isolates used in this study are part of an already established culture collection (Keller-Costa *et al*., 2017) available in our laboratory and derived from the gorgonian *Eunicella labiata*. Each of the 36 isolates has a unique 16S rRNA gene sequence.

Prior to chitinolytic activity assays, all strains were re-activated from glycerol stocks and grown in half-strength Marine Broth (1:2 diluted in artificial seawater, MB 1:2).

2. Preparation of colloidal chitin

Colloidal chitin (CC) was prepared from β-chitin powder (Sigma-Aldrich Química, S.L. Sintra, Portugal) following the procedures of Hsu & Lockwood (1975) and Kuddus & Ahmad (2013) with slight modifications. Briefly, 5g of chitin powder were slowly added to 150 mL of concentrated HCl (37%) and kept for 40 min at room temperature, with rigorous stirring. The solution was then transferred slowly to ice-cold distilled water (dH_2O) for CC precipitation. CC was filtered through coarse filter paper and washed by re-suspending it in 2L of dH_2O. The pH of this solution was then adjusted to 3.5 using 10M NaOH. A CC cake was then collected by filtering it through coarse filter, using a vacuum filtration system, autoclaved at 121ºC for 15 min and the sterile CC stored at 4ºC until it was used as substrate.

3. Chitin-degradation activity screening

In a preliminary assay, the 36 strains were tested for chitinase activity on CC agar medium plates containing artificial seawater (ASW), 0.15% potassium dihydrogen phosphate (KH_2PO_4), 1.5% agar and different concentrations of colloidal chitin (0.2%, 0.3% or 0.5%) and yeast extract (0.05% or 0.1%) or casamino acids (0.1%) as additional nutrient source. Since chitin haloes were only observed on medium consisting of 0.05 % yeast extract and 0.5% colloidal chitin, this medium composition was used for all further chitin agar plate assays. The CC agar plates were divided into four quadrants and, using a sterile micropipette tip, a small slot was stamped in each quadrant. Each of the four slots on one CC agar plate was then inoculated with 10 µL of a liquid culture (grown until late exponential phase on MB 1:2) of the same bacterial strain (4 replicates per plate). For each strain, at least two CC plates were prepared and monitored, making in total 8 replicates per isolate. One CC agar plate without any inoculation was kept as a negative control to monitor eventual contaminations. All plates were incubated at room temperature for two weeks (14 days). The whitish turbidity of the CC medium allows for visual evaluation of chitin degradation through clearing zones (haloes) around the inoculation spot. A semi-quantitative analysis of chitin-degrading activity was performed, by measuring the radius of the haloes produced (one measurement if the clearing zones were a regular circle and an average of four measurements if the clearing zones were asymmetric). Clearing zones with a radius up to 0.5 cm were classified with one plus (+), between 0.5 – 1 cm with two

plusses (++), and zones with a halo radius larger than 1 cm with three plusses (+++).

4. Chitinolytic activity assays

Chitinase enzyme activity was measured fluorometrically using a specific chitinase assay kit (Sigma-Aldrich Química, S.L., Sintra, Portugal) and a multi-mode microplate reader (Filter Max F5, Molecular Devices). The enzymatic activity was measured as the release of 4-methylumbelliferone (4-MU) from various 4-MU labelled GlcNAc-substrates. Exo-chitinase (EC 3.2.1.52) activities were detected using the substrates: **1)** 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (**M2133**) and **2)** 4-methylumbelliferyl N,N′-diacetyl-β-D-chitobioside hydrate (**M9763**) to detect N-acetyl- β-glucosaminidase (release of GlcNAc monomers) and chitobiosidase (release of GlcNAc dimers) activity, respectively. Endo-chitinase (EC 3.2.1.14) activity was detected using substrate **3)** 4-methylumbelliferyl β-D-N,N′,N″-triacetylchitotriose (**M5639**; release of GlcNAc trimers). All assays were performed at substrate concentrations of 0.5 mg/mL.

