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

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Biotechnology

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“However difficult life may seem,

There is always something you can do and succeed at.

It matters that you don’t just give up.”

- Stephen Hawking

To the kindest, the brightest, my dad.

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Resumo

Os corais são conhecidos por viverem em associação com uma complexa comunidade microbiana. Os simbiontes produzem muitos dos produtos naturais e enzimas, com interesse biotecnológico, encontrados em extractos de corais. A quitina é o polímero mais abundante do oceano e a sua hidrólise é mediada por enzimas quitinolíticas – quitinases – com inúmeras aplicações. Neste trabalho, a capacidade quitinolítica de 36 simbiontes bacterianos isolados do coral *Enicella labiata* (Thomson, 1927), é estudada recorrendo a genómica e ensaios biológicos *in-vitro*. As abundâncias relativas de genes quitinolíticos de 20 metagenomas microbianos de octocorais e do seu ambiente envolvente são investigadas. Foi observada degradação da quitina para isolados de *Aquimarina* (*Bacteroidetes*), *Vibrio* e *Enterovibrio* (*Gammaproteobacteria*). Foi detectada actividade extracelular da enzima endo-quitinase (EC 3.2.1.14) em todas as estirpes de *Aquimarina* e *Vibrio,* com actividades de 0.335 Unidades/L e 0.53 Unidades/L, respectivamente. Encontraram-se variações amplas no número de genes que codificam quitinases nos géneros investigados, bem como uma alta diversidade de sequências de quitinases (*chiA*) nestes géneros. A frequência de genes envolvidos na degradação da quitina em microbiomas de octorais foi semelhante à da água do mar e de sedimentos. Contudo, um aumento significativo nos domínios de ligação à quitina foi encontrado nos microbiomas dos octocorais. A composição taxonómica dos organismos que degradam quitina é diferente entre os microbiomas acima mencionados, de acordo com uma análise da diversidade do gene *chiA*. Para concluir, organismos filtradores como os octocorais são uma fonte valiosa de bactérias taxonomicamente diversas que degradam quitina e, potencialmente, novas enzimas bio-catalíticas.

Palavras-chave: quitinases; quitina; octocorals; microorganismos; biotecnologia.

Abstract

Corals 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, being 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 applications. Here, the chitinolytic capacity of 36 bacterial symbionts isolated from the octocoral *Enicella labiata* (Thomson, 1927), is addressed using genomics and several *in-vitro* bioassays. Moreover, relative abundances of chitinolytic genes within 20 microbial metagenomes of octocorals and their surrounding environments is investigated. Strong chitin-degradation was observed for 12 strains of the genera *Aquimarina (*Bacteroidetes*), Vibrio* and *Enterovibrio (Gammaproteobacteria).* 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 chitinase (*chiA*) sequences. Metagenomic analyses 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 and its degradation. Moreover, the taxonomic composition of chitin-degrading microorganisms differed between the microbiomes of gorgonian corals, sediments and seawater as revealed by a metagenomics-centred analysis of *chiA* gene diversity. In conclusion, suspension-feeding octocorals are a valuable source of taxonomically diverse chitin-degrading bacteria and potentially novel biocatalyst enzymes.

Keywords: chitinase; chitin; octocorals; microorganisms; biotechnology.

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4-MU – 4-methylumbelliferone

a.a. – Amino acids.

ABC – ATP-binding cassette.

ASW – Artificial sea water.

BLAST – Basic local alignment search tool.

CC – Colloidal chitin.

EBI – European bioninformatics institute.

EL – *Eunicella labiata*.

EV – *Eunicella verrucosa*.

GlcNAc – N-acetylglucosamine

GTA – Gene transfer agents.

HCl – Hydrochloric acid.

iBB – Institute for bioengineering and biosciences.

IP – Interpro.

IST – Instituto superior técnico.

KH_2PO_4 – Potassium dihydrogen phosphate.

LGT – Lateral gene transfer.

MB – Marine broth.

MGE – Mobile genetic elements.

NCBI – National center for biotechnology information.

NaOH – Sodium hydroxide.

OD_{600} – Optical density at 600 nm.

OTU – Operational taxonomic unit.

PTS – Phosphotransferase system.

PUL – Polysaccharide utilization loci.

RAST – Rapid annotation using subsystem technology.

RCF – Relative centrifugal force.

RT – Room temperature.

SD – Sediment.

Sus – Starch uptake system.

SW – Seawater.

INTRODUCTION

The ocean represents two thirds of the planet surface and it remains quite unexplored, as well as the vast diversity of marine ecosystems and their potential applications. Coral reefs are the most diverse of all marine ecosystems and although there is still much to unveil about their diversity, microorganisms are likely the most varied element of these communities (Rohwer et al., 2002). Microorganisms are the most abundant and diverse biological entities on this planet and usually live in consortia through highly dynamic interactions. Studying these consortia is of great importance since it will lead to a better understanding of their ecological role in bio-chemical cycling and -ecosystem functioning and, hopefully, to the identification of new biosynthetic pathways and metabolites (Dinsdale *et al.,* 2008). Therefore, one of the main focuses of marine research is to understand the composition and ecology of coral microbiomes.

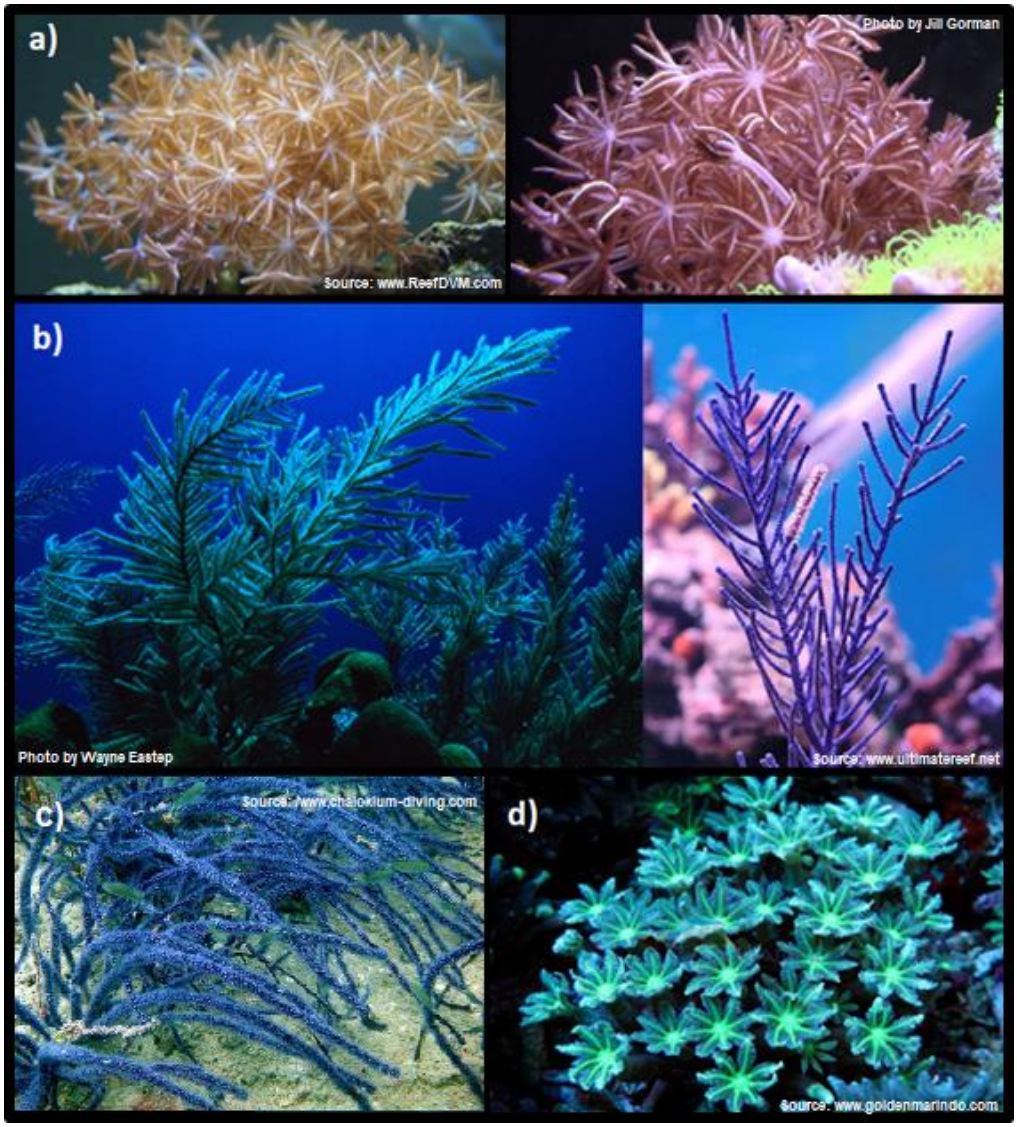


Figure 1. Under-water photographs of some of the most prolific soft coral species, regarding bioactive compounds production: a) *Xenia* sp. (source: www.ReefDVM.com (left); photo by Jill Gorman (right)); b) *Pseudopterogorgia* sp. (photo by Wayne Eastep (left); source: www.ultimatereef.net); c) *Junceella* sp. (source: www.chaloklum-diving.com); d) *Clavularia* sp. (source: www.goldenmarindo.com)

Corals (phylum Cnidaria, class Anthozoa), the most important members of coral reefs (see **Figure 1**), 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 are a topic of interest within the scientific community, not only due to the current environmental concern of coral bleaching caused by the loss of their "zooxanthellae" symbionts under elevated water temperatures, but also due to the potential pharmaceutical, medical or biotechnological value of new natural products and enzymes synthesized by corals and their symbionts (Bayer *et al.,* 2013; Hernandez-Agreda *et al*., 2016).

1. Marine biotechnology

In marine biotechnology, marine organisms are exploited to produce or modify specific natural products for industrial applications. Until recently, the pharmaceutical industry was largely based on the chemical synthesis of drugs. Natural products are revolutionizing drug production as they are more promising for an efficient and economic development of new pharmaceutics than extensively synthesized chemicals (Kong *et al*., 2010). Data on the sources of new drugs from 1981 to 2014 show that more than half of the drugs approved during this period were based on natural products, including from marine invertebrates (Kurtböke, 2017).

Over previous decades, there was an exponential increase in the number of new natural products isolated from marine sources and marine invertebrates of the phyla Porifera (sponges) and Cnidaria (mainly octocorals), followed by Mollusca, Arthropoda and Echinodermata, are among the most prolific ones (Mehbub *et al.,* 2014). Yet in the very last decade the numbers of novel natural products derived from marine microbes, particularly fungi and bacteria has been steadily increasing and are now surpassing marine invertebrates (see **Figure 2**). Most interestingly, nearly 50% of these microbial producers are host-associated, particularly with octocorals and marine sponges (Raimundo *et al*., submitted; Blunt *et al*., 2015).

These numbers reveal that the interest in and knowledge about natural products from marine (host-associated) microbes is growing. Marine natural products are now known to have a wide range of therapeutic properties, such as antimicrobial, anti-inflammatory, antioxidant, anticancer, anticoagulant, wound healing, immune modulator and other medicinal effects. They are the basis of many active ingredients of novel marine drugs, contributing to the development of the medical and pharmaceutical field (Senthilkumar & Kim, 2013).

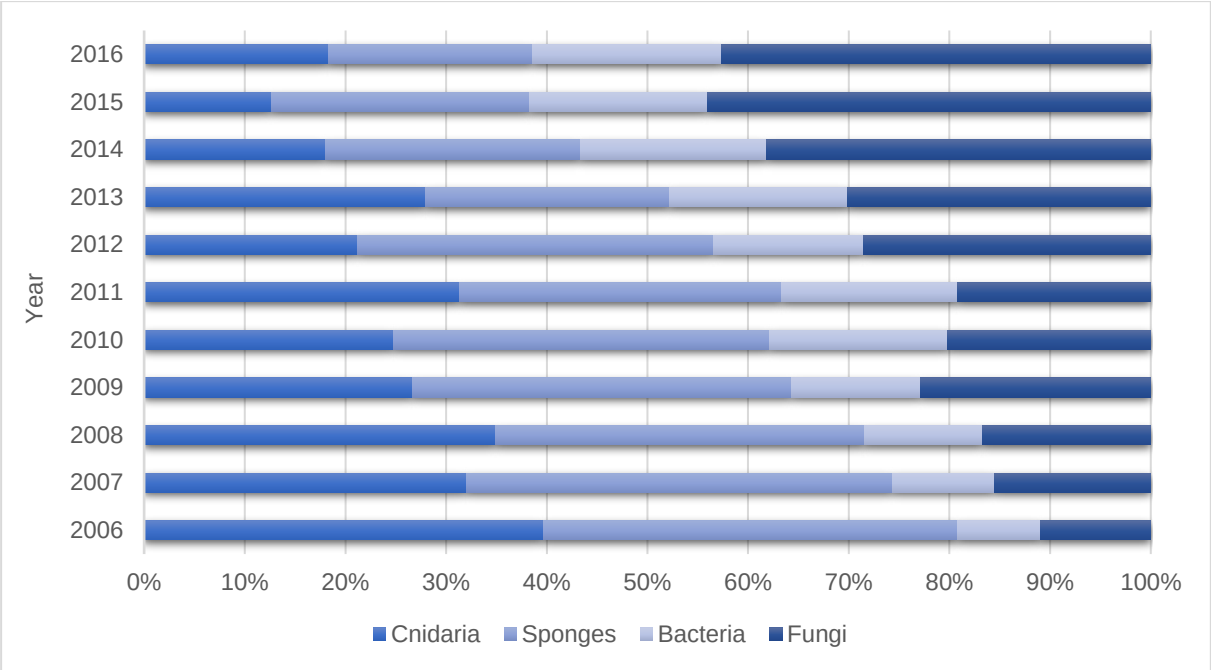


Figure 2. Overview of new marine natural products discovered in cnidarians, sponges, bacteria and fungi between 2006 and 2016. Data were retrieved from review articles published by Blunt *et al*., from 2008 to 2018.

A major milestone for the increasing interest in natural products of marine origin was the discovery of spongouridine and spongothy-midine by Bergmann and Feeney, in 1951, which are the first marine-derived bioactive compounds, both isolated from the marine sponge *Tectitethya crypta* (Bergmann & Feeney, 1951). Microbial symbionts are likely the true producers of many of these products, as evidenced for several polyketides such as the potential Anti-Alzheimer agents Bryostatins (Davidson *et al.,* 2001) or the cytotoxic Onnamide A (Piel *et al*., 2004). It is now believed that most of the biologically active compounds derived from marine invertebrates, like sponges, bryozoans and corals, are produced by their microbial associates and, compared with bioactive compounds from terrestrial organisms, marine natural products are novel regarding their bioactivities and chemistry (Kong *et al*., 2010).

2. Coral holobiont – diversity and function

Although coral-microbe associations have been described since the 1970s (Ducklow & Mitchell, 1979), a deeper exploration of this field was only accomplished in the beginning of this century, due to the development of new sequencing technologies, and it is currently an important and outgrowing research topic (Rohwer *et al.,* 2001; Wegley *et al.,* 2007; Bayer *et al*., 2013; Hernandez-Agreda *et al*., 2016; Peixoto *et al.*, 2017).

Bacteria are dominant members of coral microbiomes, representing up to 80 – 90 % of the coral-associated microbiota (Mouchka *et al*., 2010). *Proteobacteria* is usually the most represented phylum, with orders like *Rhodobacterales, Vibrionales, Alteromonadales* and *Oceanospirillales* being the most common ones. Other dominant phyla of the coral bacteriome are *Actinobacteria, Cyanobacteria* and *Bacteroidetes* (Bourne *et al.,* 2016). Also, *Firmicutes, Verrucomicrobia* and *Spirochaetes* are frequently

present but less abundant members of coral microbiomes (Mouchka *et al*., 2010; Ainsworth *et al*., 2015; Wegley *et al*., 2007; Hernandez-Agreda *et al.,* 2016).

Some reports suggest that 99% of microorganisms in natural habitats are uncultivatable (Whitman *et al*., 1998), meaning that many of the studied communities have a lot more diversity than initially estimated and described by cultivation-dependent methods. This uncultivability is because many cultivation media were designed to target only specific (often human microbiome related) species, do not mimic natural environmental conditions, or, traditionally contain ingredients in high concentrations (e.g. high carbon content, agar as gelling agent) that inhibit the growth of many environmental bacteria (Streit & Schmitz, 2004). Despite still being a challenge for the study of coral-associated microbes, efforts are being done towards the cultivation of these so-called "unculturable" microorganisms and several studies describing the diversity and function of the coral microbiome have already been published (reviewed in Bourne *et al.,* 2016). They revealed that coral-microorganism associations play key roles in the development and survival of the host.

Through photosynthesis, *Symbiodinium* associates supply up to 95% of the coral's carbon requirements (Muscatine *et al.*, 1984). In addition, we can find bacterial symbionts that are able to fix nitrogen (Lesser *et al.,* 2004; Lema *et al.,* 2014), metabolize sulfur (Raina *et al*., 2009), participate in biogeochemical cycling (Kimes *et al.,* 2010) and even produce antibiotic compounds (Reshef *et al*., 2006; Ritchie *et al*., 2006). The production of these bioactive compounds by symbiotic microbes is likely involved in the control of coral pathogens and it is believed that they are also used as a defence mechanism to keep predators away, to avoid the colonization and overgrowth of the coral surface by macroalgae or bacterial biofilms (Slattery *et al*., 1995; Pawlik *et al.,* 1987). The *Symbiodinium* and other microalgae symbionts produce dimethylsulfoniopropionate (DMSP), that is a compound that can be degraded by specific bacteria and is believed to control bacterial populations on the coral surface (Raina *et al.,* 2009). Mycosporine-like amino acids (MAA) are also produced by algae symbionts and protect the coral tissue against ultraviolet radiation (Dunlap & Shick, 1998). Quorum Sensing (QS) signal molecules have also been described, and they allow microbial interactions within the holobiont, the control of bacterial colonization, pathogenesis and extracellular enzyme production (Tait *et al.,* 2010; Sharp & Ritchie, 2012; Certner & Vollmer, 2015). In this context, the new term "the coral holobiont" was introduced and it refers to a complex assemblage including the coral animal and all its associated microorganisms as an ecological unit (Rohwer *et al.,* 2002; Rosenberg *et al.*, 2007). Overall, the association between microbes and corals seems to have important functional benefits to the coral holobiont (Rohwer *et al*., 2001; Bayer *et al*., 2013; Ainsworth *et al*., 2015).

3. Chitin and its biotechnological potential

Chitin (see **Figure 3**) is the polymer of (1→4)-β-linked N-acetylglucosamine (GlcNAc). Right after cellulose, it is the second most abundant polymer in nature, being the most abundant in the marine environment (Paulsen *et al.,* 2016). It was discovered in France, in 1811, by the scientist Henri Braconnot, but the industrial and commercial interest on this enzyme only started in the 1970s, with a consequent increase in the knowledge of this molecule (Roberts, 1992). Chitin can be classified in three

crystalline forms: alpha- (α-), beta- (β-), and gamma- (γ-) form, differing in the orientation of chitin micro-fibrils, being the alpha-form the most common in nature, with an antiparallel orientation (Carlstrom, 1957). Compared to β-chitin, with a parallel orientation of chitin micro-fibrils, α-chitin is a much more stable molecule due to the formation of stronger inter-molecular connections, hence harder to degrade (Kurita *et al*., 2001). γ-chitin consists of a mixture between the α- and β-forms of chitin, being rare in nature (Rudall & Kenching, 1973).

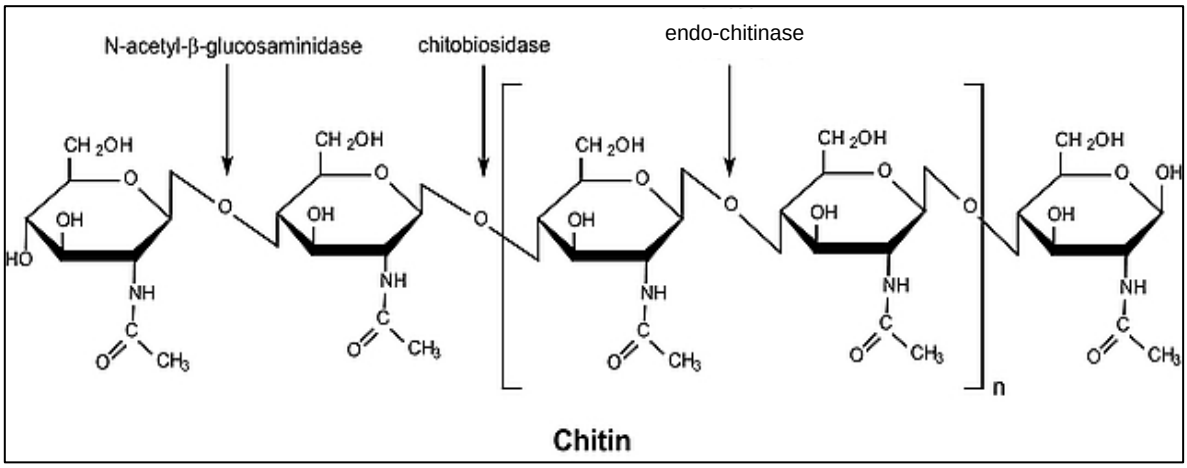


Figure 3. The chitin polymer and points of action of chitinolytic enzymes. The endo-chitinases bind to chitin and randomly cleave glycosidic linkages in chitin and chitodextrins in a non-processive mode, generating chito-oligosaccharides and free ends on which exo-chitinases (N-acetyl-β-glucosaminidases and chitobiosidades) and exo-chitodextrinases can then act. Adapted from *SIGMA-Aldrich* (www.sigma-aldrich.com).

The global production of chitin is surprisingly high. It can be extracted from insects or marine organisms, but the main source for its industrial production are crustacean shells retrieved from the industrial processing of surimi, being Japan, USA and China the top producers (Roberts, 1992).

In the marine environment, chitin does not accumulate because it is hydrolyzed by marine microorganisms that can utilize chitin as a carbon, nitrogen and/or energy source (Beier & Bertilsson *et al.,* 2013). The process of degrading chitin itself is termed chitinoclastic and when this degradation is mediated by chitinolytic enzymes, the chitinases, that hydrolyze the β-1,4 glycosidic bonds between the GlcNAc residues, producing chito-oligosaccharides, it is called chitinolytic. The majority of chitinases belong to the family 18 of glycosyl hydrolases and can be separated, according to amino acid (a.a.) sequence similarities, into three subfamilies: A, B and C (Hjort *et al.,* 2014; Karlsson & Stenlid, 2009; Suzuki *et al.,* 2002; Henrissat & Bairoch, 1993). Orikoshi and colleagues have proposed a division into groups A – D (Orikoshi *et al*, 2005), being *chiA* the best known of these chitinolytic enzymes (Brurberg *et al*., 1996). Giving the catalytic specificity, 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 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 is also frequently acting in the periplasmatic space of the bacterial cell (Beier & Bertilsson, 2013).

In the biotechnological field, chitin and its derivates present applications in the food, medical and agricultural sectors (Beier & Bertilsson *et al.,* 2013) since they have hypoallergenic, antibacterial, 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). They can be used in the pharmaceutical sector for the preparation of important chito-oligosaccharides and N-acetyl D-glucosamines, single cell proteins, in the isolation of protoplasts from fungi and yeast, hence for the control of pathogenic fungi and of malaria transmission (Dahiya *et al.,* 2006). They are also being studied for their use in novel drug delivery systems (Hata *et al*., 2000; Nsereko & Amiji, 2002; Wang *et al.,* 2018; Tejada *et al*., 2017; Zhao *et al*., 2017; Alkhader *et al*., 2017; Xing *et al*., 2017; Komenek *et al.,* 2017; Shevtsov *et al*., 2018; Xu *et al.,* 2018). 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).

