# UNIVERSIDADE DE LISBOA

# INSTITUTO SUPERIOR TÉCNICO

## Uncovering the chitin degradation potential of the microbiomes of marine sponges and octocorals

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**Thesis approved in public session to obtain the PhD Degree in**

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**Jury final classification: Pass with Distinction**

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**Resumo**

A quitina é o polissacárido mais abundante nos oceanos e o segundo mais abundante do planeta, depois da celulose. O conhecimento atual sobre os microrganismos, enzimas catalíticas, proteínas acessórias e vias metabólicas envolvidas na degradação de quitina deriva maioritariamente de estudos dependentes de cultivo e continua a ser limitado no que respeita aos ambientes marinhos. No entanto, recentes estudos ligados à investigação metagenómica sugerem que muitos dos micróbios capazes de degradar quitina são até agora incultiváveis. Desta forma, o entendimento atual da comunidade científica sobre o processamento de quitina na natureza poderá representar apenas a escassa ponta de um imenso icebergue por desvendar, apesar da relevância deste biopolímero para fisiologia dos organismos e funcionamento dos ecossistemas. Neste contexto, surgiu esta tese que junta métodos dependentes e independentes de cultivo, utilizando microrganismos associados a microbiomas marinhos de esponjas, corais, sedimentos e água do mar envolvente, com o intuito de aumentar o conhecimento atual sobre esta temática e investigar o potencial destes ecossistemas na degradação e transformação de quitina em compostos mais simples e de elevado valor biotecnológico, comparativamente com os atuais processos enzimáticos comerciais e industriais e, eventualmente, acrescentando valor ao sector da biotecnologia azul.

Assim, esta tese revê o conhecimento actual geral de ecofisiologia relacionado com a classificação, estrutura e papel biológico das enzimas capazes de degradar quitina e das vias metabólicas nos ambientes marinhos e terrestres, com especial destaque para os complexos processos quitinoliticos (isto é, com degradação de quitina). Na sequência de um capítulo introdutório (Capítulo 1) a respeito dos organismos e vias metabólicas envolvidas no metabolismo da quitina, segue-se um capítulo de revisão da literatura (Capítulo 2) em que são especialmente abordadas a relevância destes processos para os ciclos de carbono e nitrogénio dos diferentes ambientes, e as relações simbióticas entre hospedeiros e microrganismos em

sistemas modelo dessas relações, tais como rizo- e endo-esfera em plantas-solo e esponjas marinhas e corais no ambiente aquático, tendo em atenção processos mediados tanto por enzimas provenientes de arqueias como por bactérias. Este capítulo examina também interações microbianas inter-especificas que ocorrem durante o catabolismo da quitina (como por exemplo alimentação bacteriana cruzada e relações antagónicas entre fungos e bactérias), bem como a sua relevância para a organização funcional dos ecossistemas. Adicionalmente, é ainda abordada a utilização potencial de quitinases e produtos derivados da degração de quitina entre os diversos setores de aplicações biotecnológicas, desde reutilização de produtos sem valor comercial para transformação em produtos de valor acrescentado, a aplicações ligadas a sistemas agrícolas, industria e preservação alimentar e às áreas da saúde humana e diagnóstico, numa acção que deverá ser coordenada entre investigadores, empreendedores e utilizador final.

De seguida surge um capítulo de investigação original (Capítulo 3) que integra análises dependentes e independentes de cultivo para examinar o potencial de degradação e utilização de quitina e seus derivados nos microbiomas de esponjas marinhas, octocorais, sedimentos e água do mar envolventes. Neste capítulo é também investigado se diversas espécies podem trabalhar em conjunto nestes microbiomas e se estes são taxonómica e funcionalmente distintos, discutindo a hipótese de alimentação cruzada e o seu papel dentro destes habitats. Com este capítulo foi demonstrado que bactérias cultiváveis pertencentes aos géneros *Aquimarina, Enterovibrio, Microbulbifer*, *Pseudoalteromonas*, *Shewanella* e associadas aos hospedeiros anteriormente referidos têm a capacidade para degradar quitina coloidal *in vitro*. Além disso, uma monitorização do genoma de simbiontes cultiváveis pertencentes a espécies de *Vibrio* e *Aquimarina*, em particular, possuem diversos genes codificadores de endo- e exo-quitinases, levando-nos a inferir a sua capacidade para degradar o polimero de quitina em fragmentos mais pequenos – dímeros e oligomeros – o que é consistente com os resultados dos ensaios enzimáticos. Também com base nestes ensaios podemos afirmar que as espécies de

*Alphaproteobacteria* se especializam mais frequentemente na utilização do monómero de quitina – N-acetil-glucosamina. Também neste capítulo a realização de ensaios filogenéticos determinou um elevado grau de diversidade intra-específica de múltiplos genes codificadores de endo-quitinases nos genomas de estirpes de *Aquimarina* e *Vibrio*, sugerindo uma versatilidade no que concerne ao catabolismo de quitina nestes organismos. Posteriormente, foi analisada a abundância e distribuição de genes relacionados com o metabolismo de quitina em 30 metagenomas microbianos, sequenciados através da tecnologia Illumina, tendo-se demonstrado que a comunidade endosimbiótica de *Spongia officinalis* é enriquecida em genes codificadores de deacetilases de polissacáridos. Isto sugere que o microbioma destas esponjas marinhas possui a capacidade de transformar quitina na sua forma deacetilada quitosano, um composto extremamente versátil em termos biotecnológicos. Pelo contrário, relativamente à abundância de genes codificadores de endo-quitinases e proteínas de ligação a quitina, octocorais saudáveis mostraram valores semelhantes com os ambientes envolventes enquanto octocorais com tecido necrotizado demonstraram valores inferiores aos octocorais saudáveis e aos referidos ambientes. Estas metodologias independentes de cultivo permitiram ainda revelar a riqueza surpreendente de comunidades quitinolíticas associadas a estes organismos hospedeiros e a sua estrutura, bem como papéis putativos de simbiontes não cultiváveis como *Gammaproteobacteria* e *Chloroflexi* nesses processos de degradação e de novas enzimas quitinoliticas em simbiontes cultiváveis nos quais esse papel não era conhecido (como o caso do género *Aquimarina*) e que poderão no futuro ser exploradas pelo sector da biotecnologia azul.

Posteriormente, no Capítulo 4 são investigados os processos metabólicos de degradação de quitina em microbiomas não cultivados através da reconstrução de genomas assemblados de metagenomas (MAGs) com origem nos metagenomas microbianos de esponjas marinhas (filo Porifera) e água do mar envolvente. As esponjas marinhas encontram-se amplamente

distribuídas por entre todos os ambientes marinhos, e abrigam vastas comunidades bacterianas de elevada diversidade filogenética com diferentes simbiontes procariotas não cultiváveis até aos dias de hoje. Portanto, estas comunidades simbióticas constituem um sistema modelo interativo e complexo para estudos funcionais, e neste caso em concreto das diversas vias de degradação de quitina. Um total de doze metagenomas provenientes de amostras de *Spongia agaricina*, *Spongia officinalis* e água do mar envolvente foram assemblados, tendo sido obtidos cento e trinta e sete MAGs considerados de boa e de alta qualidade. Após classificação taxonómica, foi possível identificar entre estas MAGs doze filos procariotas e dezoito classes diferentes, sendo *Proteobacteria* o filo mais representado no conjunto de resultados, com um número total de trinta e uma MAGs neste grupo. A presença de domínios codificadores de endo-quitinases foi identificada em doze das cento e trinta e sete MAGs, representando 8.76% do total e onde se encontravam MAGs pertencentes a seis classes diferentes, nomeadamente *Anaerolineae*, *Dehalococcoidia*, *Gammaproteobacteria*, *Rhodothermia*, *Bacteroidia* e *WGA-4E* (filo *Poribacteria*). Adicionalmente, a presença de domínios codificadores de exo-quitinases, deacetilases e de N-acetilglucosaminidases foi demonstrada em quarenta e sete MAGs (33.58%), cento e sete MAGs (78.10%) e setenta e três MAGs (53.28%), respetivamente.

Ainda relativamente aos domínios codificadores de enzimas envolvidas na degradação e utilização de quitina e dos seus sub-produtos, é de realçar que todas as MAGs classificadas como *Rhodothermia* apresentam todos os domínios analisados, o que sugere que os membros desta classe poderão assumir um papel extremamente importante do metabolismo de quitina nestes habitats.

Neste capítulo foi ainda abordada a diversidade filogenética das endo-quitinases pertencentes à família dezoito das hidrolases glicosídicas (GH18), utilizando para isso sequências de aminoácidos dos seus genes codificadores presentes nos MAGs e nos genomas

de isolados bacterianos que lhes são mais semelhantes, abrangendo diversas classes taxonómicas. Um grupo filogenético principal, apresentando 99% de suporte, é formado exclusivamente por sequências de aminoácidos de endo-quitinases provenientes de MAGs pertencentes à ordem *Rhodothermales* e derivadas de esponjas marinhas.

As análises genómicas compreendendo cinquenta e dois genomas pertencentes a essa ordem demonstram que as sequências provenientes dos MAGs obtidos nesta tese formam grupos filogenómicos compostos exclusivamente por bactérias não cultivadas até ao momento, e sugerem a possível existência de pelo menos três novas famílias dentro da ordem *Rhodothermales*. Especificamente, nesta tese duas novas famílias pertencentes à ordem *Rhodothermales* são formalmente propostas em função de sua robustez filogenética, nomeadamente as famílias *Candidatus* Spongiachitinolyticaceae e *Candidatus* Irciniachitinolyticaceae, em referência à sua proveniência (os géneros de esponja marinha *Spongia* e *Ircinia*, respetivamente) e ao seu potencial de degradação da quitina.

De um modo global, estas descobertas abrem novas linhas de investigação não só sobre os mecanismos de degradação e utilização de quitina, mas também no que respeita ao conhecimento atual sobre simbiontes bacterianos pertencentes aos filos *Chloroflexota*, *Bacteroidota* (p.ex., o género cultivável *Aquimarina*), *Rhodothermota* (classe *Rhodothermia*, ordem*Rhodothermales*) e *Proteobacteria* (classe *Gammaproteobacteria*) e dos seus potenciais papéis essenciais nos microbiomas das esponjas marinhas e octocorais.

**Palavras chave:** esponjas marinhas, octocorais, microbioma, quitinases, metagenomas

**Abstract**

Marine environments are an untapped source of novel enzymes with biotechnological potential across several fields. A group of enzymes attracting recent research interest are the chitinases, as their substrate, chitin, ranks as the most abundant polysaccharide in the oceans being widespread in nature and among several living organisms. Moreover, the products of chitinolytic activity, chito-oligosaccharides, possess several properties of interest in applied biotechnology. However, knowledge of the structure and diversity of chitin-degrading communities across marine microhabitats is scarce. This thesis integrates cultivation-dependent and -independent approaches to shed light on the chitin processing potential within the microbiomes of marine sponges, octocorals, sediments and seawater, placing emphasis on the discovery and inferred metabolism of chitin-degrading symbionts of the abovementioned animals.

Cultivatable host-associated bacteria from several genera including *Aquimarina* and *Vibrio* were found to be able to degrade colloidal chitin *in vitro*. *Vibrio* and *Aquimarina* species, particularly, possessed several endo- and exo-chitinase-encoding genes underlying their capacity to cleave the large polymer, while *Alphaproteobacteria* species were found to specialize in the utilization of N-acetylglucosamine more often. A cultivation-independent approach relying on the functional profiling of unassembled reads from the microbial metagenomes of sponges, octocorals, seawater and sediments revealed distinctive abundance distributions of genes involved in chitin metabolism across the surveyed microbiomes. While an enrichment of polysaccharide deacetylase encoding genes was unveiled in the *Spongia officinalis* microbiome, suggesting capability of transforming chitin into its deacetylated – and biotechnologically valuable – form chitosan, the relative abundance of endo-chitinase and chitin-binding proteins was more pronounced in the octocoral microbiome, indicating that the degradation of chitin via hydrolysis may be a relevant process in these symbiotic communities.

Furthermore, putative roles for uncultivated *Gammaproteobacteria* and *Chloroflexi* symbionts of corals and sponges, respectively, in chitin processing within sessile marine invertebrates were revealed by homology-based analysis of unassembled microbial metagenomes.

To enable a deeper understanding of the taxonomy, function and metabolism of uncultivated microbial symbionts involved in chitin degradation / utilization processes, a genome-resolved metagenomics approach was employed to functionally compare metagenome-assembled genomes (MAGs) retrieved from seawater and from the marine sponges *Spongia officinalis* and *Spongia agaricina*. Microbial metagenome assembly and binning procedures resulted in 137 MAGs of good- to high-quality that were functionally screened, unveiling the presence of endo-chitinase domains in a select group of 12 MAGs classified in to the bacterial classes *Rhodothermia*, *Chloroflexi*, and WGA4-E (*Poribacteria*) – all obtained from marine sponges - and *Flavobacteriia* and *Gammaproteobacteria* - obtained from seawater. In contrast, exo-chitinase domains as well as deacetylases and N-acetylglucosamine utilization domains were found on MAGs from multiple and diverse bacterial classes. This indicates that hydrolysis of the large chitin polymer through the action of endo-chitinases is a bottleneck function within the marine sponge microbiome, presumably performed by a narrow range of symbionts. It further allows hypotheses to be made on potential cross-feeding among the few chitin degraders and the many utilizers of chitin derivatives (e.g., chitin oligosaccharides and N-acetylglucosamine) within the sponge symbiotic consortium. Remarkably, sponge symbionts of the order *Rhodothermales* (class *Rhodothermia*) possessed a coding potential for all the chitin degradation and utilization features screened for in this thesis. Moreover, they were found to consistently code for heparinases, sulfatases, chondroitinases and multiple carbohydrate binding modules, hinting at a versatile polysaccharide metabolism and specific roles for these symbionts in the processing of glycosaminoglycans present in the sponge mesohyl. Finally, genome-wide assessments consistently revealed the existence of distinct

clusters of so-far unculturable *Rhodothermales* symbionts specific to marine sponges likely representing at least three novel families within this bacterial order. Based on these findings, two novel families within the *Rhodothermales* order are herein proposed, strongly supported by phylogeny and average nucleotide identity assessments, denoted family *Candidatus* Spongiachitinolyticaceae and family *Candidatus* Irciniachitinolyticaceae to refer to their provenance (the marine sponge genera *Spongia* and *Ircinia*, respectively) and inferred ability to hydrolyse chitin.

In conclusion, these thesis' findings suggest differential chitin degradation and processing pathways across marine micro-niches, opening new avenues to the study of chitin degradation and utilization in marine ecosystems and shedding light on emerging, presumably essential roles in chitin metabolism played by bacterial symbionts in the phyla *Chloroflexota*, *Bacteroidota* (*e.g.*, the culturable genus *Aquimarina*), *Rhodothermota* (class *Rhodothermia*, order *Rhodothermales*) and *Proteobacteria* (class *Gammaproteobacteria*) in association with marine sponges and octocorals.

**Keywords:** marine sponges, octocorals, microbiomes, chitinases, metagenomes

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"Words are, in my not-so-humble opinion, our most inexhaustible source of magic. Capable of both inflicting injury, and remedying it." – **Albus Dumbledore**

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Today I am not the same person who started this journey a few years ago. It was a hell of a ride (sometimes literally…)! But I want you all to know that I am grateful to everyone who was part of it.

– Mischief managed.

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**Figure 3.3 Relative abundance of chitin processing-encoding genes across marine biotopes.** The analysis involved the screening of all microbial metagenomes for InterPro (IPR) database entries corresponding to the presence of GH18 endo-chitinase **(a, f, k)**, chitin-binding protein (**b, g, l**), exo-chitinase (**c, h, m**), N-acetylglucosamine utilization (**d, i, n**), and polysaccharide deacetylase (**e, j, o**) coding sequences (CDSs) (see Additional file 1: Table S5 for details on the InterPro entries used here). The relative abundances (mean ± SE) of IPR entries pertaining to each functional category (calculated

High resolution figures can be found online at:

https://drive.google.com/drive/folders/16MraFJqb0KiDAtLFMuDTx7FT-xpTPD0w?usp=sharing

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**List of acronyms and abbreviations**

**4-MU** – 4-methylumbelliferone

**A** - Adenine

**ANI –** Average nucleotide identity

**ANOVA** – Analysis of variance

**ASW** - Artificial Seawater

**ATP** – Adenosine triphosphate

**BLAST –** Basic local alignment search tool

**bp –** base pairs

**C** - Cytosine

**CBPs –** Chitin-binding proteins

**CC –** Colloidal chitin

**CDSs –** Coding sequences

**ChiP** – Chitoporin

**CMC** – N,O-carboxymethyl-chitosan

**CMTMC** – O-carboxymethyl-N,N,N-trimethyl-chitosan

**CMFASW –** calcium/magnesium free artificial seawater

**COS –** Chitooligosaccharides

**Cr(IV)** – Chromium

**Cu(II)** – Copper

**DNA –** Deoxyribonucleic acid

**dNTPs –** Deoxynucleoside triphosphates

**EC** – Enzyme commission

**Fe(II)** – Iron

**G** - Guanine

**GH –** Glycosyl hydrolases

**GlcN –** Deacetylated residues from chitosan

**GlcNAc –** N-acetylglucosamine

**$(GlcNAc)_2$ –** N,N'-diacetylchitobiose

**$(GlcNAc)_3$** – Chitotriose

**$(GlcNAc)_4$** – Chitotetroses

**GMOs –** Genetically modified organisms

**GTDB –** Genome Taxonomy Database

**GTR –** Generalised Time Reversible model

**HGT** – Horizontal gene transfer

**HMA –** High microbial abundance

**HQ –** High quality

**IPR –** InterPro Functional Annotation

**IUBMB –** International Union of Biochemistry and Molecular Biology

**LMA –** Low microbial abundance

**MAGs** – Metagenome-assembled genomes

**MB –** Marine Broth

**MCL** – Maximum Composite Likelihood

**MCP** – Microbial cell pellet

**MG-RAST** – Meta-Genome Rapid Annotation using Subsystems Technology

**MPC –** 5-methyl pyrrolidinone chitosan

**MW –** Molecular weight

**NCBI** – National Center for Biotechnology Information

**OTU** - Operational Taxonomic Unit

**PCoA** - Principal Coordinates Analysis

**PCR –** Polymerase chain reaction

**Pfam –** Protein family database

**PGPMs –** Plant growth-promoting microbes

**PTS** – Phosphoenolpyruvate transferase system

**RAST –** Rapid Annotation using Subsystem Technology tool

**rRNA –** Ribossomal Ribonucleic Acid

**RT** – Room temperature

**Sd –** Sediment

**Sp –** Sponges

**Sw –** Seawater

**T** – Timine

**TC-DNA** - Total Community DNA

**TMC** – N,N,N-trimethyl-chitosan

**U** - Uracile

**US –** United States

**UV –** Ultraviolet

**Zn(II)** – Zinc

# Chapter 1

## General Introduction

# General Introduction

## 1.1 Aims of the study and thesis outline

This PhD thesis examines the marine sponge and octocoral microbiomes, two highly versatile sources of new natural products and biocatalysts, for their coding potential of novel chitinases and related enzymes involved in chitin degradation. It sheds light on the chitin degrading capacities of understudied, cultured and uncultured marine bacteria, opening new avenues for the discovery of chitinases that may have the potential to outperform current commercial enzymes and to foster the development of blue biotechnology for chitin waste.

The overarching aim of this thesis is to improve the current knowledge of the chitin degradation potential and of the diversity of chitin degraders (that is, those that breakdown the large chitin polymer) and utilizers (that, is, those that metabolize the dimer chitobiose and/or the monomer N-acetylglucosamine) across different marine biotopes, with emphasis on the chitin degrading and utilizing consortia inhabiting marine sponges and octocorals. To reach these objectives, a blend of cultivation-dependent and independent approaches was employed, as summarized below, and explained in more details in **Chapters 3 and 4** of this thesis. Thus, the following, specific objectives were delineated and addressed in Chapter 3: (i) to determine the chitinolytic activity of culturable sponge- and octocoral-associated bacteria, assessing the genetic machinery underpinning chitin degradation among the most efficient chitin degraders, (ii) to test the hypothesis that the taxonomic composition of the culturable, chitinolytic consortium of sponges and octocorals differs from that of the prevailing, uncultivated or so-far unculturable symbionts in each animal, (iii) to examine the abundance distributions of genes involved in chitin breakdown and utilization in seawater, marine sediments,

sponges and octocorals and determine whether prevailing chitin processing pathways and microorganisms differ among these biotopes, while in Chapter 4, (iv) to employ innovative metagenome-resolved genomics procedures to connect the taxonomy and function ("who does what?") of chitin-degrading and utilizing symbionts of marine sponges, drawing a map of the total chitin processing capacity within the sponge symbiotic consortium in a cultivation-independent fashion, (v) to unveil potentially novel or/and currently unculturable bacterial chitin degraders, highlighting the phylogeny and putative ecological roles of symbionts thus far not known to degrade chitin were addressed.