From a freshly grown liquid culture ("pre-inoculum"), 200 µL of all strains were re-inoculated into 20 mL of MB 1:2, followed by RT incubation at 45 rpm on an orbital shaker (Fisher Scientific) and grown for 4 days (72h), until they reached late stationary phase (monitored by measuring optical densities at 600 nm (OD_{600})).

To prepare enzymatic samples, 250 µL of these bacterial cultures were harvested after 24h, 48h, 36h or 72h and centrifuged for 15 min at 5,000 RCF, followed by removal of the cell pellet and transfer of the supernatant to a new 1.5 mL microtube. All enzymatic activities are extracellular activities as measurements were performed on culture supernatants and, 10 µL of supernatant were applied in each test.

All assays were performed at pH 5 in the assay buffer (A8730) provided with the kit for 60 min at 37 °C in a total reaction volume of 100 µL. The reaction was stopped by the addition of 200 µL sodium carbonate solution (**S2127**) and the fluorescence of released 4-MU was measured in the microplate reader immediately after the end of the reaction, at excitation (λ_{ex}) and emission (λ_{em}) wavelengths of 360 and 465 nm, respectively. The enzyme activity was expressed as the amount of 4-methylumbelliferone released per minute, where one Unit of chitinase activity corresponds to the release of 1 µmole of 4-MU from the appropriate substrate per minute, per litre.

For all readings, the released amount of 4-MU (MW=176,17 g/mol) was converted to units of chitinase activity / L using a 4-MU standard curve. To generate this standard curve, a 4-MU standard stock solution (40 mg/mL; **M3570**) provided with the kit was diluted 100, 1,000 and 10,000 times, to prepare three standard working solutions with concentrations of 400 µg/mL, 40 µg/mL and 4 µg/mL, respectively. To prepare 400 µg/mL, 1 µL of standard stock solution was added to 99 µL of stop solution (**S2127**), for 40 µg/mL, 10 µL of the 400 µg/mL solution were added to 90 µL of stop solution (**S2127**) and for 4 µg/mL, 10 µL of the 40 µg/mL were added to 90 µL of stop solution (**S2127**). The 4 µg/mL and the 40 µg/mL working solutions were then used to produce the standard curve with final concentrations of 0,076 nmol/mL (4ng in 0.3mL assay vol.), 0,152 nmol/mL (8ng in 0.3mL assay vol.), 0,76 nmol/mL (40ng in 0.3mL assay vol.) and 1,52 nmol/mL (80ng in 0.3mL assay vol.), respectively. The respective fluorescence values (at 465nm) were subtracted by a standard (assay buffer) blank for standard curve used to quantify enzyme activity (Units/L) in the samples. All fluorescence values of the samples were first subtracted by a substrate blank (substrate solution of 0.5mg/mL). An exo- and endo-chitinase mixture purified from the fungus

Trichoderma viride (C6252) served as a positive control.

5. PCR amplification of *chiA* genes

Genomic DNA from the 36 bacterial isolates was already available in the laboratory from the study of Keller-Costa *et al.,* 2017. To amplify the *chiA* gene from the genomic DNA, of the 36 strains, the primer pair *chiA*_F2 / *chiA*_R2 (Hobel *et al.,* 2005) was used, which generate amplicons of approximately 240 bp in size. Primer sequences were as follows: *chiA*_F2, 5'-CGT GGA CAT CGA CTG GGA RTW YCC-3' and *chiA*_R2, 5'-CCC AGG CGC CGT AGA RRT CRT ARS WCA-3' (Hobel *et al.,* 2005). Amplification was carried out using 50-µL PCR reactions. Each reaction mixture consisted of 27,76 µL ultrapure water, 5 µL of 10x reaction buffer (Bioline®), 5 µL of 2mM deoxynucleoside triphosphates (dNTPs), 3.74 µL of 50 mM $MgCl_2$ (Bioline®), 4 µL of 50% acetamide, 1 µL of 10 µM of each primer, and 0.5 µL of 5 U/µL Taq DNA polymerase (Bioline®). For all the strains, except EL24 and EL43, 2 µL of template DNA (ca 20 ng/µL) was used in the reactions. For EL43 template DNA volume was increased to 4 µL after amplification with 2 µL failed. For strain EL24 a reaction without acetamide and with 3 µL of template DNA was used. The reaction mixture was denatured at 95ºC for 3 min followed by 35 thermal cycles with 45 sec of denaturation at 95°C, 45 sec of primer annealing at 47°C, and 90 sec of elongation at 72°C. A final extension was performed at 72ºC, for 8 min.