Chitinolytic activities and genes have already been reported in some corals. For instance, a study on the gorgonian coral *Gorgonia ventalina* revealed that crude extracts from the coral contained detectible levels of exo-chitinase activity (Douglas *et al.,* 2007). More recently, Keller-Costa and colleagues found 19 chitinase-encoding genes on the genome of one *Aquimarina* sp. strain, isolated from the gorgonian *Eunicella labiata*, suggesting that the strain is probably capable of degrading chitin (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 (*A. digitifera, Galaxea fascicularis, Goniastrea aspera, Montipora digitata, Pavona divaricata, Pocillopora damicornis* and *Porites australiensis)*, which may indicate that chitinases are widely distributed in the coral-holobiont (Yoshioka *et al.,* 2017).

4. Distribution of chitin degradation pathway related genes

The chitinolytic pathway starts with the hydrolysis of the chitin polymer into oligomers, dimers and monomers outside the cell and in the periplasmatic space, continues with the subsequent integration of chitin monomers into the central metabolism and ends with fructose-6-phosphate (fructose-6-P), acetate and ammonium as final products (see **Figure 4**).

First, the chitin polymer is broken down by extracellular chitinases (***chiA***) outside the cell, with the help of chitin binding proteins (**CBP**) that adhere to the substrate. Afterwards, the resulting $(GlcNAc)_{n>2}$ oligosaccharides are transported into the periplasmic space via a specific porin (**Omp**) while the monomer GlcNAc and dimer N,N-diacetylchitobiose are believed to enter the periplasm with the help of nonspecific porins (**ChiP** or **YbfM**). Once in the periplasm, chitin oligosaccharides are degraded by periplasmic chitinodextrinases and β-N-acetylglucosaminidases. $(GlcNAc)_2$ is transported into the cytosol with the help of an ABC-type transporter (**ChbABC**), while GlcNAc is transported there through

a phosphotransferase system (PTS) transporter (**NagE**) and, consequently, phosphorylated (Hunt *et al.,* 2008).

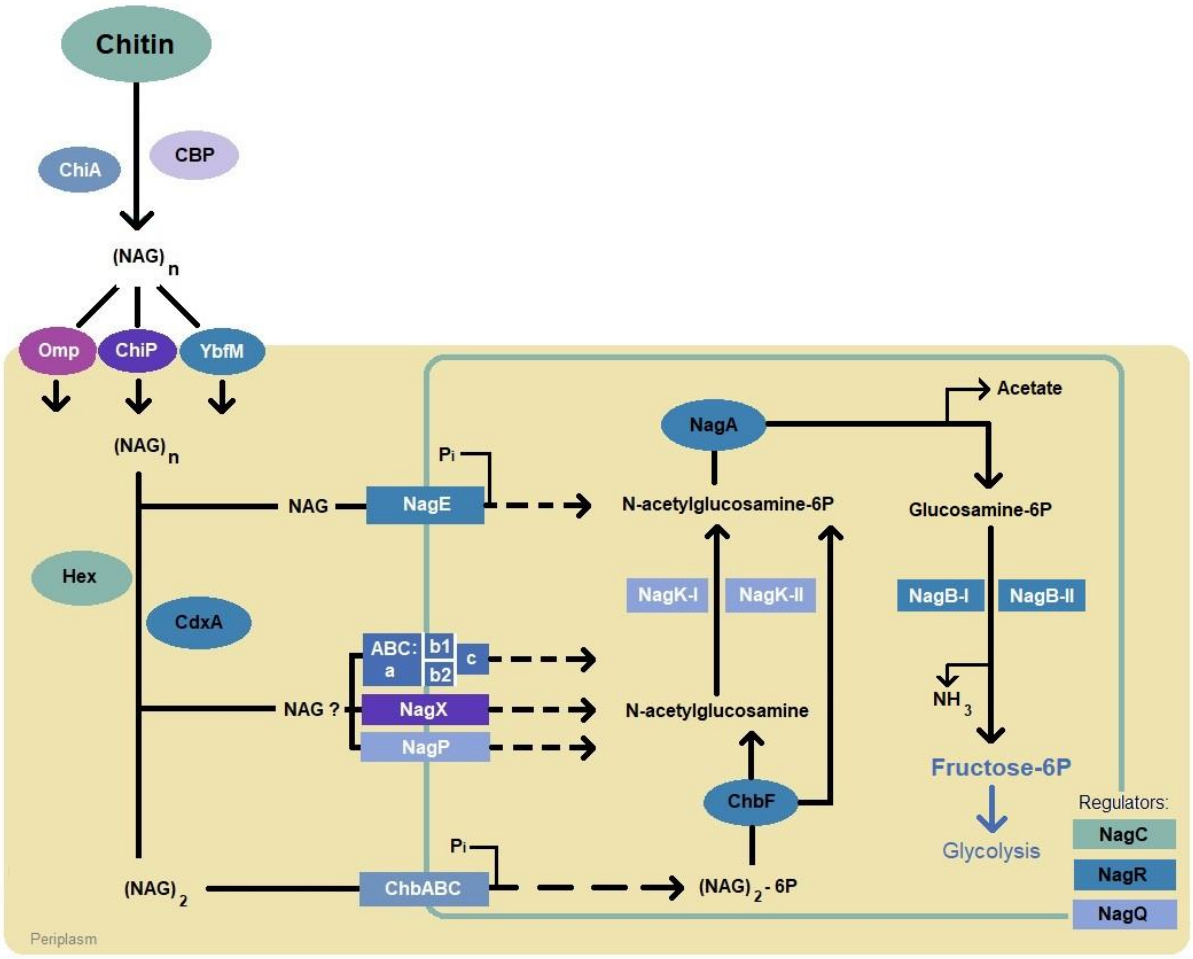


Figure 4. Schematic overview of chitin and N-Acetyl-D-Glucosamine utilization in a *Vibrio* sp. cell. Adapted from RAST (http://rast.nmpdr.org) and modified after Hunt *et al.,* 2008. ***chiA***: chitinase (EC 3.2.1.14), **CBP**: chitin binding protein, **Omp**: N-acetylglucosamine-regulated TonB-dependent outer membrane receptor, **YbfM**: N-acetylglucosamine-regulated outer membrane porin, **CdxA**: Chitodextrinase precursor (EC 3.2.1.14), **NagEa / Eb / Ec**: PTS system, N-acetylglucosamine-specific IIA / IIB / IIC component (EC 2.7.1.69), **ABCa**: N-Acetyl-D-glucosamine ABC transport system ATP-binding protein, **ABCb1 / b2**: N-Acetyl-D-glucosamine ABC transport system, permease protein 1 / 2, **ABCc**: N-Acetyl-D-glucosamine ABC transport system, sugar-binding protein, **NagX**: N-acetylglucosamine related transporter, NagX, **NagP**: N-acetyl glucosamine transporter, **ChbA / B / C**: PTS system, chitobiose-specific IIA / IIB / IIC component (EC 2.7.1.69), **ChbF** :Chitobiose-specific 6-phospho-beta-glucosidase ChbF (EC 3.2.1.86), **NagK**: Predicted N-acetyl-glucosamine kinase (EC 2.7.1.59), **NagA**: N-acetylglucosamine-6-phosphate deacetylase (EC 3.5.1.25), **NagB**: Glucosamine-6-phosphate deaminase (EC 3.5.99.6), **NagC**: N-acetylglucosamine-6P-responsive transcriptional repressor NagC, ROK family, **NagR**: Predicted transcriptional regulator of N-Acetylglucosamine utilization, LacI family, **NagQ**: Predicted transcriptional regulator of N-Acetylglucosamine utilization, GntR family.

Once in the cytosol, $(GlcNAc)_2$ is converted into 2(GlcNAc-6-P) by an N,N-diacetylchitobiose phosphorylase (**NagE**), a GlcNAc-1P-mutase (**middle**) and a predicted GlcNAc-specific ATP-dependent kinase (**ChbABC**). All the GlcNAc-6-P generated is then converted into fructose-6-P with the help of an

N-acetylglucosamine-6-phosphate deacetylase (**NagA**) and a glucosamine-6-phosphate deaminase (**NagB-I / NagB-II**; Hunt *et al.,* 2008).

Overall and to sum up, the chitinolytic pathway can be divided into the following major steps:

1. Presence of the chitin polymer.
2. Endo- and exo-chitinases action.
3. Presence of the chitin monomer.
4. Uptake of GlcNAc.
5. Phosphorylation of GlcNAc.
6. Deacetylation of GlcNAc-6P.
7. Deamination-isomerization of GlcN-6P to F-6P.

5. The wonders of octocorals

Octocorals (phylum Cnidaria, class Anthozoa, subclass Octocorallia) are a diverse group of marine invertebrates that are known to be abundant members of tropical and temperate reefs and to occur in coastal, shallow-water areas as well as the deep sea. Their role in temperate waters is extremely important, since these corals are habitat formers, which with their unique shape and 3-dimensional structure, construct and modify environments. As ecosystem engineers they create new niches and habitats for other species to colonize and thereby increase the biodiversity of the whole ecosystem (Curdia *et al.,* 2013). Octocoral communities can also serve as nursery areas and contribute to an energy flow in the marine environment, from pelagic to benthic zones, by enhancing the settlement of particles (Bryce *et al.,* 2018; Gili & Coma 1998), which is due to their ecological role as benthic suspension feeders. As filter-feeding organisms, octocorals depend on food sources available in the water column, such as suspended particulate organic matter, phytoplankton, prokaryotes, diatoms, dinoflagellates and ciliates. This suspended matter can be derived from detrital matter, resuspended sediment and excretory products from other animals, which makes it extremely diverse in terms of the organic compounds present (Ribes *et al*., 2003).

Underlying the success of octocorals is the use of diverse natural products that allow their survival and prosperity (Berrue & Kerr, 2009). Most descriptions of coral-derived natural products stem from the order Alcyonacea (commonly known as “soft corals”, including the “sea fans” and “sea whips”) (Rocha *et al.,* 2011). Unique secondary metabolites, particularly diterpenoids, sesquiterpenoids and steroids have been isolated and identified from various octocoral species. These natural products possess a wide range of biological activities such as anti-tumour, antiviral, antifouling and anti-inflammatory (Wei *et al*., 2013). These compounds are known to act, in the coral holobiont as grazing or feeding deterrents to both invertebrates (Gerhart, 1986; Lasker *et al*., 1988; Harvell & Fenical, 1989; Fenical & Pawlik, 1991; Van Alstyne & Paul, 1992) and fish (Lasker, 1985; Pawlik *et al*., 1987; Sammarco *et al.,* 1987; Harvell *et al*. 1988; Harvell & Fenical, 1989; Fenical & Pawlik 1991).

Enicella labiata (Thomson, 1927) is an octocoral from the family Gorgoniidae ("gorgonians") (Costello *et al.,* 2001) that can be found in shallow waters of the Atlantic and the Mediterranean (Coll *et al*., 2010), and is the most abundant gorgonian along the Southern and South-Eastern coast of Portugal (see **Figure 5**; 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). For these reasons, this species was the model host organism of this thesis. In a previous study (Keller-Costa *et al.*, 2017), three *Eunicella labiata* specimens had been sampled at ca. 18-m depth in the Atlantic Ocean, offshore of the Algarve region, South Portugal (36°58'47.2''N, 7°59'20.8''W) and a permanent culture collection of 175 bacteria (consisting of 36 unique 16S-phylotypes) associated with *E. labiata* had been established. Keller-Costa and colleagues also demonstrated that many of these bacterial isolates are indeed dominant members of the *E. labiata* microbiome, which suggests that they may fulfil relevant functions in this holobiont. This culture collection can now be investigated regarding its physiological capacities, symbiont-related features and biotechnological potential.

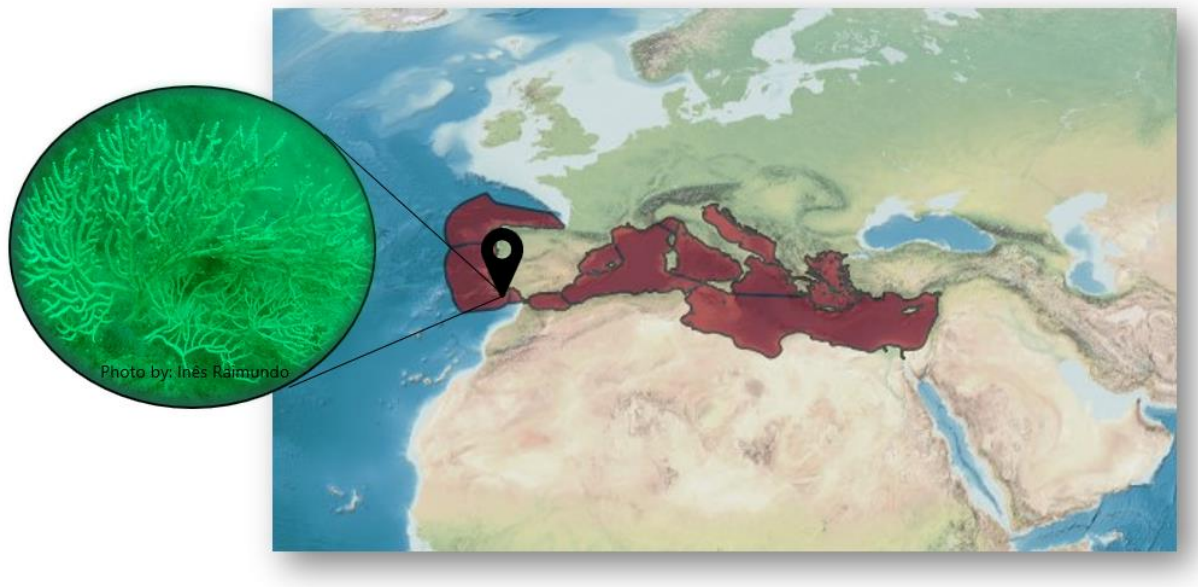


Figure 5. The gorgonian coral *Eunicella labiata* and its respective geographic distribution. Photo of *Eunicella* sp. taken by Inês Raimundo, Jardim das Gorgónias, Sesimbra, Portugal; Map source: World Register of Marine Species (http://www.marinespecies.org).

OBJECTIVES

In the present work, the chitinolytic activity of 36 phylogenetically distinct phylotypes of this *E. labiata* derived culture collection has been assessed. Firstly, a colloidal chitin agar plate assay was used to test the *E. labiata* strains for their ability to degrade chitin *in-vitro*. Secondly, the enzymatic activity of endo-chitinases was quantified fluorometrically for some of the strains. Thirdly, a polymerase chain reaction was performed to detect the presence of the endo-chitinase-encoding gene *chiA* in the 36 bacterial strains and a phylogenetic analysis undertaken to explore the diversity of this gene. Then, a genome-wide screening for genes related to chitin catabolism was undertaken for 15 gorgonian-derived bacterial isolates, to explore the functioning and diversity of this pathway. Moreover, the relative abundances of chitinolytic genes and the diversity of the *chiA* gene within the 20 microbial metagenomes of three octocoral species and their surrounding environments were investigated, enabling assessment of chitin degradation potential in corals, sediments and seawater in a cultivation-independent manner. **Figure 6** presents the workflow employed in this study.

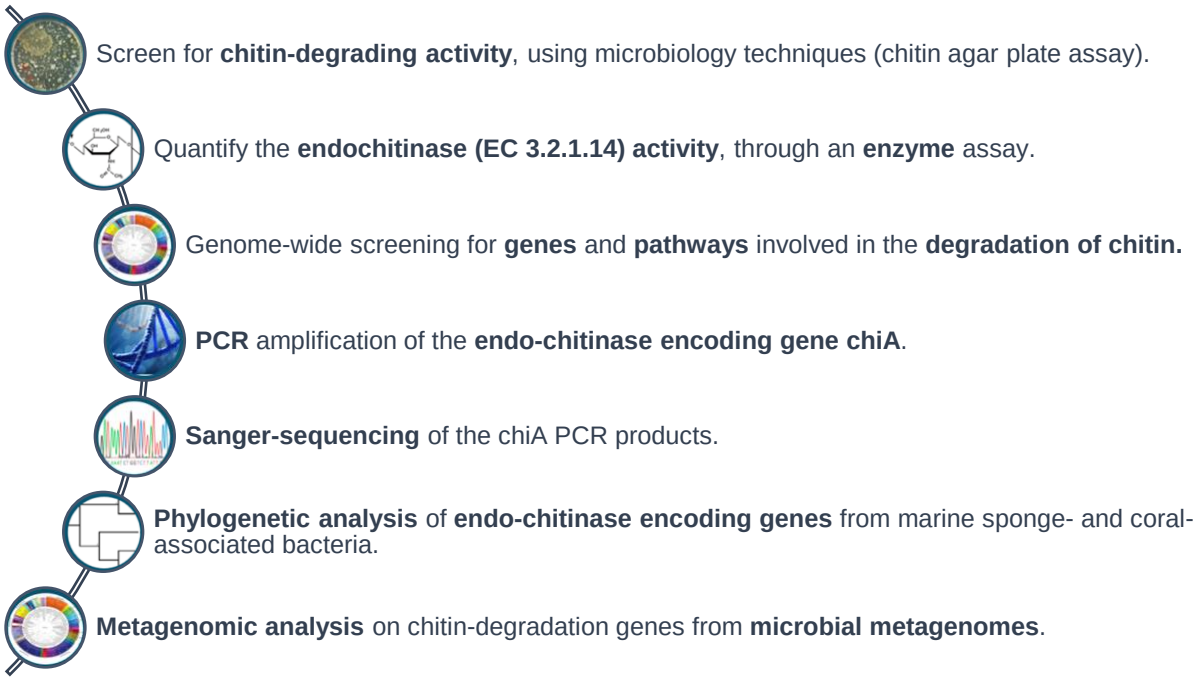


Figure 6. Schematic overview of the workflow followed to investigate the chitinolytic capacities of the *E. labiata* derived bacterial isolates in this Master Thesis.

SUMMARY OF HYPOTHESES AND RESEARCH QUESTIONS

The main research questions and hypotheses addressed in this thesis are listed below along with the corresponding methodology employed to approach them.

A. "Because of the suspension-feeding behaviour of gorgonian corals, their associated microbiomes are likely hotspots for chitin degradation."

This hypothesis is addressed by comparing the relative abundances of chitinolytic related genes in the microbial metagenomes of gorgonian corals, seawater and sediments.

B. "Bacterial associates from the gorgonian coral *Eunicella labiata* are able to degrade chitin *in-vitro*."

Chitinolytic capacity is determined for diverse bacteria isolated from gorgonian corals using colloidal chitin degradation and endo-chitinase activity bioassays.

C. "Can the endo-chitinase (EC 3.2.1.14) gene *chiA* be used as a phylogenetic marker? Or is it not as conserved as the 16S rRNA gene? Can it be used as a robust indicator of chitinolytic activity among diverse bacteria?"

Deep phylogenetic assessments are undertaken to compare 16S rRNA gene and *chiA* gene tree topologies and infer whether the former gene can be a useful phylogenetic marker or is rather prone to lateral gene transfer.

D. "The chitinolytic community within gorgonian, sediment and seawater microbiomes differ in taxonomic compostion and structure."

To determine whether this hypothesis is true, *chiA* genes detected in the microbial metagenomes of corals, sediment and seawater were subjected to taxonomic assignments and analysis of diversity.

MATERIALS AND METHODS

1. Bacterial strains

The 36 bacterial isolates (see **Table 1**) 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 sequences, i.e. they are phylogenetically distinct.

Table 1. Genus-level classification of the 36 bacterial isolates used in this study, derived from the gorgonian *Eunicella labiata*.

Strain ID	Colour of the Colony	Genus	Taxonomic Class
EL01	beige	*Ruegeria*	*Alphaproteobacteria*
EL04	beige	*Ruegeria*	*Alphaproteobacteria*
EL08	beige	*Ruegeria*	*Alphaproteobacteria*
EL09	beige	*Ruegeria*	*Alphaproteobacteria*
EL10	beige	*Ruegeria*	*Alphaproteobacteria*
EL11	beige	*Ruegeria*	*Alphaproteobacteria*
EL12	salmon	*Shewanella*	*Gammaproteobacteria*
EL15	beige	*Kiloniella*	*Alphaproteobacteria*
EL22	beige	*Vibrio*	*Gammaproteobacteria*
EL24	yellowish	*Enterovibrio*	*Gammaproteobacteria*
EL26	beige	*Roseovarius*	*Alphaproteobacteria*
EL27	beige	*Pseudophaeobacter*	*Alphaproteobacteria*
EL30	beige	*Ruegeria*	*Alphaproteobacteria*
EL33	orange	*Aquimarina*	*Flavobacteriia*
EL36	beige	*Vibrio*	*Gammaproteobacteria*
EL37	beige	*Enterovibrio*	*Gammaproteobacteria*
EL38	beige	*Vibrio*	*Gammaproteobacteria*
EL41	beige	*Vibrio*	*Gammaproteobacteria*
EL43	orange	*Aquimarina*	*Flavobacteriia*
EL44	beige	*Sulfitobacter*	*Alphaproteobacteria*
EL46	beige	*Ruegeria*	*Alphaproteobacteria*
EL49	beige	*Vibrio*	*Gammaproteobacteria*
EL53	beige	*uncl. Rhodobacteraceae*	*Alphaproteobacteria*

EL57	beige	*Aliivibrio*	*Gammaproteobacteria*
EL58	beige	*Aliivibrio*	*Gammaproteobacteria*
EL62	beige	*Vibrio*	*Gammaproteobacteria*
EL67	beige	*Vibrio*	*Gammaproteobacteria*
EL112	beige	*Vibrio*	*Gammaproteobacteria*
EL119	beige	*Roseovarius*	*Alphaproteobacteria*
EL122	beige	*Ruegeria*	*Alphaproteobacteria*
EL129	beige	uncl. *Rhodobacteraceae*	*Alphaproteobacteria*
EL138	yellow	*Sphingorhabdus*	*Alphaproteobacteria*
EL143	salmon	*Labrenzia*	*Alphaproteobacteria*
EL163	yellow	*Sphingorhabdus*	*Alphaproteobacteria*
EL179b	beige	*Roseovarius*	*Alphaproteobacteria*
EL199	beige	*Kiloniella*	*Alphaproteobacteria*

Prior to chitinolytic activity assays, all strains were re-activated from glycerol stocks (50 µL of culture) in 20 mL of half-strength Marine Broth (1:2 diluted in artificial seawater, MB 1:2), followed by room temperature (RT) incubation at 45 rpm on an orbital shaker (Fisher Scientific), until they reached late exponential growth phase.

2. Preparation of colloidal chitin

Colloidal chitin (CC) was prepared from α-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 CC (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 the medium

consisting of 0.05 % yeast extract and 0.5% CC, 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 fluorimetrically 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-substrats. 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. To prepare the enzymatic samples, 250 µL of a bacterial culture grown for 24h, 48h, 36h or 72h in liquid MB 1:2 were centrifuged for 15 min at 5,000 RCF, followed by removal of the cell pellet and transfer of the supernatant to a new 1.5mL microtube. All enzymatic activities are extracellular activities as measurements were performed on the culture supernatant and, 10 µL of supernatant were applied in each test.

All assays were performed at pH 5 using the buffer (**A8730**) provided with the kit for 60 min at 37 °C in a total reaction volume of 100 µL, unless otherwise stated.

The reaction was stopped by the addition of 200 µL sodium carbonate solution (**S2127**) and the fluorescence of released 4-MU was measured 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 then subtracted by a standard (assay buffer) blank and used to produced the standard curve which was then used for enzyme activity (Units/L) calculations.

As an alternative to the standard curve and with the aim of validating the values obtained using the former, a formula given in the protocol of the kit (here referred to as SIGMA formula) was used (see **Equation 1**).

$$Units/L = \frac{(\mathrm{FLUsample} - \mathrm{FLUblank}) \times 1.529 \times 0.3 \times \mathrm{DF}}{\mathrm{FLUstandard} \times \mathrm{time} \times \mathrm{Venz}}$$

Equation 1. Calculation of Enzyme activity in Units/L: 1 Unit corresponds to 1 µmol of substrate converted per minute (in 1 L of culture supernatant).