The contents of this thesis are presented in five main chapters. **Chapter 1** provides an overview of the current knowledge of chitin degradation in the marine environment, developing further on the potential for chitin turnover within benthic ecosystems. It introduces the reader into the different enzymes involved in chitin hydrolysis and utilization, the typical microorganisms conducting chitin degradation in the marine realm into the microbiomes of marine sponges and corals, the main research targets in this thesis. **Chapter 1** presents the fundamental basis and hypotheses underlying the search for chitin-degrading microorganisms associated with marine sponges and corals and develops on state-of-the-art metagenomic approaches that enable the analysis of the diversity and function of microbial communities in a cultivation-independent fashion, in line with the methodologies thoroughly employed in research **Chapters 3 and 4**.

**Chapter 2** extends and deepens the overview on chitin degradation provided in **Chapter 1**, and thoroughly reviews the classification, structure and biological role of chitin-degrading enzymes and metabolic pathways in both marine and terrestrial environments, with emphasis on complex, host-microbe symbioses and chitinolytic processes (i.e., chitin hydrolysis) mediated by bacterial and archaeal chitinases. This

chapter further examines interspecific microbial interactions during chitin breakdown (e.g., bacterial cross-feeding, bacterial-fungal antagonism) and their relevance for organismal and ecosystem functioning. Finally, it also addresses the potential use of chitinases and chitin-derived products across several sectors of applied biotechnology, from waste management to agricultural ecosystems and human health. The contents on chitin-degrading capacities and biotechnology potential of microbial communities summarized in **Chapter 2** have been compiled and submitted for publication as a review article.

The third chapter of this thesis (**Chapter 3**) integrates cultivation-dependent and -independent analyses to shed light on the potential degradation and utilization of chitin and its derivatives in the microbiomes of marine sponges, octocorals, sediments and seawater. Using analyses of unassembled metagenomes, it determines that chitin degrading assemblages within these microbiomes are taxonomically and metabolically distinct and discusses the hypothesis of inter-species cross-feeding and its role within these biotopes. This study also uncovers a high degree of diversification of chitinase genes in culturable marine bacteria as well as putative novel chitinolytic enzymes in the genus *Aquimarina* that could be explored in future blue biotechnology-oriented studies. This chapter has been published in the international, multidisciplinary Journal *Microbiome*.

**Chapter 4** delves deeper into chitin-degradation pathways in uncultured microbes by reconstructing metagenome-assembled genomes (MAGs) from the microbial metagenomes of marine sponge and seawater microhabitats. One hundred and thirty-seven high and medium quality MAGs, spanning 18 bacterial and archaeal classes, are examined for the presence, abundance and diversity of chitin degradation encoding genes. Endo-chitinase (EC 3.2.1.14) encoding genes were present on 12 MAGs affiliated with

six bacterial classes, from *Gammaproteobacteria* to *Poribacteria*, with a higher number of gene copies present in *Dehalococcoidia* (phylum *Chloroflexi*). Moreover, all the MAGs recovered from the *Rhodothermia* class (phylum *Bacteroidetes*) presented one copy of the chitinase gene, suggesting a consistent role of these symbionts in chitin processing within the sponge holobiont. Overall, these results indicate that only a small portion of the total marine sponge microbiome can degrade the large chitin polymer by means of hydrolysis (endo-chitinolytic activity). In contrast, genes coding for exo-chitinases (EC 3.2.1.52), which are involved in the hydrolysis of smaller chitin oligosaccharides (COS) derived from endo-chitinolytic activity, were found in a broad range of marine sponge symbionts, being present on 47 MAGs from 13 bacterial classes. This suggests that even if only a few symbionts present chitinolytic activity, a large part of the community can utilize the degradation products, consistent with the hypothesis that chitin degradation and utilization cascades may promote coexistence of diverse marine sponge symbionts and may play a fundamental role in carbon and nitrogen cycling in benthic ecosystems. This study uncovers for the first time the potential role of members of the phyla *Chloroflexi*, *Poribacteria* and *Bacteroidetes* (specifically, symbionts in the *Rhodothermales* order within the *Rhodothermia* class) in chitin degradation within marine sponges. Comprehensive, genome-wide comparative analyses revealed that the putative chitinolytic *Rhodothermales* symbionts of the keratose marine sponges examined in Chapter 4 (*Spongia officinalis* and *Spongia agaricina*) most likely represent two novel families composed exclusively by so-far uncultured and sponge-specific bacteria. These novel bacterial lineages also include symbionts of other sponge species collected from different geographical locations, shedding new light on the distribution, degree of specificity and potential roles of *Rhodothermales* symbionts of marine sponges in a large biogeographical context.

A final general discussion and conclusion chapter (**Chapter 5**) is presented, portraying the participation of symbionts of marine sponges and corals in chitin degradation and utilization, spanning microbial taxa involved in chitin and COS hydrolysis, chitin deacetylation and N-acetylglucosamine utilization within these sessile marine invertebrates. Their roles in the corresponding microbiomes are placed into context regarding the versatile polysaccharide catabolic potential usually reported from these systems which, nevertheless, have been seldom approached or studied from the standpoint of chitin catabolism. A summary of a novel diversity of chitin-degrading microorganisms uncovered in this thesis is provided, which has been enabled using advanced, cultivation-independent bioinformatics tools that permit the construction of nearly complete bacterial genomes from metagenome sequences. The complementarity between the methodological tools employed throughout the thesis to study chitinolytic microorganisms in marine samples, involving both cultivation-dependent and -independent approaches and addressing their weaknesses and strengths, is discussed. Finally, methodologies and approaches to harness the metabolism of so-far unculturable chitin degrading microorganisms are envisaged.

### 1.2 Current knowledge of chitin degradation in marine ecosystems and potential role of benthic communities in the process

Marine environments are a great reservoir of untapped resources of potential use in biotechnology. There is currently an increasing demand of several industries for biocatalysts, from pharmaceutical, biomedical or food processing (Barzkar *et al.* 2018; Jahromi and Barzkar 2018). The increased value of biocatalyst enzymes is related with the fact that most industrial processes are carried out in severe physicochemical conditions, often not adjusted to the optimum conditions required for the activation and

activity of the so-far used enzymes in terms of pH, temperature, and salinity range (Stefanidi and Vorgias 2008; Makhdoumi *et al.* 2015). Moreover, marine organisms are currently being intensively subjected to bioprospection campaigns for novel natural products that could be used, for example, as inspiration for the development of new drugs to overcome the current antibiotic resistance crisis (Dieterich *et al.* 2022; Paoli *et al*. 2022), or which could find applicability as antifouling and disease control agents in marine biotechnology and aquaculture (Borges *et al*. 2021; Tara Foundation, 2022). Through a myriad of properties, marine-derived biocatalysts, secondary metabolites, and materials may find applicability across a broad range of economic activities and industrial sectors, including waste management and bioremediation, agriculture, aquaculture, and the food industry, biomedicine and the pharmaceutical industry, among others (Tara Foundation, 2022).

One class of enzymes and catalytic process of relevance in the ocean that is attracting increasing attention are chitinases and chitin degradation, respectively. As further discussed in Chapter 2., chitin, the polymer of (1→4)-β-linked N-acetylglucosamine (GlcNAc), is the most abundant polysaccharide in the marine environment (Paulsen *et al.* 2016). Chitin is indeed widespread in nature and among diverse living organisms, with the first description of chitin isolation dating back to 1811, in mushrooms (Braconnot 1881).

Although the production rates of chitin are estimated to be about $10^{10}$–$10^{11}$ tonnes annually in the aquatic biosphere alone (Gooday 1990), chitin does not accumulate in aquatic habitats as it is constantly hydrolysed by microorganisms that can use it as a carbon, nitrogen and/or energy source (Beier and Bertilsson 2013). A major part of chitin degradation in aquatic ecosystems is accomplished by bacteria, either free-living in the water column or in association with host-organisms (Gooday 1990; Poulicek, Gaill and

Goffinet 1998). Remarkably, elemental carbon and nitrogen were earlier presumed to become virtually unavailable in the ocean in a relatively short time if chitin would not be metabolized into biologically useful forms, since these two elements are usually limiting in the marine ecosystem (Johnstone 1908). Such pioneering considerations about the fate of chitin bear testimony to the importance of the polymer, the enzymes involved in its breakdown, the bacteria producing chitin catalysts for carbon and nitrogen cycling and to the functioning of the entire marine ecosystem (Souza *et al.* 2011). Seki (1965) reported that almost all chitin produced in the surface waters of the global ocean may be completely transformed in between 50 to 140 days depending on the temperature, with shorter recycling times for temperatures around 25°C and major delays for temperatures around 15°C. For extreme conditions, complete chitin degradation may take until 370 days in intermediate waters at 5°C, and until 500 days in deep waters (Seki 1965). Poulicek and Jeauniaux (1989) reported that most marine sediments bear low or very low chitin biomass, with around 67% of analysed samples showing under 100 μg of chitin per g of decalcified sediment and hypothesized that this observation could be explained by the very active degradation of chitin compounds, almost at the same rate as it is produced. A study in the York River Estuary, Virginia, which reported that 88% to 93% of particulate chitin can be readily mineralized to $CO_2$ in the water column, while 55% to 65% may be fully mineralized in sediments (Boyer 1994). Other studies also showed that chitin degradation rates differ between environments where the process is occurring (Hillman, Gooday and Prosser 1989a; Montgomery, Welschmeyer and Kirchman 1990; Gooday *et al.* 1991; Kirchman and White 1999; Ravaux *et al.* 2003).

Chitin degradation is a specific process often mediated by chitinolytic enzymes, named chitinases. These enzymes, isolated for the first time in 1911 from orchid bulbs by Bernard (Bernard 1911), can be divided in two types: endo-chitinases (EC 3.2.1.14), that

cleave chitin randomly at internal sites, generating diverse oligomers of GlcNAc such as chitotriose and chitotetraose; and exo-chitinases (EC 3.2.1.52), that can be further divided into two subtypes: chitobiosidases, which catalyse the progressive release of chitobiose, starting at the non-reducing end of the chitin microfibril; and N-acetyl-β-glucosaminidases, or chitobiases, which cleave the oligomeric products of endo-chitinases and chitobiosidases, generating monomers of GlcNAc (Cohen-Kupiec and Chet 1998). More detail on this topic is provided in Chapter 2 of this thesis.

Chitinases can play various roles in organismal physiology such as digestion of chitin-containing organisms in the food chain, cuticle turnover and cell differentiation (Khoushab and Yamabhai 2010a). Moreover, their presence in bacteriophage sequences suggests that endochitinase-encoding genes may be laterally transferred by viruses, mostly for morphogenesis and pathogenesis functions, more specifically cell wall biogenesis and degradation, respectively (Berini *et al.* 2016; Nguyen *et al.* 2016). Based on amino acid sequence similarity, endo-chitinases can be mainly divided into GH families 18 and 19 (Brzezinska *et al.* 2014). GH family 18 (GH18) is found in a wide range of organisms including bacteria, fungi, arthropods, plants (Itoh *et al.* 2002) and Archaea (Adrangi and Faramarzi 2013). GH18 enzymes act through a substrate-assisted catalysis, which retains the anomeric configuration of the substrate (Itoh *et al.* 2002). They are the most widespread endo-chitinase family found among bacteria, and can be further grouped into subfamilies A, B and C, based on the homology of amino acid sequences within their catalytic domain (Frederiksen *et al.* 2013). GH family 19 (GH19) is found in some plants (Kojima *et al.* 2005; Grover 2012) and bacteria (Kojima *et al.* 2005; Frederiksen *et al.* 2013) and their catalytic mechanism is a general acid-base process that inverts the anomeric configuration of the hydrolysed GlcNAc residue (Frederiksen *et al.* 2013).

Due to their widespread distribution and activity, chitinases have been attracting interest from the biotechnological sector. Potential and already-in-use applications of these enzymes and their degradation products, as well as their main fields of utilization, are presented in Chapter 2. Here, a few examples are provided to illustrate the potential of marine-derived chitinases in applications of interest to the human health and/or food production sectors. For instance, Han *et al.* (2009) reported on the antifungal effect of a chitinase isolated from the sponge-associated bacterium *Streptomyces sp*. DA11 against the human opportunistic pathogen *Candida albicans* and the phytopathogenic fungus *Aspergillus niger*. Likewise, García-Fraga *et al.* (2015), isolated a chitinase from the marine bacterium *Pseudoalteromonas tunicata* CCUG 44952 which showed anti-fungal activity against the abovementioned phytopathogenic and human pathogenic fungi (García-Fraga *et al.* 2015). Moreover, the autecology of chitin-producing microorganisms might also provide us with insights into the widespread occurrence and broad activity / affinity spectrum of marine chitinases. The cosmopolitan bacterium *Vibrio harveyi* which, probably due to the need of hydrolysing natural chitin in a large variety of forms and embedded into many different matrices, is capable of excreting about ten chitinases when grown on chitin in the laboratory (Svitil *et al.* 1997). The produced chitinases were found to show different responses according to the structure of the chitin they encounter. Moreover, *V. harveyi* showed higher growth rate and more chitinase activity when in the presence of β-chitin than in the presence of α-chitin (Svitil *et al.* 1997). As you may found mentioned in further detail in Chapter 2, chitin occurs in three different crystalline forms – α, β and γ – which differ from each other mainly in the arrangement of the microfibrils' chains. Those chains are parallel in the β-structure but antiparallel in the α-structure (Beier 2010).

Marine microorganisms bear an extraordinary importance in sustaining oceanic habitats and represent a major pillar of the aquatic biosphere. Namely, metabolic pathways mediated exclusively by marine microorganisms promote the functioning of several biochemical cycles that other organisms would be unable to complete otherwise (Finlay, Maberly and Cooper 1997; Hunter-Cevera, Karl and Buckley 2005). Chitinolytic bacteria are among keystone organisms in the ocean as they play a pivotal role in nutrient cycling and carbon and nitrogen release (Gooday 1990). Consequently, several studies have reported on the isolation of chitinolytic bacteria from many marine sources throughout the years. One of the primordial studies was performed by Campbell and Williams (1951), resulting in the identification of chitinases in eight then-new species of marine bacteria belonging to the genera *Achromobacter*, *Flavobacterium*, *Micrococcus*, and *Pseudomonas*. Later studies also reported the presence of chitinolytic bacteria in marine fish (Lindsay and Gooday 1985; Fukasawa *et al.* 1992; Itoi *et al.* 2007) and whales (Herwig and Staley 1986), probably because of the need of host animals to feed on zooplankton, constituted by chitin. Nevertheless, chitinolytic bacteria have also been found in other animal hosts such as marine sponges (Han *et al.* 2009) and in the exoskeleton of shrimp (Brzezinska *et al.* 2008) and crabs (Juarez-Jimenez *et al.* 2008) besides being usually documented as free-living microorganisms in the marine and freshwater column (Aumen 1980; Donderski 1984; Ramaiah *et al.* 2000; Fenice *et al.* 2007). Collectively, these studies revealed a high diversity of culturable, marine bacterial species involved in the chitin breakdown, including *Alteromonas* spp. (Tsujibo *et al.* 1993; Orikoshi *et al.* 2005), *Microbulbifer degradans* (Howard *et al.* 2004), *Moritella marina* (Stefanidi and Vorgias 2008), *Salinivibrio costicola* (Aunpad and Panbangred 2003), and *Streptomyces* spp. (Han *et al.* 2009), besides a firm documentation of chitinolytic activity as a conserved feature in the genus *Vibrio* - for instance, *Vibrio*

*alginolyticus* (Suginta 2007), *Vibrio anguillarum* and *Vibrio parahaemolyticus* (Hirono, Yamashita and Aoki 1998), *Vibrio cholerae* (Li and Roseman 2004; Meibom *et al.* 2004), *Vibrio furnissi* (Bassler *et al.* 1991b; Keyhani and Roseman 1999), *Vibrio harveyi* (Svitil *et al.* 1997), *Vibrio proteolyticus* (Itoi *et al.* 2007), and *Vibrio vulnificus* (Somerville and Colwell 1993). The presence of chitinase encoding genes was also reported in the family *Flavobacteriaceae* (*Bacteroidetes*, *Flavobacteriia*), including the genus *Aquimarina* (Keller-Costa *et al.* 2016) which is approached in this thesis as a chitinolytic bacterial taxon regularly isolated from marine sponges and corals (Raimundo *et al.* 2021 - Chapter 3). More information about the sampling location and the species used may be seen on **figures 1.1** and **1.2**.

Because of the abundance of chitin in the marine environment, it is reasonable to hypothesise that benthic, filter-feeding organisms such as marine sponges and corals may have evolved efficient mechanisms for chitin breakdown and utilization (Raimundo *et al.* 2021). One possible strategy may rely on the activity of chitinolytic, symbiotic microorganisms that inhabit these animals to contribute to overall chitin turnover within marine holobionts. Owing to their presumed importance for the host, some community-level studies aiming to connect diversity and function for a wide range of microbial taxa found in sponges (e.g., Slaby *et al.* 2017; Engelberts *et al.* 2020; Robbins *et al.* 2021) and corals (Robbins *et al.* 2019) have already been conducted. However, scientific knowledge regarding the chitinolytic capacity of sponge- and coral-associated microorganisms is currently still very limited, and scarce are the studies using molecular approaches to uncover the chitin processing capacity of animal-associated microbiomes in marine ecosystems.

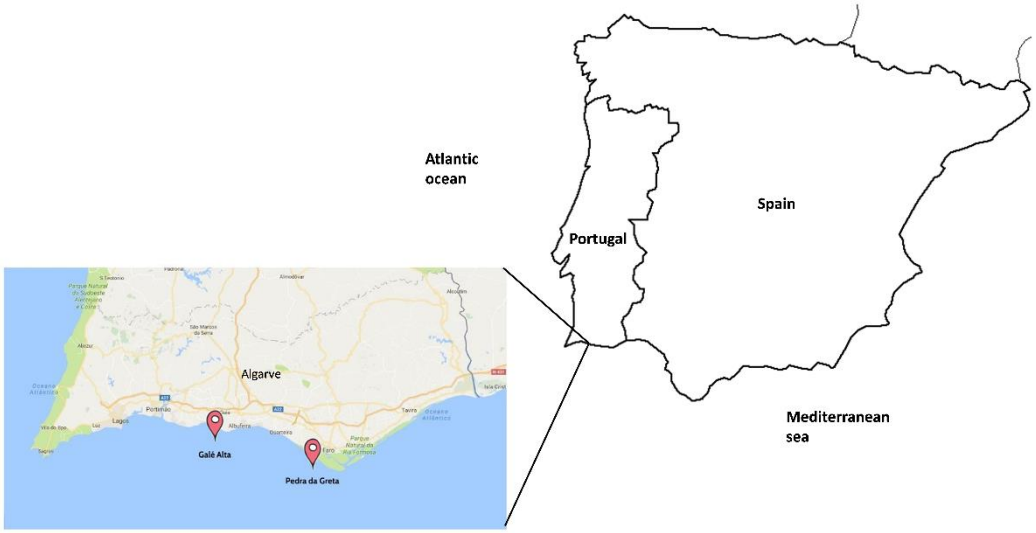


**Figure 1.1** Sampling locations of the specimens used in this thesis, along the southern (Algarve) coast of Portugal. Marine sponges *Ircinia variabilis* and *Sarcotragus spinosulus*, sources of the bacterial isolates used in cultivation-dependent analyses (Chapter 3) were sampled at Galé Alta (37°04′09.6N, 8°19′52.1W) in June 2010 (Esteves *et al.* 2013). *Spongia officinalis* and *Spongia agaricina* used in metagenomic, cultivation-independent analyses (Chapter 3 and 4) were sampled at Pedra da Greta (36° 58′ 47.2N, 7° 59′ 20.8 W) in May 2014 (Karimi *et al.* 2017) and October 2016 (sampling performed in this PhD thesis), respectively. All the octocoral specimens, used as source of bacterial isolates and in metagenomics analyses (Chapter 3), were sampled as well at Pedra da Greta, in June 2014 (Keller-Costa *et al.* 2021).