All PCR products were subjected to electrophoresis on 1.2% agarose gels and visualized under UV light.

6. Sequencing of the amplified *chiA* genes

The PCR amplified *chiA* genes from the bacterial isolates were Sanger sequenced. PCR products were purified on Sephadex G-50® columns and then shipped for sequencing at STAB VIDA (Caparica, Portugal). Nucleotide sequences were quality-checked, trimmed and edited using the sequence scanner software v. 2.0 from Applied Biosystems®. Only high-quality sequences were used in downstream phylogenetic analyses.

7. Phylogenetic analysis

Partial *chiA* sequences corresponding to the PCR products were compared directly with the nucleotide collection (nr/nt) and the whole-genome shotgun contigs (wgs) databases using the nucleotide BLAST from NCBI. They were also compared to endo-chitinase sequences obtained from bacterial genomes from a variety of gorgonian and marine sponge bacteria from our laboratory available on the Rapid Annotation Using Subsystem Technology (RAST), 2.0 server (http://rast.nmpdr.org; Aziz *et al.,* 2008).

All *chiA* sequences were aligned using the program MEGA7 (Kumar *et al.,* 2016), with the ClustalW method. Phylogenetic tree was created in MEGA7 using a maximum likelihood method, recurring to partial deletion with a 95% cut-off. Prior to tree construction, an analysis for the most suitable evolutionary model was performed and it was found that Tamura Nei Model was most suitable. To validate the tree, 100 bootstrap repetitions were generated. The percentage of

trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. (Kumar *et al.,* 2016).

8. Relative abundance of genes involved in chitin degradation in gorgonian, sediment and seawater metagenomes

Metagenomic DNA from 3x *sediment* samples, 4x *seawater* samples and three gorgonian species (3x healthy *Eunicella gazella,* 3x diseased *Eunicella gazella*, 4x *Eunicella verrucosa* and 3x *Leptogorgia sarmentosa*) sampled of the coast of Faro (Algarve, *Portugal*)

in summer 2014, was previously shot-gun sequenced on an Illumina HiSeq 2500 platform in the framework of the research project EXPL/MAR-EST/1664/2013. A functional analysis of this metagenome dataset (comprising 20 metagenomes) was performed using the European Bioinformatics Institute (EBI) metagenomics platform (https://www.ebi.ac.uk/metagenomics; Mitchell *et al.*, 2017) and an IntrerPro (IPR) output table with functional categories was retrieved. The number of sequence reads in the IPR table was then Hellinger transformed (i.e. square root of relative abundance of reads). Several IPR entries related to the same chitin degrading-related function (i.e. chitin-binding protein, chitinase, chitobiase, etc) were joined and summed together to represent the relative abundance of each inspected function in the corresponding metagenome. Thereafter, mean relative abundance values and standard errors where calculated for each analysed function in each micro-environment and statistical analysis was performed. Normality was tested using the Shapiro-Wilk-Test. A non-parametric Kruskal-Wallis-Test on ranks was used followed by a Dunn's post-hoc test if significant, since data did not follow a normal distribution.