Where FLU_{sample} is the fluorescence of the sample; FLU_{blank} is the fluorescence of the blank (containing only substrate working solution); 1.52 is the final concentration of 4-MU in nmole/ml corresponding to the 80ng standard (i.e. 80ng/0,3mL/176g/mol); 0.3 is the final reaction volume (mL) after addition of the stop solution; DF corresponds to the enzyme dilution factor (here always 1); $FLU_{standard}$ is the fluorescence of the 80ng standard solution minus the fluorescence of the standard blank (only assay buffer); time is the time of incubation in min (here always 60 min); V_{enz} is the volume of the sample in millilitre (here always 0.01 mL).

In every assay a substrate blank (substrate working solution of 0.5 mg/mL only) and an assay buffer blank as well as a positive control, an exo- and endo-chitinase mixture isolated and purified from the fungus *Trichoderma viride*, were measured and, in the enzyme activity calculations, whether they were made through the standard curve or the SIGMA formula, all values of fluorescence obtained in the microplate reader were first blank subtracted.

4.1. Chitinase assay with non-induced strains (i.e. without chitin in the growth medium)

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), until they have reached stationary phase or late exponential phase (monitored by measuring optical densities at 600 nm (OD600)).

4.2. Chitinase assay with induced strains (i.e. with chitin in the growth medium)

Aquimarina sp. strains EL33 and EL43 that were used for the induction assay were re-inoculated in 20 mL of either CC liquid medium or chitin powder liquid medium and incubated as mentioned above, until they have reached the stationary phase or late exponential phase (monitored by OD_{600} measurements). To prepare both chitin media, artificial seawater (ASW) and 0.15% potassium dihydrogen phosphate (KH_2PO_4) were mixed with either CC (0.2%) or chitin powder (0.2%). The strains were incubated in each medium for 10 days, at RT and 45 rpm orbital shaking, and 0.25 mL of liquid culture were sampled on days 2, 3, 4, 7, 8, 9 and 10 after inoculation and supernatants collected as described earlier. In both media, pronounced clearance of the initial whitish turbidity caused by the chitin in the medium was observed over time for both strains, indicating chitin consumption by the bacteria.

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 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. These changes in the protocol were made so that the amplification result was as clear as possible.

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.

Moreover, metagenomic DNA from 3x *sediment* samples, 3x *seawater* samples and two gorgonian species (6x *Eunicella labiata* and 2x *Eunicella verrucosa*) sampled off the coast of Faro (Algarve, *Portugal*) in spring 2015 and 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 off the coast of Faro in summer 2014 were also subjected to PCR amplification of *chiA* genes as described above for bacterial isolates. DNA concentrations of each metagenomic sample were measured using Nanodrop and metagenomic template DNAs were standardized to concentrations of about 40ng per 50 µL reaction. To increase the yield of PCR products and to produce more visible bands on agarose gel, after the usual *chiA* PCR (see above), a second, "reconstituting" PCR was then performed using the same PCR, conditions but with 15 instead of 35 cycles and with 2 µL of the PCR products from the first PCR as template.

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

6. Sanger sequencing of the amplified *chiA* genes

The PCR amplified *chiA* genes of bacterial isolates were subjected to sequencing. Briefly, PCR products were purified using Sephadex®, whereby the samples were loaded on Sephadex G-50® columns, prepared in microtiter plates and then centrifuged. The effluent containing the purified DNA was collected 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 with endo-chitinases sequences obtained from bacterial genomes from a variety of gorgonian coral and marine sponge bacterial cultures of our laboratory 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), by the ClustalW method. Sequences that were ambiguous during and after the alignment process were excluded from further phylogenetic analysis. The phylogenetic trees were created in MEGA7 using a maximum likelihood method, recurring to partial deletion with a 95% cut-off. An analysis for the most suitable evolutionary model was performed after each alignment and before constructing the tree and each phylogenetic tree was constructed with its respective most suitable DNA model. To validate the trees, 100 bootstrap repetitions were generated. In all phylogenetic trees, the percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. All trees are drawn to scale, with branch lengths measured in the number of substitutions per site (Kumar *et al.,* 2016).

A calculation of the nucleotide distances within and between phylogenetic clusters (groups) was also performed. The groups were created based on the phylogenetic clusters present in each tree. Also, percentages of identical aligned nucleotides were compared, using the p-distance method, for sequences having identical deduced a.a. sequences. Here, we have included both the substitutions, transitions and transversions, and the missing data treatment was performed recurring to complete deletion, except in the case of the final alignment and phylogenetic tree, where the complete deletion methodology was not feasible. In this particular case, the pairwise deletion option was first used, as complete deletion eliminates all sites that comprise a gap in any part of the alignment. After this test,

MEGA was unable to calculate the distance between two and all of the sequence pairs in the alignment. These pairs were identified and removed from the alignment. A complete deletion option was then used, and the p-distance calculation performed, after removing these two sequences.

8. Taxonomic classification of chitinase-encoding genes from gorgonian, sediment and seawater microbial 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. The (unassembled) sequencing data of these 20 microbial metagenomes are available in public databases under the study accession number PRJEB13222 (ERP014771) and privately (to our group) on the Metagenomics Analysis Server MG-RAST (https://www.mg-rast.org; Meyer *et al.,* 2008). A functional assignment was performed on this platform, using default parameters, and all sequence entries that were assigned by MG-RAST into the carbohydrate subsystem for each of the 20 metagenomes were downloaded. A screening on those sequences was performed to extract the ones corresponding to chitinases. Sequences from the replicate samples of the same habitat were pooled and then blasted against the NCBI nucleotide database (e-value = 10.0) and the resulting alignment files were analysed using the MEGAN6 software package (for microbiome analysis; Huson *et al.,* 2016) to obtain a taxonomic assignment for each chitinase sequence retrieved from the metagenome dataset.

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

A functional analysis of the 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.

In the analysis of the microbial metagenomes of *Eunicella gazella, Eunicella verrucosa, Leptogorgia sarmentosa*, sediment and seawater, 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.

In the analysis of the microbiomes from healthy versus necrotised *Eunicella gazella* tissue, a paired t-test was used when data were homocedastic. Paired tests were used because the same *E. gazella* specimens were sampled twice (healthy branch of colony versus necrotic branch of same colony).

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 (CC) was performed. Preliminary tests showed that most pronounced chitin hydrolysis occurs on agar medium supplemented with 0.5% CC and 0.05% yeast extract and this composition was subsequently used in all experiments. Of the 36 strains, 12 were found to degrade chitin forming typical clearing zones (haloes) around the inoculation spot (**Table 2** and **Figure 7**, see also **Appendix A.**). 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 larger than 1.0 cm (**Table 2** and **Appendix A.**), 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.

Table 2. Chitin-degrading activity of 12 bacterial isolates**.** Semi-quantitative analysis of chitinase activity was performed by measuring the size of clearing zones (haloes), from the centre of the inoculation spot to the edge of the clearing zone. (+) halo-radius up to 0.5 cm, (++) halo-radius from 0.5 – 1 cm and (+++) halo-radius ≥1 cm.

Strain ID	Genus	Chitin-degrading activity	
		After seven days of incubation	After 14 days of incubation
EL24	***Enterovibrio***	++	**+++**
EL37	***Enterovibrio***	++	**+++**
EL22	***Vibrio***	++	++
EL36	***Vibrio***	+	++
EL62	***Vibrio***	+	++
EL67	***Vibrio***	+	++
EL38	***Vibrio***	++	++
EL49	***Vibrio***	++	++
EL41	***Vibrio***	++	**+++**
EL112	***Vibrio***	**+++**	**+++**
EL33	***Aquimarina***	**+++**	**+++**
EL43	***Aquimarina***	++	**+++**

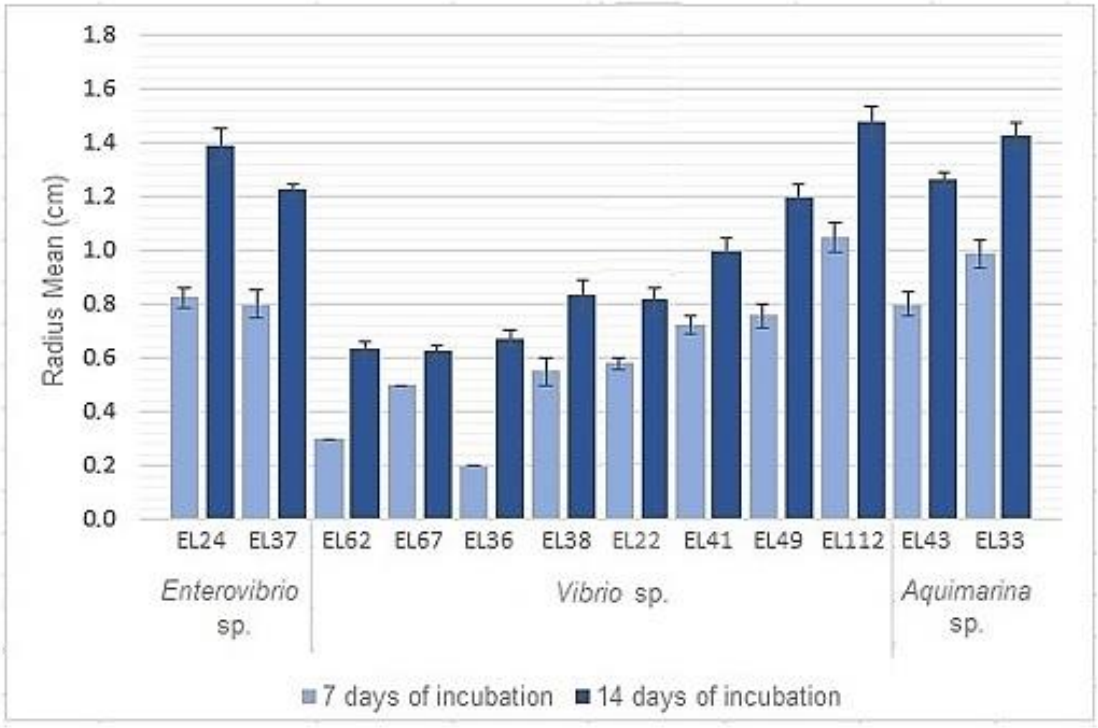


Figure 7. Chitin-degrading activity of 12 bacterial strains isolated from the gorgonian *E. labiata*, presented as the mean halo size (radius in cm) and respective standard error (*N=8*) for each strain after 7 days (light blue) and 14 days (dark blue) of incubation.

2. Chitinase enzyme assay

2.1. Assay without prior chitin-based induction of chitinase expression

A chitinase enzyme assay was performed to verify that the chitin-degrading capacity observed on chitin-agar plates is due to the activity of chitinases and to quantify this chitinase activity.

A 4-methylumbelliferone (4-MU) standard curve was obtained using various 4-MU concentrations in a range that was found adequate for the here tested samples and used since then for the calculation of the enzyme activities (**Figure 8**).

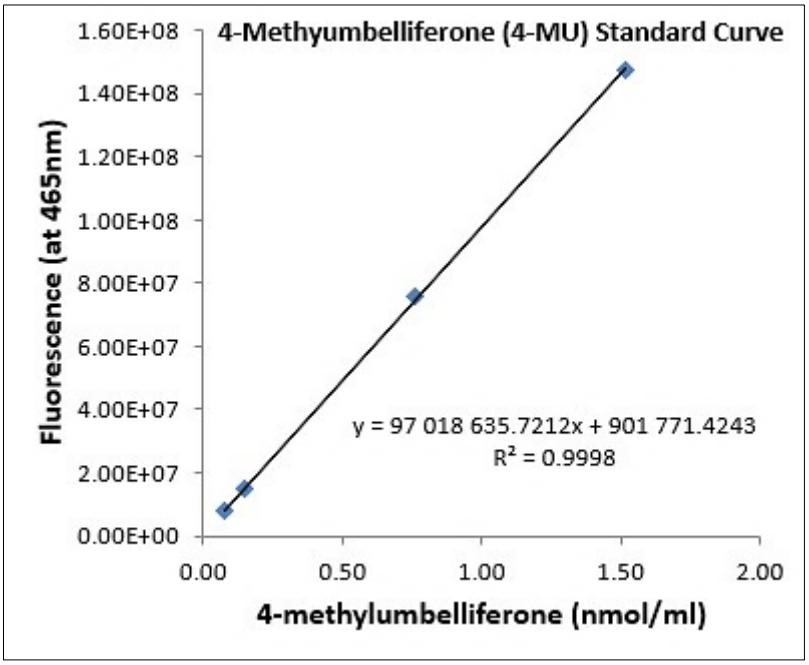


Figure 8. Standard curve of 4-Methylumbelliferone and respective linear regression line with its equation and R square value.

The kit contained three different substrates: **1)** 4-methylumbelliferyl N-acetyl-β-D-glucosaminide, **2)** 4-methylumbelliferyl N,N′-diacetyl-β-D-chitobioside hydrate and **3)** 4-methylumbelliferyl β-D-N,N′,N″-triacetylchitotriose. The first enzyme assays tested all 3 substrates on the *Aquimarina* sp. strains EL33 and EL43. These tests revealed that substrate 3 (detecting endo-chitinase activity) yielded higher activity values (**Table 3**). Hence, all further tests described here focus exclusively on endo-chitinase activity, using substrate 3.

Table 3. Exo- and endo-chitinolytic activity of *Aquimarina* strains, grown for 24h and 36h, and respective OD_{600} values.

	Growth	Standard curve			SIGMA formula		
		Substrate 1	Substrate 2	Substrate 3	Substrate 1	Substrate 2	Substrate 3
	OD_{600}	*Units/L*	*Units/L*	*Units/L*	*Units/L*	*Units/L*	*Units/L*
Positive control	–	0.302	0.254	0.135	0.298	0.251	0.136
EL33 24h	0.532	0	0.005	0.048	0.002	0.009	0.052
EL43 24h	0.582	0	0.001	0.027	0.004	0.005	0.030
EL33 36h	1.788	0	0.013	0.014	0.003	0.017	0.018
EL43 36h	1.748	0	0.021	0.335	0.004	0.025	0.330

Substrates 1 and 2 are screening for exo-chitinase activity (EC 3.2.1.52; β-N-acetylglucosaminidase and chitobiosidase, respectively), whereas substrate 3 is detecting endo-chitinase (EC 3.2.1.14) activity. Chitinolytic activities estimated with 4-MU standard curve (**Figure 7**) and with the SIGMA formula (**Equation 1**) are compared. The strains were tested without prior chitin-based induction (i.e. no chitin was present in the growth medium).

All activity results calculated with the SIGMA formula were in the same range and comparable with values obtained with the standard curve. Since the standard curve-based activity calculations are considered more precise, all activity values present from here on are based on standard curve calculations.

To confirm that the endo-chitinase activities detected in the *E. labiata* isolates indeed resulted from the growing bacterial strains, enzyme activity was measured also in the liquid growth medium (MB 1:2), which showed, as expected, zero enzymatic activity and hence served as a blank control. In addition, *Ruegeria* sp. strain EL09 (*Alphaproteobacteria*) was chosen as a negative control for endochitinolytic activity. Since it did not show any chitin degrading activity on the CC agar plates we also did not anticipate any activity in the chitinase assay. As expected, there was no activity detected for *Ruegeria* EL09 (OD_{600}: 1.252, probably late exponential phase). On the other hand, *Vibrio* sp. strain EL36, one of the most active chitin degraders on the CC agar plate assay, showed activity (0.297 Units/L, when grown for 24 h (OD_{600}: 1.952, probably late exponential phase), and 0.530 Units/L, when grown for 73h (OD_{600}: 1.280, probably late stationary phase), even higher than the activities obtained for the two *Aquimarina* strains (**Table 4**).

Table 4. Endo-chitinase (EC 3.2.1.14) activity of *Vibrio* sp. EL36 and *Ruegeria* sp. EL09 isolated from *E. labiata*.

	Endo-chitinase activity	
	Units/L	*Units/L*
Positive Control	0.025	0.029
***Ruegeria* sp. EL09 73h**	0	0
***Vibrio* sp. EL36 24h**	0.297	0.302
***Vibrio* sp. EL36 73h**	0.530	0.535
MB 1:2	0	0

The substrate used for this assay was substrate 3, that screens for endo-chitinase activity. Chitinolytic activities were estimated using the 4-MU standard curve (**figure 7**). The strains were tested without prior chitin-based induction (i.e. no chitin was present in the growth medium).

2.2. Induction of chitinase production by adding chitin to the growth medium

The assays described above (**Tables 3** and **4**) had been performed on strains growing in MB 1:2 without an apparent induction of the production of the chitinase proteins. To test whether endo-chitinase activity would increase when chitinase production was stimulated by the availability of chitin as carbon and energy source in the growth medium, the *Aquimarina* sp. strains EL33 and EL43 were inoculated in liquid CC (0.5 %) and chitin powder (0.5 %) media, respectively. The two *Aquimarina* strains were grown on both media for 10 days, and samples were taken on days 2, 3, 4, 7, 8, 9 and 10 after inoculation (**Table 5**).

Aquimarina sp. strain EL43 inoculated in the chitin powder medium had no activity during the first three days post inoculation; slight activity was observed on day 4 and then it increased continuously until day 10. In contrast, *Aquimarina* sp. strain EL33 showed no activity on chitin powder medium, until day 9 and 10, for which 0.01 Units/L and 0.15 Units/L were obtained, respectively.

On colloidal chitin medium, *Aquimarina* sp. strain EL43 had again no activity during the first three days post inoculation, but it increased throughout the days until an activity value of 0.40 Units/L on day 10. As for EL33, there was no activity registered until 10 days after the inoculation, for which an activity of 0.15 Units/L was measured.

Table 5. Endo-chitinase (EC 3.2.1.14) activity of *Aquimarina* sp. strains upon induction of chitinase production with colloidal chitin and chitin-powder containing growth media.

Strains grown for	EL43 chitin powder		EL43 colloidal chitin		EL33 chitin powder		EL33 colloidal chitin	
	Standard Curve	SIGMA formula	Standard Curve	SIGMA formula	Standard Curve	SIGMA formula	Standard Curve	SIGMA formula
	Units/L	**Units/L**	**Units/L**	**Units/L**	**Units/L**	**Units/L**	**Units/L**	**Units/L**
2 Days	0	0	0	0	0	0	0	0
3 Days	0	0	0	0	0	0	0	0
4 Days	0.005	0.009	0.245	0.250	0	0	0	0
7 Days	0.093	0.097	0.354	0.360	0	0	0	0
8 Days	0.181	0.186	0.322	0.327	0	0	0	0
9 Days	0.265	0.270	0.440	0.446	0.004	0.009	0	0
10 Days	0.351	0.356	0.399	0.405	0.428	0.433	0.145	0.150

The substrate used for this assay was substrate 3, that screens for endo-chitinase (EC 3.2.1.14) activity. Chitinolytic activities were estimated using a 4-MU standard curve as well as the formula (**Equation 1**) provided by the SIGMA-Aldrich kit.

2.3. Overall activities

After all the manipulations of the experimental conditions described above, a final and optimized protocol of the chitinolytic enzyme assay, without prior chitin-based induction and using substrate **3**, was established, and endo-chitinase (EC 3.2.1.14) activity measurements for all the remaining chitin-degrading strains (from the chitin agar plate assay) were performed (see **Table 6**). Since the strains are from different species with consequently different growth curves, chitinase activities were measured in bacterial cultures grown for both 24h and 36h, and the respective highest endo-chitinase activity value is presented for each strain (**Table 6**).

Table 6. Endo-chitinase activity of the remaining chitin-degrading strains, grown for 24h or 36h, and respective OD_{600} values.

	Genus	Standard curve		
		Grown for (h)	OD_{600}	Chitinase activity *Units/L*
EL22	***Vibrio***	36	0.363	0.956
EL24	***Enterovibrio***	36	0.254	0.663
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
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

The substrate used for this assay was substrate 3, that screens for endo-chitinase (EC 3.2.1.14) activity. Chitinolytic activities were estimated using the 4-MU standard curve.

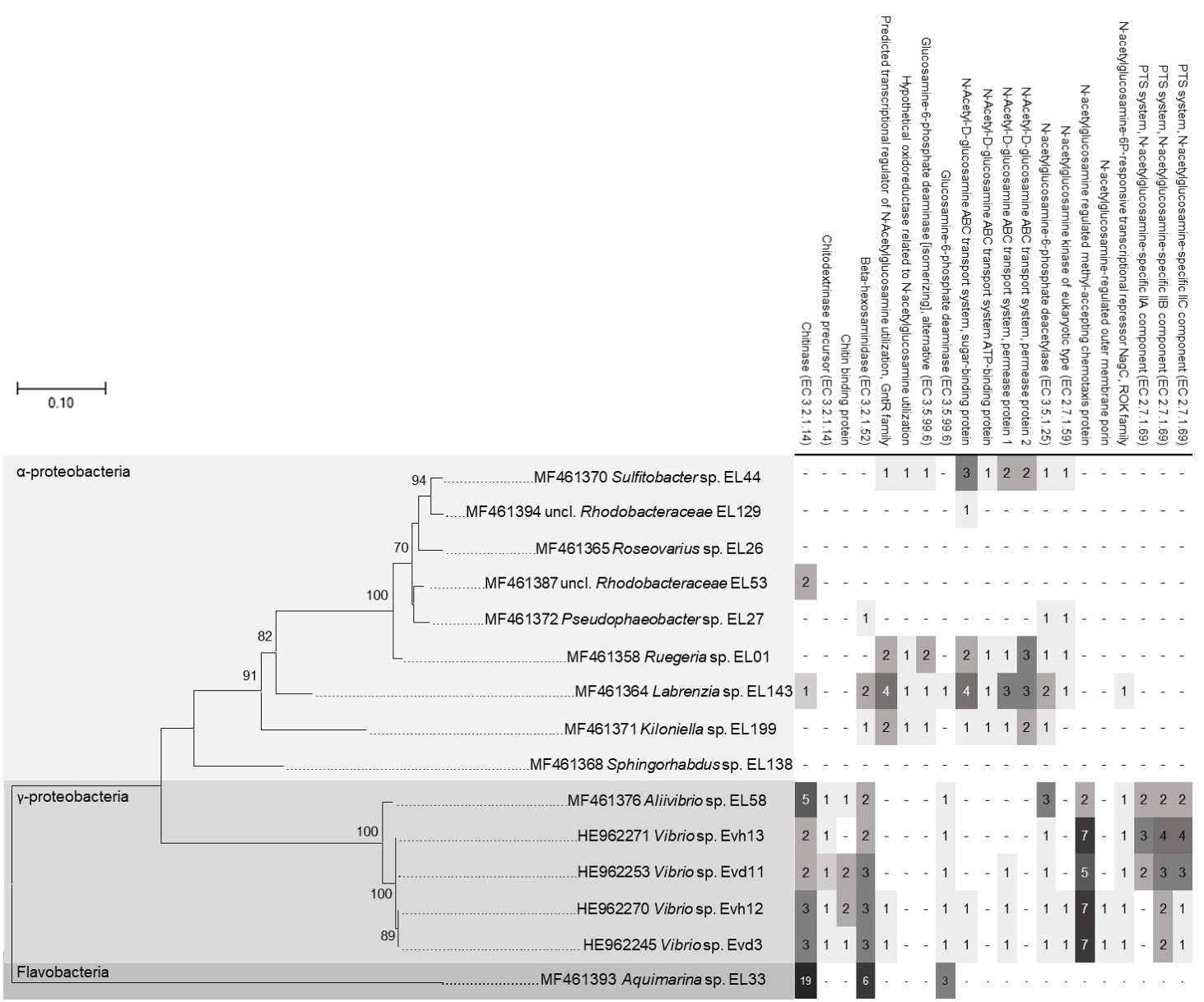
3. Distribution of chitin degradation pathway related genes among bacterial symbionts of *Eunicella* corals

In this section, we aim to explore how conserved was the chitin degradation pathway among the bacteria isolated from two gorgonians studied in our lab, *Eunicella labiata* and *Eunicella verrucosa*.