To fill this knowledge gap, this thesis addresses chitin degradation potential within the microbiomes of marine sponges and octocorals based on the above-mentioned hypothesis. To do so, the marine sponges *Ircinia variabilis*, *Sarcotragus spinosulus*, *Spongia agaricina* and *Spongia officinalis*, all belonging to the order Dictyoceratida in the class Demospongiae (phylum Porifera) and the octocorals *Eunicella labiata*, *Eunicella gazella*, *Eunicella verrucosa* and *Leptogorgia sarmentosa* (subclass Octocorallia, class Anthozoa, phylum Cnidaria) were used as model host organisms in this thesis, and the chitinolytic capacity of their bacterial communities was approached using diverse methodologies encompassing cultivation-dependent and independent techniques.

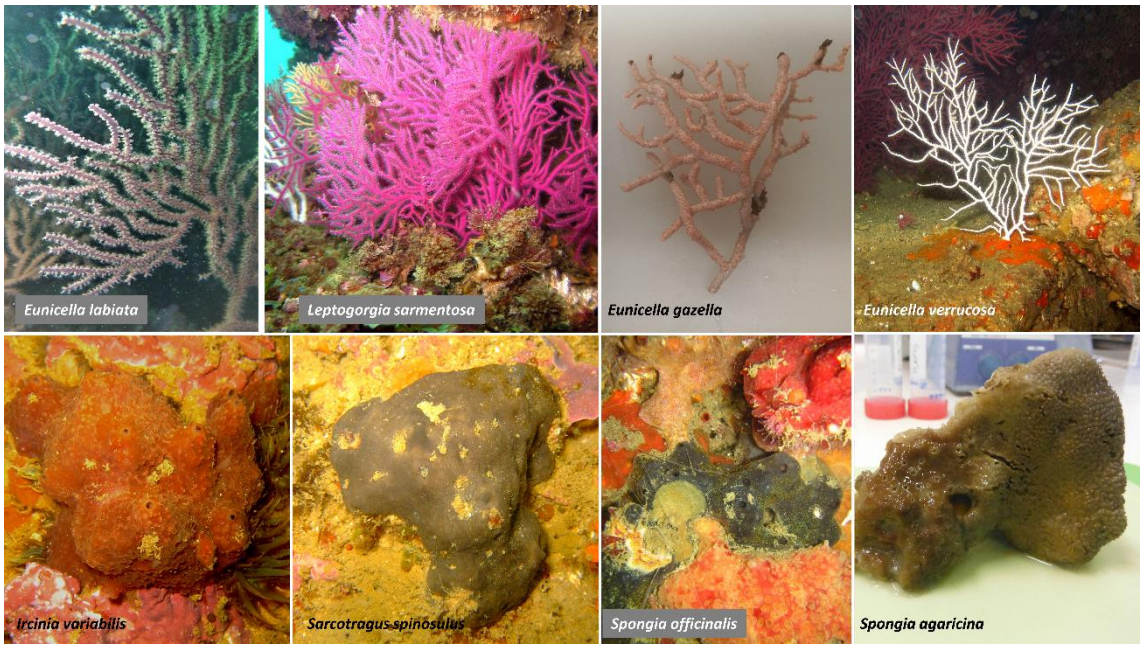


**Figure 1.2** Pictures of octocoral and sponge species used in this study. Underwater pictures of the octocorals *Eunicella labiata*, *Leptogorgia sarmentosa* and *Eunicella verrucosa* were taken by Adela Belackova, Pedro Veiga and Jorge Gonçalves (Centre of Marine Sciences, Algarve University), respectively. The laboratory picture of the octocoral *Eunicella gazella* was taken by Tina Keller-Costa. Underwater pictures of the marine sponges *Ircinica variabilis, Sarcotragus spinosulus and Spongia officinalis* were kindly provided by Dr. Jorge Gonçalves, and the laboratory picture of *Spongia agaricina* belongs to Rúben Silva's personal collection. *Eunicella labiata* and marine sponges *Ircinia variabilis* and *Sarcotragus Spinosulus* were source of the bacterial isolates analysed for chitinolytic activity in this thesis (Chapter 3). Octocoral specimens of *Leptogorgia sarmentosa, Eunicella gazella* (healthy and necrotised tissue) and *Eunicella verrucosa* were used for the octocoral metagenome dataset (project ID PRJEB13222, Keller-Costa *et al.* 2021 analysed in Chapter 3 (unassembled metagenomes). Specimens of *Spongia officinalis* were used for the sponge metagenome dataset (project ID PRJEB11585, Karimi *et al.* 2017) analysed in Chapter 3 (unassembled metagenomes). The same *S. officinalis* samples along with extra *Spongia agaricina* specimens, were used in Chapter 4 for the assembly, binning and construction of prokaryotic MAGs.

## 1.3 Marine sponges and their microbial symbionts

The term holobiont is usually used to refer to the assemblages of various species forming ecological units, as it happens for instance in the case of an animal host and all its associated symbiotic microorganisms (Rosenberg *et al.* 2007), both internally – endosymbionts - and externally - ectosymbionts. Marine sponges and octocorals, the host organisms targeted in this thesis, are examples of animals that fit in this description, not only due to the usually high diversity and abundance of their microbial symbionts, but

also because of manifold host-microbe mutualistic associations which are believed to drive holobiont functioning, and of molecular interactions shown to play fundamental roles in the establishment of complex symbioses (Rosenberg *et al.* 2007; Webster and Thomas 2016; Peixoto *et al.* 2017).

Marine sponges (phylum Porifera) are widely distributed around the planet (**Figure 1.3**) from cold and/or temperate waters to tropical reefs and can even be found in several freshwater habitats (Aboul-Ela *et al.* 2012). They are commonly denominated "living fossils" because they are among the oldest multicellular metazoans. Sponges are grouped into four different taxonomic classes: Hexactinellida, also known as glass sponges, Calcarea or calcareous sponges, Homoscleromorpha, and Demospongiae or demosponges, which is the most abundant and diverse of all sponge classes (Aboul-Ela *et al.* 2012). Marine sponges are benthic, sessile organisms, commonly attached to solid substrates, such as rocks or shells. They occur across a wide depth range from thousands of meters in the deep ocean to the intertidal shores (Hentschel, Usher and Taylor 2006; Thomas, Kavlekar and LokaBharathi 2010). Their internal body is constituted primarily by a predominantly collagenous matrix known as the mesohyl, which houses (i) choanocyte chambers responsible to take up particles in suspension acquired through filtering activity (see more below), (ii) archaeocytes responsible for the digestion of microorganisms captured by the sponges as food sources and (iii) free floating, non-differentiated cells and needle-like structures called spicules, that are formed by silicon or calcium carbonate and represent the sponge skeleton. The presence of spicules varies across different sponge species. In members of the Demospongiae class, a collagenous, fibrous protein known as spongin is also present in the mesohyl. The sponge mesohyl is surrounded and held together by the pinacoderm, an external layer of epithelial cells, usually referred to as pinacocytes (Hentschel *et al.* 2012). The pinacoderm presents

several pores called ostia, which are inhalant openings that connect the external environment to the choanocyte chambers present in the mesohyl. Within the choanocyte chambers, particles in suspension pumped into the sponge body through the ostia are taken up by choanocytes, which collectively form the choanoderm, through flagellar beating. Microorganisms that make part of the sponge diet, including planktonic bacteria, unicellular algae, and viruses (Reiswig 1975; Pile *et al.* 1996; Hadas *et al.* 2006), are then translocated to the inner mesohyl layer, where they are taken up and digested by archaeocytes (Hentschel *et al.* 2012). Marine sponges are in fact famous for their remarkable filter feeding ability, pumping up to 24 000 litres of water per kg of sponge per day (Vogel 1977).

**Figure 1.3** Simplified mapping on the world distribution of marine sponges (Porifera), adapted from van Soest *et al.* (2012). Represented in blue are the geographical locations possessing sponge species richness (that is, number of species) values below 100. In green are the locations possessing sponge richness values ranging between 100 and 200 and in orange the locations where the total number of marine sponge species is higher than 200.

In complex symbiotic relationships, the host animal can benefit from several modes of interaction. In marine sponges these involve polysaccharide breakdown and nutrient metabolism and acquisition (e.g., provision of essential amino acids and vitamins – see for instance Karimi *et al.* 2017; Slaby *et al.* 2017 and Robbins *et al.* 2021), stabilization of the sponge skeleton, processing of metabolic waste (such as ammonia; see e.g., Radax *et al.* 2012) and symbiont-driven secondary metabolite production, which might contribute to the host's chemical defence (Piel *et al.* 2004; Lopanik, Targett and Lindquist 2006), among others (see Taylor *et al.* 2007; Hentschel *et al.* 2012 and Webster and Thomas 2016 for comprehensive reviews on the matter). The sponge-associated microorganisms benefit from shelter and an increase in food availability resulting from the continuous pumping activity of the host, along with an extra supply of nitrogen resulting from the excretion of ammonia by the sponge (Taylor *et al.* 2007; Hentschel *et al.* 2012).

Microbial symbionts that inhabit the sponge mesohyl may comprise up to 60% of the animal's biomass and include mostly bacteria, but also archaea, fungi and even viruses (Hentschel *et al.* 2012). Nevertheless, while some sponge species were observed to harbour highly dense microbial communities (Reiswig 1974), others were found to present significantly fewer symbiotic microorganisms (Reiswig 1974). Hentschel and colleagues further studied this dichotomy, coining the terms high microbial abundance (HMA) and low microbial abundance (LMA) sponges (Hentschel *et al.* 2003) to distinguish among sponge species housing differentially abundant microbiomes, as bacterial densities in HMA sponges can contain up to $10^8$–$10^{10}$ bacteria per gram of sponge wet weight and be two to four orders of magnitude higher than in seawater (Hentschel, Usher and Taylor 2006) and in the LMA sponges (Hentschel, Usher and Taylor 2006). While LMA microbiomes have been found to be mainly composed by

*Proteobacteria* and *Cyanobacteria* (Hentschel, Usher and Taylor 2006; Weisz *et al.* 2007; Schmitt *et al.* 2011; Giles *et al.* 2013; Gloeckner *et al.* 2014), HMA microbiomes have been considered to present more complex taxonomic profiles at the phylum level (Moitinho-Silva *et al.* 2017), and an apparent stability within the same host species throughout time (Hardoim *et al.* 2014) and space (Erwin *et al.* 2012; Thomas *et al.* 2016). Several bacterial phyla have been recognized to contain typical sponge symbionts, with species belonging to *Proteobacteria*, *Nitrospira*, *Cyanobacteria*, *Bacteroidetes*, *Actinobacteria*, *Chloroflexi*, *Planctomycetes*, *Acidobacteria*, *Gemmatimonadetes*, and *Verrucomicrobia* typically making up a large portion of the microbiome of HMA sponges (Brinkmann, Marker and Kurtböke 2017). In addition to the abovementioned phyla, the candidate phyla *Poribacteria* (Fieseler *et al.* 2004) and *Tectomicrobia* (Wilson *et al.* 2014) are examples of novel taxa discovered in association with marine sponges using metagenomics tools. Both candidate phyla possess consistent patterns of association with HMA sponges, being enriched and possessing a high frequency of occurrence in these hosts (Fieseler *et al.* 2004; Wilson *et al.* 2014), although they may be found in free-living (e.g., seawater, sediments) and other host-associated settings (Taylor *et al.* 2012; Karimi *et al.* 2017; Podell *et al.* 2018). While the *Poribacteria* has been recognized to potentially play an important role in polysaccharide degradation with the sponge mesohyl (Siegl *et al.* 2011; Kamke *et al.* 2013; Slaby *et al.* 2017) the phylum *Tectomicrobia* has been so far widely acknowledged for its remarkable versatility regarding the biosynthesis of bioactive natural products (Wilson *et al.* 2014; Lackner *et al.* 2017).

Marine sponges are continuously exposed to changes in environmental conditions, such as shifts in temperature, pH, habitat structure, ocean currents, and to deleterious biotic interactions such as competition for substrate with other sessile organisms, predation by fish and gastropods, poisoning, infection, or overgrowth. To overcome the

danger of such changes and biotic interactions for their survival, the production of small bioactive chemical compounds is presumably essential. These compounds are secondary metabolites whose biosynthesis are likely advantageous against a variety of host diseases or natural enemies, conferring marine sponges a reputation of key marine reservoir of bioactive natural products (Taylor *et al.* 2007). In the past 20 years, several metagenomics-based studies suggested that many, if not most, of the secondary metabolites detected in these animals are most likely produced by their microbial symbionts rather than from the marine sponges themselves (Piel *et al.* 2004; Taylor *et al.* 2007; Thomas, Kavlekar and LokaBharathi 2010; Hentschel *et al.* 2012; Wilson *et al.* 2014). Thus, a potential role for these metabolites in competitive microbe-microbe interactions within the sponge microbiome cannot – and should not - be ruled out. Collectively, sponge-associated microbes, culturable or not, have been consistently observed to possess a profuse ability to synthetise diverse secondary metabolites, boosting research interest in the bioprospection of the marine sponge microbiome for novel natural products and bioactivities (Piel *et al.* 2004; O' Halloran *et al.* 2011; Hentschel *et al.* 2012; Wilson *et al.* 2014; Karimi *et al.* 2017; Silva *et al.* 2022).

Specifically, regarding natural product biosynthesis, marine sponges from the Dictyoceratida order – which comprise the sponge species investigated in this thesis - are considered an important source of compounds of both ecological and biotechnological relevance (Hardoim and Costa 2014a). Besides metagenomics- and synthetic biology-based methods (Piel *et al.* 2004; Piel 2011), classical cultivation-dependent approaches proved to be helpful in the study of the biosynthetic potential of a portion of the marine sponge microbiome (O' Halloran *et al.* 2011; Esteves *et al.* 2013; Karimi *et al.* 2019). On this note, culturable species in the *Aquimarina* genus (family *Flavobacteriaceae*) have been attracting research interest due to their promising biosynthetic capacities and

antimicrobial activities (Silva *et al.* 2019, 2022), culminating with the recent structure elucidation of the polyketide cuniculene (Helfrich *et al.* 2019) and of the small antibiotic peptides aquimarins (Dieterich *et al.* 2022) produced by *Aquimarina* strains originally isolated from the Dictyoceratida sponges *Ircinia variabilis* and *Sarcotragus spinosulus* (Esteves *et al.* 2013). Interestingly, as usual among species of the *Flavobacteriaceae* family, members of the genus *Aquimarina* are capable of hydrolysing chitin, and in this thesis both sponge- and octocoral-associated *Aquimarina* strains have been investigated for their chitin degradation abilities.

### 1.4 Corals and their microbial symbionts

Like marine sponges, corals (phylum Cnidaria, class Anthozoa) are also widely distributed throughout the world's oceans (Veron *et al.* 2015), despite the existence of an epicentre of diversity in the coral triangle in the western Pacific Ocean and secondary centres in the Red Sea and northern Madagascar regions (**figure 1.4**) (Veron *et al.* 2015). Their diversity is known to be highest in tropical regions, but cold-water corals can also be found from temperate to Artic regions to littoral waters and the deep sea (Roberts *et al.* 2009). Corals can be classified into three different subclasses, namely Hexacorallia, Octocorallia and Ceriantharia. The Hexacorallia comprise hard corals, sea anemones and zoanthids, the Octocorallia comprise blue corals, soft corals, sea pens, sea whips and sea fans (gorgonians), and the Ceriantharia consist of tube-dwelling anemones (Veron *et al.* 2015).

Hard corals, also known as stony corals, hexacorals, or scleractinian corals, secrete a hard calcium carbonate exoskeleton and represent the world's primary reef-builders. In contrast, most of Octocorallia do not secrete such massive exogenous carbonate matrix and are flexible. However, there is the case of the blue corals can secrete an exoskeleton

and gorgonians (order Alcyonacea, family Gorgoniidae) possessing an inner horny-like skeleton of a protein complex called gorgonin, contrarily to the soft corals harbouring only some internal calcite sclerites. Particularly in the hard corals of shallow reefs, the symbiotic relationship is usually marked by the presence of a keystone, photosynthetic intracellular dinoflagellate microalgal symbiont of the family Symbiodiniaceae. Through photosynthesis, this symbiont provides the coral with most of the nutrients needed for survival, especially with carbohydrates (Cooper *et al.* 2014; van de Water, Allemand and Ferrier-Pagès 2018). In addition to Symbiodiniaceae, a diverse cohort of prokaryotes, microeukaryotes and even viruses make up the coral microbiome (Peixoto *et al.* 2017).

Corals are formed by colonies of polyps that can live on their own, if attached to a hard substratum (Roberts *et al.* 2009). Those polyps are small and saclike soft-bodied organisms, with an opening surrounded by venomous tentacles called nematocysts. During the night, when Symbiodiniaceae symbionts cannot provide photosynthates, the tentacles are extended allowing them to catch zooplankton and organic particles. Polyps can also produce a structure using calcium and carbonate ions from seawater, to protect their soft body from predators and to offer a substrate for the new polyps to attach themselves (Roberts *et al.* 2009). Another clear distinguishing feature between hexacorals and octocorals lies in the symmetry of their polyps, which is eight-fold in all octocorals and six-fold in all hexacorals.

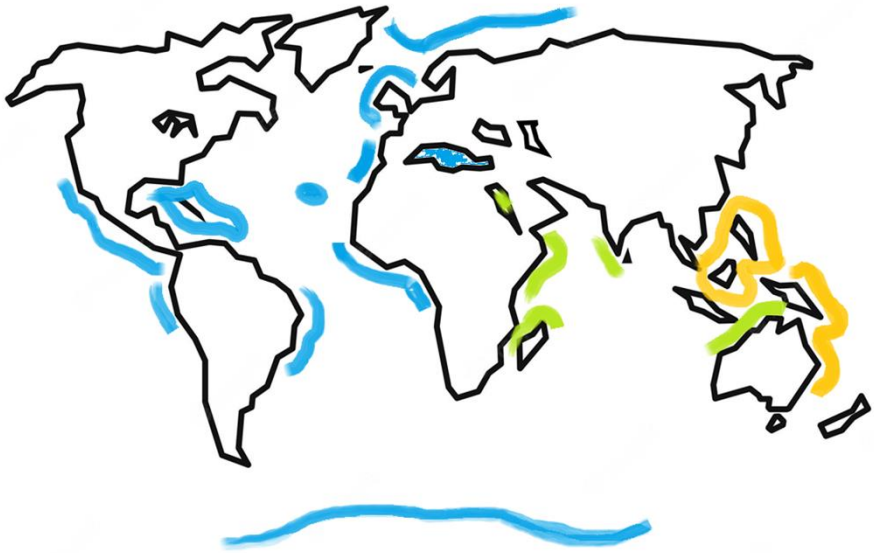

**Figure 1.4** Simplified mapping on the global distribution of corals, adapted from Veron *et al.* (2015). Represented in blue are the geographical locations presenting coral species richness values below 100. In green are the locations presenting coral species richness ranging between 100 and 400, and in orange the locations where coral species richness is higher than 400.

Like marine sponges, octocorals are benthic, sessile organisms specialized in chemical defence, derived from secondary metabolites biosynthesis. Interestingly, some of these compounds possess diverse pharmacological properties. This is true, for instance, for the cytotoxic agent eleutherobin and for pseudopterosins, both metabolites with anti-inflammatory and analgesic properties (Look *et al.* 1986). Although the origin of these metabolites is not always clear, there is increasing evidence in the literature that several bioactive compounds from corals are (once again, like in marine sponges) produced by their associated microorganisms as reviewed in Raimundo *et al.* 2018 and Sang *et al.* 2019. In 2013, for example, approximately 47% of the marine microbial producers of novel metabolites were isolated from host organisms, with sponges and octocorals being the most frequent hosts (Raimundo *et al.* 2018). In this way, the origin of the compounds is not always unknown. For instance, in the case of pseudopterosins, it has been shown that certain Symbiodinaceae strains produce these compounds in the lab from scratch

without the presence of coral, suggesting that the true pseudopterosin producer may be the dinoflagellate symbiont (Mydlarz *et al.* 2003).