RESULTS

1. Colloidal Chitin Agar Plate Assay

To test whether the cultured bacterial symbionts were able to degrade chitin, an assay with agar plates containing colloidal chitin was performed. Of the 36 strains, 12 were found to degrade chitin forming typical clearing zones (haloes) around the inoculation spot (**Figure 1**). The *Bacteroidetes* strains *Aquimarina* sp. EL33 and EL43 and the *Gammaproteobacteria* strains *Enterovibrio* sp. EL24 and EL37 and *Vibrio* sp. EL49 and EL112 formed the largest haloes with radius above 1.0 cm, hence being potential targets for more quantitative and detailed experimental analysis. In contrast, none of the *Alphaproteobacteria* strains, including representatives of the genera *Ruegeria, Labrenzia, Sphingorhabdus, Kiloniella, Roseovarius, Pseudophaeobacter* and *Sulfitobacter* showed any chitin-degrading activity in this plate assay.

2. Chitinase Assay

A chitinase enzyme assay was performed to verify if the chitin-degrading capacity observed on chitin-agar plates is due to the activity of chitinases and to quantify this chitinase activity. In a preliminary chitinase enzyme assay all 3 chitin substrates were tested on *Aquimarina* sp. EL33 and EL43 culture supernatants. These tests revealed that substrate 3 (4-methylumbelliferyl β-D-N,N′,N″-triacetylchitotriose; detecting endo-chitinase activity) yielded higher activity values. Hence, all further tests described here correspond exclusively to endo-chitinase (EC 3.2.1.14) activity. To confirm that the endo-chitinase activities detected in the *E. labiata* isolates indeed resulted from the growing bacterial strains, activity was measured also in liquid growth medium (MB 1:2), which showed, as expected, zero enzymatic activity (negative control). In addition, *Ruegeria* sp. strain EL09 (*Alphaproteobacteria*) was chosen to corroborate our results. Since EL09 did not show any chitin degrading activity on the CC agar plates we also did not anticipate any activity in the endo-chitinase assay and, indeed, no activity was detected for the strain. In contrast, *Vibrio* sp. strain EL36, one of the most active chitin degraders on the colloidal chitin agar plate assay, showed activity (0.297 units/mL, when grown for 24 h (OD_{600}: 1.952, probably late exponential

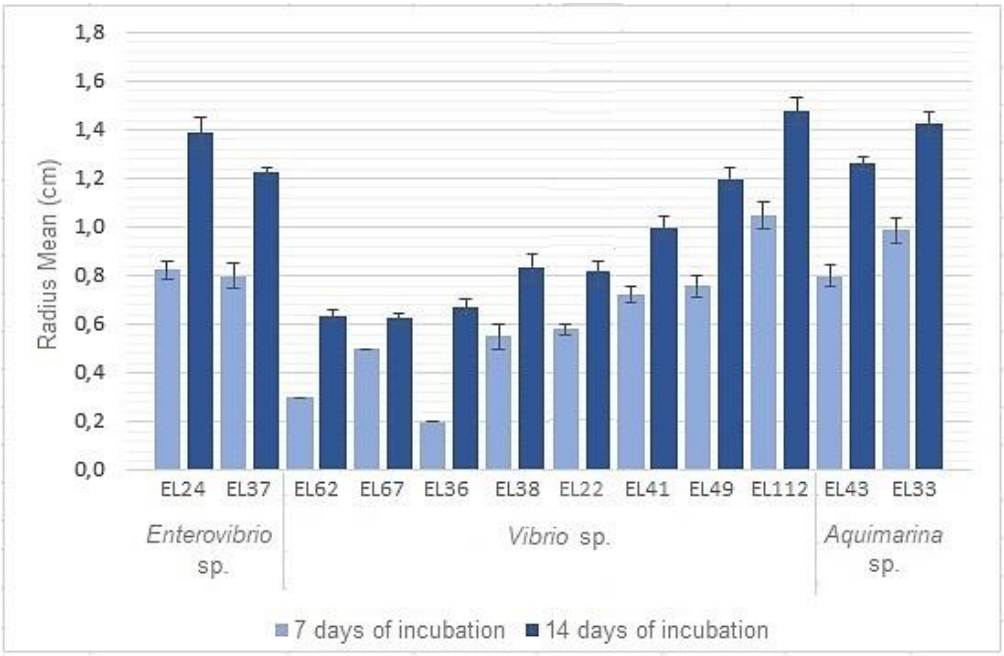

Figure 1. Chitin-degrading activity of 12 bacterial strains isolated from the gorgonian *E. labiata*, presented as the mean halo size (radius in cm) and respective standard error (*N=8*) for each strain after 7 days (light blue) and 14 days (dark blue) of incubation in chitin-agar plates.