The phylogenetic relationships of the sequenced genomes were contrasted with the presence and absence of genes related to the chitin-degradation pathway (**Figure 9**) to better understand how this pathway varies between different species and how it changes with and responds to phylogeny. The conservation of the chitin degradation pathway is clear and most evident among the sequenced *Vibrionaceae* genomes, which suggests that chitin metabolism is an ancestral feature of the *Vibrios* (**Figure 9**). Gene distribution patterns change considerably when it comes to *Alphaproteobacteria*, mainly in terms of the genes that are present, in comparison with *Vibrio* species, and not so much in terms of gene copy number. Among the *Alphaproteobacterial* genomes, the genes are mostly the same, except for two strains (*Roseovarius* sp. EL26 and *Sphingorhabdus* sp. EL138) that do not possess any chitin-degradation-related genes and other two strains that only possess two different chitin-related genes.

The only gorgonian-derived *Bacterooidetes* (*Flavobacteria*) isolate with its genome sequenced is *Aquimarina* sp. strain EL33 which, representing a different phylum (*Bacteroidetes*) compared to the remaining isolates (which are all *Proteobacteria*). Strain EL33 presents a very different set of genes involved in the chitin degradation pathway, with for instance a very high copy number of endo-chitinases (19). However, despite having this high number of endo- and exo-chitinase-encoding genes, there are not any ATP-binding cassette (ABC) transporters, phosphotransferase system (PTS) components or binding proteins related to chitin-degradation in this genome. Overall, the here analysed genomes show taxon-specific segregations in the type and copy number of chitinase-encoding genes.

Figure 9. Distribution of chitin pathway related genes among sequenced bacterial genomes isolated from the corals *Eunicella labiata* and *Eunicella verrucosa.* The phylogenetic tree was based on a maximum likelihood analysis of the 16S rRNA gene. Numbers at nodes represent values based on 100 bootstrap replicates. On the right, a table with chitin metabolism-related genes and their functions. Values in each cell are numbers of that gene in the corresponding genome. Fewer gene copies are highlighted in light-grey shading and the colour darkens as the gene numbers increase.



4. *chiA* gene polymerase chain reaction amplification from genomic DNA

A *chiA* gene-specific PCR was performed for all 36 strains used in this study (see **Table 1**) but amplicons of correct size and sufficient quality for sequencing could only be obtained for 12 strains (see **Table 7 and Figure 10**).

Table 7. Chitin degrading activity of the 36 isolates on the colloidal chitin agar plates ((+) halo-radius up to 0.5 cm, (++) halo-radius from 0.5 – 1 cm and (+++) halo-radius ≥1 cm) and their respective *chiA* PCR results ((+) *chiA* amplicon of correct size and sufficient quality obtained, (-) no amplification). Ambiguous results are highlighted with light grey.

Strain ID	Genus	Chitin Degrading Activity		*chiA* PCR results
		7 days incubation	14 days incubation	
EL01	***Ruegeria***	-	-	**-**
EL04	***Ruegeria***	-	-	**-**
EL08	***Ruegeria***	-	-	**-**
EL09	***Ruegeria***	-	-	**-**
EL10	***Ruegeria***	-	-	**-**
EL11	***Ruegeria***	-	-	**-**
EL12	***Shewanella***	-	-	**-**
EL15	***Kiloniella***	-	-	**-**
EL22	***Vibrio***	++	++	**+**
EL24	***Enterovibrio***	++	**+++**	**+**
EL26	***Roseovarius***	**-**	**-**	**-**
EL27	***Pseudophaeobacter***	**-**	**-**	**-**
EL30	***Ruegeria***	**-**	**-**	**-**
EL33	***Aquimarina***	**+++**	**+++**	**+**
EL36	***Vibrio***	+	++	**+**
EL37	***Enterovibrio***	++	**+++**	**+**
EL38	***Vibrio***	++	++	**+**
EL41	***Vibrio***	++	**+++**	**-**
EL43	***Aquimarina***	++	**+++**	**+**
EL44	***Sulfitobacter***	**-**	**-**	**-**
EL46	***Ruegeria***	**-**	**-**	**-**
EL49	***Vibrio***	++	**++**	**+**
EL53	***uncl. Rhodobacteraceae***	**-**	**-**	**-**
EL57	***Aliivibrio***	**-**	**-**	**+**

EL58	***Aliivibrio***	**-**	**-**	**-**
EL62	***Vibrio***	+	++	**+**
EL67	***Vibrio***	+	++	**+**
EL112	***Vibrio***	**+++**	**+++**	**+**
EL119	***Roseovarius***	**-**	**-**	**-**
EL122	***Ruegeria***	**-**	**-**	**-**
EL129	**uncl. *Rhodobacteraceae***	**-**	**-**	**-**
EL138	***Sphingorhabdus***	**-**	**-**	**-**
EL143	***Labrenzia***	**-**	**-**	**-**
EL163	***Sphingorhabdus***	**-**	**-**	**-**
EL179b	***Roseovarius***	**-**	**-**	**-**
EL199	***Kiloniella***	**-**	**-**	**-**

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 the chitin agar medium. All amplified ***chiA*** fragments were sequenced and subjected to phylogenetic analysis.

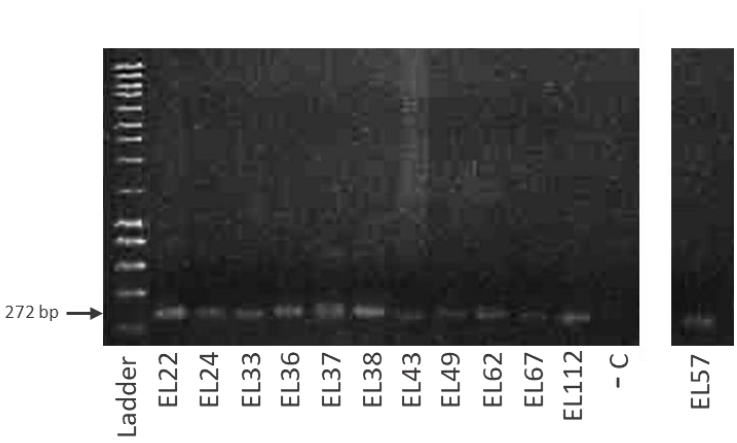


Figure 10. Positive results of the *chiA* PCR amplification on a 1.2% agarose gel.

5. 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. The first phylogenetic analysis contained all the previously sequenced *chiA* PCR products from the bacterial isolates, from the previously mentioned amplification and sequencing processes (see **Figure 11**, **a)**). The majority of the *Vibrio* strains included in this analysis clustered together and tightly with 100% bootstrap support (**Cluster I**). **Cluster II** is also very well supported, containing three *chiA* sequences from *Vibrio* strains plus one from an *Aquimarina* sp., strain EL43. Indeed, all *chiA* sequences related to the *Vibrionaceae* family gathered together in large, very well supported cluster with a bootstrap support value of 95% (**Cluster III**). Four of the six analysed *Aquimarina chiA* gene sequences also formed a very well bootstrap-supported cluster (**Cluster IV**), separate from the *Vibrionaceae* cluster. However, the *chiA* sequence of *Aquimarina* sp. Aq107, a strain isolated from the marine sponge *Sarcotragus spinosulus*, branches deeply between cluster III and IV.

However, the *chiA* phylogeny does not follow quite the same topology as the 16S rRNA gene (a highly conserved phylogenetic marker widely used in bacterial taxonomy and evolutionary biology; see **Figure 11**, **b)**). Indeed, several *chiA sequences,* such as those from *Aquimarina* sp. EL43 and *Enterovibrio* sp. EL24, did not cluster according to their 16S rRNA-based identity but within *Vibrio chiA* sequences.

After this, the p-distance method was used to calculate the proportion of nucleotide sites at which two sequences being compared are different (see **Appendix B.**, **Table 1**). For the *chiA* results, isolates from the same bacterial species share virtually no difference in the nucleotide sites. However, when comparing *Aquimarina* strains with *Vibrio* or *Enterovibrio* strains, the percentage distance rises, and this even happens among *Aquimarina* species. Additionally, when calculating distances within and between clusters (groups) for this dataset, we found that the distance within the *Vibrio* strains cluster was 0.053, and within the *Aquimarina* strains cluster, 0.222. The distance between these two clusters was 0.567 (see **Table 8**).

The distances for the 16S rRNA gene were considerably smaller than the ones obtained for the same isolates, using the *chiA* gene (see **Appendix B.**, **Table 2**). Moreover, distances between and within clusters were also calculated being 0.013 within the *Vibrionaceae* strains cluster, 0.026 within the *Aquimarina* strains cluster and 0.292 between clusters (see **Table 8**).

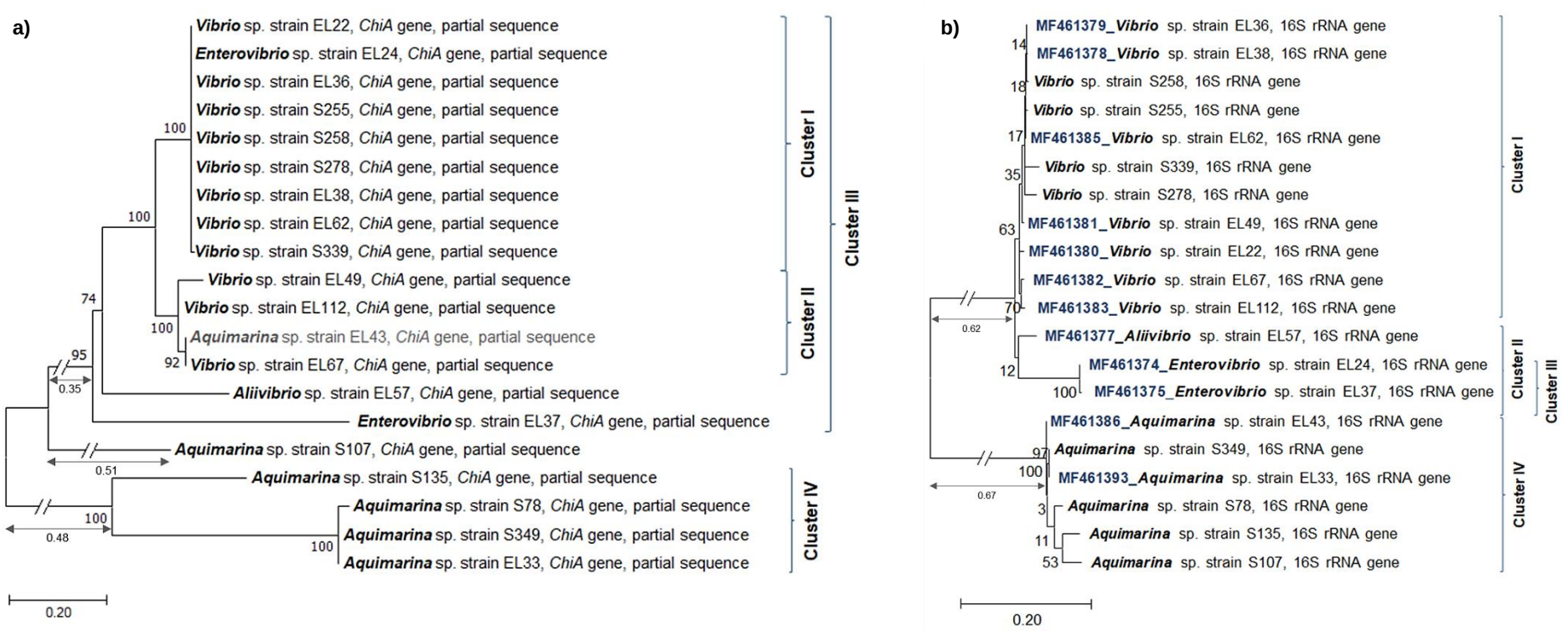


Figure 11. a) Maximum likelihood phylogenetic inference, based on the Tamura-Nei model (Tamura & Nei, 1993), of *chiA* genes amplified from *E. labiata*-associated bacteria. The tree with the highest log likelihood (-1120.54) is shown. The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 13.58% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 20 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 145 positions in the final dataset; **b)** Maximum likelihood phylogenetic inference based on the Kimura 2-parameter model (Kimura, 1980), of 16S rRNA genes from the same *Vibrionaceae* and *Aquimarina* strains presented in the *chiA* tree on the left (**a**). The tree with the highest log likelihood (-2882.26) is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.2721)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 20 nucleotide sequences. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 773 positions in the final dataset.

Table 8. Estimates of evolutionary divergence (p-distances) within and between phylogenetic groups calculated for *chiA* and 16S rRNA gene sequences.

	***chiA* PCR products**	**16S rRNA gene**
	Within-group distance	
***Vibrio* cluster (I + II)**	0.053	0.013
***Aquimarina* Cluster (IV)**	0.222	0.026
	Between-group distance	
***Vibrio* vs. *Aquimarina* Cluster**	0.567	0.292

The number of base differences per site from averaging over all sequence pairs within each group and between-groups are shown. All positions containing gaps and missing data were eliminated.

To complement the previous phylogenetic analysis, a more detailed exploration of the phylogenetic relatedness and distances of the *chiA* sequences within the *Vibrionaceae* family, with all *chiA* sequences available for this group was performed. The *chiA* 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 with chitinase sequences from bacterial genome sequences available in our laboratory on the RAST server. All the sequences that gave reliable best hits and alignments were included in the phylogenetic analysis and aligned with the *chiA* PCR products. Due to high dissimilarities between the *chiA* sequences from different taxa, separate phylogenetic analyses were made for *Vibrionaceae* and *Aquimarina* to allow in depth investigation of the diversity and phylogenetic relatedness of this gene within each taxon. All 16 analysed *Vibrio* spp. *chiA* sequences as well as the *chiA* sequences of *Enterovibrio* EL24 clustered together with 100% bootstrap support (**Cluster I**, **Figure 12, a)**). All here analysed *Aliivibrio* spp. *chiA* sequences clustered together (**Cluster II**), including one *chiA* PCR product, one chitinase sequence from an *Aliivibrio* strain (EL58) isolated from the coral *E. labiata*, one from *Aliivibrio fischeri* MJ11 and another one from *Aliivibrio fischeri* ES114, and separate from *Vibrio* spp.

The *chiA* phylogeny presents a similar topology as the 16S rRNA gene (see **Figure 12**, **b)**), except for the *Enterovibrio* strain sp. EL24 which is on a different position, among the *Vibrio* strains in the 16S rRNA tree, and in a distant position from all the other strains.

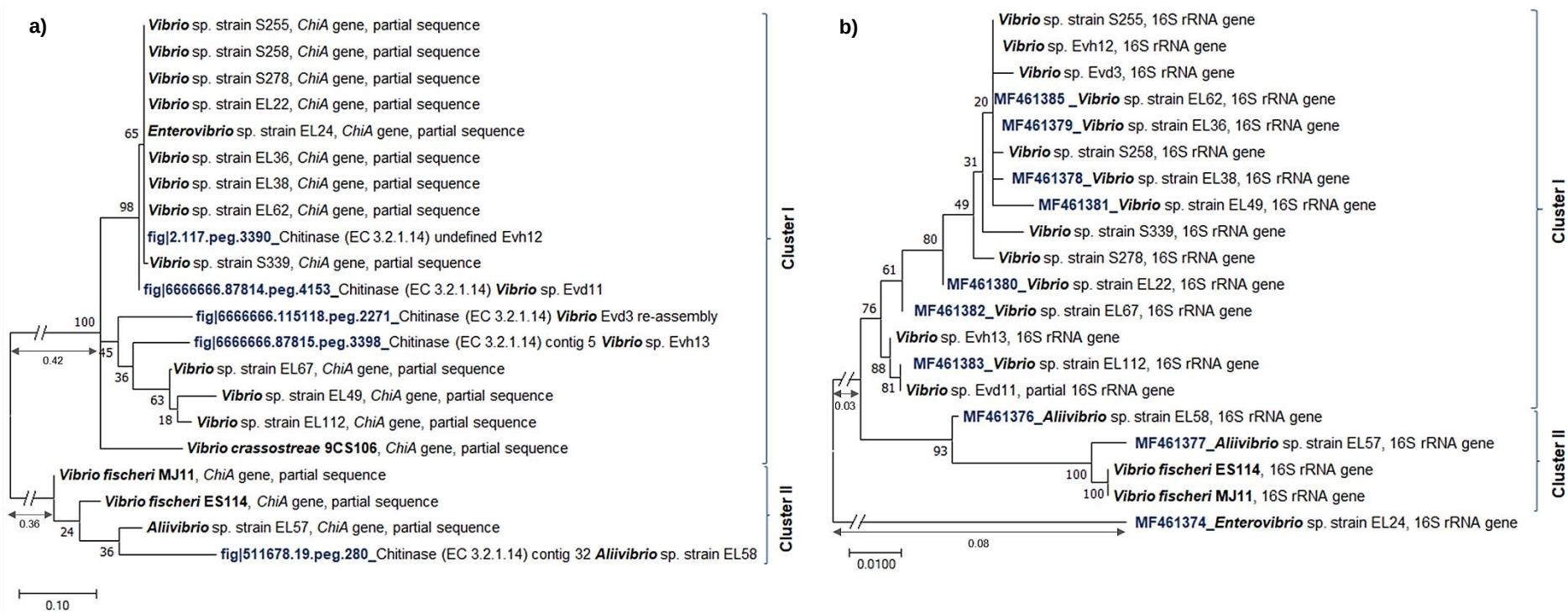


Figure 12. a) Phylogenetic inference of *chiA* genes of the *Vibrio* strains (except EL37) and respective best hits retrieved from NCBI (BlastN) and from alignments with chitinases from sequenced genomes obtained from RAST. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). The tree with the highest log likelihood (-837.13) is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.3169)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.The analysis involved 21 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 174 positions in the final dataset; **b)** Maximum likelihood phylogenetic inference of the 16S rRNA gene from the *Vibrio* strains represented in the phylogenetic tree on the left. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). The tree with the highest log likelihood (-1287.11) is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.0500)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 20 nucleotide sequences. There were a total of 549 positions in the final dataset.

Afterwards, the p-distance method was used to compare the analysed *chiA* sequences (see **Appendix B.**, **Table 3**) and, since only *Vibrio* and *Enterovibrio* isolates were investigated, the proportion of different nucleotide sites was in the majority low. However, *V. fischeri* MJ11, *V. fischeri* ES114 and the chitinases retrieved from the RAST server for *Alliivibrio* sp. strain EL58 and *Vibrio* sp. strain Evh13 presented slightly higher difference proportions. Within-group p-distances calculated for the *Vibrionaceae* and *Aliivibrio* clusters were 2.1% and 9.6%, respectively, while the distance between these two clusters was 29% (see **Table 9**).

The distances for the 16S rRNA gene (see **Appendix**, **Table 4**) were always low, which was expected since all strains belong to the same family, *Vibrionaceae*. Moreover, the within cluster distances were investigated and, compared to the *chiA* phylogeny, major differences were found among the *Allivibrio* strains cluster (1.8%) and between the *Allivibrio* and the *Vibrio* cluster (4.5%; see **Table 9**). The most outstanding result is the between-group distance obtained for *chiA* and 16SrRNA gene, with *chiA* presenting an extremely high distance (29%, compared to 4% in the 16S rRNA; **Table 9**).

Table 9. Estimates of evolutionary divergence (p-distances) within and between phylogenetic groups calculated for *chiA* and 16S rRNA gene sequences.

	***chiA* PCR products**	**16S rRNA gene**
	Within-group distance	
***Vibrio* cluster**	0.021	0.024
***Aliivibrio* Cluster**	0.096	0.018
	Between-group distance	
***Vibrio* vs. *Aliivibrio* Cluster**	0.290	0.045

The number of base differences per site from averaging over all sequence pairs within each group and between-groups are shown. All positions containing gaps and missing data were eliminated.

Again, to explore the phylogenetic relatedness and distances of the *chiA* sequences within the *Aquimarina* strains in more detail, phylogenetic inference of all *chiA* sequences available for this group was performed. Sequences from *Aquimarina chiA* PCR products, best hits and good alignments with chitinases from RAST genomes were used (see **Figure 13, a)**). The first cluster on the tree is very well supported, with a bootstrap value of 100 and it only contains *Aquimarina* sequences and a partial sequence from the genome of *Photobacterium damselae* (**Cluster I**). The last cluster is also well supported, with a bootstrap value of 86 and it contains sequences from the *chiA* PCR products best hits on BLAST *Brevibacillus brevis*, *Paenibacillus* sp and *Stenotrophomonas*, being phylogenetically distant from the first cluster (**Cluster II**). In between these two groups, a small cluster was formed containing two *Aquimarina chiA* PCR products, with one strain from *E.labiata* (EL43) and the other from a marine sponge (Aq107).

In the 16S rRNA tree (**Figure 13, b)**), all *Aquimarina* strains clustered tightly together, with a bootstrap value of 100, except *Aquimarina longa* strain SW024 (**Cluster I**), which branched separate of

the *Aquimarina* cluster. As expected, the Gammaproteobacteria *Stenotrophomonas* and *Photobacterium*, cluster together with strong bootstrap (99%) support (**Cluster III**).

Some p-distances for *Aquimarina* strains and their best hits on NCBI were the highest from our set of data, which means that these are the most distant sequences here reported (see **Appendix B.**, **Table 5** and **6**). The within-group distances of the *chiA* sequences forming the *Aquimarina* cluster were much larger than the distances of the 16S rRNA gene sequences within the *Aquimarina* cluster (**Table 10**).

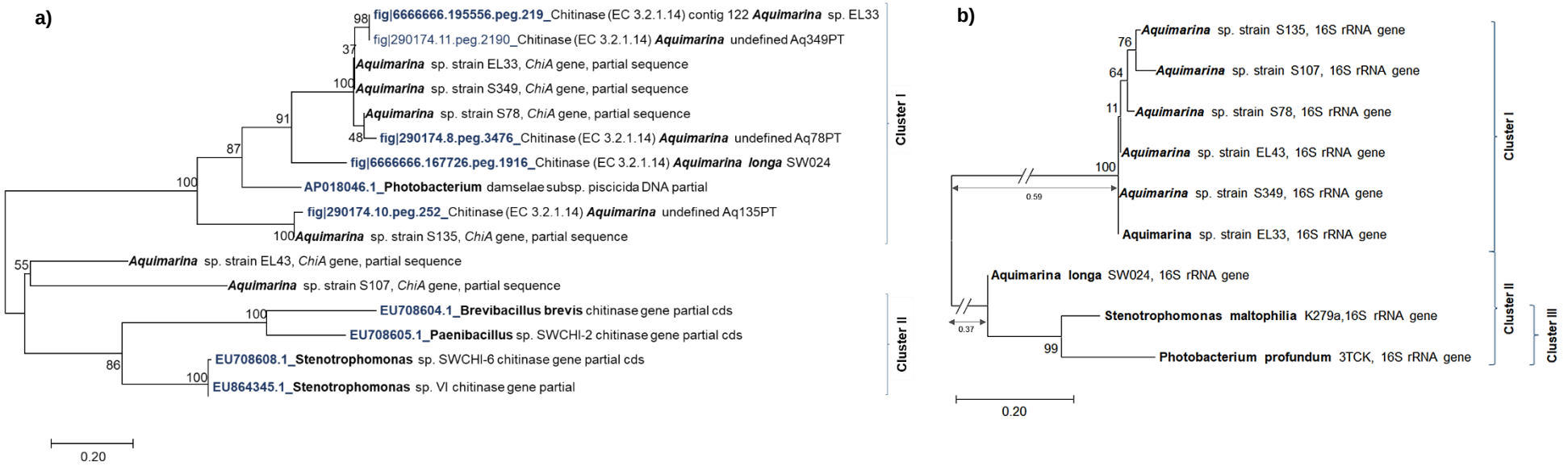


Figure 13. a) Phylogenetic inference of the *chiA* PCR products from all *Aquimarina* strains, and respective best hits from NCBI and from successful alignments with chitinases sequences from the sequenced and available genomes on RAST. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). The tree with the highest log likelihood (-1431.87) is shown. The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 12.74% sites). The analysis involved 17 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 143 positions in the final dataset; **b)** Maximum likelihood phylogenetic inference of 16S rRNA gene from the *Aquimarina* strains represented in the phylogenetic tree on the left. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). The tree with the highest log likelihood (-2342.94) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 9 nucleotide sequences. There were a total of 535 positions in the final dataset.