Other roles of the coral-associated microbiome include nutrition and disease-control in healthy corals, as reviewed by Rosenberg *et al.* (2007). As mentioned earlier the Symbiodiniaceae symbionts are crucial for carbon provision to corals, with 60-80% of the carbon fixed from their photosynthetic activity being transferred to the host. Furthermore, it has been estimated that the coral-associated bacteria provide up to 50% of the total nitrogen needs of the host (Rosenberg *et al.* 2007). Moreover, the symbiotic community was reported to play an important role in coral survival during coral bleaching. This condition is caused by the disruption of the mutualistic interaction between the host and the Symbiodiniaceae symbionts mainly by heat waves and higher than usual seawater temperatures, leading to an expulsion of Symbiodiniaceae from coral.. The loss of the Symbiodiniaceae symbiont and consequently of its photosynthetic pigments causes the loss of colour in the coral and exposure of the white calcium carbonate skeleton underneath (making the animal look bleached) (Rosenberg *et al.* 2007). However, endolithic microbes may help during these bleaching events and protect the corals through their own photosynthetic activity, keeping the coral alive until a possible re-colonization of *Symbiodiniaceae* takes place (Peixoto *et al.* 2017). Additionally, frequent coral symbionts such as *Vibrio* spp. (despite their low abundance when looking at metagenomic studies) were discussed to play a role in other ecological functions such as secreting potentially quorum-sensing signalling molecules to control processes of colonization, pathogenesis, and extracellular enzyme production (Peixoto *et al.* 2017). Moreover, other *Gammaproteobacteria* symbionts of the orders *Oceanospirllales* and *Alteromonadales*, for instance, which are more abundant than *Vibrio* spp. in several different coral species worldwide, were also associated with

emerging evidence of involvement in nutrient cycling, coral health indication and benefit for the coral during bleaching events or pathogen attacks (Bourne, Morrow and Webster 2016; Rosado *et al.* 2018).

Recently, a better understanding of the taxonomy and function of the coral microbiome was enabled through shotgun metagenomics and reconstruction of Metagenome Assembled Genomes (MAGs)- of the symbiotic consortium of the hard coral *Porites lutea* (Robbins *et al.* 2019), and the octocorals Eunicella verrucosa, E. gazella and Leptogorgia sarmentosa (Keller-Costa *et al.*, 2021 and 2022). Robbins *et al.* 2019 presented an integrated genomic exploration of the coral-associated symbionts, through the analysis of one *Cladocopium* (the photosynthetic dinoflagellate) and 52 high-quality bacterial and archaeal MAGs. The recovered prokaryotic MAGs included the bacterial phyla *Acidobacteriota*, *Actinobacteriota*, *Bacteroidota*, *Chloroflexota*, *Dadabacteria*, *Gemmatimonadota*, *Latescibacterota*, *Nitrospirota*, *Poribacteria*, *Proteobacteria* and the archaeal phylum *Thaumarchaeota*, representing not only much of the taxonomic diversity observed in previous studies of coral species where 16S rRNA genes were surveyed, but also phyla that have been overlooked in those previous studies, such as *Poribacteria*, which had not been identified before in *Porites*. Many of these bacterial and archaeal phyla were found to possess multiple genome features involved in amino acid, vitamin, and carbon provision to their host, and in retaining the nutrients in oligotrophic water environments. Additionally, they may be also implied in diverse mechanisms for transformation of ammonia, nitrate, and urea within the coral holobiont, among others (Robbins *et al.* 2021).

## 1.5 Use of metagenomics tools to address the diversity and function of microbial communities in nature

Traditionally, early genomic studies of microbial symbionts were limited to the microbes culturable in the laboratory (Taylor *et al.* 2007). However, culturable microorganisms may only comprise a small fraction of the symbiotic consortium of marine sponges (Hardoim *et al.* 2014) and corals, although in the latter case it seems that a somewhat larger portion of the symbiotic consortium, may be culturable, as suggested for the octocoral host *Eunicella labiata* (Keller-Costa *et al.* 2017)., with a culturable fraction of around 60%, against around 10-14% in the marine sponges (Hardoim *et al.* 2014; Keller-Costa *et al.* 2017). Also for seawater, a recent study showed the possibility to culture around 45% for the highest efficiency of cultivation, in marine agar (Rodrigues and de Carvalho 2022). Those are just a few examples of the high compelling evidence accumulated during the past 30 years or so, supporting the fact that for the majority of the natural environments examined thus far, many of the microorganisms observable under the microscope cannot be grown under laboratory conditions. The expression “the great plate count anomaly” was first used by Staley and Konopka in 1985 to explain the difference between the number of cells observed under the microscope and the numbers of viable colonies from natural environments obtained on agar medium plates (Harwani 2012). The so-called plate count anomaly can be explained in several ways. For instance, an otherwise culturable species may not appear on the plate because of its growth state when collected in nature. Additionally, to grow microorganisms properly outside their natural habitat, many species require very specific conditions of nutrient concentrations in the media, pH conditions, incubation temperatures or levels of oxygen (Köpke *et al.* 2005). Moreover, if a given organism has a low prevalence or presents a slow growing

behaviour, there is a high probability that it becomes overlooked or outcompeted by other species during cultivation. Also, some genetically distinct species are sometimes phenotypically indistinguishable, leading to errors in diversity estimates of culturable bacteria when such estimates are solely based on phenotyping techniques (Harwani 2012).

In addition to all these factors, because many microorganisms are oligotrophic in their natural habitat, they may need fastidious conditions to be successfully cultivated. In mixtures of organisms cultured together, there may also exist competition for nutrients and growth may be inhibited, for instance, by bacteriocins released from other bacteria, or by antibacterial compounds within the culture medium (Tamaki *et al.* 2005). Also, within natural bacterial communities there are mechanisms - such as quorum sensing and release of signalling molecules - that are involved in the regulation and survival of the bacteria which are not easy to replicate *in vitro* (Nichols *et al.* 2008), requiring specialized techniques to promote cultivation of microorganisms depending on such factors for growth (Wirsen *et al.* 2002).

It was only in the more recent era of molecular biology and bioinformatics that the documentation of the diversity and phylogeny of uncultured bacteria not grown in laboratory conditions became tractable. Several, key advances in biochemistry and molecular biology were however needed before the modern field of metagenomics could be developed in the shape, form, and extent of impact as it is known today. The pioneering ribosomal RNA (rRNA)-based phylogeny of the living world proposed by Woese and Fox (1977) was not only instrumental to improve the general perspective of the tree of life, then suggested to comprise three fundamental domains (Bacteria, Archaea and Eukarya), but also established rRNAs as the quintessential molecular clock to approach the diversity and evolution of (micro) organisms across all branches of life (Woese 1987), an influential

legacy that persists to this date. The emergence of the dideoxynucleotide chain termination ("Sanger") method for nucleic acid sequencing (Sanger, Nicklen and Coulson 1977) along with the invention of the polymerase chain reaction (PCR) method in 1983 for the fast amplification of DNA molecules *in vitro* have sharply facilitated the description of DNA sequences (including the genes coding for rRNA molecules) from culturable microorganisms, promoting the implementation of innovative, molecular-based studies of organismal phylogeny and evolution. The early studies of Torsvik and team (Torsvik, Goksoyr and Daae 1990; Torsvik *et al.* 1998), relying on DNA reassociation rates to estimate bacterial diversity in environmental samples such as soils and sediments, were fundamental to boost molecular, cultivation-independent studies of microbial communities based on the analysis of "total-community" DNA (TC-DNA) extracted from environmental or host tissue samples. Simultaneously, the first TC-DNA "cloning-and-sequencing" (Giovannoni *et al.* 1990) and "fingerprinting" studies (Muyzer, de Waal and Uitterlinden 1993) of environmental samples have been published, inaugurating a new era in the study of microbial community diversity and function. Cloning-and-sequencing and DNA fingerprinting methods, such as PCR-DGGE, SSCP, and t-RFLP (see Oros-Sichler (2007) for a comprehensive review on DNA fingerprinting methods) relied heavily on PCR-based analysis of rRNA encoding genes (most frequently, 16S rRNA genes) to describe and compare the structure of microbial (especially prokaryotic) communities in different samples. The use of these methods dominated the field of molecular microbial ecology for nearly two decades, and boosted the discovery of manifold, novel lineages of uncultured or so-far unculturable bacteria and archaea, greatly expanding general knowledge of the tree of life (Rappé and Giovannoni 2003).

The application of the abovementioned techniques enabled greater knowledge of the microbiomes of marine sponges and corals, revealing that these symbiotic communities are sharply distinct in terms of taxonomy and structure from those of the environmental surroundings (Hardoim *et al.* 2009; Morrow *et al.* 2012), are often host species-specific (Erwin *et al.* 2012; Hardoim *et al.* 2012; Morrow *et al.* 2012) and change according with the developmental stage of the host (Apprill *et al.* 2009). In the case of marine sponges, these techniques helped revealing a vast diversity of microbial lineages either "enriched" or specifically associated with these animals (Simister *et al.* 2012), being the discovery of the candidate phylum *Poribacteria* (Fieseler *et al.* 2004) the most prominent example. Regarding coral-associated microbial communities, cloning-and-sequencing and DNA fingerprinting techniques were as well important to reveal associations between coral bleaching events and shifts in host-associated microbial communities (Bourne *et al.* 2008), contributing to better understanding of disease emergence and dysbiosis states in these animals (Rosenberg *et al.* 2008).

Despite the remarkable advances enabled by cloning-and-sequencing and DNA fingerprinting procedures, these cultivation-independent techniques suffered from throughput hurdles that sharply limited their capacity to describe complex microbial communities beyond their most abundant members. These limitations were overcome by next-generation sequencing (NGS) techniques that allowed the exploration of microbial communities with high-throughput, more affordable price-range, and more precise and practical methodology. In fact, NGS techniques bypass the time-consuming and throughput-limiting procedure of constructing environmental DNA clone libraries prior to either amplicon or untargeted shotgun metagenome sequencing (see definitions below), which were required by less-resolving sequencing technologies (e.g., the Sanger sequencing method). Nowadays, a broad palette of NGS technologies, encompassing both

"short-read" (e.g., Illumina sequencing) and "long-read" (e.g., Nanopore and Pac-Bio sequencing) approaches is available. They constitute the standard tools for deep, cultivation-independent analyses of the diversity and function of microbial communities via either amplicon sequencing (also referred to as "metabarcoding" and normally used in taxonomic profiling) or full metagenome sequencing approaches (Raes, Foerstner and Bork 2007).

Briefly, amplicon sequencing relies on sequencing regions of an amplified gene to identify community members and define the taxonomic composition of a given sample, when typical phylogenetic marker genes are used as targets in PCR amplification. In this case, specific PCR primers are used to amplify informative genome regions from different taxonomic groups. Usually, these regions are the 16S rRNA (ribosomal RNA) gene for *Bacteria* and *Archaea* (Bahram *et al.* 2019), the 18S rRNA gene and/or ITS (internal transcribed spacers) regions for micro-*Eukarya*, particularly microfungi and microalgae (Tedersoo *et al.* 2015). Amplicon sequencing is still a widely used approach since the workflows are easy, there are several platforms available for data analysis e.g., QIIME2, mothur and LotuS (Schloss *et al.* 2009; Hildebrand *et al.* 2014; Bolyen *et al.* 2019), and the approach is cost-effective, as a small fraction of reads may be enough to identify the prevailing taxa, depending on the complexity of the community. However, amplicon sequencing has also severe limitations, such as low taxonomic resolution, variations in the copy number of the rRNA genes (Edgar 2017; de Oliveira Martins *et al.* 2020), as well as taxonomic biases related to using PCR amplifications (Tedersoo *et al.* 2015; Bahram *et al.* 2019). Moreover, when targeting rRNA genes as mentioned above, this technology only provides information on the taxonomy, but not the functions putatively carried out by the targeted microbiota. To minimize those biases, several bioinformatic algorithms (e.g., PICRUSTt, Tax4Fun) were developed to infer the functional potential

of communities based on taxa detected via rRNA gene amplicon sequencing (Douglas *et al.* 2020). However, these predictions are inherently difficult to make without ancillary metagenomic data.

Untargeted, metagenome sequencing approaches are required to obtain sequences from all sorts of genes, instead of from single phylogenetic markers such as 16S/18S rRNA genes often used in amplicon sequencing, allowing functional profiles from the total microbial community to be annotated and simultaneous assessment of community diversity. A further analytical advance involves the use of full shotgun metagenome sequencing data in the construction of singularized metagenome-assembled genomes (MAGs) to link taxonomy and function within complex microbial communities. Both functional metagenomics and genome-resolved metagenomics (that is, the construction and analysis of MAGs) have already been well employed in the study of the marine sponge microbiome, allowing novel insights into sponge-symbiont relationships (Thomas, Kavlekar and LokaBharathi 2010; Fan *et al.* 2012; Liu *et al.* 2012) and boosting new knowledge of the function, ecology, and phylogeny of the uncultured sponge symbiont diversity (Bayer *et al.* 2018; Engelberts *et al.* 2020; Haber *et al.* 2021; Taylor *et al.* 2021). A schematic representation on the functional metagenomics study workflow may be seen in **Figure 1.5**. Perhaps the first comprehensive metagenome-resolved genomics study of the marine sponge microbiome was published by Slaby *et al.* (2017), in which 37 MAGs spanning 13 of the most typical sponge-associated bacterial phyla / candidate phyla, such as *Poribacteria*, *Chloroflexi*, *Bacteroidetes*, *Acidobacteria*, *Actinobacteria*, *Proteobacteria* and *Gemmatimonadetes*, among others, were reconstructed from the host species *Aplysina aerophoba* and analysed. The authors verified that, in comparison with reference genomes, the genomes of sponge symbionts were usually enriched in features enabling bacterial defence, such as restriction-

modification and toxin-antitoxin systems. When comparing the nutrient utilization profiles of the symbionts, the authors depicted the formation of two functional guilds, one formed by bacteria able to metabolize carnitine and the other sulphated polysaccharides, which are compounds regularly present in the mesohyl of sponges (Slaby *et al.* 2017). Another clear example of metagenome-resolved genomics for a comprehensive characterization of the marine sponge microbiome is the study of Engelberts *et al.* (2020), which described the likely metabolic activities of diverse taxa associated with the sponge *I. ramosa*, with special focus on those involved in carbon and nitrogen cycling. Based on the analysis of 259 MAGs, the authors suggested that carbon and nitrogen fixation and other essential functions that had previously been attributed to only a restricted group of bacterial symbionts are in fact widespread across diverse taxa including *Thaumarchaeota* (family *Nitrosopumilaceae*), *Nitrospirota*, *Cyanobacteriota* and *Gammaproteobacteria*, while other pathways, such as ammonia and sulfite oxidation, are limited to specific sole taxa, such as *Thaumarchaeota* to perform the ammonia oxidation step of nitrification and *Gammaproteobacteria* regarding sulfite oxidation. In comparison with the wealth of information gathered by metagenome-resolved genomics of the marine sponge microbiome, MAGs-based studies of the coral microbiome remain few, and perhaps limited, thus far, to the inaugural effort by Robbins *et al.* (2019) in disentangling the taxonomy and function of microbial symbionts of the calcified coral *Porites lutea*. As mentioned above, this influential study comprised the analysis of the reconstructed genomes of the dinoflagellate symbiont *Cladocopium* sp. and of 52 further archaeal and bacterial genomes and, beyond highlighting molecular mechanisms involved in coral-prokaryote symbioses, placed emphasis as well on the role of the coral-associated prokaryotic consortium in the provision of B-vitamins, amino-acids, and fixed carbon to the coral host. Most recently, other studies disclosed octocoral species microbiome

functions, by reconstructing it using shot-gun metagenome functional profiling (Keller-Costa *et al.* 2021) and metagenome-resolved genomics (Keller-Costa *et al.* 2022a). Those studies shed light on MAGs of dominant *Endozoicomonadaceae* symbionts to be present on all octocoral species and in 90% of the healthy coral specimens, favoring the current idea of these symbionts as potential indicators of coral health. Additionally, dominant symbionts participation in several functional roles such as carbon, nitrogen and sulfur cycling, amino acid and b-vitamin provision, chemical defense, and oxidative and osmotic stress protection were suggested (Keller-Costa *et al.* 2021, 2022a).

Through the different chapters, this thesis combines both cultivation-dependent and -independent approaches (including shotgun metagenome sequencing and metagenome-assembled genomes) to unveil the chitin utilization potential within sessile marine invertebrates that include several different species of marine sponges and octocorals, contributing with new knowledge about in-animal biogeochemical cycling and expanding common understanding of the diversity of chitin degrading microorganisms and communities in natural environments.

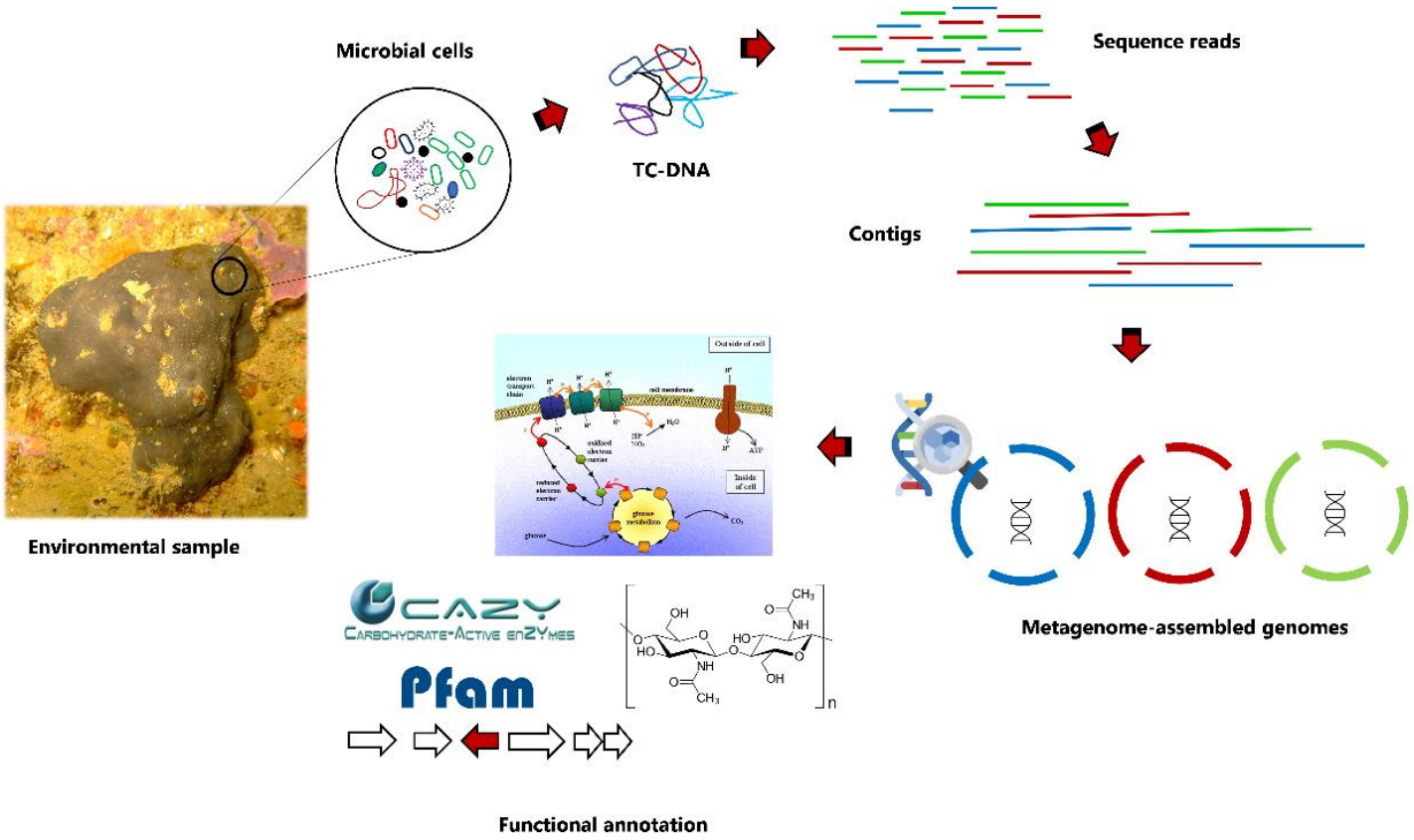


**Figure 1.5** Basic workflow for the retrieval of prokaryotic MAGs from a given microbial source. The illustration displays a picture of the marine sponge *Sarcotragus spinosulus (*credit: Dr. Jorge Gonçalves, Centre of Marine Sciences, Algarve University), source of some of the bacterial isolates analysed for chitinolytic activity in this thesis (Chapter 3). After separation of microbial cells form the sponge host (see Chapters 3 and 4) total-community DNA (the "microbial metagenome") is extracted, fragmented and subjected to high-throughput, next generation sequencing using, for instance, a short-read sequencing technology such as Illumina. The short reads are then assembled into longer, contiguous sequences (contigs), fundamentally based on the overlapping regions between those reads. Contigs possessing highly similar relative abundance ("coverage") in the total metagenome and patterns of tetranucleotide frequencies across their lengths are very likely to originate from the same prokaryotic species, and are therefore binned into one single genome (MAG) thought to be representative of that species. MAGs can thereafter be subjected to any sort of genome-wide screenings, annotation and comparative analyses as usually performed for isolate genomes. The figure illustrates the search for endochitinase-encoding genes / protein domains using the Cazy and Pfam databases as performed in this thesis for MAGs retrieved from *Spongia officinalis* and *Spongia agaricina.*

# Chapter 2

## Chitin degradation in a diverse microbial world: from metabolic pathways to organismal interactions and biotechnological applications

Contents of this chapter were submitted as an original review article to the journal *FEMS Microbiology Ecology*.