phase), and 0.530 units/mL, when grown for 73h (OD_{600}: 1.280, probably late stationary phase), even higher than the activities obtained for the two *Aquimarina* strains.

All chitin-degrading strains had their endo-chitinase activity measured after both 24h and 36h, and the respective highest endo-chitinase activity value is presented for each strain (**Table 1**).

3. *chiA* PCR amplification from genomic DNA

A *chiA* gene-specific PCR was performed for all 36 strains used in this study but amplicons of the correct size and quality for sequencing could only be obtained for 12 strains. In general, a positive *chiA* PCR result appears to be a good predictor of the capability to degrade chitin *in-vitro*. However, one *Vibrio* strain (EL41), gave a negative PCR result but was able to degrade chitin on the chitin agar plate assay. On the contrary, one *Aliivibrio* strain (EL57), gave a positive PCR result but did not show any chitin degrading activity on chitin agar medium.

4. Phylogenetic analysis of the *chiA* gene

Phylogenetic inference with a maximum likelihood approach was used to investigate how closely related these *chiA* genes of different bacterial species are (see **Figure 2**). Several *chiA* sequences formed taxon-specific clusters that follow – at least in part – 16S rRNA gene based phylogenies, However, some *chiA* sequences and clusters disregarded 16S rRNA gene phylogeny and did not align with their taxonomic affiliation. E.g. *Aquimarina* sp. Aq107 isolated from a marine sponge was clustering within a *Vibrionaceae* cluster (**Cluster III**). Also, most *Aliivibrio chiA* sequences clustered separate (**Cluster IV**) and away from the large *Vibrionaceae* group (**Cluster III**), contradicting 16S rRNA gene-based phylogeny.

5. Chitin-degradation related genes in microbial metagenomes

The relative abundances of chitin degradation-related-genes in the microbial metagenomes of *Eunicella gazella, Eunicella verrucosa, Leptogorgia sarmentosa*, sediment and seawater were estimated (see **Figure 3**). Chitin-binding

Table 1. Endo-chitinase activity of all chitin-degrading strains, grown for 24h or 36h, and respective OD_{600} values.

	Genus	Grown for (h)	OD_{600}	Chitinase activity *Units/L*
EL22	***Vibrio***	36	0.363	0.956
EL24	***Enterovibrio***	36	0.254	0.663
EL33	***Aquimarina***	24	0,532	0.048
EL36	***Vibrio***	36	0.313	0.440
EL37	***Enterovibrio***	24	0.188	0.024
EL38	***Vibrio***	36	0.372	1.259
EL41	***Vibrio***	24	0.250	0
EL43	***Aquimarina***	36	1,748	0.335
EL49	***Vibrio***	24	0.407	0.474
EL62	***Vibrio***	24	0.428	0.323
EL67	***Vibrio***	24	0.405	1.235
EL112	***Vibrio***	36	0.248	0

chitobiase (the enzyme that hydrolyses GlcNAc dimers into monomers) encoding genes presented a significant difference between environments, with overall p-values of ≤0.05 and 0.01, respectively (see **Figure 3**, **a)** and **b)**). The relative abundance of genes encoding for chitin-binding proteins was significantly higher in the *Eunicella gazella* microbiome than in sediment (p≤0.001). There was also a higher abundance of chitin-binging protein encoding genes in *E. verrucosa* compared to sediment (p=0.03). In contrast, the relative abundance of chitobiase encoding genes was significantly reduced in all three gorgonians compared to sediment (*E. gazella* vs. sediment: p=0.02; *E. verrucosa* vs. sediment: p≤0.01; *L. sarmentosa* vs. sediment: p=0.02). The relative abundance of chitobiase encoding genes was also significantly reduced in *Eunicella verrucosa* compared to seawater (p=0.02). However, relative abundances of chitinase-encoding genes were statistically similar in corals, seawater and sediment (see **Figure 3**, **c)**).