Table 10. Estimates of evolutionary divergence (p-distances) within and between phylogenetic groups calculated for *chiA* and 16S rRNA gene sequences.

	***chiA* PCR products**	**16S rRNA gene**
	Within-group distance	
***Aquimarina* cluster**	0.214	0.133
***Best Hits* Cluster**	0.400	0.187
	Between-group distance	
***Aquimarina* vs. *Best Hits* Cluster**	0.548	0.515

The number of base differences per site from averaging over all sequence pairs within each group and between-groups are shown. All positions containing gaps and missing data were eliminated.

Finally, to obtain a more global picture of the diverstity of the *chiA* endo-chitinase gene, a *chiA* tree combining all sequences explored in the previous *chiA* specific phylogenetic inferences was created (see **Figure 14**).

Several sequences formed taxon-specific clusters that are congruent with 16S rRNA gene phylogeny. However, there were also some sequences and clusters that did not show congruence with 16S rRNA gene phylogeny, such as the *chiA* sequence of *Aquimarina* sp. strain Aq107, isolated from a marine sponge, that was placed into an *Aliivibrio* cluster (**Cluster IV**) close to the *Vibrionaceae* cluster (**Cluster III**). Also, the cluster composed of *Aquimarina* strains (**Cluster V**) is in this tree more closely related to the *Aliivibrio* and the *Vibrionaceae* **Clusters IV** and **III**, respectively, than the other *Aliivibrio* cluster (**Cluster VI**).

Figure 14. Phylogenetic inference of *Aquimarina* and *Vibrio chiA* PCR sequences, best hits of these sequences from alignments on NCBI (blastN) with sequences of chitinases from reference and sequenced genomes from RAST. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura & Nei, 1993). The tree with the highest log likelihood (-2447.93) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 7.8551)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 43 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 129 positions in the final dataset.

6. *chiA* gene polymerase chain reaction amplification from metagenomic DNA

To extend our knowledge of the abundance of chitinases in the marine environment, we have conducted a PCR experiment using metagenomic DNA extracted from microbial pellets prepared from samples of the gorgonian corals *Eunicella labiata*, *Eunicella verrucosa*, *Eunicella gazella* and *Leptogorgia sarmentosa* and their surrounding seawater and sediment. The results show that there are indeed endo-chitinase (*chiA*) encoding genes present in all coral species and in sediment and seawater (see **Figure 15**, **16** and **17**). Despite the ubiquity of the *chiA* gene across several samples representing distinct environments, the bands observed after agarose gel electrophoresis were relatively faint, suggesting that only a small portion of the total microbial community in each habitat carries the target gene. Future Southern blot-hybridization and real-time PCR assays can be helpful in determining whether differential proportions of the *chiA* gene are observed across the inspected habitats. Below, a metagenomics-centred approach is applied to reveal the *chiA* gene diversity (**section 7**) and proportions (**section 8**) of several genes involved in the degration of chitin across gorgonian corals, seawater and sediments.

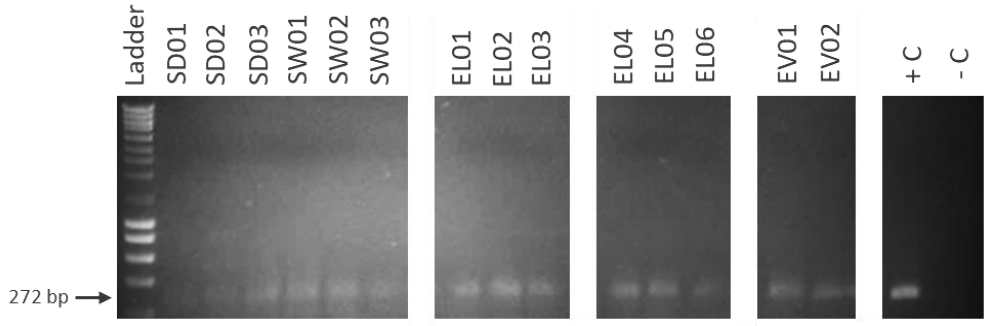


Figure 15. PCR amplification of *chiA* genes from metagenomic DNA samples of sediments (SD), seawater (SW), *Eunicella labiata* (EL) and *Eunicella verrucosa* (EV).

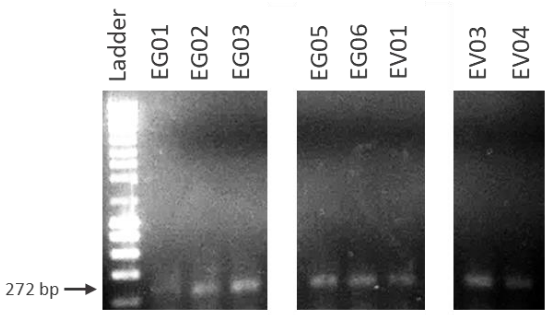


Figure 16. PCR amplification of *chiA* genes from metagenomic DNA samples of *Eunicella gazella* (EG) and *Eunicella verrucosa* (EV).

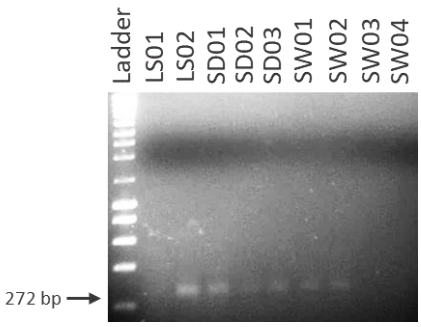


Figure 17. PCR amplification of *chiA* genes from metagenomic DNA samples of *Leptogorgia sarmentosa* (LS), sediments (SD) and seawater (SW).

7. Taxonomic classification of endo-chitinases encoding genes retrieved from microbial metagenomes of gorgonians, sediment and seawater

To further explore the diversity of chitinase genes in gorgonian corals and their surrounding environment, taxonomic analysis of endo-chitinase-encoding genes identified in, microbial metagenomes was undertaken (**Figure 18** and **19**). The proportion of sequences classified as chitinase-encoding genes that could not align with any other sequence present in the whole NCBI database was extremely high for every sample (see **Table 11**).

Table 11. Proportion of sequences classified on MG-RAST as chitinase-encoding genes possessing no or identifiable hits, after blast searches performed on NCBI.

	Eunicella gazella (healthy)	*Eunicella gazella* (necrotic)	*Eunicella verrucosa*	*Leptogorgia sarmentosa*	Sediment	Seawater
Proportion of sequences with no hits (%)	88.3	74.1	89	98.4	97	82.6
Proportion of classified sequences (%)	11.7	25.9	11	1.6	3	17.4

For those sequences that were classifiable (**Figure 18** and **19**), the taxonomic diversity was higher in sediment and seawater, than in the gorgonian microbiomes which are commonly influenced by host-selective processes. *Vibrionales* was the dominant order among the chitinase sequences present in the gorgonian microbiome. On the contrary, *Flavobacteriales* related endo-chitinase sequences were only identified in sediment and seawater. Another interesting aspect is that no eukaryotic-like chitinase sequences were found in the coral micro-environments, on the contrary of what happens in both seawater and sediment. Curiously, the *Eunicella verrucosa* taxonomic profile is different from the other two gorgonian species, with a high proportion of chitinase sequences identified as *Alteromonadales* and *Thiotrichales*.

At the species level, most *chiA* sequences from corals with hits on NCBI were classified as *Vibrio splendidus* and *V. crassostreae*. The only identifiable *Flavobacteria* hits (the family to which the *Aquimarina* genus belongs) were retrieved from sediment and seawater metagenomes, and affiliated with *Maribacter* sp.

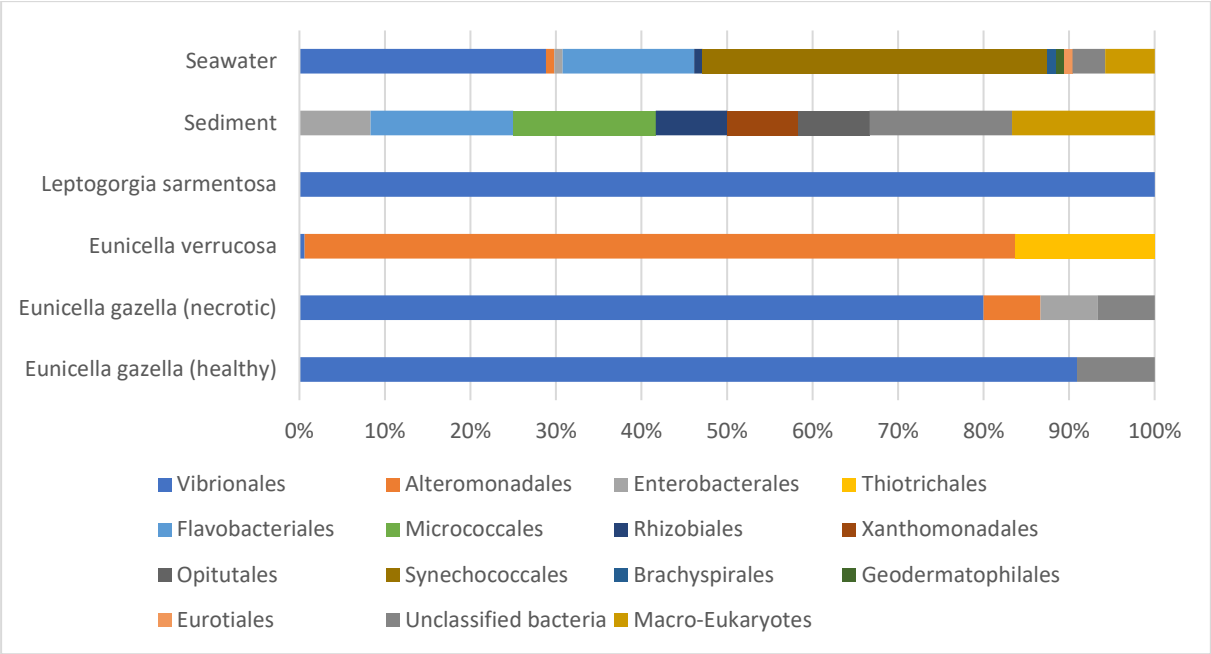


Figure 18. Closest NCBI BlastN hits (analysed in the software package MEGAN) of the endo-chitinase sequences retrieved from the microbial metagenomes of sediment, seawater and three gorgonian coral species. Sequences from the replicate samples of the same habitat were pooled in this analysis. The taxonomic classification is presented at order level; sequences with "no hits" to the NCBI BlastN database were not included in this graph.

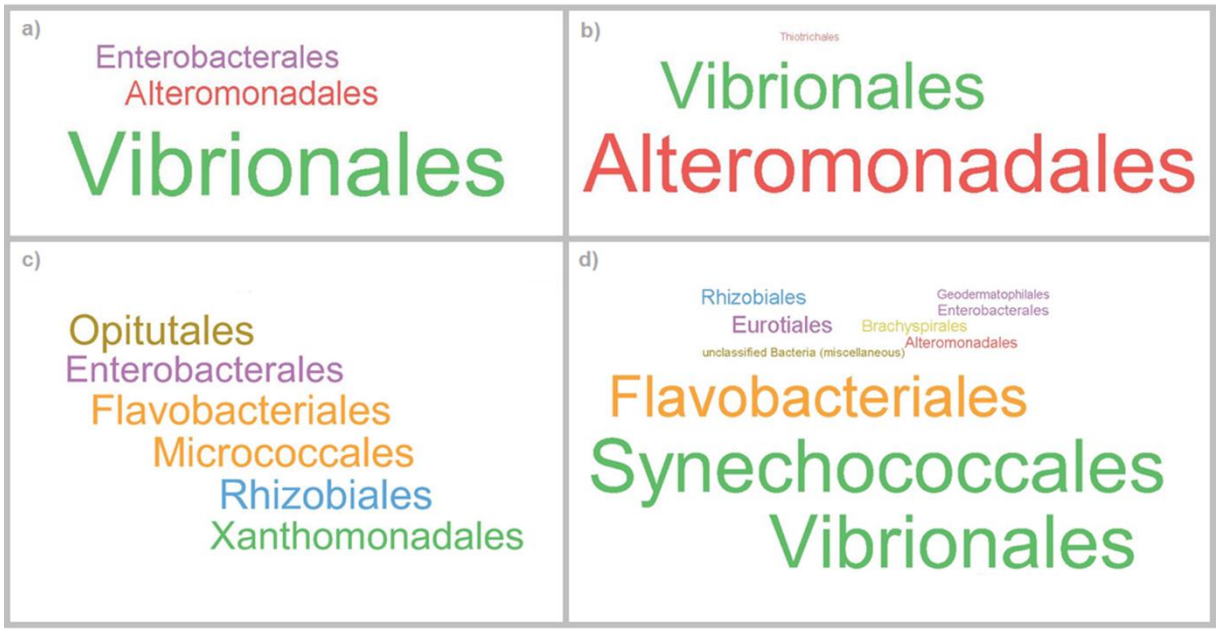


Figure 19. Word cloud with the microbial blast results of the chitinase sequences retrieved from the microbial metagenomes of a) *Eunicella gazella* (necrotic), b) *Eunicella verrucosa*, c) sediment and d) seawater. Blast results from the metagenomes of both *Eunicella gazella* (healthy) and *Leptogorgia sarmentosa* corresponded to only one order, *Vibrionales*, hence no comparison could be made to create a word cloud for these micro-habitats. Size of the letters is proportional to the number of sequences that were classified at the order level.

8. Relative abundance of chitin-binding, chitobiase and chitinase genes in microbial metagenomes of gorgonians, seawater and sediment

To obtain a glimpse on whether gorgonian corals could be a hotspot for chitin-degradation, the relative abundance of chitin degradation-related-genes in the microbial metagenomes of *Eunicella gazella, Eunicella verrucosa, Leptogorgia sarmentosa*, sediment and seawater was estimated (see **Figure 20**).

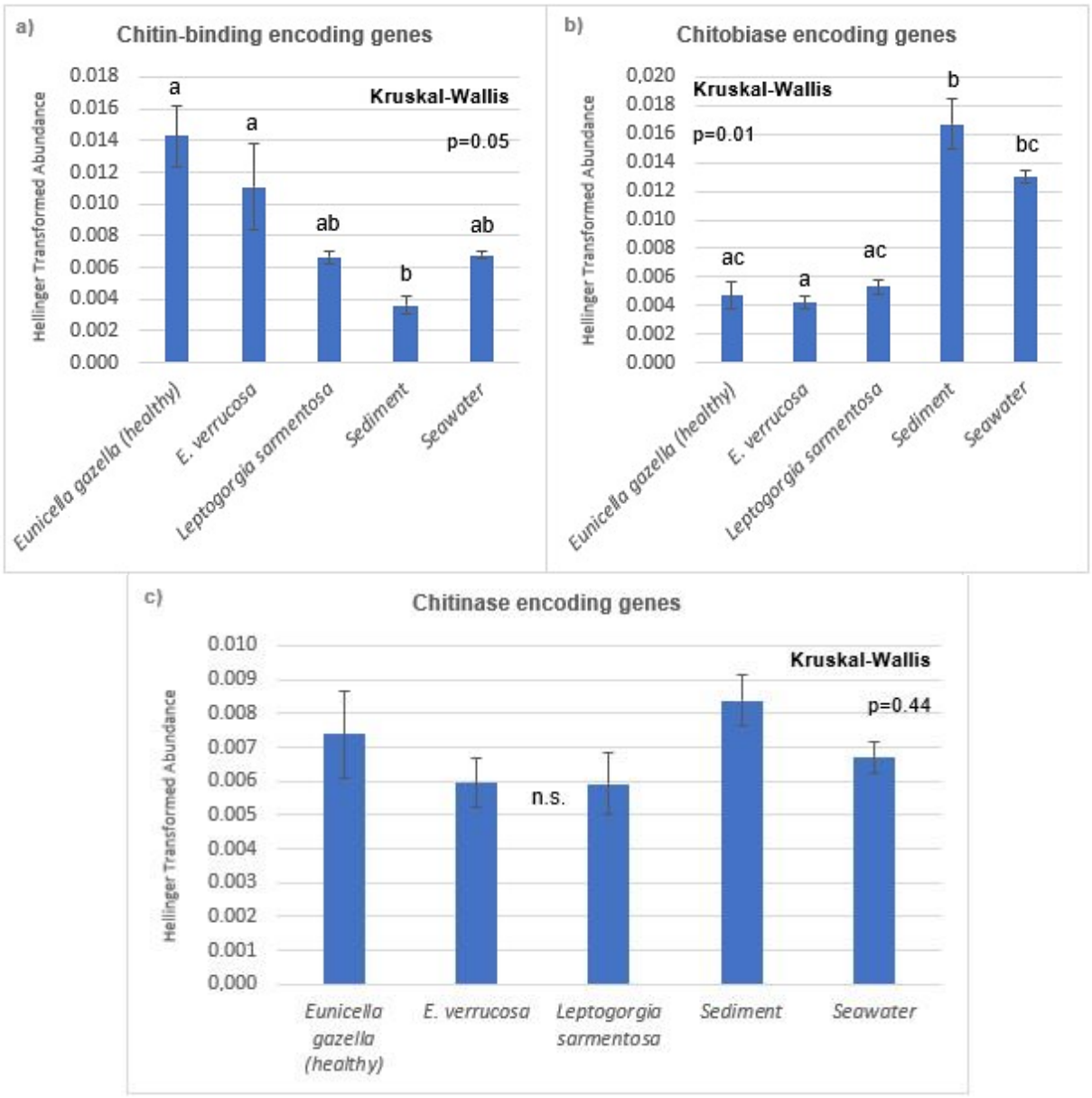


Figure 20. Relative abundance of genes involved in chitin degradation present in gorgonian, sediment and seawater microbial metagenomes. InterPro (IPR) relative abundances (mean ± SE) after Hellinger transformation are shown. Letters above error bars indicate significant differences (Kruskal-Wallis-Test on Ranks, followed by Dunn's post-hoc test if significant). n.s.: not significant.

Chitin-binding and chitobiase (the enzyme that hydrolyses GlcNAc dimers into monomers) encoding genes presented a significant difference between environments, with p-values of 0.05 and 0.01, respectively (see **Figure 20, a)** and **b)**). The relative abundance of genes encoding for chitin-binding proteins was significantly higher in the *Eunicella gazella* microbiome than in sediment ($p \leq 0.001$; see **Figure 20**, **a)**). There was also a strong tendency for a higher abundance of chitin-binging protein encoding genes in *E. verrucosa* compared to sediment (p=0.03; see **Figure 20**, **a)**). 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: $p \leq 0.01$; *L. sarmentosa* vs. sediment: p=0.02; see **Figure 20**, **b)**). The relative abundance of chitobiase encoding genes was also significantly reduced in *Eunicella verrucosa* compared to seawater (*E. verrucosa* vs. seawater: p=0.02; see **Figure 20**, **b)**). As for the chitinase-encoding genes, no significant differences were found.

Moreover, the relative abundance of chitin-degradation related genes in the microbiomes of healthy *Eunicella gazella* tissue was compared with that of the microbiomes from necrotised *Eunicella gazella* tissue (see **Figure 21**). Again, the relative abundance of chitin-binding and chitobiase genes presented significant differences between the healthy versus diseased coral states (see **Figure 21**, **a)**, **b)**), whereas chitinase-encoding genes did not differ significantly (see **Figure 21**, **c)**).

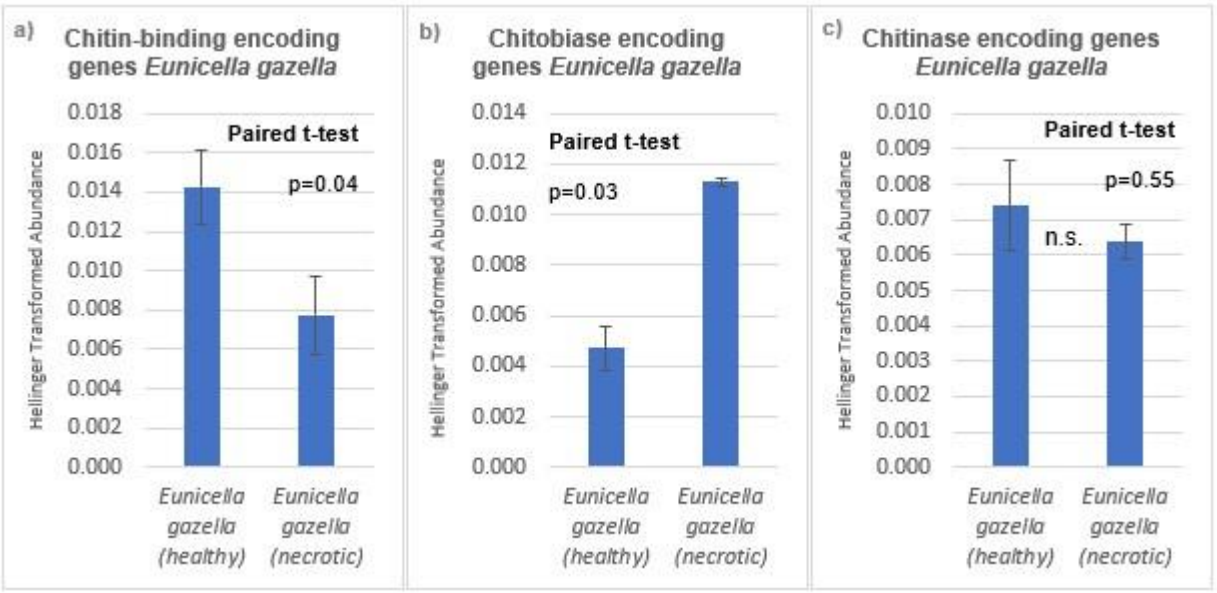


Figure 21. Relative abundance of chitin degradation related genes present in the microbial metagenomes retrieved from healthy versus necrotic *Eunicella gazella* samples. InterPro (IPR) relative abundances (mean ± SE) after Hellinger transformation. n.s. not significant.

DISCUSSION

1. *In-vitro* results

Chitin degradation is an important chemical process in the marine environment and chitinolytic enzymes or chitin-degrading microorganisms are being increasingly studied due to their industrial and medical applications. Here we analysed 36 phylogenetically distinct bacteria, all previously isolated from the gorgonian coral *Eunicella labiata,* and found that 12 of these strains (all belonging to the genera *Aquimarina* (*Bacteroidetes*), *Enterovibrio* and *Vibrio* (*Gammaproteobacteria*)) are indeed capable of degrading chitin. Chitin agar plate assays were previously performed in *Aquimarina longa* (Xu *et al*., 2015), using a protocol from Hsu & Lockwood (1975), in *A. addita* sp. (Yi & Chun, 2011) and in *A. salinaria* sp. (Chen *et al.,* 2012), both using a protocol from Smibert (1994) and the three *Aquimarina* species were found to be chitin degraders. Regarding *Vibrios*, a study from Suginta and colleagues also reported chitinolytic activity, for *Vibrio alginolyticus*, *V. carchariae, V. harveyi, V. campbellii, V. nereis* and *V. aestuarianus*, using a chitin agar plate assay (Suginta *et al.,* 2000). For the genus *Enterovibrio*, no reports on their chitin degrading ability have been found.