# Chitin degradation in a diverse microbial world: from metabolic pathways to organismal interactions and biotechnological applications

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**Keywords:** chitinases, chitosan, deacetylases, host-microbe interactions, cross-feeding, chitinolytic bacteria

**Running title:** Chitin degradation by microorganisms

## 2.1 Abstract

Chitin is the second most abundant polysaccharide on Earth, after cellulose, and the most abundant in the marine environment. Current knowledge of the microorganisms, catalytic enzymes, accessory domains, and metabolic pathways involved in chitin degradation, although extensive, derives mostly from cultivation-dependent studies. However, recent metagenomics surveys suggest that many chitin-degrading microbes have thus far not been cultivated. Therefore, our understanding of chitin turnover in nature remains rather limited despite the relevance of this major biopolymer for organismal physiology and ecosystem functioning. In this context, this review examines our current knowledge of the ecophysiology of chitin degradation processes in marine and terrestrial ecosystems and their biotechnological valorisation. Particularly, the relevance of chitin breakdown to carbon and nitrogen cycling, host-microbe, and microbe-microbe interactions in model host-microbiome systems such as the plant rhizo- and endosphere, marine sponges, and corals is addressed. We discuss possible interspecies cross-feeding among microorganisms mediated by chitin metabolism highlighting the importance of chitin-derived metabolites and chitinases in the biological control of phytopathogens and in a range of biotechnological applications. To foster the successful exploitation of microbial metabolism of chitin for improved human health, environmental preservation, food production and waste management, coordinated action among researchers, entrepreneurs, and end-users is required.

**One sentence summary:** Chitin degradation and utilization are fundamental processes underpinning organismal and ecosystem health which bear biotechnological potential that can be enlarged by harnessing the metabolism of diverse naturally-occurring chitinoclastic microorganisms.

## 2.2 Introduction

Chitin is the second-most abundant natural polymer on Earth after cellulose (Tran, Barnich and Mizoguchi 2011), and the most abundant in the marine environment (Paulsen *et al.* 2016). Chitin is formed by β-1,4 bonds of N-acetylglucosamine (GlcNAc) units, and its structural subunit is formed by two monomer units rotated 180° to each other, referred to as the disaccharide N,N'-diacetylchitobiose ($(GlcNAc)_2$). Chitin builds helices that assemble into microfibrils, stabilized by hydrogen bonds between the amine and the carbonyl groups (Beier 2010). It occurs in three different crystalline forms – α, β and γ – which differ from each other mainly in the arrangement of the microfibrils' chains (Beier 2010). Those chains are parallel in the β-structure but antiparallel in the α-structure, the latter being the most common form found in nature (Purushotham *et al.* 2012)because its arrangement favours strong hydrogen bonding (Rathore and Gupta 2015) and, consequently, higher stability (Yadav *et al.* 2019). The γ-structure occurs as a mixture of the other two (Rathore and Gupta 2015), containing two parallel and one anti-parallel chains (Khoushab and Yamabhai 2010). Both β and γ structures can be converted into α, but the opposite has never been described (Khoushab and Yamabhai 2010). Chitin is insoluble in water due to its intermolecular hydrogen bonds, although some chitin-based derivatives such as its deacetylated-form, namely chitosan (with between 60 to 80% of acetyl groups removed) are water-soluble (Khoushab and Yamabhai 2010).

With global chitin production rates estimated at $10^{12} – 10^{14}$ tonnes per year (Yadav *et al*. 2019), chitin is a major constituent of fungal cell walls, yeasts, marine diatoms, crustaceans, and zooplankton and it is typically difficult to degrade in the natural environment (Tran, Barnich and Mizoguchi 2011; Patel and Goyal 2017). A major role of chitin as a structural component of living systems is in protecting these organisms from harsh environmental conditions and from anti-parasite immune responses of their

eventual hosts (Tran, Barnich and Mizoguchi 2011). Moreover, in marine ecosystems, its insolubility in water contributes to the formation of "marine snow" (Biancalana *et al.* 2017). Chitin-enriched suspended particles, however, rarely reach the ocean floor. As these particles slowly sink, they are degraded by chitinoclastic bacteria that use chitin as a source of carbon and nitrogen for their metabolism (Li and Roseman 2004; Zobell and Rittenberg 1937). Although temporary accumulation of chitin may occur (Beier 2010), the lack of reports on quantitatively significant, long-term accumulation of chitin in marine ecosystems suggests that the degradation and utilization processes must be ubiquitous and highly active in these environments (Biancalana *et al.* 2017).

The study of the metabolic pathways of chitin degradation, and of the organisms, enzymes, transport proteins and regulatory molecules involved in these processes constitute an increasingly attractive field of research (Bouacem *et al.* 2018; Li *et al.* 2017; Subbanna *et al.* 2018; Wakita *et al.* 2017; Zhang *et al.* 2017). In recent years, chitin, its by-products such as chitosan, and chitin-degrading enzymes, have been shown to display a broad range of activities of relevance to diverse commercial sectors such as the pharmaceutical and food industries (**Figure 2.1**). This evidence, coupled with chitin's widespread distribution and abundance in nature, currently underpins much research interest in biotechnological applications based on the metabolism and properties of chitin and its derivatives (Nagpure, Choudhary and Gupta 2014; di Rosa *et al.* 2016; Ilangumaran *et al.* 2017). The metabolic pathways of chitin degradation in terrestrial environments have been largely explored (Geisseler *et al.* 2010; Hjort *et al.* 2013; Wieczorek, Hetz and Kolb 2014; Cretoiu *et al.* 2015; Shinya *et al.* 2015; Langner and Göhre 2016), and several studies have shed more light on chitin breakdown and transformation in aquatic ecosystems, particularly in freshwater environments (Beier and Bertilsson 2011, 2013; Beier *et al.* 2012). However, despite our current capacity to

effectively describe microbial communities owing to advances in DNA sequencing technologies, we still barely understand the full diversity and taxonomic composition of chitin-degrading microbial communities across aquatic and terrestrial biomes, especially of those which remain recalcitrant to laboratory cultivation. Moreover, the extent to which prevailing metabolic pathways and microorganisms involved in chitin breakdown shift at the microscale because of changes in biotic and abiotic features has been poorly investigated. Addressing such fundamental questions is certain to foster a more effective exploitation of microbial activities linked to chitin metabolism in modern biotechnological applications.

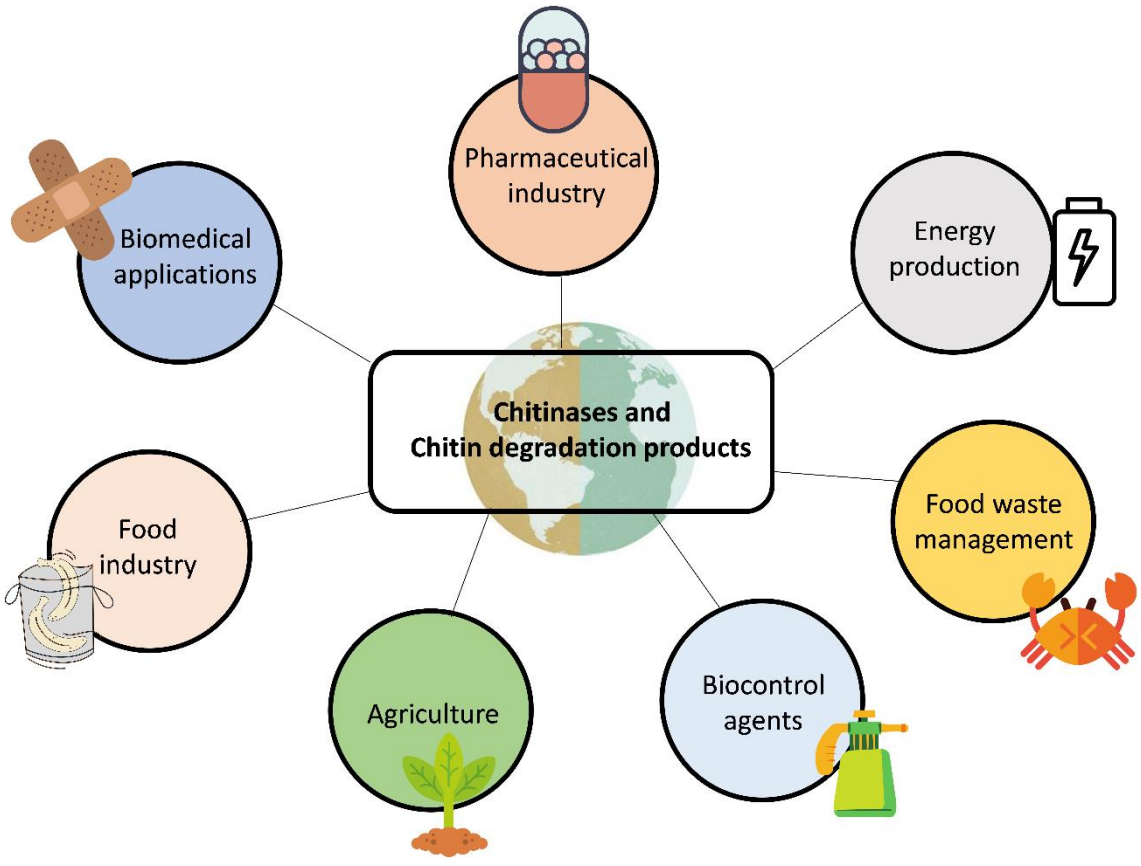


**Figure 2.1** Chitinases and chitin degradation products are promising sources for applications across various commercial sectors such as the pharmaceutical industry (e.g., drug delivery systems), biomedicine (e.g., wound healing materials), the food industry, (e.g., bioplastic packings - bags and films), agriculture and biocontrol (e.g., disease suppression), waste management (e.g., degradation and value-increase of shellfish waste), and energy production (e.g., increased utilisation of fermentation products in microbial fuel cells).

In this study we first review and update our current knowledge of the classification, structure and biological role of chitin-degrading enzymes and metabolic pathways in terrestrial and marine ecosystems. Second, we examine our emerging understanding of chitinolytic processes (i.e., chitin hydrolysis) as mediators of complex host-microbe symbioses and their relevance to interspecific microbial interactions (e.g., bacterial cross-feeding, bacterial-fungal antagonism) and organismal and ecosystem functioning. Finally, we address the potential use of chitinases and chitin-derived products across several sectors of applied biotechnology, from waste management to agricultural ecosystems and human health.

## 2.3 Microbial enzymes, proteins, and metabolic pathways involved in chitin degradation

### 2.3.1 Exo- and endo-chitinases

Chitin degradation is generally referred to as a chitinoclastic process, independently of the mechanisms involved. When the degradation of chitin occurs by enzyme-catalysed hydrolysis of the β-glycoside bond, the process is called chitinolytic (Beier and Bertilsson 2013) and the enzymes involved in the hydrolysis process are referred to as chitinases (di Rosa *et al.* 2016).

The location where chitinases hydrolyse the bond within chitin molecules (i.e., internal or endpoints) can further be used to distinguish two types of chitinolytic enzymes: endo-chitinases (EC 3.2.1.14) and exo-chitinases (EC 3.2.1.52) (Dahiya, Tewari and Hoondal 2006). Endo-chitinases randomly cleave at internal sites of the chitin molecule, generating soluble oligomers of low molecular mass, such as chitotetriose (Brzezinska *et al.* 2013), chitotriose, chitobiose and diacetylchitobiose (Rathore and Gupta 2015). Exo-

chitinases comprise two sub-types, chitobiosidases which catalyse the progressive release of the dimer chitobiose from the non-reducing edge of the polymer, and N-acetylglucosaminidases, or chitobiases, which hydrolyse endo-chitinase- and chitobiosidase-derived oligomers, generating GlcNAc units (Cohen-Kupiec and Chet 1998; Dahiya, Tewari and Hoondal 2006). All exo-chitinases are included in the common set of β-N-acetyl-hexosaminidases (EC.3.2.1.52) by the Nomenclature Committee of IUBMB (International Union of Biochemistry and Molecular Biology) (Brzezinska *et al.* 2013) . Endo-chitinases (EC 3.2.1.14) are glycosyl hydrolases (GH), whose size usually varies between 20 kDa and 90kDa (Rathore and Gupta 2015). However, some exceptions may occur, such as a specific chitinase of 130 kDa known as *Tk*-ChiA described by Chen *et al.* (2019). GH are important carbohydrate active enzymes (CAZymes) and are known to have an important role on the enzymatic processing of not only chitin but also other complex carbohydrates (Berlemont and Martiny 2016). Endo-chitinases are commonly found among bacteria (Bai *et al.* 2016; Yang *et al.* 2016), archaea (Chen *et al.* 2019; Nishitani *et al.* 2018; Sorokin *et al.* 2015), fungi (Jenin *et al.* 2016; Khan *et al.* 2015), higher plants (Volpicella *et al.* 2014; Ray *et al.* 2016), insects (Li *et al.* 2016; Liu *et al.* 2017) and other invertebrates (Kucerova *et al.* 2016), and vertebrates including mammals (Mack *et al.* 2015; Janiak, Chaney and Tosi 2018) and humans specifically (Berlemont and Martiny 2016). Endo-chitinases can play various roles in organismal physiology such as digestion of chitin-containing organisms in the food chain, cuticle turnover and cell differentiation (Khoushab and Yamabhai 2010b). Endo-chitinases usually belong to GH families 18 and 19 (Brzezinska *et al.* 2013), yet GH family 23 also comprises enzymes with endo-chitinolytic activity, including an endo-chitinase recently found in the archaeon *Thermococcus chitonophagus* (Horiuchi *et al.* 2016). In contrast, exo-chitinases usually belong to GH families 3 and 20. It has been suggested that endo-chitinases classified into

distinct GH families differ in their 3D-structure and catalytic mechanism (Paulsen *et al.* 2016), and have likely evolved from different ancestors (Tran, Barnich and Mizoguchi 2011). GH family 18 (GH18) endo-chitinases are found in a wide range of organisms including bacteria, fungi, arthropods, plants (Itoh *et al.* 2002) and archaea (Andronopoulou and Vorgias 2004; Nishitani *et al.* 2018; Chen *et al.* 2019). GH18 enzymes act through a substrate-assisted catalysis which retains the anomeric configuration of the substrate (Itoh *et al.* 2002). They are the most widespread endo-chitinase family found among bacteria, and can be classified into subfamilies A, B and C according to the homology of amino acid sequences within their catalytic domain (Frederiksen *et al.* 2013). GH19 endo-chitinases are found in some plants (Kojima *et al.* 2005; Grover 2012) and bacteria (Frederiksen *et al.* 2013; Kojima *et al.* 2005) and their catalytic mechanism is a general acid-base process that inverts the anomeric configuration of the hydrolysed GlcNAc residue (Frederiksen *et al.* 2013). All endo-chitinase families have signal peptides, indicating the possibility to the enzymes to be secreted and to function outside the cells (Khoushab and Yamabhai 2010).

### 2.3.2 Chitin hydrolysis is dictated by chitinases and accessory molecules

Chitinases are generally required for four main processes: 1) to help build and remodel the cell walls and exoskeleton of chitin-bearing organisms to maintain and support body size and shape, 2) to digest the insoluble chitin polymer into smaller metabolites which can then be used as source of nutrients, 3) to degrade the protective chitin-coated shield of microbial pathogens as a defence mechanism, preventing the infection by those organisms, and 4) to facilitate parasitism and pathogenesis whenever host organisms contain chitin (Dahiya, Tewari and Hoondal 2006; Adrangi and Faramarzi 2013; Rathore and Gupta 2015). Generally, organisms can possess multiple endo- and/or exo-chitinases

(Horiuchi *et al.* 2016; Lin *et al.* 2017; Suzuki *et al.* 2002; Zhang *et al.* 2015) to increase the efficiency of chitin degradation processes (Dahiya *et al.* 2006; Suzuki *et al.* 2002). In bacteria, the presence of multiple chitinases is most likely explained by lateral gene transfer, gene duplication and post-transcriptional protein modifications (Hult *et al.* 2005). Chitin hydrolysis and intake of chitooligosaccharides (COS) into the cell usually rely on the aid of accessory molecules that facilitate chitin-docking and transmembrane import such as chitin-binding proteins and chito-porins, respectively. For instance, one of the most effective bacteria for chitin degradation, the soil-dweller *Serratia marcescens,* possesses several GH18 chitinases (ChiA, ChiB, ChiC) and a chitin-binding protein (CBP21, see more below) that are important not only for nutrition, but also for virulence towards plant and insect hosts (Toratani *et al.* 2008). Several marine bacteria, such as the *Vibrio* species, also possess a very efficient chitin degradation machinery which enables them to exploit the high abundance of chitin in marine ecosystems (Li *et al.* 2017; Svitil *et al.* 1997). *V. harveyi*, for instance, possesses several type A chitinases along with a chito-porin channel termed VhChiP, which mediates COS transport through the outer membrane (Suginta *et al.* 2013). Moreover, Berlemont and Martiny also showed that GH targeting chitin were more abundant in marine environments microbiomes, including mats, freshwater, sponges and corals when compared with other environments.

In addition to the enzymes interacting directly with the chitin substrate, multiple other proteins are involved in sensing, chemotaxis, and gene expression during bacterial chitin degradation. The roles played by chitin-binding, transport, and chemotaxis proteins in the process of chitin degradation by bacteria are addressed in more detail below.

### 2.3.3 Chitin-binding proteins are characteristic accessory molecules for chitin hydrolysis among diverse bacteria

Many chitinases possess domains that help recognition and binding to insoluble chitin, therefore increasing the accessibility to the substrate (Kojima *et al.* 2005; Manjeet *et al.* 2013), promoting its interaction with the enzyme and enhancing degradation efficiency (Hashimoto *et al.* 2000; Kojima *et al.* 2005). These domains, known as chitin-binding modules, can also occur as individual proteins, so-called chitin-binding proteins (CBPs) (Vaaje-Kolstad *et al.* 2005, 2010), which possess no catalytic activity. In nature, chitin-binding domains are involved in root colonization, pathogen defence, and polysaccharide biosynthesis (Guillén, Sánchez and Rodríguez-Sanoja 2009). Chitinases and CBPs often interact synergistically as the latter bind to the insoluble chitin substrate and thus enhance substrate accessibility to the former (Manjeet *et al*., 2013;). Moreover, CBPs can also play a role in the regulation of chitinolytic gene expression (Li and Roseman 2004; Vaaje-Kolstad *et al.*, 2010).