DISCUSSION

This study found octocoral associated bacteria of the genera *Vibrio*, *Enterovibrio* and *Aquimarina* to be efficient chitin degraders while diverse *Alphaproteobacteria* associates were generally observed as non-degraders. These results are in accordance to previous studies that reported *Aquimarina longa* (Xu *et al.,* 2015), *A. addita* (Yi & Chun, 2011) and *A. salinaria* (Chen *et al*., 2012) as able to grow on chitin. Another study reported chitinase activity in several *Vibrio* sp. isolates (Suginta *et al.,* 2000). All strains that were able to degrade chitin on agar plates, also showed detectable levels of extracellular endo-chitinase (EC 3.2.1.14) activity. With the revealed presence of endo-chitinase activity in the understudied genus *Aquimarina*, one of the future steps will be the purification of endo-chitinase proteins from octocoral-derived *Aquimarina* strains, to achieve more accurate quantifications of their endo-chitinase activity in Units/mg enzyme and to investigate enzyme performance under varying environmental conditions (e.g. pH and temperature). While a previous study reported the molecular mass of an endo-chitinase from *Vibrio cholerae* (88.7 kDa; Connell *et al.,* 1998), to our knowledge, no studies have so far investigated the molecular mass, structure and properties of *Aquimarina*-

Figure 2. Phylogenetic inference based on the Tamura-Nei model (Tamura & Nei, 1993) of *Aquimarina* and *Vibrio chiA* PCR sequences, best sequence hits of these products from allignments on NCBI (blastN) and sequences of chitinases from reference and sequenced genomes obtained from RAST. The analysis involved 43 nucleotide sequences and a total of 129 positions in the final dataset.

-derived chitinases. Moreover, the conditions used in this assay (pH 5, 37ºC etc) are optimized to detect chitinase activities in terrestrial fungi and mammalian cells, while they may not be ideal for microorganisms that thrive in habitats with considerably higher pH and lower temperatures. Future assays should investigate the optimum pH and temperature conditions of endo-chitinases from marine bacteria and, particularly, *Aquimarina* spp.

Generally, the strains that showed chitin degrading ability on agar plates had a *chiA* gene on their genome that was amplifiable with the *chiA* specific primer pair used in this study. However, *Aliivibrio* sp. EL57 showed no chitin degrading activity but presented a positive *chiA* PCR result. This may be due to the inactivation of the *chiA* gene (or another gene obligatory for chitin degradation) or an insufficient up-regulation of its expression under the culture conditions used in this study. On the contrary, *Vibrio* sp. EL41 had a negative *chiA* PCR result despite being a chitin degrader. Possibly, the *chiA* PCR primer set used in this study did not match the chitinase sequences of this *Vibrio* strain.