The chitinase enzyme assay with the three different substrates revealed that substrate 3, 4-methylumbelliferyl β-D-N,N′,N″-triacetylchitotriose (specific for the detection of endo-chitinase (EC 3.2.1.14) activity), yielded more activity than the other two substrates (specific for exo-chitinase activities) in both *E. labiata* derived *Aquimarina* sp. strains. This is in line with the genomic information available for *Aquimarina* sp. EL33, showing that much more endo-chitinase (*N* = 19) than exo-chitinase (*N* = 6) encoding genes are present on the EL33 genome (Keller-Costa et al., 2016). In the case of *Vibrionaceae*, the ratio between the number of exo- and endo-chitinases present in their genomes is much more equilibrated (Hunt *et al.,* 2008). Moreover, all activities measured in this study were extracellular activities of chitinase enzymes secreted by the bacterium into the growth medium, since the cells were not lysed before the experiment, but assays were performed with culture supernatants. Endo-chitinases are enzymes that typically act outside the cell, cleaving large water insoluble chitin polymers into smaller soluble oligomers (Beier & Bertilsson, 2013). In one study of *Serratia marcescens*, endo-chitinases were secreted into the medium, whereas the washed and sonicated cell pellet showed no detectable chitinase activity (Béguin & Aubert, 1994). Yet, the exo-chitinase *N-acetylglucosaminidase* is, in bacteria, frequently found to act in the periplasmic space on soluble chitin oligomers and dimers that have been taken up via specific transport proteins (Beier & Bertilsson, 2013). Since cells were not lysed before the assays, it seems plausible that relatively little exo-chitinase activity was detected in the *Aquimarina* strains. We are currently screening for intracellular exo-chitinase activity (from cell pellet extracts) in the same 36 bacterial isolates.

No endo-chitinase (EC 3.2.1.14) activity was detected in the culture supernatants of the alpha-proteobacterium *Ruegeria* sp. EL09 – in line with the lack of any chitin-degrading ability on chitin agar plates. On the other hand, *Vibrio* sp. strain EL36, one of the strongest chitin degraders of the chitin agar plate assay, showed endo-chitinase activities even higher than the ones obtained for the two *Aquimarina* strains. This is the first report of endo-chitinase-specific activity for members of the genus *Aquimarina*. A previous study from Yu and colleagues had already reported chitin-degrading ability for several *Aquimarina* strains, including *A. megaterium*, which is the closest type strain to our *Aquimarina* isolates

(Yu *et al*., 2014), but a specific detection and quantification of endo-chitinase (EC 3.2.1.14) activity and a deeper analysis of the genes involved, such as the endo-chitinase *chiA*, was not yet reported in the literature. However, bacterial chitinase activity values available in the literature are usually reported as untits (U) per mg of purified chitinase protein (see **Table 12**), while in this study we measured activites directly in culture supernatants as Units per L (with unknown amounts of proteins); hence, the values are not directly comparable.

Table 12. Overview of the specific endo-chitinase (EC 3.2.1.14) activity values in other bacterial species (Units per mg of purified chitinase proteins excreted into chitin-containing culture medium).

Organism	Specific Activity	Observations	Reference
Pseudomonas sp.	0.0069 unit / mg protein	Purified chitinase CHT1 from culture supernatants with shrimp shell wastes (chitin substrate)	Wang *et al.,* 2008
Aeromonas hydrophila	0.076 µmol N-acetyl-D-glucosamine / min / mg enzyme	Purified protein. Chitinase activity assayed in a CC supplemented medium	Ohtakara *et al.,* 1988
Bacillus circulans	0.15 unit / mg of protein 0.16 unit / mg of protein	Purified protein. Chitinase activity assayed in purified chitin and CC supplemented media, respectively	Wiwat *et al*., 1999
Serratia marcescens	0.181 µmol N-acetyl-D-glucosamine / min / mg enzyme	Purified protein. Chitinase activity assayed in a CC supplemented medium	Ohtakara *et al.,* 1988
Bacillus cereus	0.62 µmol reducing sugars / min / mg enzyme	Purified protein, from culture supernatants with shrimp shell powder	Wang et al., 2009

When our *Aquimarina* sp. strains were grown on chitin-supplemented media, endo-chitinase activities increased throughout time and were most evident on days 9 and 10. On CC, *Aquimarina* sp. strain EL43 had its maximum activity on day 9 while in strain EL33 activity still increased from day 9 to day 10. On chitin powder, endo-chitinase activity of EL43 was also still increasing from day 9 to 10, which might be because the insoluble chitin powder generally is a harder-to-access substrate (and hence also less preferred) than the hydrated and activated colloidal chitin (CC). Chen and colleagues have studied chitinase expression in *Aeromonas schubertii* and found that fine (small) chitin particles suppress chitinase activity in the culture supernatant due to a higher reaction area (larger particle surface) for reducing sugars and a consequent higher concentration of hydrolysed chitin products (oligomers) that can provoke a negative feed-back loop and suppress endo-chitinase expression in the cell (Chen *et al.,* 2014).

Overall, the endo-chitinase activities of the induced *Aquimarina* strains (grown on chitin-supplemented media) remained in the same range of the values obtained for the non-induced strains (grown on MB 1:2 without chitin) and no significant improvement of endo-chitinase activity was found. One explanation for this might be the possible competition between the soluble chitin-oligomers present in the chitin-containing growth medium (that cannot be removed from the supernatant by simple

centrifugation) and the 4-methylumbelliferone substrate fed to the enzyme during the activity assay, which may result in a reduction of the activity measured. One way to overcome this problem could be the use of an inducer substrate other than chitin. However, the study by Chen and colleagues, compared several carbon sources when testing for chitinase induction, including glucose, GlcNAc, GlcN, sorbitol, sucrose, cellulose, starch, chitosan and chitin itself, being the latter the most suitable substrate, suggesting that chitinases are responsive to specific substrates and that chitin is the best-known substrate to promote the activation of chitinase-encoding genes (Chen *et al.,* 2014). Instead of directly using the culture supernatant for the enzyme assay, extraction and purification of the chitinase protein would be a more accurate way to quantify chitinase activity (as Units per mg of enzyme). However, since the purification of a protein is more laborious and time-consuming, it is hardly feasible as a first activity screening approach for a large set of bacterial strains – which was the aim of this thesis – since this study revealed the presence of endo-chitinase activity in the understudied flavobacterial genus *Aquimarina*, one of the next steps in our research group will be the purification of endo-chitinase proteins from our *Aquimarina* strains, which may be achieved using chromatography methods, such as an affinity chromatography. A previous study had already reported the molecular mass of an endo-chitinase from *Vibrio cholerae* (88.7 kDa; Connell *et al.,* 1998), however, to our knowledge, no studies have so far investigated the molecular mass, structure and properties of *Aquimarina*-derived chitinases available in the literature.

Moreover, the conditions of the enzyme assay can be extensively manipulated in the future, according to the organism being studied, as a mean to improve the measured activities. This enzyme assay kit was designed to detect chitinase activity in fungal and bacterial growth media and it was tested on *Trichoderma viride*, which serves as the positive control for the kit, on some mammalian cell lines, human macrophages and rat organ tissues, hence not being optimized for bacteria or marine microorganisms. In addition, a previous study showed that chitinases obtained from the marine *Streptomyces* sp. DA11 strain isolated from the marine sponge *Craniella australiensis* had higher activities at pH 8 (Han *et al.,* 2009). Among marine bacterial taxa, chitin degradation pathways are most studied and best understood in the genus *Vibrio* (Hirono *et al*., 1998; Suginta *et al.,* 2000; Revathi, *et al.,* 2012; Rao *et al*., 2013; Svitil *et al*., 1997). The production and characterization of chitinases from *Vibrio* spp. was already assessed in a report from Revathi and colleagues, where the authors have tried to find pH and temperature optima for chitinase activity and have found an optimal pH between 6.0 and 6.5 and an optimum temperature of 45ºC (Revathi *et al.,* 2012). In addition, Rao and colleagues have found that *V. alginolyticus* and *V. harveyi* showed chitinase activity at pH 5.0, 7.0 and 9.0, and at 25ºC (Rao *et al.,* 2013). All these evidences suggest that chitinases can act differently according to the producing organism and tests should be made prior to each assay to determine the most accurate pH and temperature conditions for chitinase activity, for each bacterial isolate, if precise measurements are to be registered for the organism in question. Yet as a first approach – to determine endochitinolytic activity for a diverse panel of bacteria, the methodology used here, essentially following the supplier's recommendations, was found to be robust given the corroboration observed with the CC plate assay.

2. Genomic analysis of the chitin degradation pathway

Genome-wide inspections revealed that many genes related to the chitin-degradation pathway that were detected in the *Vibrionaceae* isolates were absent in the genomes of the *Alphaproteobacteria* and *Flavobacteria* isolates analysed in this study. This concerned especially chitin binding proteins, the chitodextrinase precursor, PTS genes and N-acetylglucosamine-regulated methyl-accepting chemotaxis proteins. Among the genes annotated with chitin-related functions, some of them have an inconsistent distribution of copy numbers within *Vibrionaceae* genomes, which may indicate that apart from the chitin degradation core, there is some flexibility in the gene contents and some gene redundancies.

The set of genes related to chitin catabolism that is present in the *Aquimarina* genome is quite different from the one found in *Proteobacteria*, with many chitinase genes (19), six beta-hexosaminidase and three glucosamine-6-phosphate deaminase genes being found on the *Aquimarina* genome. A previous *in silico* analysis of the genome of *Aquimarina* sp. strain EL33 identified 19 chitinase-encoding genes (all endo-chitinases, EC 3.2.1.14; Keller-Costa *et al*., 2016), corroborating our results. Also, another study on *Aquimarina longa* revealed the presence of seven genes encoding chitinases (Xu *et al*., 2015). Regarding *Vibrio* spp., several studies report on the existence of chitinase-encoding genes on the genome of this genus or on *in vitro* chitinase activity from several *Vibrio* species (Svitil *et al.,* 1997; Suginta *et al*., 2000; Mansson *et al.,* 2011). Regarding *Enterovibrio* sp., no reports have been found.

Only very few studies on *Aquimarina* physiology and genomics are present in the literature and the chitin degradation pathway has never been described for this taxon. However, the insights gained in this study suggest that the genes involved in chitin degradation of *Aquimarina* are quite different from the other taxa analysed here. For example, the *Aquimarina* genome lacks the common PTS- or ABC-transport systems for chitin monomers, dimers and / or oligomers and the chitin transporters are yet to be discovered in this genus. Other genomic studies on *Bacteroides* showed the presence of many so-called Polysaccharide Utilization Loci (PULs), known to be involved in cell surface polysaccharide capture and hydrolysis (Foley *et al.,* 2017). The most studied PUL-encoded polysaccharide uptake system is the Starch Uptake Systems (Sus) encoded in the Sus operon (Shipman *et al.,* 2000; Foley *et al.,* 2017). The Sus system is unique to Gram-negative *Bacteroidetes* phylum and various analogues of the system exist that seem to target numerous, diverse polysaccharides while PTS- or ABC-transport systems seem to be widely absent in this taxonomic group (Shipman *et al.,* 2000; Foley *et al.,* 2017). It is also believed that Sus systems may be involved in the binding of other polysaccharides, such as xylose and chitin (Nicole Koropatkin, personal communications). An *in-silico* analysis of the genome of *Aquimarina* sp. EL33, on RAST, confirmed the presence of three Sus genes, SusA, SusC and SusD. Sus A corresponds to an alpha-amylase, neopullulanase, that is in the periplasmic space and hydrolyses oligosaccharides before their transport to the cytoplasmic membrane; SusC and SusD are both outer membrane proteins and are physically associated, being crucial genes for starch binding and growth on starch. An also interesting fact is that a whole-genome analysis of the genome of *B. thetaiotaomicron* found 101 SusC/SusD paralog pairs, and each of these pairs is believed to target a specific glycan. Interestingly, some marine *Bacteroidetes* have also shown the presence of these paralogs (Koropatkin

et al., 2008; Flint *et al.,* 2008; Foley *et al*., 2016). A better understanding of the chitin degradation pathway in *Aquimarina* could open doors to develop new mechanisms to degrade polysaccharides, such as chitin, for all the applications mentioned previously in this study (Koropatkin, *et al.,* 2008).

Aliivibrio sp. EL58 contains several chitin-degradation related genes on its genome, including five chitinase-encoding genes. This corroborates results obtained for *A. salmonicida*, where genes belonging to the chitinolytic pathway were found, including three chitinase-encoding genes (Hjerde *et al.,* 2008). Some *Vibrio* strains present multiple gene copies encoding for the N-acetylglucosamine-regulated methyl-accepting chemotaxis protein and N-acetylglucosamine-specific PTS, which may indicate that more protein or enzyme can be produced, resulting in a higher efficiency in the fulfilment of the functions, or that, if the gene copies are heterogenous, the enzymes may have slightly different specificities. Moreover, it is known that in *Vibrios* the chitinolytic process starts with the detection of chitin oligosaccharides and / or GlcNAc by chemotactic sensors (Li *et al.,* 2004), which explains the presence of N-acetyl-glucosamine-regulated methyl-accepting chemotaxis proteins in all *Vibrio* genomes. An also interesting aspect is that *Alphaproteobacteria* isolates present N-acetyl-D-glucosamine permease proteins and N-acetyl-D-glucosamine binding proteins related to ABC-transport systems whereas not a single PTS encoding gene was found. This finding can be corroborated by a comparative genomic study from Yang and colleagues that showed the existence of variations and non-orthologous dislocations of components from transport mechanisms, especially the PTS-transport of GlcNAc, characteristic of *Vibrionales*, that is replaced by permeases and ABC cassettes (Yang *et al.,* 2006).

When chitin degrading activity was observed in only some cells from bacterial cultures, it was hypothesized that those cells that did not present chitinase activity were feeding on the excess hydrolysis products produced by the cells with chitinase activity (Shapiro, 1998) and this has been described for several strains that are able to degrade chitin (Gaffney *et al*., 1994; Chernin *et al*., 1998; DeAngelis *et al*., 2008). As a survival mechanism, it would be a great adaptative strategy if part of the cell population had chitinases up-regulated and would further supply hydrolysis products to their kin, through a mechanism called cross-feeding (Smith *et al*., 1992; Kirchman & White, 1999). In open environments, such as the ocean, this release of hydrolysis products would trigger interspecifc cross-feeding which, in the case of chitin degrading activity, seems plausible since, as reported on previous studies, some bacteria that grow on GlcNAc (Kaneko & Colwell, 1978) or $(GlcNAc)_2$ (Keyhani & Roseman, 1997) do not possess enzymes for chitinolytic activity. Other studies have already compared the ratio of chitin degrading bacteria vs. chitin consuming bacteria in aquatic environments and strongly support a significant interspecific cross-feeding during chitin breakdown (reviewed in Beier & Bertilsson, 2013). Also, the chitin-user community, i.e. all chitin degraders and all organisms that rely on cross-feeding processes, can play a key role regarding chitin destination. For instance, chitin consumers likely use a higher percentage of GlcNAc to produce their cell wall (Konopka, 2012), whereas chitin-degraders may use these substrates to produce energy (Beier & Bertilsson, 2013).

All these findings can be applied to the present study, suggesting a potential coupling between *Gammaproteobacteria* and *Aquimarina* sp. with *Alphaproteobacteria* in the cycling of chitin, where the former two are catabolizing the polymer, while the latter benefit from this breakdown to further process

GlcNAc residues. These organisms are all found in the coral microbiome and a potential syntrophy between them could lead not only to a better efficiency in the chitin cycling but also in the cycling of carbon and nitrogen (Beier & Bertilsson, 2013) hence, positively affecting the functioning of the gorgonian microbiome and surrounding ecosystem.

Additionally, although the *Alphaproteobacteria* analysed in this study did neither degrade chitin on agar plates nor show chitinase activity in the enzyme assay (tested for *Ruegeria*), several *Alphaproteobacterial* taxa possessed genes involved in the chitin degradation pathway and, *Labrenzia* sp. EL143 and *Rhodobacteraceae* bacterium EL53 even possessed endo-chitinase (EC 3.2.1.14) encoding genes. Cottrell and colleagues have found that *Alphaproteobacteria* that contain the *chiA* gene were not able to grow on chitin, which we could also corroborate with our chitin agar plate assay, and this result can suggest that, despite being present, the chitin degrading pathway might not be functional, hence it can represent a possible lateral gene transfer (LGT) event to a strain that did not present a complete chitin-related metabolic pathway (Cottrell *et al.,* 2000). Also, it is possible that, in the strains where chitinase genes are present but no *in vivo* activity was detected, the gene could have always been present, as an ancestral gene, and once the chitin degradation machinery was not needed anymore, from one point onwards, the bacterium started to lose those genes.

3. PCR of the *chiA* gene, its phylogenetic analysis and comparison with 16S rRNA gene

Most of the strains that showed chitin degrading ability on the plates had a *chiA* gene on their genome that was amplifiable with the *chiA* specific primer pair used in this study. There were only two exceptions: firstly, *Aliivibrio* sp. EL57 that showed no chitin degrading activity on the plate but presented a positive *chiA* PCR result. This could be due to the inactivation of the *chiA* gene (or another gene obligatory for chitin degradation) or an insufficient up-regulation of *chiA* gene expression under the culture conditions used in this study. Secondly, *Vibrio* sp. EL41 that, on the contrary, had a negative *chiA* PCR result despite degrading chitin on the plate. This may be due to the *chiA* PCR primer set used in this study, which may not match these *Vibrio* chitinase sequences or the strain may be able to degrade chitin by another pathway that does not require specifically the *chiA* gene. Previous studies reported great difficulties in designing new primer pairs for capturing additional *chiA* sequences in strains that were positive for growth on chitin but did not amplify with the “usual” primer sets used. This suggests that either *chiA* is not essential for chitin degradation or that the gene presents a higher diversity than what was previously expected (Hunt *et al*., 2008). Difficulties in the PCR amplification of the *chiA* gene have been reported repeatedly in various scientific articles due to the high heterogeneity of this gene (Cottrell *et al.,* 2000; Ramaiah *et al.*, 2000; LeCleir *et al.,* 2004). The phylogenetic analysis conducted in this study shed light on this remarkable heterogeneity, revealing that the *chiA* gene is much more divergent between the bacterial isolates studied than the 16S rRNA gene, which suggests that the *chiA* gene is evolving at a much faster rate. Also, Hunt and colleagues found that, when a different set of genes (hsp60, a chromosomal gene that codes for an heat shock protein (Kwok *et al.,* 2002); mdh, a chromosomal housekeeping gene that codes for malate dehydrogenase and is not subjected to LGT (Boyd *et al.,* 1994; Denamur *et al.,* 2000); adk, that codes for adenylate kinase, being ubiquitous, with a well conserved function and less likely to be horizontally transferred (according to the complexity

hypothesis, where proteins are less prone to lateral transfer events; Coenye & Vandamme, 2005; Counago & Shamoo, 2005)) were 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*, which makes sense since the three genes used are thought not to be horizontally transferred and this phylogeny reflects a vertical evolution of the lineages, that are phylogenetically close. The genomes of *Photobacterium* are known to have divergent *chiA* sequences, suggesting that processes of LGT and gene duplication occur in core chitin degradation genes, including the *chiA* gene (Hunt *et al*., 2008). Other studies have also reported the occurrence of lateral gene transfer (LGT) or duplication of this gene, and on how difficult it is to create and interpret phylogenies based on it (Cottrell *et al.,* 2000).

As for the phylogenetic analysis performed, *chiA* phylogeny was compared to 16S rRNA gene phylogeny and we were able to explore how the accurate taxonomic phylogeny changes when genes not necessarily related to taxonomic classification are studied. For example, *Aquimarina* sp. EL43 was placed among the *Vibrionaceae* cluster, which suggests that this *Aquimarina* strain may have received a *chiA* gene, for example from a *Vibrio* strain by LGT. The evolutionary distances (calculated from proportions of nucleotide differences) both, within the *Vibrio* and within the *Aquimarina* clusters are much higher in the respective *chiA* phylogeny than in the 16S rRNA phylogeny. For example, the pairwise distance between *Aquimarina* sp. S78 and *Aquimarina* sp. S107 was 0.566 (i.e. only 43.4% nucleotide sequence similarity) in the *chiA* gene alignment and 0.040 (96% nucleotide sequence similarity) in the 16S rRNA gene alignment (see **Appendix B.**, **Table 1** and **2**), which clearly demonstrates how heterogenous the *chiA* gene is. Likewise, the *chiA* gene phylogeny of the *Vibrionaceae* strains does not follow completely the 16S rRNA gene phylogeny of this family. For example, *Enterovibrio* sp. EL24 groups together with other *Vibrio* spp. strains in the *chiA* phylogeny whereas it forms a unique branch, separate from *Vibrio* spp. and *Allivibrio* spp. in the 16S rRNA gene tree. Again, this may suggest the occurrence of a LGT event, when a *Vibrio* and an *Enterovibrio* strain exchanged genetic information, e.g. during conjugation. The distance between the *Vibrio* and the *Allivibrio* cluster, was higher in the *chiA* gene tree (0.290, i.e. 71% nucleotide sequence similarity) as compared to the 16S rRNA gene tree (0.045, i.e. 95,5% nucleotide sequence similarity), again showing that the *chiA* gene is more heterogeneous and divergent among different species.

Darwin's theory (Darwin & Irvine, 1904) states that natural selection corresponds to the unequal individual reproductive success in a population so that the fittest, i.e. most adapted, individuals gave rise to more offspring and, consequently, the traits that are responsible for this higher adaptation become dominant in the next generation, and this cycle is repeated endlessly. Additionally, Dawkins stated ''if you look at the way that natural selection works, it seems to follow that anything that has evolved by natural selection should be selfish'', and he defends that ''by definition a copying error is to the disadvantage of the gene which is miscopied. [However, if] it is to the advantage of the selfish mutator that induces it, the mutator can spread through the gene pool'', which means that if a mutator gene, with copying errors, has a negative effect on other genes, its mutant alleles can still be selected and propagated due to its own selfishness, especially under high selective pressure (Dawkins, 1976). These notions gave rise to the concept of "selfish" gene evolution, that constitutes the standard model by which genes are inherited, through a vertical transfer process, via mitosis or binary fission, in eukaryotes and

prokaryotes, respectively. However, genes can also be exchanged between different genomes, through a process of LGT (Orgel & Crick, 1980). This genetic exchange can enhance the adaptation of organisms to specific environmental conditions and it is often perceived as an altruistic behaviour, from the point of view of the organism that is sharing the gene since its fitness does not improve once the transfer has been made (Fournier *et al.,* 2015). Previous studies have suggested that genes that were vertically transferred code for essential cellular processes, whereas the horizontally transferred, non-essential, genome codes for several secondary metabolites that can constitute mechanisms to fight specific toxins or antibiotics or that can confer the ability to exploit a specific niche (Hacker & Carniel, 2001; Norman *et al*., 2009). These findings are of great interest for the topics addressed in this study which focusses on coral microbiomes and the evolution of chitinase genes. In a context where the community is exposed to a high selective pressure (e.g. high amount of chitin entering coral organisms through filtering activity), the existence of few specialists that could, after some generations of huge pressure on the community function, dominate the system might not solve the problem. But, if the ability to degrade chitin could spread throughout the coral-associated microbial community and diverse organisms were able to perform this chitin-degrading function it could lead to a higher resilience and functional redundancy in the community. Our results and previous studies on the possibility that *chiA* gene can be horizontally transferred (Hunt *et al*., 2008; (Cottrell *et al.,* 2000) may suggest that this mechanism can indeed be happening, despite being merely speculative. Future studies of the coral microbiome shall focus on the gene content that usually traffics about on plasmids ("the metamobilome") to enlighten our knowledge of the traits more likely to be spread throughout the community, including genes involved in antibiotic resistance and secondary metabolites production.