Several bacterial genera such as *Aeromonas*, *Arthrobacter*, *Bacillus*, *Chromobacterium*, *Clostridium*, *Pseudomonas*, *Serratia*, *Streptomyces* and *Vibrio* present CBPs of different sizes and with varying substrate preferences (Manjeet *et al*., 2013). These proteins play an important role in increasing chitinolytic activity not only by interacting with chitin oligomers but also by triggering signal regulatory cascades within the cell. Complete lysis of insoluble chitin typically occurs by a sequence of cleavage events taking place at different cellular locations (e.g., extracellular space, periplasm and/or cytoplasm) and catalysed by different enzymes (Pakizeh, Moradi and Ghassemi 2021) (see below).

### 2.3.4 Chitin deacetylation is widespread in nature and generates biotechnology-valuable chitosan

In addition to chitin degradation by endo- and exo-chitinases, which produces COS of varying sizes and monomers of N-acetylglucosamine, chitin can be broken down through deacetylation leading to the production of the polymer chitosan (Pakizeh, Moradi and Ghassemi 2021; Amer *et al.* 2022; Huq *et al.* 2022). This process is mediated by another group of enzymes known as chitin deacetylases (EC 3.5.1.41) (Zhao *et al.* 2010). In addition, COS deacetylases (EC 3.5.1.105) produce partially deacetylated chitobiose (Grifoll-Romero *et al.* 2019). These enzymes are part of the carbohydrate esterase family 4, which also includes peptidoglycan N-acetylglucosamine deacetylases (EC 3.5.1.104), peptidoglycan N-acetylmuramic acid deacetylases (EC 3.5.1.-), poly-β-1,6-N-acetylglucosamine deacetylase (EC 3.5.1.-), and some acetyl xylan esterases (EC 3.1.1.72) (Grifoll-Romero *et al*., 2019). As for the previously presented enzymes, deacetylases are also widespread in a variety of insects, fungi, and bacteria (Zhao *et al.* 2010). *Vibrio* and *Shewanella* species are the marine bacteria most often described as possessing chitin deacetylases, sharing around 50-60% deacetylase amino acid sequence homology (Grifoll-Romero *et al*., 2019). Chitosan resulting from deacetylation processes can further be subjected to deamination, generating cellulose-like molecules (Kucerova *et al*., 2016). These chitin-derived by-products, which possess great potential for biotechnological applications, can be further hydrolysed to N-glucosamine and/or glucose by chitosanases (EC 3.2.1.132) and/or cellulases (EC 3.2.1.4), respectively (Xia, Liu and Liu 2008). Cellulases can also bind directly to chitin or chitosan (Xia, Liu and Liu 2008). While cellulases can degrade chitosan even without previous deamination, the same has not been shown for chitin (Kucerova *et al*., 2016). Chitosan can also be degraded by specific chitinases, depending on the deacetylation degree of the molecule (Beier and

Bertilsson 2013). Only a few, early studies have compared both processes (i.e., chitinolytic *vs.* non-chitinolytic) and suggested that chitin degradation by deacetylation/deamination might be more relevant in terrestrial than in aquatic environments (Hillman, Gooday and Prosser 1989b; Gooday 1994). However, a recent genome- and metagenome-based study revealed deacetylase encoding genes to be more frequent than endo-chitinase encoding genes among cultivated bacterial symbionts of marine sponges. Also, deacetylase encoding genes were revealed to be enriched in the microbial metagenome of marine sponges (Raimundo *et al.* 2021), indicating that multi-omics approaches bear great potential in advancing our understanding of the abundance distribution of genes underlying chitin breakdown in distinct marine habitats.

In conclusion, chitin-degrading pathways involve several enzymes (Li *et al.* 2007) and constitute complex processes whose regulation remains partially unclear. Since diverse bacterial species dictate chitinolytic processes mediated by chitinases in marine and terrestrial ecosystems, they will serve as model systems for chitin hydrolysis in the following section, as the general processes are similar in other organisms (Merzendorfer and Zimoch 2003; Zhang *et al.* 2012).

### 2.4 The ecophysiology of chitin hydrolysis by bacteria

Generally, chitin degradation by chitinases starts by bacteria binding to chitin (Li *et al.*, 2007; Li and Roseman 2004), although a previous step of sensing and moving towards the substrate is usually required, particularly in the marine environment (Keyhani and Roseman 1999). Chemotaxis, broadly defined as the directed movement of an organism in response to a chemical gradient (Kearns 2010), is the common sensing and searching mechanism through which bacteria scavenge for chitin (Mandel *et al.* 2012; Sun *et al.* 2015; Datta *et al.* 2016). In the marine environment, bacteria such as *Vibrio furnissii* are

attracted to chito-oligosaccharides of multiple sizes (Bassler *et al.* 1991; Bassler *et al.* 1991), which may be leaked by chitinous organisms during moulting or from haemolymph from sick or dying organisms (Keyhani and Roseman 1999). In soils, *Serratia marcescens* recognizes chitin in the extracellular space due to the presence and uptake of dimers of N-acetyl-glucosamine $(GlcNAc)_2$ (Hult *et al.* 2005). After sensing, bacteria adhere to the chitin substrate to increase its availability to their chitinases (Tran, Barnich and Mizoguchi 2011). This process can be specific and mediated either by adhesins (Keyhani and Roseman 1999) or, more commonly, by CBPs (Vaaje-Kolstad *et al*. 2005). Alternatively, the adhesion process can be non-specific, mediated by a variety of polymers and exopolysaccharides through ionic and hydrophobic interactions, also involved in biofilm formation (Keyhani and Roseman 1999).

Following adhesion, the insoluble chitin polymer is cleaved into smaller, water-soluble oligomers (Kucerova *et al*. 2016). This step takes place in the extracellular space (Suginta *et al.* 2013), mostly by the action of endo-chitinases (Suginta *et al.* 2000, 2013). In addition to the catalytic domain, some of these enzymes, such as chitinase A from *Vibrio harveyi*, a GH18 chitinase, also contain structural binding domains that facilitate the sliding of the bound chitin chain towards the catalytic domain and its entry into the substrate-binding groove (Pantoom, Songsiriritthigul and Suginta 2008). This further suggests that chitin-binding and catalytic domains work synergistically during chitin breakdown (Suginta *et al*. 2013). In contrast, the short, water-soluble COS do not require interaction with chitin-binding domains to reach the active site (Songsiriritthigul *et al.* 2008).

In *Vibrio* spp. and other *Gammaproteobacteria*, as the chitin chain is catabolized to oligosaccharides, the already cleaved chito-fragments are transported through the outer membrane into the periplasmic space in two different ways, according to their sizes (Li

*et al.* 2007). While monomers and dimers of GlcNAc enter the periplasm by nonspecific porins (Hunt *et al.* 2008) such as sugar-porins or glycoporins (Keyhani, Li and Roseman 2000), longer oligosaccharides are transported by a specific porin, usually referred to as chitoporin or *ChiP* (Keyhani *et al*. 2000; Li and Roseman 2004). Once in the periplasm, the translocated oligomers are hydrolysed to GlcNAc and $(GlcNAc)_2$ (Suginta *et al*. 2013) by exo-chitinases (Li *et al*. 2007) such as β-N-acetyl-glucosaminidase (Keyhani and Roseman 1996) and chitobiosidases (Svitil *et al.* 1997). Cleavage into $(GlcNAc)_2$ is particularly important because it is a crucial player in a two-component signalling system that regulates the expression of chitinolytic genes and chitinase secretion (Li *et al.* 2007; Li and Roseman 2004). Periplasmic $(GlcNAc)_2$ has high affinity to CBPs present in the inner membrane, which are associated with a ArcB type hybrid sensor kinase, designated *ChiS* (Kwon *et al.* 2000), whose inactivation was found to reduce extracellular chitinase activity (Meibom *et al.* 2004). As long as CBPs are connected with *ChiS*, the latter remains locked in an inactive conformation (Meibom *et al*., 2004). Dissociation of CBPs upon binding to $(GlcNAc)_2$ activates the *ChiS* sensor (Suginta *et al*. 2013). This sensor up-regulates the expression of approximately 50 genes, most of which are involved in chitin catabolism, including the extracellular chitinase (Li and Roseman 2004). During expression, *ChiS* remains active given that COS are generated (Meibom *et al*. 2004). Finally, when chitin is completely depleted, the periplasmic CBP binds again to the *ChiS* sensor locking it into the inactive configuration and the system is turned off (Li and Roseman 2004).

Specifically for species in the phylum *Proteobacteria*, the transport through the inner membrane occurs according to the type of molecule to be translocated (Suginta *et al*. 2013): $(GlcNAc)_2$ is actively transported by the $(GlcNAc)_2$ ABC-type permease (Bouma and Roseman, 1996; Li and Roseman, 2004), whereas GlcNAc is transported by the

phosphoenolpyruvate transferase system (PTS) (Bouma and Roseman 1996; Keyhani *et al.* 1996). When $(GlcNAc)_2$ arrives to the cytosol, it is phosphorylated into two molecules of GlcNAc-6-phosphate by a N,N-diacetylchitobiose phosphorylase (Park, Keyhani and Roseman 2000), a predicted GlcNAc-specific ATP-dependent kinase (Bassler *et al*. 1991; Keyhani and Roseman 1999) and a GlcNAc-1P-mutase (Li and Roseman 2004). Thus, GlcNAc-6P is produced either during uptake by the PTS or by the N,N-deacetylchitobiose phosphorylase action (Kucerova *et al*. 2016). It is later converted into fructose-6-phosphate by the activity of an N-acetylglucosamine-6-phosphate deacetylase and a glucosamine-6-phosphate deaminase (Heidelberg *et al.* 2000), also generating ammonium ($NH4^+$) and acetate (Bassler *et al.* 1991; Keyhani and Roseman 1996; Li *et al*. 2007) as by-products.

The deacetylated residues (GlcN) from chitosan degradation can also be incorporated into the chitin catabolic cascade (Hunt *et al.* 2008). Past research on *V. cholerae* demonstrated that the gene VC1280, adjacent to a cellobiose PTS transporter, was up-regulated upon addition of $(GlcN)_2$ (Meibom *et al.* 2004). Once in the cytoplasm, the 1-4 linkage between the glucosamine residues can be broken by a chitobiase (Park, Wang and Roseman 2002) and enter the normal chitin degradation pathway (Hunt *et al.* 2008). The cytoplasmic GlcN may then be phosphorylated by an ATP-dependent glucosamine kinase (Park *et al.* 2002), generating again fructose-6-P (Hunt *et al.* 2008).

### 2.5 Microbial chitin degradation and its relevance in host-microbe and microbe-microbe interactions

Arguably, the most described organismal relationships mediated by chitinolytic processes are host-pathogen interactions, such as in the cases of *Aphanomyces astaci* (water mold) infesting crayfish (Soderháll and Unestam 1975), or *Vibrio* species (*V.*

*cholerae*, *V. parahaemolyticus*, and *V. vulnificus*) infecting the blue crab *Callinectes sapidus* (Sullivan and Neigel 2018). Nonetheless, chitinolytic organisms can also be involved in mutualistic relationships, as exemplified by the interaction between the sugar-beet root maggot (*Tetanops myopaeformis*) and the bacteria *Serratia liquefaciens* and *S. marcescens*, which help adult flies emerge from the puparium by digesting chitin present in that structure (Iverson *et al.* 1984). Another example of interspecific, mutualistic relationship involving chitinolytic organisms includes the endogenous gut microbiota and their animal hosts (Kucerova *et al.* 2016). Chitin present in the diet of multicellular organisms (that feed on e.g., fungi or invertebrates), including humans, is broken down by bacteria to be further assimilated by their hosts (Janiak 2016; Muzzarelli *et al.* 2012; Yang *et al.* 2015). Endogenous gut microbes that assist their hosts in chitin digestion include bacteria from the gastrointestinal tract of whales (Seki and Taga 1965), bovines (Patton and Chandler 1975), or *Xanthomonas* and *Curtobacterium* from the springtail (Collembola) *Folsomia candida* (Borkott and Insam 1990), to name a few. Moreover, the mutualistic plant bacterium *Rhizobium leguminosarum* can produce short chitin oligomers with four or five units that activate $Ca^{2+}$ spiking in root epidermal cells (Walker, Viprey and Downie 2000), a mechanism generally used by these bacteria to enter their host (Capoen *et al.* 2009). Also, when the mutualistic fungus *Rhizophagus irregularis* colonizes plant roots, it needs to undergo cell wall remodelling to adapt to the new growing conditions. Since chitin is one of the components of the fungal cell wall, this remodelling is mediated by the action of chitinases provided by the roots (Balestrini and Bonfante 2014). Thus, a wide breadth of beneficial host-microbe interactions is mediated or influenced by chitin degradation processes. Below we summarize recent, multi-omics driven research that sheds new light on the relevance of these processes for foundational symbioses in terrestrial and marine ecosystems.

### 2.5.1 Chitin hydrolysis may fuel intra- and interspecific bacterial cross-feeding

The high amount of chitin produced every year in nature and the absence of long-term accumulation suggest that chitinolytic organisms, especially bacteria, may degrade more chitin polymers than what they need for their own biological activities (Beier and Bertilsson 2013). For example, although *Pseudoalteromonas* strain S91 has a great ability to hydrolyse chitin, only a reduced number of cells within the bacterial population degrade chitin to use as a source of carbon and nitrogen, while the remaining cells utilize the chitin oligomers that become available (Baty *et al.* 2000). Additionally, multicellular cooperation is common among bacteria (Shapiro 2003), including associations between chitinolytic and non-chitinolytic strains (Chernin *et al.* 1998; DeAngelis, Lindow and Firestone 2008). Thus, substrate cross-feeding of chitin derivatives may occur between species (Kaneko and Colwell 1977; Keyhani and Roseman 1997) (**Figure 2.2**). In aquatic environments, although only 0.1 - 5.8% of all prokaryotes have been estimated to be chitinolytic (Cottrell *et al.* 2000; Beier *et al.* 2012), at least 4 – 40% of the bacteria utilize chitin-derived products in their metabolism (Riemann and Azam 2002), reinforcing the hypothesis of interactions between different bacterial taxa enabled by chitin degradation processes. Efficient collection of chitin oligomers by bacteria occurs in a limited distance of approximately 10 μm from the polymeric source (Vetter *et al.* 1998), and this observation strengthens cross-feeding hypotheses raised recently for highly abundant, host-associated microbial assemblages (Raimundo *et al.* 2021, see below). For longer distances between receiving organisms and hydrolysis products, high affinity to the substrate is needed. However, it is still unknown whether this affinity increases the efficiency of substrate consumption or not (Kucerova *et al.* 2016).

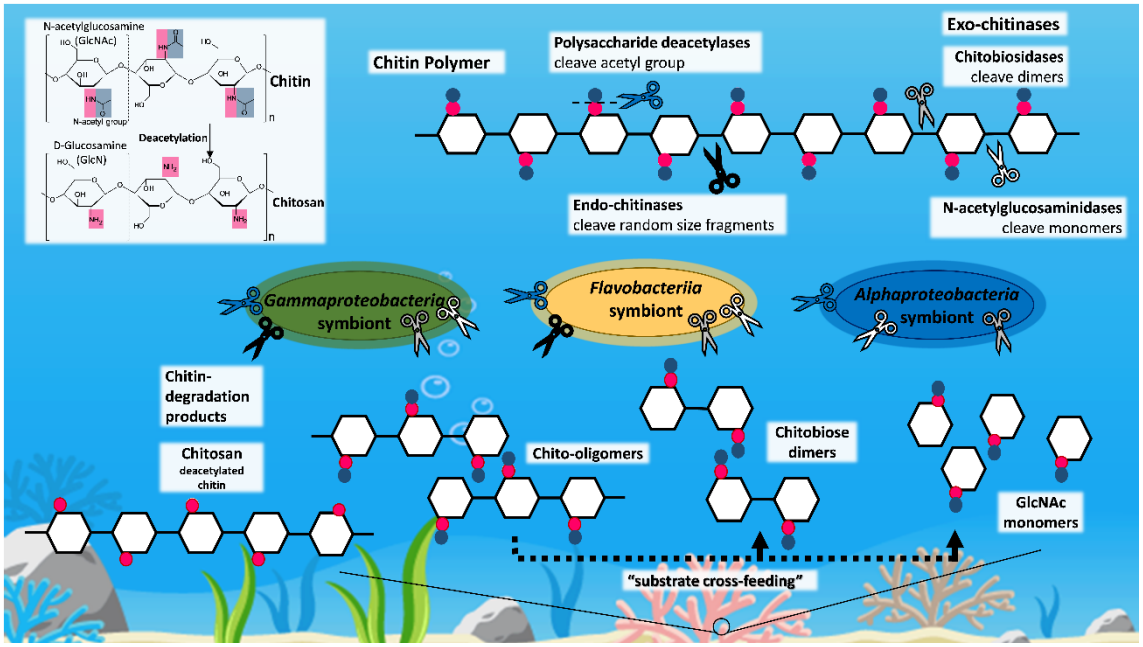


**Figure 2.2** Representation of possible substrate cross-feeding among marine host-associated bacteria. The inlet on the top left displays the basic structures of chitin and its deacetylated form, chitosan. On the top centre-right, the chitin polymer is illustrated, with coloured scissors representing different enzymes involved in chitin degradation processes, namely endo-chitinases (black) deacetylases (blue), and two types of exo-chitinases (grey and white). Recent research (Raimundo *et al.*, 2021) suggests that *Gammaproteobacteria* and *Flavobacteriia* symbionts of corals, including so-far uncultured lineages, possess the ability to degrade the large chitin polymer into smaller chito-oligosaccharides, which could be taken up by specific lineages of *Alphaproteobacteria* through the activity of exo-chitinases in an example of substrate cross-feeding as defined by Smith *et al.* (2019). In this scheme, each hexagon represents a monomer of N-acetylglucosamine, with the amine and the acetyl groups represented by the pink and blue dots, respectively. Besides chitinase activity, which underpins chitin hydrolysis into chito-oligomers, chitin can also be converted into chitosan by the action of deacetylases, which seem to be widespread among bacterial symbionts of marine sponges and octocorals.

### 2.5.2 Multi-omics approaches illuminate chitin metabolism in complex marine symbioses

In a recent study (Raimundo *et al.* 2021), bacterial symbionts of marine sponges and octocorals were analysed for the presence of endo- and exo-chitinase activities. *Gammaproteobacteria* and *Flavobacteriia* species (e.g., *Pseudoalteromonas*, *Shewanella*, *Vibrio*, and *Aquimarina*) were able to degrade the chitin polymer and possessed both endo-and exo-chitinase activities. In addition, *Alphaproteobacteria*

species (e.g., *Ruegeria*, *Roseibium* - synonym *Labrenzia* - and *Pseudovibrio*) displayed exo-chitinase activity only, suggesting that they may take advantage of the chito-oligomer products released by endo-chitinases from *Gammaproteobacteria* and *Bacteroidetes* spp. present in marine host-associated microbial communities. The latter may be an example of possible substrate cross-feeding (Smith *et al.* 2019) during the processing of chitin within benthic marine invertebrates (Raimundo *et al.* 2021) (**Figure 2.2**). Further, by using bacterial cultivation combined with metagenome sequencing, the abovementioned study not only highlighted high chitinolytic activity among less studied, culturable bacterial symbionts such as *Aquimarina* spp. (see also Keller-Costa *et al.* 2016), but has also revealed a possible role of novel, so-far unculturable, lineages of *Gammaproteobacteria*, *Flavobacteriia* and *Chloroflexi*, in chitin breakdown and turnover within eukaryotic hosts. Particularly intriguing is the emerging ecology of profuse chitinolytic species in the *Aquimarina* genus (*Flavobacteriia*) whose genomes possess diverse GH encoding genes, including multiple endo-chitinase gene copies (Silva *et al.* 2019; Raimundo *et al.* 2021). Culturable *Aquimarina* species have been already identified as etiological agents of disease in the economically valuable lobster *Homarus americanus* (Quinn *et al.* 2012) and in the red alga *Delisea pulchra* (Kumar *et al.* 2016a). Endo-chitinases and diverse glycoside hydrolases, respectively, have been suggested to be implicated in disease development (Quinn *et al.* 2012). More recently, the enrichment of an uncultured *Aquimarina* sp. population in necrotic octocoral tissue was revealed by a comprehensive metagenomics survey (Keller-Costa *et al.* 2021), highlighting the opportunistic behaviour of this chitin-degrading bacterial group. Chitinolytic activity was further found to promote the expression of genes involved in host colonization and secondary metabolite biosynthesis, including the antibiotics andrimid and holomycin in the *Vibrionaceae* species *Vibrio coralliilyticus* and *Photobacterium galatheae* (Giubergia

*et al.* 2017). To advance our understanding of the contribution of chitinolytic processes to beneficial and deleterious host-microbe interactions, future studies should examine more microbial taxa and model systems to address the hypothesis of a concerted regulation of chitin catabolism and secondary metabolite biosynthesis for improved host colonization and invasion.