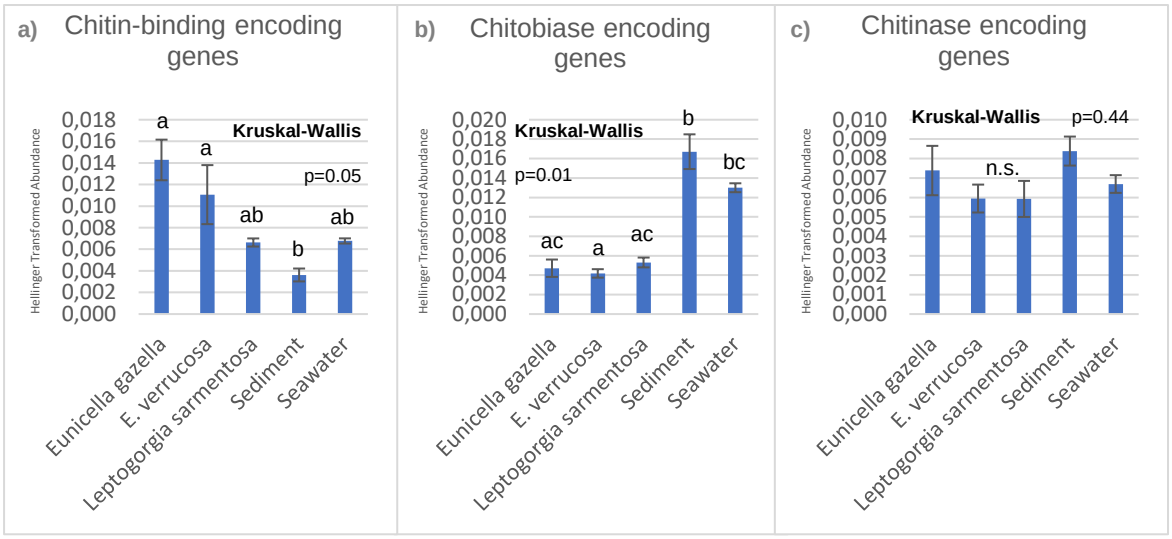

Figure 3. Relative abundance of genes involved in chitin degradation present in gorgonian, sediment and seawater microbial metagenomes. InterPro (IPR) relative abundances (mean ± SE) after Hellinger transformation shown. Letters above error bars indicate significant differences (Kruskal-Wallis-Test on Ranks, Dunn's post-hoc test if significant).

The phylogenetic analysis conducted in this study shed light on the remarkable heterogeneity of the *chiA* gene, revealing that it is much more divergent than the 16S rRNA gene, which suggests that the it is evolving at a much faster rate. Also, Hunt and colleagues found that, when a different set of genes (hsp60, mdh and adk) was used to create a phylogeny including *Vibrio* isolates that had grown on chitin but had negative *chiA* PCR results, some *Vibrio* clustered together with *Photobacterium*. Genomes of *Photobacterium* are known to have divergent *chiA* sequences, suggesting that processes of lateral gene transfer (LGT) and/or gene duplication may occur in core chitin degradation genes, including the *chiA* gene (Hunt *et al*., 2008). Other studies also reported on the occurrence of LGT events and duplication of the *chiA* gene, and on how difficult it is to construct phylogenies based on it (Cottrell *et al.,* 2000). Here, most *Aliivibrio chiA* sequences formed a separate cluster, apart from other *Vibrionaceae chiA* sequences, while the *chiA*s from *Aquimarina* sp. strains EL43 and Aq107 clustered among *chiA* sequences of *Vibrio* and *Enterovibrio*. Such heterogeneity of the *chiA* gene eliminates the possibility of using it as a reliable phylogenetic marker gene. Moreover, this high genetic variability suggests that the endo-chitinase (EC 3.2.1.14) *chiA* gene is not essential for survival. Yet its presence can be a competitive advantage in chitin-rich micro-habitats, offering more versatile nutrient scavenging abilities. The metagenomic analysis performed revealed that octocoral microbiomes had an enrichment in chitin-binding genes, that enhance the cells' capacity to adhere to chitin substrates, which can indicate a higher chitin-processing efficiency in the octocoral micro-habitats. However, no difference was found for the relative abundance of chitinase-encoding genes, which is in line with PCR results from this study where the endo-chitinase *chiA* gene was amplified from all micro-habitats. Chitobiase-encoding genes (responsible for the cleavage of N-acetylglucosamine dimers into monomers) were higher in sediment and seawater, suggesting a higher efficiency in the processing of the cleaved oligomers in these habitats. In conclusion, suspension-feeding octocorals are a valuable source of taxonomically diverse chitin-degrading bacteria and potentially novel biocatalyst enzymes.

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