In the *chiA* phylogenic tree, most *chiA* PCR products amplified from the bacterial isolates share their sequence with one of the multiple endo-chitinase genes detected on the genomes of these same isolates by *in-silico* (RAST-based) analysis. That is, within all endo-chitinase sequences that are present in the genome of one strain, there is one that aligns completely with its corresponding *chiA* PCR product (e.g. the *chiA* sequence of *Aquimarina* sp. S135 clustered together with endo-chitinase gene fig|290174.10.peg.252 from its own genome). According to the description on RAST, all the sequences identified as chitinases from our genomes are *chiA* endo-chitinases (that is, family 18 glicosyl-hydrolases genotype A) and, when blasting those sequences against a nucleotide database (BlastN on NCBI) no different closest hits were found (such as c*hiB*, c*hiC* or *chiD*; Hjort *et al.,* 2014; Karlsson & Stenlid, 2009; Suzuki *et al.,* 2002; Henrissat & Bairoch, 1993; Orikoshi *et al*., 2005). However, when constructing a phylogeny with not only chitinases sequences from our genomes (retrieved from RAST and therefore identified as endo-chitinases *chiA*) but also *chiB* and *chiC* gene sequences from diverse organisms they are all mixed together and a clear division between *chiA*, *chiB* and *chiC* sequences is not detectable (see **Appendix C.**, **Figure 1**). This may suggest that not everything that RAST identifies as endo-chitinase (EC 3.2.1.14) is indeed *chiA* or that the lack of knowledge of *chiB* and *chiC*-type chitinases is affecting the identification of chitinases by this and other alignment platforms.

Finally, a comprehensive *chiA* phylogenetic analysis (**Figure 14**) that comprised not only all *chiA* PCR products and endo-chitinase-encoding genes detected on the genomes of the bacterial isolates of this study, but also multiple *chiA* sequences retrieved from the public database was performed. Here,

most *Aliivibrio* sequences formed a separate cluster, apart from *Vibrionaceae* strains. While *Aquimarina* strains are mostly together in the same cluster, apart from the *Aquimarina* sp. strain EL43 mentioned above, and *Aquimarina* sp. strain 107, extracted from a sponge isolate, that also appears among the *Vibrio* and *Enterovibrio* strains.

Overall, the genomes analysed in this study show taxon-specific segregations in the type and copy number of chitinase-encoding genes and, within- and across-taxon diversity of *chiA*-like gene sequences. Moreover, it was found that the *chiA* gene is highly diverse and seemingly prone to higher mutation rates and LGT events. This confirmed diversity of the gene, even more highlighted when compared with the 16S rRNA gene, eliminates the possibility of using it as a phylogenetic marker gene. However, it can be helpful when a deeper phylogeny relationship is required, for instance, with an enough resolution to infer on species and sub-species levels. All this analysis led to the conclusion that the endo-chitinase (EC 3.2.1.14) *chiA* gene is not essential for survival but it presents, undoubtedly, a competitive advantage under certain environmental pressures and / or conditions, being this the reason to be so prone to LGT events.

One of the best-known features of bacteria is their overwhelming genotypic and phenotypic diversity, that results in different metabolic traits and, consequently, different lifestyles (Ochman *et al.,* 2000). This diversity is often due to the transfer of genes by transformation, conjugation or transduction through this process called LGT, that plays a crucial role in evolution. Advances in sequencing technologies over the past few years have allowed the sequencing of whole genomes from prokaryotic organisms and, through sequences comparison, LGT phenomena were discovered. Mobile genetic elements (MGE) and gene transfer agents (GTA) are known to help in this process and, despite marine microorganisms being difficult to cultivate and not so accessible as clinical bacterial isolates, there are already some studies on the occurrence of LGT events in the oceans, including some specific studies describing these events in particular organisms, in coastal environments, in the water column and in deep sea systems, in the sediment, on coral reefs and in open ocean systems (Deschamps *et al*., 2014; McDaniel *et al*., 2012.; Labonté *et al.,* 2015; Sobecky & Hazen, 2009; Degnan, 2014). LGT is now known to be extremely important for the evolution of marine microorganisms and, consequently, of marine microbial communities and ecological processes. In a time where environmental stresses, including climate change, are happening, this mobilome represents the most important source of gene flux for an increasing genetic plasticity and adaptability and chances of survival and reproduction in the sea (McDaniel *et al*., 2010). Thus, LGT processes are extremely useful and can be an evolutionary adaptation that will be selected and transmitted through generations, allowing the survival of the species that do so.

As previously mentioned, the gorgonian coral microbiome could benefit from lateral transfer of genes responsible for chitin degradation, such as the endo-chitinase *chiA*, to different community members. To address this, a prediction of LGT candidates in our prokaryotic genomes, available on RAST, could be done. Several methods have previously been reported to do so and they can be generally separated in two groups: composition-based methods, that screen for atypical characters present in gene sequences (Becq *et al*., 2010), and phylogeny-related methods, that screen for incongruences between the taxonomic phylogenetic tree of a specific species and the orthologous gene tree of the gene in

question (Beiko & Ragan, 2008; Podell & Gaasterland, 2007). Composition-based methods assess the similarity between genome sequences from the host, since they tend to be more alike, and recently acquired genes. These genes generally present characters in the gene sequence composition (such as GC content, codon usage, etc) that differ from the host genome (Dufraigne *et al*., 2005). But this is not as simple as it seems, for instance, it is known that genes that have suffered LGT can adjust their sequence to the base composition and codon usage of the host genome (Lawrence & Ochman, 1997) and that genes that are present in the same genome can still present some dissimilarity. In turn, phylogeny-related methods depend on related orthologous genes. Despite being extremely laborious, these methods will likely produce an accurate result, mainly because genome sequences are always being uploaded and updated (Beiko & Hamilton, 2006). This is still an extremely recent field of study and there are not yet flawless and totally accurate methodologies to determine what are the genes that have actually been subjected to LGT events, but this last phylogeny-related method could be used by our research group, in future studies, to better understand the role of the endo-chitinase (EC 3.2.1.14) *chiA* gene in the coral-microbiome, how it get there, and how it is evolving.

4. Metagenomic analysis (*chiA* PCR amplification and taxonomic classification)

Analyses of metagenomic DNA obtained from microbial pellets derived from seawater, sediments and the gorgonians *Eunicella labiata*, *Eunicella verrucosa*, *Eunicella gazella* and *Leptogorgia sarmentosa*, confirmed the presence of *chiA* endo-chitinases in all these communities. For the gorgonian coral *Eunicella labiata* we had anticipated this result as this study showed that some of its dominant bacterial associates possess chitinase-encoding genes in their genomes and are capable of degrading chitin *in vitro*. However, this metagenomic analysis shows that the *chiA* gene is ubiquitous in the microbiomes of octocorals and their surrounding environment. Seawater is undoubtedly a vehicle for the transport and dispersal of bacteria between marine environments. Chitin breakdown is a process that is well known to occur in the water column mediated e.g. by planktonic *Vibrio* species (Huq *et al*., 1983; Meibom *et al.,* 2005), and the detection of *chiA* genes in the seawater metagenomes analysed in this study corroborates this prespective. Although the microbial community composition residing in octocorals is clearly distinct from seawater (e.g. Keller-Costa *et al.,* 2017; van de Water *et al.,* 2018), the latter likely acts as a source for the recruitment of new microbial associates as octocorals are suspension-feeding organisms that collect bacteria that are present in the water column attached to organic particles (marine snow) and (zoo)plankton on which they feed (van de Water *et al*., 2018). Moreover, a recent metagenomics study on the microbiome of marine sponge conducted in our laboratory has shown that sponge microbial communities are more similar to sediment than seawater microbiomes, suggesting that the surrounding sediment likely acts as an important source for microbial recruitment (Karimi *et al.,* 2017). This may also be true for octocorals which, like sponges, are sessile organisms anchored to the seafloor.

A surprisingly unexpected result for the taxonomic classification of chitinases from the metagenomic samples was the extremely high percentage of reads for which no taxonomic classification was achieved, not even at domain or phylum level. This is clearly an indication on how understudied chitinases are in general and how little we know about them. It also suggests that chitinases

heterogeneity is much likely even higher than what we have just seen in our phylogenetic analysis, which again demonstrates that we do not possess enough knowledge to use endo-chitinase genes as phylogenetic markers.

Among the chitinase sequences that were classified, less diversity was found in corals than in sediment and seawater. This tendency can be corroborated by a previous study from Keller-Costa and colleagues that found a significantly higher number of prokaryotic phylotypes (Operational Taxonomic Units (OTUs)) in seawater, compared to the gorgonian *Eunicella labiata* (Fig. S2A, Supporting Information, Keller-Costa *et al.,* 2017), suggesting that lower chitinase diversity in gorgonians reflects the lower taxonomic diversity of these communities in comparison with that of the surrounding bacterioplankton. Other studies also found that bacterial communities differ from seawater and coral surfaces (Frias-Lopez et al., 2002; Rohwer et al., 2002; Bourne & Munn, 2005). Studies on the bacterial diversity of corals are increasing and, it is now known that their microbial communities are as diverse and distinct as marine sponges (Webster *et al*., 2010). Much more literature is available for marine sponges and several studies had already reported the difference in the microbial diversity of sponges, seawater and sediment micro-habitats (de Voogd *et al.,* 2015; Cleary *et al*., 2015.; Bayer *et al.,* 2014). For example, a study from Hardoim and colleagues report a higher bacterial diversity in seawater when compared to the marine sponges *Sarcotragus spinosulus* and *Ircinia variabilis* (Hardoim *et al.,* 2014). Also, Thomas and colleagues have found that generally diversity measures decrease from sediments to seawater samples and from the latter to marine sponges' samples (Thomas *et al.,* 2016).

Regarding the taxonomic classification at the species level, *Vibrio splendidus* and *V. crassostreae* were among the most abundant ones in the coral-derived chitinase sequences. This result makes absolute sense since both species correspond to the best *chiA* hits to our *Vibrio* sp. isolates, when blasting them against a nucleotide database (NCBI). On the contrary, taxonomic assignment of metagenomic *chiA* sequences did not lead to the detection of any *Aquimarina*-derived *chiA* genes in the dataset. Most likely this is because chitinases sequences from *Aquimarina* spp. are yet unstudied and no annotated *Aquimarina* specific *chiA* sequences are currently present in public nucleotide databases. In sediment and seawater, *Maribacter* sp. appears as the only *Flavobacteria* species classified, most likely also reflecting the limited availability of *chiA* sequences derived from several marine bacteria other than *Vibrio* species and closely related *Gammaproteobacteria*.

As for the relative abundance of the chitin degradation genes across the different micro-habitats inspected, octocoral microbiomes show an enrichment of chitin-binding genes, that enhance the cells' binding capacity to chitin substrates and chitinases, a crucial step for chitin degradation to occur. In the case of *Eunicella gazella*, 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 the PCR results from this study where the endo-chitinase *chiA* gene is present in all micro-habitats (corals, seawater and sediment). Chitobiase-encoding genes (responsible for the cleavage of N-acetylglucosamine dimers into monomers) were higher in sediment and seawater, which suggests higher efficiency in the processing of the cleaved water-soluble oligomers in these habitats.

As for the comparison between the healthy and the diseased corals, significant differences were found for chitin-binding and chitobiase genes and the *chiA* gene composition in diseased corals

resembles that of seawater and sediment from the previous analysis. This is an interesting finding that may suggest that when a coral is diseased its microbial community is disrupted and replaced. The coral might lose its ability to select and maintain advantageous bacterial taxa as well as its capacity to filter-feed which suggests that bacteria that are dispersed in the water column and / or attached to suspended particles may arrive to the coral surface and colonize it.

Gorgonians are part of the benthic suspension feeders' communities that establishes a boundary between the substratum and the water column. In temperate marine biomes, these communities are known to regulate carbon flux, from pelagic to benthic zones and in the coast of Portugal there is a high abundance of gorgonian corals. By removing large amounts of energy, derived from suspended and / or dissolved organic particles, from the water column these organisms are among the most efficient in the ocean in uptaking and processing energy from marine ecosystems (Gili & Coma, 1998). It seems plausible that gorgonians are also key players in chitin breakdown, not only because chitin particles are part of the suspended organic matter that is present in the water column but also due to the existence of previous studies on the microbial processes behind chitin breakdown that revealed that chitin presents a significant and critical connection between the carbon and nitrogen cycles in the marine environment (Souza *et al.,* 2011). Our data strongly suggests that a different microbial community of chitin degraders is present in each micro-environment and, thus, a differential capacity to process chitin is likely to exist even though the abundance of the genes involved in chitin breakdown may not significantly differ from one micro-habitat to the other.

CONCLUDING REMARKS

This study highlights the existence of chitinases in octocoral associated bacteria. Compared with chitinases derived from terrestrial organisms, marine chitinases can have a higher pH and salinity tolerance, which can be of great interest for their previously mentioned biotechnological, medical and environmental applications. Here we showed that *Aquimarina* strains are a novel source of these enzymes and, if we continue to study them, distinctive properties may be discovered, not only from a still quite unexplored environment, the ocean, but also from a bacterial species with so much characteristics and capabilities left to be unveiled.

The metagenomic analysis also showed that so much more is yet to be disclosed about these unique micro-habitats, their associated microbiota and respective functional assignments. Octocorals are indeed a promising source of taxonomically diverse chitin-degrading bacteria and, consequently, of novel chitinolytic enzymes.

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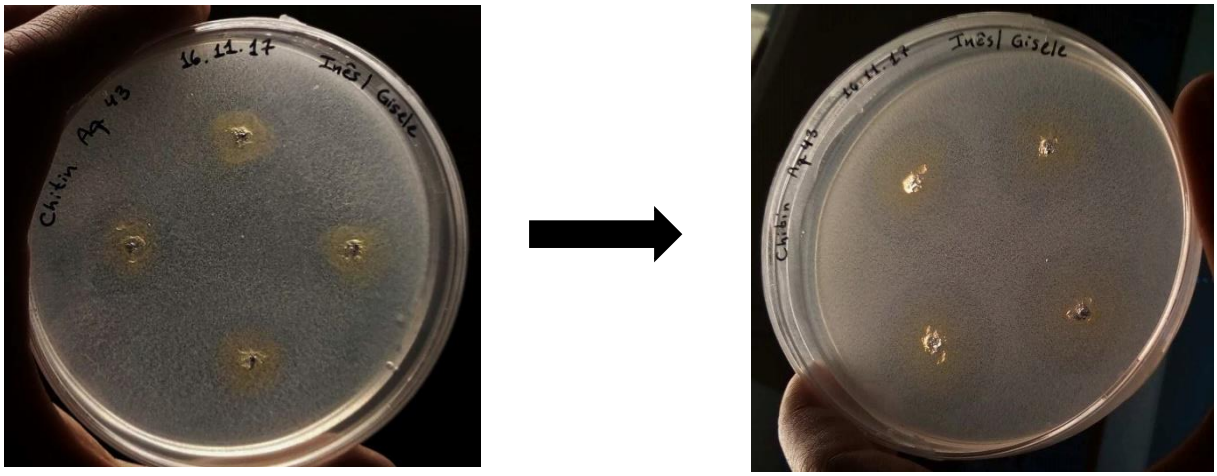
APPENDIX

A. Chitin Agar Plate Assay positive results at 7 days and 14 days of incubation, respectively.

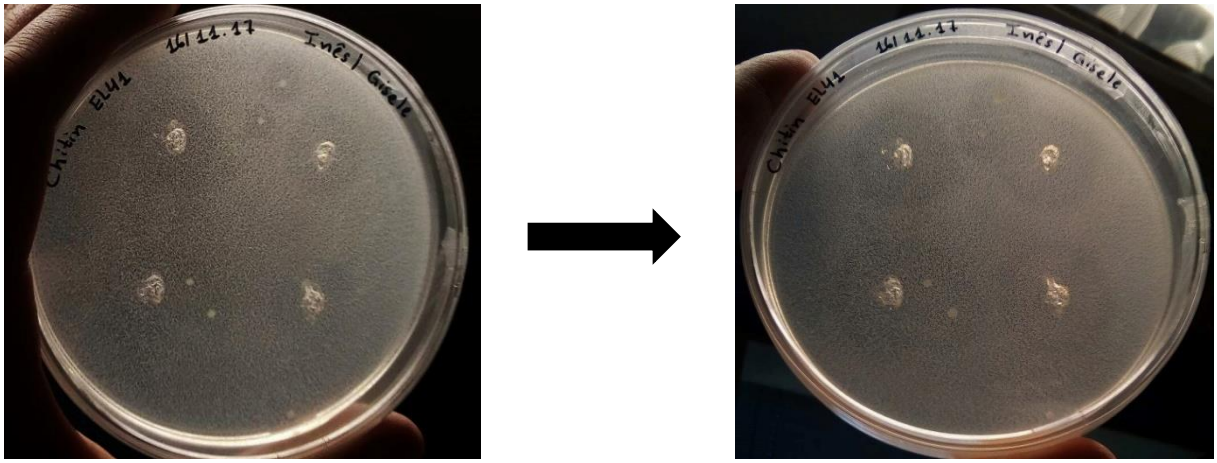
Aquimarina sp. (EL43)



Aquimarina sp. (EL33)

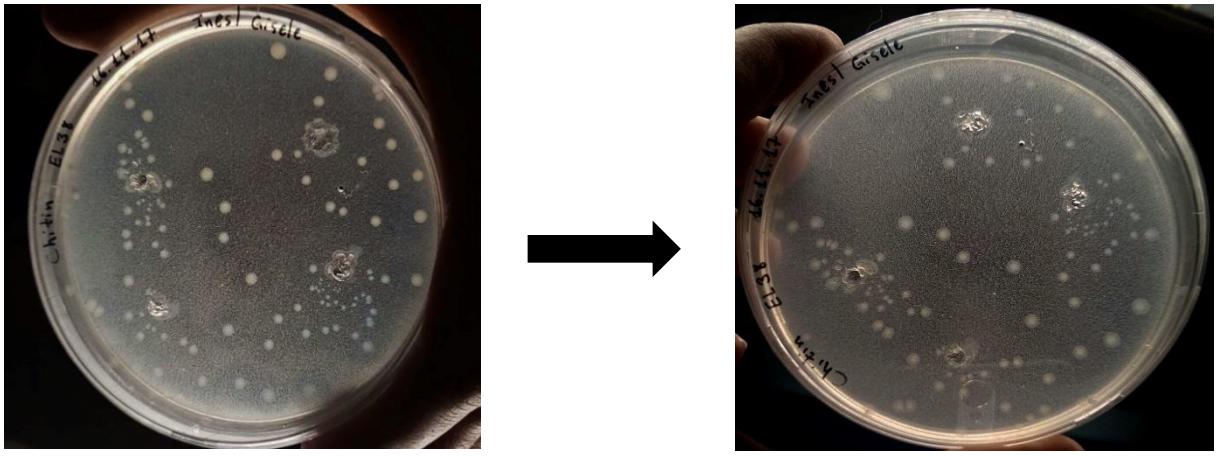
Vibrio sp. (EL112)

Vibrio sp. (EL41)



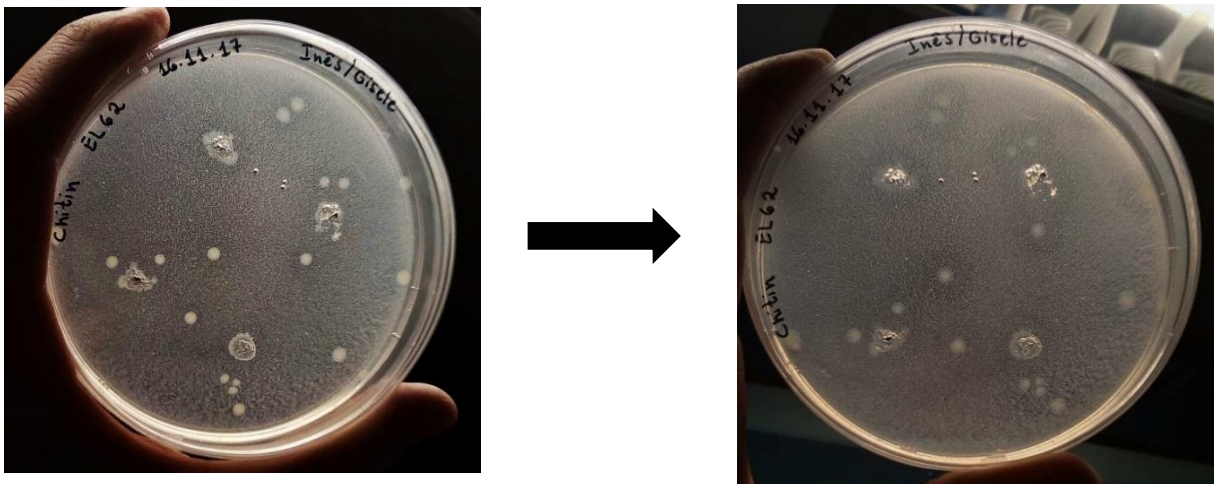
Vibrio sp. (EL49)

Vibrio sp. (EL38)



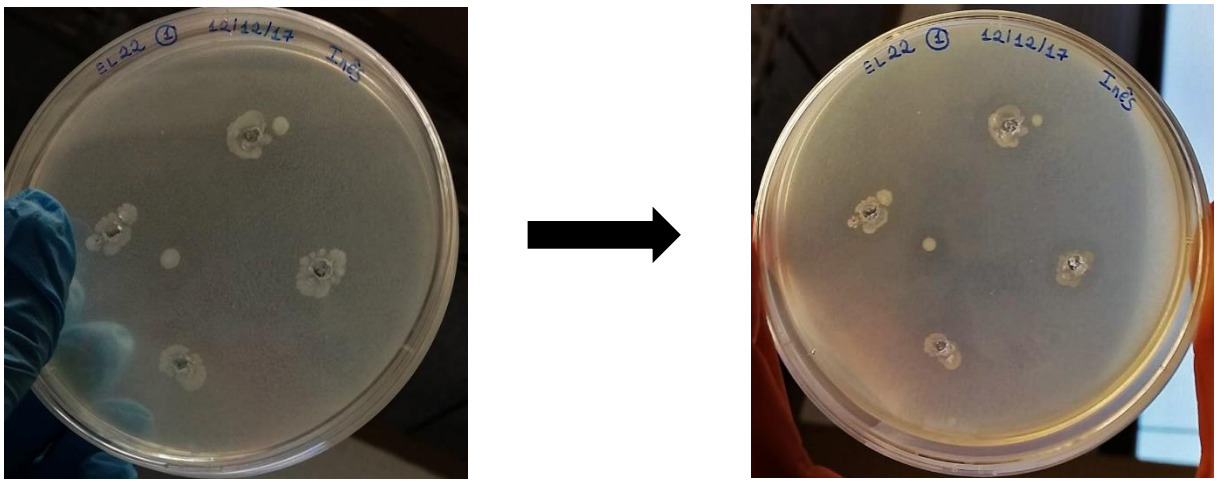
Vibrio sp. (EL67)

Vibrio sp. (EL62)

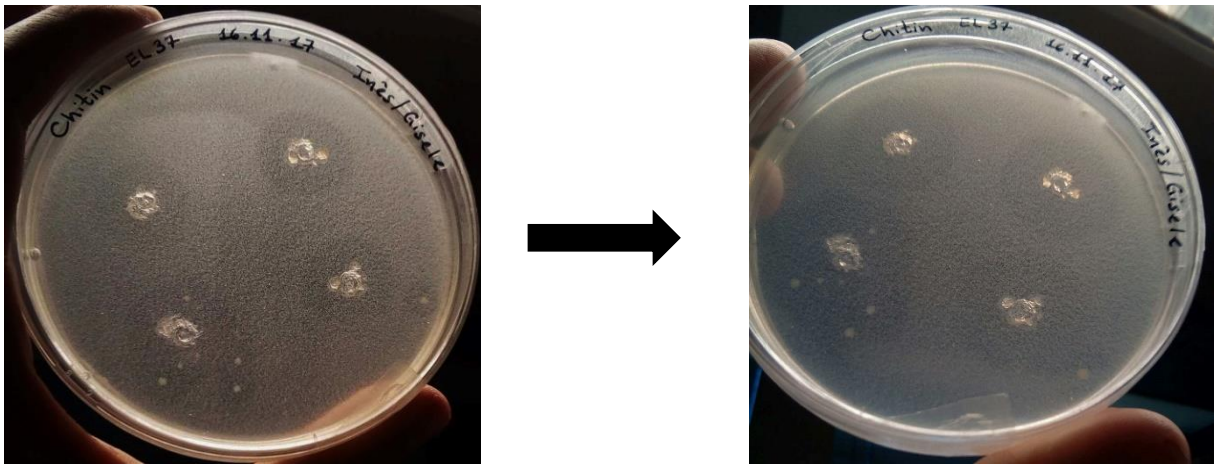


Vibrio sp. (EL36)

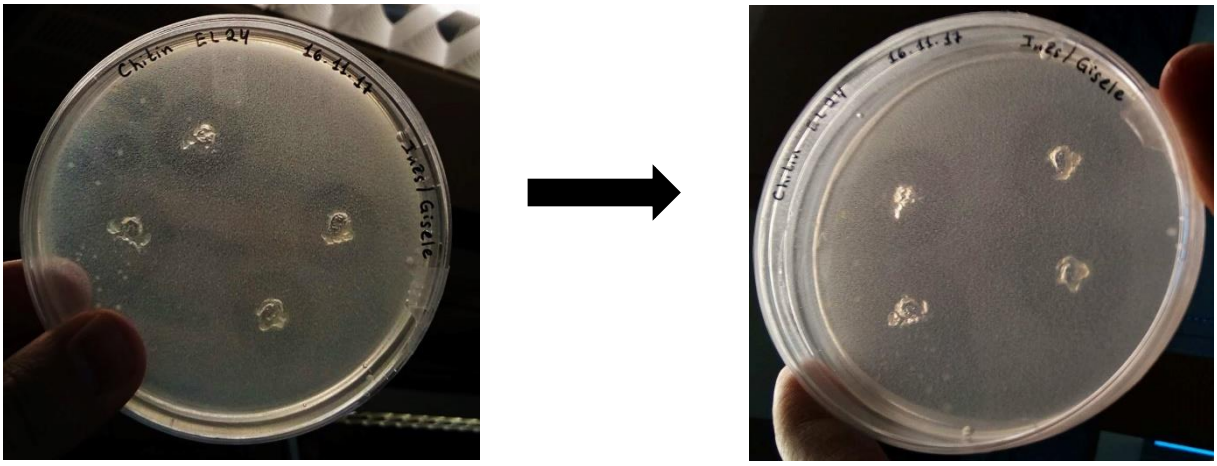
Vibrio sp. (EL22)



Enterovibrio sp. (EL37)



Enterovibrio sp. (EL24)



B. p-distances from the phylogenetic analysis

Table 1. Estimates of Evolutionary Divergence between Sequences, through the calculation of the p-distance, of the *chiA* genes amplified from *E. labiata*-associated bacteria.