### 2.5.3 Chitinolytic endophytes boost plant disease suppression

Modern metagenomic studies have also been instrumental in documenting the diversity and relevance of chitinolytic microorganisms in terrestrial carbon cycling and plant-microbe interactions. Hjort *et al.* (2013) reported, for the first time, the heterologous expression of a novel chitobiosidase (Chi18H8) using a fosmid library of a suppressive soil metagenome. The exo-chitinase was expressed in *E. coli* and was found to possess antifungal activity against a range of crop pathogens. Strikingly, chitinolytic activities within the endosphere are now known to represent a remarkable example of tight regulation of host defence mechanisms via plant-microbe interactions. When suppressive soils containing sugar beet seedlings were inoculated with the phytopathogenic fungus *Rhizoctonia solani*, glycoside hydrolases involved in fungal cell wall degradation such as chitinases, β-glucanases and endoglucanases were all found to be enriched in the metagenomes of the endosphere microbial community along with the enrichment of uncultivated chitinolytic microorganisms in the *Chitinophagaceae*, *Flavobacteriaceae*, *Burkholderiaceae* and *Xanthomonadaceae* families (Carrión *et al.* 2019) (**Figure 2.3**). Similar to the observations of Giubergia *et al.* (2017) on the coupling between chitin catabolism and secondary metabolite biosynthesis among marine *Vibrionaceae* species, the study of Carrión *et al*. (2019) uncovered the enrichment, in the plant endosphere, of biosynthetic gene clusters underlying the production of nonribosomal peptides and

polyketides deemed fundamental for disease suppression. Taken together, these results suggest a prompt response of the plant endosymbiotic community to a soil microbial pathogen and the direct participation of chitinases as an important mechanism in this response, leading to increased host defence against pathogens. Our current understanding of the plant rhizosphere (Mendes, Garbeva and Raaijmakers 2013) and endosphere (Carrión *et al.* 2019; Dini-Andreote 2020) as first and second lines of microbiome-enabled host defence, respectively, has greatly benefited from metagenomics-based approaches in the study of host-associated microbial communities. This knowledge bears relevance to the development of more sustainable agriculture practices based on the management of soil and plant-associated microbiomes. It is expected that the continued discovery of novel natural products, chitinases and chitin-degrading microorganisms from these symbiotic consortia will play an important role in devising future microbiome-based solutions for improved plant health and crop yields across agroecosystems.

Environmental conditions suitable for chitin degradation are intimately related to chitinolytic interspecific relationships. Temperature (Manucharova *et al.* 2011; Hjort *et al.* 2013), pH (Hjort *et al.* 2013; Zhang *et al.* 2012) and salinity (Nikapitiya *et al.* 2015) strongly influence not only the efficiency of the chitin hydrolysis process, but also chitin utilization yield and the chitinolytic community composition itself (Terahara *et al.* 2009) in both terrestrial and marine ecosystems. Moreover, seasonality has also been shown to affect chitin degradation and the structure of chitinolytic communities (Metcalfe *et al.* 2002; Puissant *et al.* 2015). In these cases, however, it is not correct to attribute changes in chitin degradation efficiency to one single feature, as multiple factors that are season-dependent (e.g., substrate availability, phytoplankton blooms) interact in the environment and may simultaneously influence the final yield (Beier *et al.* 2012).

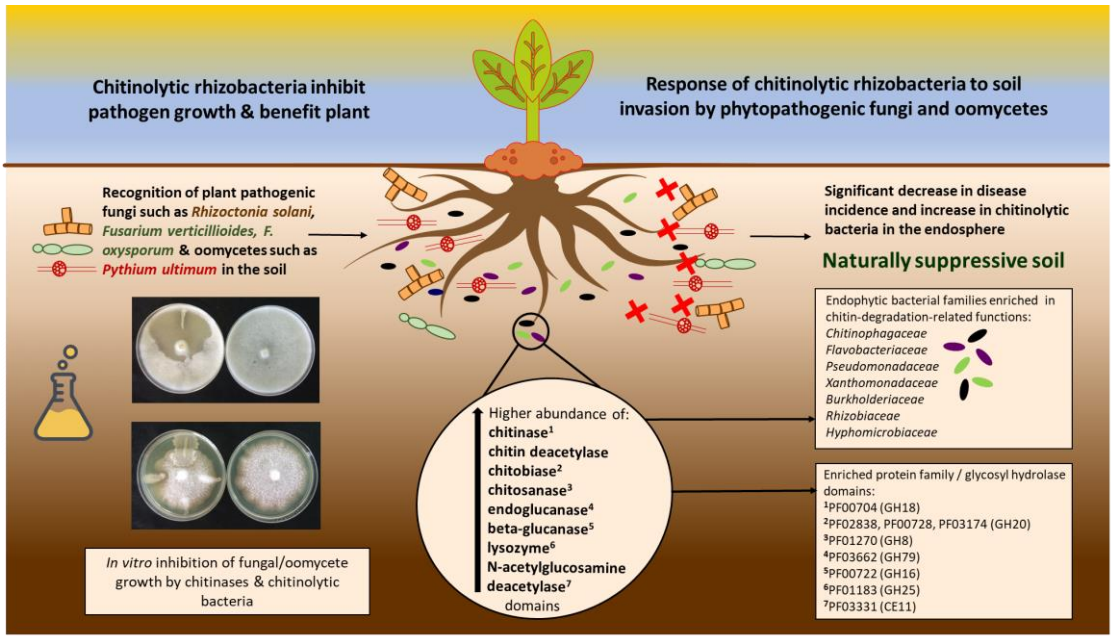


**Figure 2.3** Proposed model of response of chitinolytic rhizobacteria to soil invasion by phytopathogenic organisms such as fungi and oomycetes. The scheme represents chitinolytic bacteria (top-right inlet), chitin/carbohydrate degrading enzymes (middle inlet) and corresponding Pfam domains (bottom-right inlet) found to be enriched inside plant roots when naturally suppressive soils are inoculated with phytopathogenic fungi, after the report of Carrión *et al*. (2019) on the response of the endophytic community of sugar beet seedlings to *Rhizoctonia solani*. It also highlights (middle left panel) the well-known ability of chitinolytic rhizobacteria to inhibit fungal growth *in vitro* (e.g., Chang *et al.* 2003). The pictures illustrate the inhibition of the oomycete *Pythium ultimum* (top) and the fungus *Fusarium verticillioides* (bottom) by *Pseudomonas* strains (on the left of each panel) in comparison to control plates where the phytopathogens grow in the absence of antagonistic bacteria (on the right of each panel).

## 2.6 Biotechnological applications of chitin-derived products and chitinases

Owing to their manifold properties and ubiquitous and abundant distribution in the environment, chitin derivatives and chitinases have been mainly investigated for the development of novel biotechnologies (Nagpure *et al.* 2014). Indeed, chitinases and chitin-derived products can be applied in a range of sectors such as agriculture, waste management, human healthcare, and pharmacy, among others (Khoushab and Yamabhai 2010b; Nagpure, Choudhary and Gupta 2014; Elieh-Ali-Komi and Hamblin 2016) (**Figure 2.1**). Below we provide an overview of the use, and potential, of biocatalysts and

compounds involved in chitin metabolism across multiple sectors and spanning wide technology readiness levels.

### 2.6.1 Agriculture and biological control

In plant-microbe interactions and agroecosystems at large, chitin fragments and chitinases can play an important role in the biological control of pathogens by boosting the defence system of plants against microbial invasions (Gerbore *et al.* 2013; Carrión *et al.* 2019) (**Table 2.1, Figure 2.3**). By degrading the cuticles of insects (consisting largely of chitin) and the cell walls of phytopathogenic fungi that infest crops, chitinases produced by bacteria may reduce the proliferation of pathogens without endangering the host, humans, or other mammals (Nagpure *et al.* 2014). It has long been thought that chitinolytic rhizobacteria may play a role in plant defence against fungal phytopathogens because of their ubiquitous presence across distinct suppressive soils (Adesina *et al.* 2007) and due to their antifungal activities *in vitro*. For example, Chang *et al.* (2003) have shown that chitinases from *Bacillus cereus* YQ308 inhibited the growth of the broad host-range, phytopathogenic fungi *Fusarium oxysporum* and *F. solani*, and of the oomycete *Pythium ultimum*. Prapagdee *et al.* (2008) further reported morphological deformation and hyphal swelling of fungi grown on potato dextrose agar caused by an extracellular chitinase from *Streptomyces hygroscopius* SRA14. More recently, a combination of chitosan with methyl jasmonate was found to enhance the defence mechanisms of cherry tomato by reducing disease incidence caused by the fungus *Alternaria alternata* and lesion diameters of post-harvest fruits (Chen *et al.* 2015). Collectively, these studies and recent multi-omics approaches reporting on the response of chitinolytic rhizo/endophytic bacteria to the presence of phytopathogenic fungi in soil (Carrión *et al.* 2019; Dini-Andreote 2020), suggest belowground chitin metabolism as a pivotal factor in plant-

growth promotion and disease prevention in natural and man-made ecosystems. Notably, several patents have already been granted in Europe and the US for the utilization of chitin, chitin-derived products and/or chitinolytic microorganisms (e.g., *Brevibacillus laterosporus*) for insect pest management, plant disease control and soil bioremediation in agriculture (**Supplementary Table S2.1**).

Biological control is an attractive alternative to the use of chemical pesticides/insecticides that frequently contaminate agro-products and the soil and encourages novel agriculture practices which are less hazardous to the environment (Nagpure *et al.* 2014). Under field conditions, however, biocontrol agents are not always as efficient in pest control as chemical pesticides (Nagpure *et al.* 2014). Genetic engineering is offering new ways of biocontrol, resulting in gene expression of desired new or enhanced traits (Ortiz-Urquiza, Luo and Keyhani 2014; Lovett and Leger 2018). On the plant host side, transgenic lines of eggplant (*Solanum melongena*) expressing the class 1 (GH19) rice endo-chitinase gene showed enhanced resistance against wilt diseases caused by the phytopathogenic fungi *Verticillium dahliae* and *Fusarium oxysporum* (Singh *et al.* 2015). Likewise, transgenic potatoes (variety Desiree) over-expressing endo-chitinases showed greater resistance against *Alternaria solani* infection, with plants remaining healthy, while non-transgenic plants died three weeks after infection (Khan *et al.* 2017). Alternatively, the biosynthesis of chitinases from uncultured microbes by a heterologous host, once well established, could also function as a reliable supply of novel biocatalysts with improved antifungal activities of potential use in disease control (Hjort *et al.* 2013).

**Table 2.1** Properties of chitinases and chitin derivatives of biotechnological relevance in agriculture.

| Compound / product | Problem | Relevance | Reference |
|---|---|---|---|
| Chitin polymer | Plant diseases caused by fungal phytopathogens cause substantial crop losses worldwide | Exposure to chitin boosts defense systems of plants against microbial invasions | (Gerbore *et al.* 2013) |
| Chitin oligomers | Desensitization of tomato cells (*Lycopersicon esculentum*) to chitin in a time-dependent manner and possible effects on infection by *Rhizobium leguminosarum* | Exposure to chitin oligomers improve the defense mechanism of tomato (*Lycopersicon esculentum*) Msk8 cells | (Felix, Baureithel and Boller 1998) |
| Combination of chitosan with methyl jasmonate | Post-harvest disease induced by *Alternaria alternata* in cherry tomato | Enhances defense mechanisms of cherry tomato by reducing disease incidence and lesion diameters of fruits | (Chen *et al.* 2014) |
| Chitinases from *Bacillus cereus* strain YQ308 | Plant infestation with the phytopathogenic fungi *Fusarium oxysporum*, *F. solani* and *Pythium ultimum* | *In vitro* inhibition of the growth of these pathogenic fungi | (Chang, Chen and Wang 2003) |
| PGM chitinases | Insect and fungi pest species of plants, humans, or other mammals | Degradation of chitin from the cuticles of insects and the cell walls of phytopathogenic fungi without negative effects for the host | (Nagpure, Choudhary and Gupta 2014) |
| Chitinases and chitin-derived products | Chemical fungicides or pesticides cause negative impacts to environment | Complementation of the action of chemical fungicides or pesticides, reducing hazardous effects on the environment without negatively affecting harvest quality | (Horsch *et al.* 1997) (Feliziani, Landi and Romanazzi 2015) |
| General chitin by-products | Heavily polluted aquatic environments | Environmental/water remediation by chitosan adsorption of Cr (VI) ions in solution | (Franco *et al.* 2004) (Saifuddin and Kumaran 2005) |
| Chitosan extracted from shrimp shells | Contamination of effluents with metal ions | Adsorption of metal ions Cu(II), Zn(II), Fe(II) and Cr(IV) with rates from 65% - 99% | (Mohanasrinivasan *et al.* 2013) |

The use of genetically modified organisms (GMOs) calls for a careful safety and risk assessment and ethical considerations at the early stages of product development. But even with effective (environmental) safety management, the transition from laboratory to market can be challenging because of current legislative barriers and a general scepticism towards genetically engineered products in society. In this regard, the direct application of chitinases and chitin-derived products to complement the action of chemical fungicides or pesticides (Horsch *et al.* 1997; Feliziani, Landi and Romanazzi 2015) (**Table 2.1**) may be easier to implement than GMOs, however with the limitation that the compounds need to be repeatedly or continuously supplied. A further alternative could be exploiting the chitin degrading capacities of plant growth-promoting microbes (PGPMs) that are already marketed in plant growth and protection products. Indeed, PGPMs present one of the fastest-growing sectors in agronomy, with a predicted value of over 10 billion US $ by 2025 for the whole agricultural biocontrol industry (Berg *et al.* 2021). Importantly, most products comprise strains of genera native to plants, such as e.g., *Azospirillum, Bacillus, Pseudomonas, Rhizobium* and *Serratia* (Berg *et al.* 2021). Several are known chitin degraders themselves or have taxonomically close relatives possessing chitinolytic activity, which could benefit and accelerate product development and approval.

### 2.6.2 Waste management and environmental recovery

Chitinases can also help to counteract the continuous increase in seafood waste production, particularly waste from shells of crustaceans such as crabs and shrimps (Kaur and Dhillon 2015). Approximately half the total weight of shellfish is chitin (Hamed, Özogul and Regenstein 2016). Therefore, chitinases can be key determinants in chitinous waste removal, breaking down the insoluble polymers into bioactive oligosaccharides and monosaccharides. For example, chitinases from *Streptomyces rimosus* significantly

biodegraded chitosan (42.5% after 14 days) and shrimp shells (38.2% after 14 days) (Brzezinska *et al.* 2013). Recently, Kumar *et al.* (2018) showed that *Paenibacillus* sp. AD, isolated from seafood waste dumping sites, was able to degrade 99% of the offered shrimp waste, generating added value products such as N-acetyl glucosamine (GlcNAc), chitobiose $(GlcNAc)_2$, chitotriose $(GlcNAc)_3$, chitotetroses $(GlcNAc)_4$ and oligosaccharides of higher degree of polymerization (Kumar *et al.* 2018). Although chitinolytic-based bio-extraction of chitin from shellfish waste has been increasingly investigated and referred to as safer, cleaner, and environmentally friendly, it is not currently exploited to its maximum capacity (Kaur and Dhillon 2015), in part because the competing chemical chitin-extraction methods have lower processing time (Hamed *et al.* 2016). On this note, the search of thermostable chitinases active at high temperatures is considered a promising approach for the optimization of enzymatic chitin degradation processes at the industrial scale (Chen *et al.* 2019). In fact, current research on chitin-degrading, hyper thermophilic archaea and their utilization as model systems for bio-based processing of food waste is gaining momentum (Atomi and Reeve 2019; Chen *et al.* 2019; Horiuchi *et al.* 2016). For instance, the thermostable chitinase Tk-ChiA from the marine archaeon *Thermococcus kodakarensis* was shown by Chen *et al.* (2019) to be stable at temperatures ranging from 70 to 80 $^{o}$C and to possess dual hydrolytic activity, catalysing the breakdown of both chitin and cellulose owing to the presence of three binding domains with affinity to these substrates.

Chitin by-products can, moreover, actively assist environmental recovery by acting as bioremediation agents in heavily polluted environments (Franco *et al.* 2004; Saifuddin and Kumaran 2005). Indeed, chitosan extracted from shrimp shells was found to adsorb the metal ions Cu(II), Zn(II), Fe(II) and Cr(IV) that were present in an effluent

contaminated by the leather industry. Specifically, chitosan removed 99%, 86% and 65% of the available Cu(II), Zn(II) and Fe(II), respectively (Mohanasrinivasan *et al.* 2013).

### 2.6.3 Chitosan properties are required by manifold industrial sectors

Many pharmaceutical applications have used chitin-derived products and chitinases as key agents in disease treatment, drug delivery, and antioxidant, antimicrobial, and antitumor activity (Khoushab and Yamabhai 2010b; Kaur and Dhillon 2015; Younes and Rinaudo 2015) (**Table 2.2**). Depending on the desired application, chitin and chitin by-products can be processed as fibres, powders, gels, beads, films, sponges, solutions, or tablets (Ilium 1998; Kardas *et al.* 2012). In the development of drug delivery systems, chitosan is one of the most popular biopolymers mainly due to its wide biocompatibility, biodegradability, and low toxicity (Younes and Rinaudo 2015). Likewise, multiple applications of chitin, chitosan and their derivatives have been described in food packaging and preservation, exploiting the properties of these molecules in the development of antimicrobial agents (Rabea *et al.* 2003; Devlieghere, Vermeulen and Debevere 2004), food additives (Elbarbary, El-Sawy and Hegazy 2015; Wang *et al.* 2018), films and food coating (Dutta *et al.* 2009; Davidovich-Pinhas *et al.* 2014; Siripatrawan and Vitchayakitti 2016), and nutritional quality stabilizers (Petriccione *et al.* 2014). Notably, production of chitosan from natural sources such as shrimp and crab shells using optimized enzymatic processes is nowadays not only feasible but can also result in a good business model to feed the demand for high-quality chitosan from the agriculture, food, pharmaceutical, and biomedical sectors (see e.g., https://chitolytic.com/). We provide an overview of the properties of chitinases, COS and chitosan and their applicability to these sectors as supplementary material to this article (**Supplementary data**, **File S2.1, Tables S2.1 and S2.2**).

**Table 2.2** Properties of chitinases and chitin derivatives of biotechnological relevance in pharmacy and biomedicine.

| Compound | Problem | Relevance | Reference |
|---|---|---|---|
| Chitosan-based gastro-retentive controlled release system | Search for more effective drug delivery systems | Potential drug transporter of the beta-blocker carvedilol | (Praveen *et al.* 2017) |
| Chitosan oligosaccharide "GO2KA1" | Lack of understanding of the impact of chitosan on gut physiology | Potential to prevent hyperglycemia by inhibiting intestinal digestion and transport of glucose and by enhancing adipocyte differentiation | (Li *et al.* 2014) |
| Chitosan | Search for low toxicity compounds to improve anthropometric parameters | Body weight reduction along with improvements in body composition and anthropometric parameters (clinical study) | (Trivedi *et al.* 2016) |
| Oral administration of low-molecular-weight chitosan and its N-acetyl degradation products | Search for more effective drug delivery systems to sarcoma tumor cells | Inhibition of the growth of sarcoma 180 tumor cells and decrease of tumor weight (in mice) | (Qin *et al.* 2002) |
| Chitinases and Chitosanases | Search for more effective antitumor products | Hydrolysis of chitosan into low molecular weight products to be used in antitumor research | (Vinsova and Vavrikova 2008) |
| Water-soluble chitosan hydrolysates from *Bacillus amyloliquefaciens* strain V656 | Search for more effective antitumor products | Inhibition of tumor CT26 cells growth by fragmenting their DNA and inducing apoptosis | (Liang *et al.* 2007) |
| Chitin degradation products | Search for novel and biocompatible products to decrease inflammatory responses | Reduction of inflammatory response due to high biocompatibility. Reduced temperature elevation, erythrema, tissue injuries and abscess formation | (Minami *et al.* 1998; Li *et al.* 2014; Azuma *et al.* 2015) |
| Chitosan and its derivatives N,N,N-trimethyl-chitosan (TMC), N,O-carboxymethyl-chitosan (CMC), O-carboxymethyl-N,N,N-trimethyl-chitosan (CMTMC) and 5-methyl pyrrolidinone chitosan (MPC) | Search for novel biocompatible wound-healing materials | Potential to be used as biomaterials for wound healing (as wound dressings) | (Moura *et al.* 2014; Patrulea *et al.* 2015; Archana, *et al.* 2016) |
| Chitosan hydrogel | Search for novel biocompatible wound-healing materials | Induction of wound contraction and accelerated wound healing and closure by epithelialization and advanced granulation tissue formation | (Ishihara *et al.* 2002) |
| Copper-chitosan scaffolds implants | Search for low toxicity scaffolds for tissue regeneration | Significant improvement of bone regeneration (mice) | (D'Mello *et al.* 2015) |

## 2.7 Conclusions and outlook

Chitin, chitin derivatives and chitinases show great biotechnological potential, with many applications in several different fields and industries being developed or already in place. Technological developments have led to the optimization of well-described industrial chitin degradation processes as well as to the description of new classes of chitosanolytic enzymes (Kashyap and Deswal 2017) or even the development of new applications for already known enzymes and their degradation by-products (Salomon *et al.* 2017).