	EL22 Vb	EL24 Evb	EL36 Vb	EL37 Evb	Vb 255	Vb 258	Vb 278	Aq 135	Aq 78	Aq 107	Vb 339	Aq 349	EL38 Vb	EL43 Aq	EL49 Vb	EL57 AVb	EL62 Vb	EL67 Vb	EL112 Vb
EL22_Vb																			
EL24_Evb	0																		
EL36_Vb	0	0																	
EL37_Evb	0,421	0,421	0,421																
Vb255	0	0	0	0,421															
Vb258	0	0	0	0,421	0														
Vb278	0	0	0	0,421	0	0													
Aq135	0,517	0,517	0,517	0,648	0,517	0,517	0,517												
Aq78	0,579	0,579	0,579	0,593	0,579	0,579	0,579	0,421											
Aq107	0,517	0,517	0,517	0,538	0,517	0,517	0,517	0,572	0,566										
Vb339	0,007	0,007	0,007	0,428	0,007	0,007	0,007	0,524	0,586	0,524									
Aq349	0,559	0,559	0,559	0,586	0,559	0,559	0,559	0,428	0,021	0,559	0,566								
EL38_Vb	0	0	0	0,421	0	0	0	0,517	0,579	0,517	0,007	0,559							
EL43_Aq	0,097	0,097	0,097	0,386	0,097	0,097	0,097	0,531	0,572	0,490	0,103	0,566	0,097						
EL49_Vb	0,131	0,131	0,131	0,434	0,131	0,131	0,131	0,538	0,572	0,510	0,138	0,566	0,131	0,062					
EL57_Avb	0,297	0,297	0,297	0,462	0,297	0,297	0,297	0,510	0,552	0,497	0,290	0,531	0,297	0,303	0,317				
EL62_Vb	0	0	0	0,421	0	0	0	0,517	0,579	0,517	0,007	0,559	0	0,097	0,131	0,297			
EL67_Vb	0,097	0,097	0,097	0,386	0,097	0,097	0,097	0,531	0,572	0,490	0,103	0,566	0,097	0	0,062	0,303	0,097		

EL112_Vb	0,110	0,110	0,110	0,393	0,110	0,110	0,110	0,545	0,572	0,490	0,117	0,566	0,110	0,014	0,048	0,303	0,110	0,014	
EL33_Aq	0,559	0,559	0,559	0,586	0,559	0,559	0,559	0,428	0,021	0,559	0,566	0	0,559	0,566	0,566	0,531	0,559	0,566	0,566

The number of base differences per site from between sequences are shown. The analysis involved 20 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 145 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al.,* 2016).

Table 2. Estimates of Evolutionary Divergence between Sequences, through the calculation of the p-distance, of the 16S rRNA genes from the *Vibrionaceae* and *Aquimarina* strains represented in figure 14, b).

	Vb 339	**Vb 278**	**Vb 258**	**Aq78**	**Aq349**	**Aq135**	**Vb255**	**Aq107**	**EL22 Vb**	**EL24 Evb**	**EL33 Aq**	**EL36 Vb**	**EL37 Evb**	**EL38 Vb**	**EL43 Aq**	**EL49 Vb**	**EL62 Vb**	**EL67 Vb**	**EL112 Vb**
Vb339																			
Vb278	0,025																		
Vb258	0,022	0,009																	
Aq78	0,303	0,292	0,289																
Aq349	0,305	0,292	0,291	0,022															
Aq135	0,306	0,295	0,291	0,034	0,040														
Vb255	0,025	0,009	0,006	0,288	0,289	0,289													
Aq107	0,313	0,300	0,297	0,040	0,039	0,042	0,295												
EL22 Vb	0,025	0,009	0,006	0,291	0,291	0,292	0,012	0,300											
EL24 Evb	0,096	0,082	0,078	0,288	0,291	0,299	0,081	0,302	0,079										
EL33 Aq	0,305	0,292	0,291	0,022	0	0,040	0,289	0,039	0,291	0,291									
EL36 Vb	0,020	0,008	0,002	0,288	0,289	0,289	0,005	0,295	0,008	0,078	0,289								
EL37 Evb	0,095	0,081	0,076	0,289	0,292	0,300	0,079	0,303	0,079	0,002	0,292	0,076							

EL38 Vb	0,022	0,009	0,003	0,289	0,288	0,291	0,006	0,294	0,009	0,078	0,288	0,002	0,076						
EL43 Aq	0,300	0,288	0,286	0,020	0,005	0,039	0,285	0,044	0,286	0,291	0,005	0,285	0,292	0,286					
EL49 Vb	0,026	0,011	0,009	0,286	0,288	0,289	0,009	0,299	0,012	0,078	0,288	0,008	0,079	0,009	0,283				
EL62 Vb	0,022	0,006	0,003	0,289	0,291	0,291	0,003	0,297	0,009	0,078	0,291	0,002	0,076	0,003	0,286	0,006			
EL67 Vb	0,033	0,016	0,016	0,291	0,291	0,294	0,012	0,302	0,011	0,079	0,291	0,016	0,079	0,017	0,286	0,012	0,016		
EL112 Vb	0,036	0,019	0,019	0,294	0,289	0,295	0,022	0,300	0,014	0,075	0,289	0,019	0,075	0,017	0,288	0,016	0,019	0,009	
EL57 Avb	0,056	0,037	0,040	0,294	0,295	0,297	0,040	0,303	0,036	0,090	0,295	0,039	0,090	0,039	0,292	0,034	0,037	0,034	0,036

The number of base differences per site from between sequences are shown. The analysis involved 20 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 643 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al.,* 2016).

Table 3. Estimates of Evolutionary Divergence between Sequences, through the calculation of the p-distance, of *chiA* genes of the *Vibrio* strains (except EL37) and respective best hits retrieved from NCBI (BlastN) and from alignments with chitinases from sequenced genomes from RAST.

	Vb 255	**Vb 258**	**Vb 278**	**EL 22 Vb**	**EL 24 Evb**	**EL 36 Vb**	**EL 38 Vb**	**EL 49 Vb**	**EL 57 Avb**	**EL 62 Vb**	**V. fisch. MJ11**	**V. fisch. ES114**	**V. crass. 9CS106**	**EL 67 Vb**	**fig|2.117. peg.3390 Evh12**	**fig|511678. 19.peg.280 EL58 Avb**	**EL 112 Vb**	**fig|6666666. 87814. peg.4153 Evd11**	**fig|6666666. 115118. peg.2271 Evd3**	**fig|6666666. 87815. peg.3398 Evh13**
Vb255																				
Vb258	0																			
Vb278	0	0																		
EL22_Vb	0	0	0																	
EL24_Evb	0	0	0	0																
EL36_Vb	0	0	0	0	0															
EL38_Vb	0	0	0	0	0	0														
EL49_Vb	0,132	0,132	0,132	0,132	0,132	0,132	0,132													

EL57_Avb	0,310	0,310	0,310	0,310	0,310	0,310	0,310	0,305												
EL62_Vb	0	0	0	0	0	0	0	0,132	0,310											
V. fischeri MJ11	0,293	0,293	0,293	0,293	0,293	0,293	0,293	0,276	0,080	0,293										
V. fischeri ES114	0,282	0,282	0,282	0,282	0,282	0,282	0,282	0,287	0,086	0,282	0,052									
V. crassostreae 9CS106	0,109	0,109	0,109	0,109	0,109	0,109	0,109	0,155	0,293	0,109	0,282	0,276								
EL67 Vb	0,103	0,103	0,103	0,103	0,103	0,103	0,103	0,052	0,293	0,103	0,276	0,276	0,109							
fig|2.117. peg.3390 Evh12	0	0	0	0	0	0	0	0,132	0,310	0	0,293	0,282	0,109	0,103						
fig|511678.19. peg.280 EL58_Avb	0,276	0,276	0,276	0,276	0,276	0,276	0,276	0,316	0,109	0,276	0,138	0,109	0,270	0,305	0,276					
EL112_Vb	0,109	0,109	0,109	0,109	0,109	0,109	0,109	0,057	0,299	0,109	0,282	0,282	0,121	0,029	0,109	0,305				
fig|6666666. 87814. peg.4153 Evd11	0,006	0,006	0,006	0,006	0,006	0,006	0,006	0,132	0,305	0,006	0,293	0,282	0,103	0,098	0,006	0,270	0,103			
fig|6666666. 115118. peg.2271 Evd3	0,115	0,115	0,115	0,115	0,115	0,115	0,115	0,086	0,305	0,115	0,293	0,293	0,132	0,103	0,115	0,293	0,109	0,115		
fig|6666666. 87815. peg.3398 Evh13	0,115	0,115	0,115	0,115	0,115	0,115	0,115	0,115	0,305	0,115	0,282	0,293	0,149	0,092	0,115	0,305	0,086	0,109	0,121	
Vb339	0,006	0,006	0,006	0,006	0,006	0,006	0,006	0,138	0,305	0,006	0,299	0,287	0,115	0,109	0,006	0,282	0,115	0,011	0,121	0,121

The number of base differences per site from between sequences are shown. The analysis involved 21 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 174 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016)..

Table 4. Estimates of Evolutionary Divergence between Sequences, through the calculation of the p-distance, of the 16S rRNA gene from the *Vibrio* strains represented in the phylogenetic tree on Figure 15, b).

	EL 22 Vb	EL 24 Vb	EL 36 Vb	EL 38 Vb	EL 49 Vb	EL 57 AVb	EL 58 Avb	EL 62 Vb	EL 67 Vb	EL 112 Vb	Vb 255	Vb 258	Vb 278	Vb 339	Evh 13 Vb	Evd 11 Vb	Evh 12 Vb	Evd 3 Vb	V. fischeri ES114
EL22_Vb																			
EL24_Vb	0,084																		
EL36_Vb	0,009	0,082																	
EL38_Vb	0,011	0,082	0,002																
EL49_Vb	0,013	0,082	0,007	0,009															
EL57_AVb	0,042	0,095	0,046	0,046	0,042														
EL58_Avb	0,031	0,084	0,036	0,038	0,033	0,027													
EL62_Vb	0,009	0,082	0	0,002	0,007	0,046	0,036												
EL67_Vb	0,007	0,082	0,013	0,015	0,009	0,036	0,024	0,013											
EL112_Vb	0,015	0,080	0,020	0,018	0,016	0,042	0,024	0,020	0,007										
Vb255	0,009	0,082	0	0,002	0,007	0,046	0,036	0	0,013	0,020									
Vb258	0,007	0,082	0,002	0,004	0,009	0,047	0,036	0,002	0,013	0,020	0,002								
Vb278	0,009	0,087	0,007	0,009	0,013	0,046	0,036	0,007	0,013	0,020	0,007	0,009							
Vb339	0,015	0,091	0,009	0,011	0,015	0,051	0,042	0,009	0,018	0,026	0,009	0,011	0,013						
Evh13_Vb	0,013	0,080	0,018	0,020	0,015	0,042	0,022	0,018	0,005	0,002	0,018	0,018	0,018	0,024					
Evd11_Vb	0,015	0,080	0,020	0,018	0,016	0,042	0,024	0,020	0,007	0	0,020	0,020	0,020	0,026	0,002				
Evh12_Vb	0,009	0,082	0	0,002	0,007	0,046	0,036	0	0,013	0,020	0	0,002	0,007	0,009	0,018	0,020			
Evd3_Vb	0,013	0,086	0,004	0,005	0,011	0,049	0,040	0,004	0,016	0,024	0,004	0,005	0,011	0,013	0,022	0,024	0,004		
Vibrio_fischeri_ES114	0,042	0,097	0,047	0,049	0,044	0,009	0,026	0,047	0,035	0,040	0,047	0,047	0,047	0,053	0,038	0,040	0,047	0,051	

Vibrio_fischeri_MJ11	0,042	0,097	0,047	0,049	0,044	0,009	0,026	0,047	0,035	0,040	0,047	0,047	0,047	0,053	0,038	0,040	0,047	0,051	0

The number of base differences per site from between sequences are shown. The analysis involved 20 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 549 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016).

Table 5. Estimates of Evolutionary Divergence between Sequences, through the calculation of the p-distance, of *chiA* PCR products from all *Aquimarina* strains, and respective best hits from NCBI and from successful alignments with chitinases sequences from the sequenced and available genomes on RAST.

	fig|6666666.167726.peg.1916 Aq. Longa SW024	**fig|290174.10.peg.252 Aq 135**	**fig|6666666.195556.peg.219 EL33 Aq**	**fig|290174.11.peg.2190 Aq 349**	**EL 43 Aq**	**EL 33 Aq**	**Aq 135**	**Aq 78**	**Aq 107**	**P. damselae**	**Stenot. sp.**	**B. brevis**	**Aq 349**	**Stenot. sp.**	**Stenot. sp. SWCHI-6**	**Paenibacillus sp. SWCHI-2**
fig|6666666.167726.peg.1916 Aq. Longa SW024																
fig|290174.10.peg.252 Aq 135	0,329															
fig|6666666.195556. peg.219 EL33 Aq	0,231	0,413														
fig|290174.11.peg.2190 Aq 349	0,231	0,413	0													
EL43 Aq	0,545	0,531	0,531	0,531												
EL33 Aq	0,238	0,420	0,035	0,035	0,497											
Aq 135	0,350	0,021	0,434	0,434	0,510	0,399										
Aq 78	0,238	0,413	0,063	0,063	0,517	0,028	0,392									
Aq 107	0,580	0,608	0,552	0,552	0,427	0,517	0,587	0,531								
AP018046.1 P. damselae	0,301	0,357	0,315	0,315	0,524	0,294	0,350	0,308	0,531							
EU864345.1 Stenotrophomonas sp.	0,580	0,545	0,580	0,580	0,427	0,545	0,524	0,552	0,483	0,517						

EU708604.1 B. brevis	0,608	0,615	0,643	0,643	0,545	0,608	0,594	0,608	0,524	0,594	0,469					
Aq 349	0,238	0,420	0,035	0,035	0,497	0	0,399	0,028	0,517	0,294	0,545	0,608				
EU864345.1 Stenotrophomonas sp.	0,580	0,545	0,580	0,580	0,427	0,545	0,524	0,552	0,483	0,517	0	0,469	0,545			
EU708608.1 Stenotrophomonas sp. SWCHI-6	0,587	0,545	0,573	0,573	0,427	0,538	0,524	0,545	0,490	0,510	0,007	0,469	0,538	0,007		
EU708605.1 Paenibacillus sp. SWCHI-2	0,622	0,566	0,629	0,629	0,510	0,594	0,545	0,594	0,538	0,552	0,455	0,329	0,594	0,455	0,455	
fig|290174.8. peg.3476 Aq 78	0,238	0,413	0,035	0,035	0,545	0,056	0,420	0,028	0,559	0,322	0,580	0,636	0,056	0,580	0,573	0,622

The number of base differences per site from between sequences are shown. The analysis involved 17 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 143 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

Table 6. Estimates of Evolutionary Divergence between Sequences, through the calculation of the p-distance, of the 16S rRNA gene from the *Aquimarina* strains represented in the phylogenetic tree on Figure 16, b).

	Aq 78	**Aq 349**	**Aq 135**	***Stenotrophomonas maltophilia* K279a**	***Photobacterium profundum* 3TCK**	***Aquimarina longa* SW024**	**Aq 107**	**EL33 Aq**
Aq78								
Aq349	0,026							
Aq135	0,030	0,034						
***Stenotrophomonas maltophilia* K279a**	0,596	0,589	0,604					
***Photobacterium profundum* 3TCK**	0,593	0,591	0,600	0,187				
***Aquimarina longa* SW024**	0,550	0,538	0,557	0,164	0,230			
Aq 107	0,049	0,047	0,041	0,600	0,606	0,557		

EL33 Aq	0,026	0	0,034	0,589	0,591	0,538	0,047	
EL43 Aq	0,022	0,004	0,030	0,591	0,587	0,540	0,050	0,004

The number of base differences per site from between sequences are shown. The analysis involved 9 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 535 positions in the final dataset.

C. Phylogenetic analysis on different chitinase sequences (*chiA*, *ChiB* and *ChiC*)

Figure 1. Phylogenetic inference based on the General Time Reversible Model (Nei & Kumar, 2000) of chitinase (EC 3.2.1.14) sequences retrieved from RAST genomes, *chiA, ChiB* and *ChiC* sequences from NCBI. The tree with the highest log likelihood (-41087.44) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 19,2315)). The analysis involved 43 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 910 positions in the final dataset.

D. Growth curves for *Aquimarina* sp. and *Vibrio* sp.

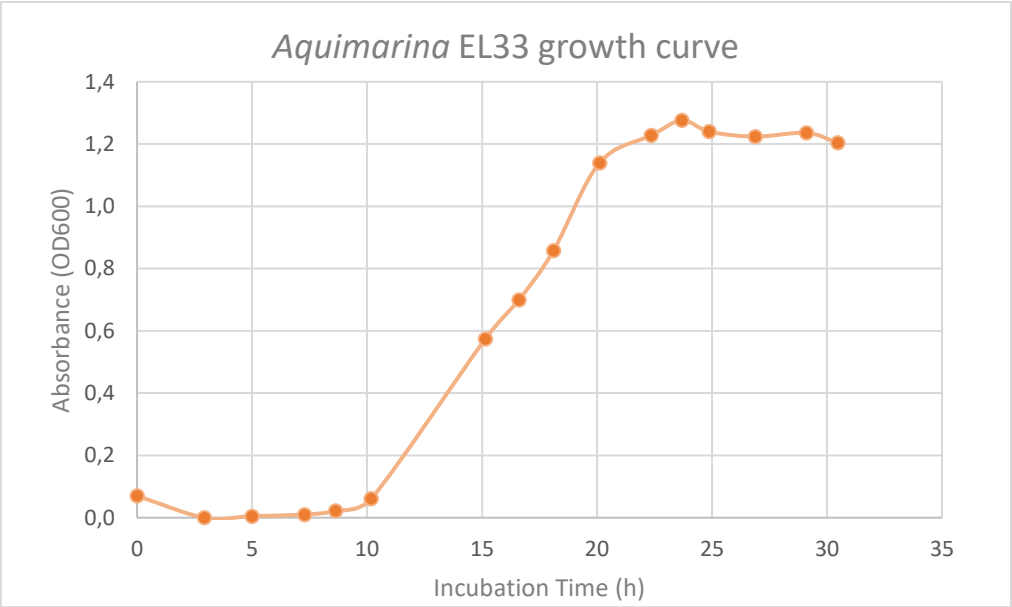


Figure 1. Growth curve for *Aquimarina* sp. strain EL33.

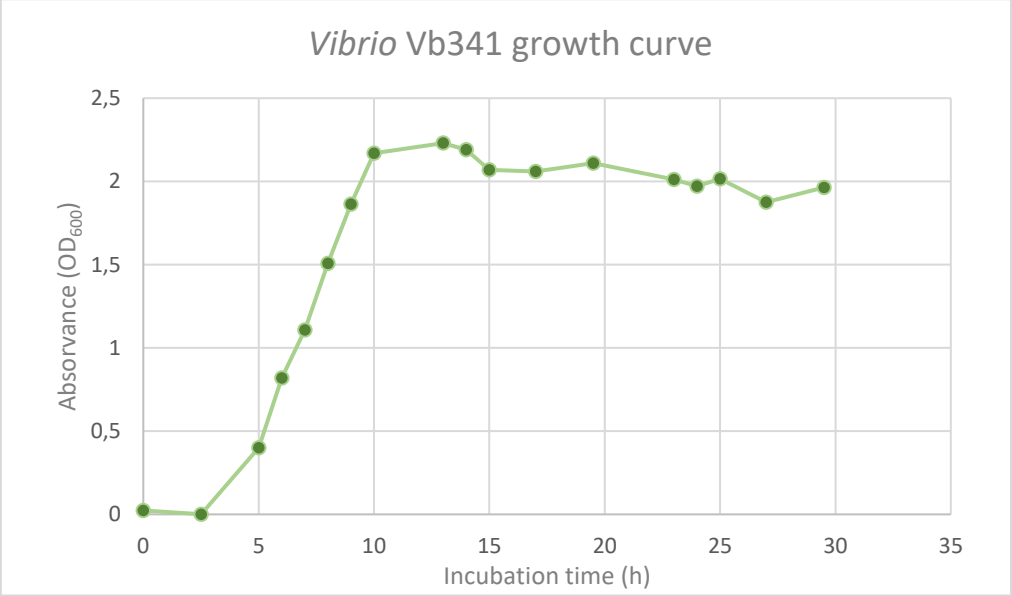


Figure 2. Growth curve for *Vibrio* sp. strain Vb431.