Much of our current knowledge on chitinolytic microorganisms and chitin degradation pathways resulted from studies of culturable microorganisms. Recent cultivation-independent approaches are unveiling a wealth of putatively novel, thus-far uncultivated chitinolytic/chitinoclastic bacteria whose metabolism and physiology are yet to be unveiled, with chitin-degrading communities shifting in composition and activity according to the microhabitats they live in and the environmental / biological triggers they are exposed to. Better understanding of their roles in foundational symbioses such as the plant-, coral- and fish-microbiomes will be instrumental to harness unique chitin metabolism – either through advanced microbial culturing practices or metabolic engineering - for improved host and ecosystem health. Improving our knowledge of novel chitin-degrading metabolism from less-studied and so-far unculturable microorganisms, including hyperthermophilic archaea, bears promise in enlarging the breadth of efficient and sustainable bio-based methods for chitin breakdown leading to the biosynthesis of added value products such as COS and chitosan with applicable properties for a variety of industrial sectors.

### 2.8 Supplementary information

High resolution figures and supplementary information pertaining to this chapter can be found online at:

https://drive.google.com/drive/folders/16MraFJqb0KiDAtLFMuDTx7FT-xpTPD0w?usp=sharing

**Additional File S2.1** Applications of chitinases, chito-oligosaccharides and chitosan in pharmacy, biomedicine, and food packaging and preservation. **Table S2.1** – Examples of patents registered to exploit the biotechnological applications of chitin and chitin derivatives. **Table S2.2** Present-day start-ups or spinoffs that explore the properties of chitinases, chitin and chitin derivatives in the development of biotechnological appliances.

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**Conflicts of interest.** None declared.

# Chapter 3

## Functional metagenomics reveals differential chitin degradation and utilization features across free-living and host-associated marine microbiomes

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**Functional metagenomics reveals differential chitin degradation and utilization features across free-living and host-associated marine microbiomes**

**Running title:** Chitin-degrading microbiomes in marine habitats.

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**Keywords:** chitinases, chitosan, metagenomics, nitrogen cycling, carbon cycling, marine sponges, octocorals, host-microbe interactions

## 3.1 Abstract

**Background:** Chitin ranks as the most abundant polysaccharide in the oceans yet knowledge of shifts in structure and diversity of chitin-degrading communities across marine niches is scarce. Here, we integrate cultivation-dependent and -independent approaches to shed light on the chitin processing potential within the microbiomes of marine sponges, octocorals, sediments and seawater.

**Results:** We found that cultivatable host-associated bacteria in the genera *Aquimarina, Enterovibrio, Microbulbifer*, *Pseudoalteromonas*, *Shewanella* and *Vibrio* were able to degrade colloidal chitin *in vitro*. Congruent with enzymatic activity bioassays, genome-wide inspection of cultivated symbionts revealed that *Vibrio* and *Aquimarina* species, particularly, possess several endo- and exo-chitinase-encoding genes underlying their ability to cleave the large chitin polymer into oligomers and dimers. Conversely, *Alphaproteobacteria* species were found to specialize in the utilization of the chitin monomer N-acetylglucosamine more often. Phylogenetic assessments uncovered a high degree of within-genome diversification of multiple, full-length endo-chitinase genes for *Aquimarina* and *Vibrio* strains, suggestive of a versatile chitin catabolism aptitude. We then analysed the abundance distributions of chitin metabolism-related genes across 30 Illumina-sequenced microbial metagenomes and found that the endosymbiotic consortium of *Spongia officinalis* is enriched in polysaccharide deacetylases, suggesting the ability of the marine sponge microbiome to convert chitin into its deacetylated – and biotechnologically versatile - form chitosan. Instead, the abundance of endo-chitinase and chitin-binding protein encoding genes in healthy octocorals levelled up with those from the surrounding environment but was found to be depleted in necrotic octocoral tissue. Using cultivation-independent, taxonomic assignments of endo-chitinase encoding genes, we unveiled previously unsuspected richness and divergent structures of chitinolytic

communities across host-associated and free-living biotopes, revealing putative roles for uncultivated *Gammaproteobacteria* and *Chloroflexi* symbionts in chitin processing within sessile marine invertebrates.

**Conclusions:** Our findings suggest that differential chitin degradation pathways, utilization and turnover dictate the processing of chitin across marine micro-niches and support the hypothesis that inter-species cross-feeding could facilitate the co-existence of chitin utilizers within marine invertebrate microbiomes. We further identified chitin metabolism functions which may serve as indicators of microbiome integrity/dysbiosis in corals and reveal putative novel chitinolytic enzymes in the genus *Aquimarina* that may find applications in the blue biotechnology sector.

### 3.2 Background

Chitin, the polymer of (1→4)-β-linked N-acetylglucosamine (GlcNAc), is the most abundant polysaccharide in the marine environment (Paulsen *et al.* 2016). Chitin does not accumulate in marine habitats as it is hydrolyzed by microorganisms that can use it as a carbon, nitrogen and/or energy source (Beier and Bertilsson 2013). This process is often mediated by chitinolytic enzymes, named chitinases, that hydrolyze the β-1,4 glycosidic bonds between the GlcNAc residues, producing chito-oligosaccharides (COS). There are two types of chitinases: endo-chitinases (EC 3.2.1.14), that cleave chitin randomly at internal sites, generating diverse oligomers of GlcNAc such as chitotriose and chitotetraose; and exo-chitinases (EC 3.2.1.52), that can be further divided into two subtypes: chitobiosidases, which catalyse the progressive release of chitobiose, starting at the non-reducing end of the chitin microfibril; and N-acetyl-β-glucosaminidases, or chitobiases, which cleave the oligomeric products of endo-chitinases and chitobiosidases, generating monomers of GlcNAc (Cohen-Kupiec and Chet 1998). Endo-chitinases are

classified into two glycoside hydrolase families, GH18 and GH19, based on amino acid sequence homology (Karlsson and Stenlid 2009). They are commonly extracellular enzymes while the exo-chitinase N-acetyl-β-glucosaminidase frequently acts inside the bacterial cell (Beier and Bertilsson 2013). The chitin derivative chitosan, formed via deacetylation, can be as well partially hydrolysed by endo-chitinases if acetylated units remain in the polymer. Both endo- and exo-chitinases and their products have properties that bear promise for the development of new appliances in the food, medical and agricultural sectors (Hudson S. 1997; Fukamizo 2000; Liang *et al.* 2007; Ngo, Kim and Kim 2008; Madhumathi *et al.* 2010; Patrulea *et al.* 2015).

Despite our awareness of the relevance of chitin degradation to biogeochemical cycling across marine (Beier and Bertilsson 2013), freshwater (Wörner and Pester 2019) and land (Wieczorek, Hetz and Kolb 2014; Wieczorek *et al.* 2019) ecosystems, current understanding of the abundance, diversity, and composition of chitin-degrading microorganisms across distinct biotopes is scarce. For marine biomes, particularly, we lack accurate documentation of how chitinolytic microbial communities - and prevailing chitin degradation pathways - may shift across environmental gradients and host-associated versus free-living settings, limiting our ability to envision and model patterns of nitrogen and carbon cycling in the oceans.

Given their remarkable filter- and detritus-feeding activities and complex microbiomes, it is tempting to hypothesize that sessile marine invertebrates host microbial symbionts which either degrade chitin or utilize its degradation products. Indeed, detectable levels of exo-chitinase activity were found in crude extracts of the octocoral *Gorgonia ventalina* (Douglas *et al.* 2007). Further, Yoshioka and colleagues identified two chitinase-like genes in the genome of the scleratinian coral *Acropora digitifera* and reported chitinolytic activity in seven coral species (Yoshioka, Tanabe and Iguchi 2017).

Therefore, chitinases may be widely distributed in the coral holobiont and could play a role in the animals' immune response against fungal infections as suggested elsewhere (van de Water, Allemand and Ferrier-Pagès 2018). *Streptomyces* sp. strain DA11, retrieved from the marine sponge *Craniella australiensis*, was found to produce antifungal chitinases (Han *et al.* 2009). Recently, chitinase-encoding genes have been identified in *Aquimarina* strains from marine sponges, corals, sediments and seawater (Keller-Costa *et al.* 2016; Silva *et al.* 2019). In addition, it is known that the remarkable chitin-degradation capacity of well-studied taxa such as *Vibrio* species is a key factor underlying their global patterns of distribution in the oceans (Mansergh and Zehr 2014) and, eventually, a generalist behaviour across free-living and host-associated habitats. Taken together, these trends support the contention that the microbiomes of sessile marine invertebrates may contribute to ecosystem functioning by serving as natural settings for chitin / COS degradation. However, this hypothesis remains largely underexplored despite the importance of chitin breakdown for carbon and nitrogen fluxes in the marine realm.

In this study, we integrate cultivation-dependent and -independent analyses to shed light on the potential degradation and utilization of chitin and its derivatives by the microbiomes of marine sponges, octocorals, sediments and seawater (hereafter designated "biotopes") and to determine whether chitin degrading assemblages within these microbiomes are taxonomically and metabolically distinct.

## 3.3 Results

### 3.3.1 Chitin degradation assays

Of the 41 marine sponge and octocoral bacterial associates tested in this study, 24 were found to degrade colloidal chitin on agar plates (**Table 3.1**). Among these, 12 were

isolated from marine sponges and 12 from octocorals, and high reproducibility was recorded among replicates (n = 8 per strain). Results were highly dependent on bacterial taxonomy instead of host origin. All *Aquimarina* strains (n = 6) (phylum *Bacteroidetes*, class *Flavobacteria*), and nearly all strains in the class *Gammaproteobacteria* – including all *Enterovibrio* (n = 2) and *Vibrio* (n = 13) strains - were able to degrade colloidal chitin regardless of their origin (**Table 3.1**). In contrast, none of the *Alphaproteobacteria* strains, encompassing eight formally described genera and three unclassified *Rhodobacteraceae* spp., as well as *Micrococcus* sp. Mc110 (*Actinobacteria*) showed any chitin-degrading activity on the agar plates.

### 3.3.2 Chitinase activity assays

Endo-chitinase (EC 3.2.1.14) activity and colloidal chitin degradation results were overall congruent (**Table 3.1**). Endo-chitinase activity was registered for 23 of the 24 strains that degraded chitin on agar plates, whereas no endo-chitinase activity was recorded for 13 of the 17 strains unable to degrade colloidal chitin on agar plates (**Table 3.1**). These few incongruencies likely result from eventual sub-optimal experimental conditions for specific strains. All *Vibrio* and *Enterovibrio* strains displayed both endo- and exo-chitinolytic activities and the capacity to utilize all three substrates used in the enzymatic bioassays (**Table 3.1**). Endo- and exo-chitinolytic activity was also recorded for most *Aquimarina* strains (**Table 3.1**). N-acetylglucosaminidase activity was documented for five of the 12 *Alphaproteobacteria* strains tested, encompassing members of the genera *Ruegeria*, *Pseudovibrio*, *Labrenzia* and *Kiloniella* (**Table 3.1**). PCR amplification of *chiA* gene fragments – targeting "group A" within glycosyl hydrolase family 18 (GH18) endo-chitinases (see methods) was considered a good indicator of

endo-chitinolytic activity by *Vibrionaceae* and *Aquimarina* strains (**Table 3.1**). However, no *chiA* gene amplicons could be retrieved for gammaproteobacterial genera out of the *Vibrionaceae* family, namely chitin-degrading strains *Shewanella* Sw66, *Microbulbifer* Mb45 and *Pseudoalteromonas* Pa284 (**Table 3.1**).

**Table 3.1** Chitin and chitin-derivative degradation capacities of marine sponge and octocoral-derived bacterial isolates.

| **Strain** | **Identity** | **Isolation source[1]** | **Chitin degra-dation[2]** | **Chitinase activity (Units/mL)[3]** | | | ***chiA* PCR[4]** | **Genome Sequence[5]** |
|---|---|---|---|---|---|---|---|---|
| | | | | **EndoS3** | **ExoS1** | **ExoS2** | | |
| **Mc110** | *Micrococcus* sp. | *Sarcotragus spinosulus* | - | - | - | - | - | **no** |
| **EL33** | *Aquimarina* sp. | *Eunicella labiata* | ++ | + | ++ | - | + | FLRG00000000.1 |
| **EL43** | *Aquimarina* sp. | *Eunicella labiata* | ++ | + | ++ | - | + | **no** |
| **Aq349** | *Aquimarina* sp. | *Sarcotragus spinosulus* | ++ | + | + | - | + | OMKB00000000.1 |
| **Aq78** | *Aquimarina* sp. | *Sarcotragus spinosulus* | ++ | + | + | - | + | OMKA00000000.1 |
| **Aq135** | *Aquimarina* sp. | *Ircinia variabilis* | + | + | - | + | + | OMKE00000000.1 |
| **Aq107** | *Aquimarina* sp. | *Sarcotragus spinosulus* | + | - | - | - | + | OMKC00000000.1 |
| **EL57** | *Aliivibrio* sp. | *Eunicella labiata* | - | + | + | - | + | - |
| **EL58** | *Aliivibrio* sp. | *Eunicella labiata* | - | ++ | + | - | - | OMPC00000000.1 |
| **EL24** | *Enterovibrio* sp. | *Eunicella labiata* | ++ | + | + | + | + | **no** |
| **EL37** | *Enterovibrio* sp. | *Eunicella labiata* | ++ | + | + | + | + | **no** |
| **EL22** | *Vibrio* sp. | *Eunicella labiata* | ++ | ++ | + | + | + | **no** |
| **EL36** | *Vibrio* sp. | *Eunicella labiata* | + | + | + | + | + | **no** |
| **EL38** | *Vibrio* sp. | *Eunicella labiata* | ++ | ++ | + | + | + | **no** |
| **EL41** | *Vibrio* sp. | *Eunicella labiata* | ++ | + | + | + | - | **no** |
| **EL49** | *Vibrio* sp. | *Eunicella labiata* | ++ | + | + | + | + | **no** |
| **EL62** | *Vibrio* sp. | *Eunicella labiata* | + | + | + | + | + | **no** |
| **EL67** | *Vibrio* sp. | *Eunicella labiata* | + | ++ | + | + | + | **no** |
| **EL112** | *Vibrio* sp. | *Eunicella labiata* | ++ | + | + | + | + | **no** |
| **Vb255** | *Vibrio* sp. | *Sarcotragus spinosulus* | ++ | + | ++ | + | + | **yes[5]** |
| **Vb258** | *Vibrio* sp. | *Sarcotragus spinosulus* | ++ | + | ++ | + | + | **yes[6]** |
| **Vb341** | *Vibrio* sp. | *Sarcotragus spinosulus* | + | + | + | + | - | **no** |
| **Vb339** | *Vibrio* sp. | *Sarcotragus spinosulus* | + | + | + | + | + | GCA_902751245.1 |
| **Vb278** | *Vibrio* sp. | *Sarcotragus spinosulus* | ++ | ++ | + | + | + | CVNE00000000.1 |
| **Cw315** | *Colwellia* sp. | *Sarcotragus spinosulus* | - | - | + | - | - | **no** |
| **Pa284** | *Pseudoalteromonas* sp. | *Sarcotragus spinosulus* | + | + | ++ | - | - | **no** |
| **EL12** | *Shewanella* sp. | *Eunicella labiata* | - | + | ++ | + | - | **no** |
| **Sw66** | *Shewanella* sp. | *Sarcotragus spinosulus* | + | + | + | ++ | - | **no** |
| **Mb45** | *Microbulbifer* sp. | *Sarcotragus spinosulus* | ++ | + | + | + | - | **no** |

| Strain | Identity | Isolation source[1] | Chitin degra-dation[2] | Chitinase activity (Units/mL)[3] | | | *chiA* PCR[4] | Genome Sequence[5] |
|---|---|---|---|---|---|---|---|---|
| | | | | EndoS3 | ExoS1 | ExoS2 | | |
| **EL27** | *Pseudophaeobacter* sp. | *Eunicella labiata* | - | - | - | - | - | OMPQ00000000.1 |
| **EL26** | *Roseovarius* sp. | *Eunicella labiata* | - | - | - | - | - | OUMZ00000000.1 |
| **EL01** | *Ruegeria* sp. | *Eunicella labiata* | - | - | - | - | - | OMPS00000000.1 |
| **Rg50** | *Ruegeria* sp. | *Sarcotragus spinosulus* | - | - | + | - | - | **no** |
| **EL44** | *Sulfitobacter* sp. | *Eunicella labiata* | - | - | - | - | - | OMPT00000000.1 |
| **EL53** | uncl. *Rhodobacteraceae* | *Eunicella labiata* | - | - | + | - | - | OMPR00000000.1 |
| **EL129** | uncl. *Rhodobacteraceae* | *Eunicella labiata* | - | - | - | - | - | ONZJ00000000.1 |
| **Ph222** | uncl. *Rhodobacteraceae* | *Ircinia variabilis* | - | - | - | - | - | **no** |
| **EL143** | *Labrenzia alba* | *Eunicella labiata* | - | + | + | - | - | OGUZ00000000.1 |
| **Pv125** | *Pseudovibrio* sp. | *Sarcotragus spinosulus* | - | - | ++ | - | - | **no** |
| **EL199** | *Kiloniella* sp. | *Eunicella labiata* | - | - | + | + | - | OMPU00000000.1 |
| **EL138** | *Sphingorhabdus* sp. | *Eunicella labiata* | - | - | - | - | - | OGVD00000000.1 |

[1]*Eunicella labiata*: octocoral; *Sarcotragus spinosulus* and *Ircinia variabilis*: marine sponges. [2]Chitin degradation observed as halo-formation on colloidal chitin-containing agar plates: ++ halo-radius ≥ 8mm, + halo-radius ≥ 1mm and < 8mm. [3]Chitinase activity measured in Units/ml on bacterial culture supernatants (EndoS3 = endo-chitinase activity) or cell extracts (ExoS1 = exo-chitinase β-N-acetylglucosaminidase activity; ExoS2 = exo-chitinase chitobiosidase activity): ++ ≥ 1 U/mL, + ≥ 0.01 and < 1 U/mL. [4]PCR-based amplification of the *chiA* gene fragments. [5]Accession numbers are provided for genome sequences available on NCBI. [5,6] Unpublished draft genomes.

### 3.3.3 Chitin metabolism encoded on the genomes of bacterial associates of marine sponges and octocorals

In general agreement with phenotypic assays, while the predicted proteomes (i.e. Pfam annotations) of *Vibrio*, *Aliivibrio* and *Aquimarina* species all possessed several endo- (EC 3.2.1.14) and exo-chitinase (EC 3.2.1.52) catalytic domains, those of *Alphaproteobacteria* species usually lacked them **(Figure 3.1**). Noteworthy was the high number of endo-chitinases of the GH18 family predicted for the three abovementioned genera, with *Vibrio* and *Aliivibrio* strains possessing types A and C GH18 chitinases, not verified among *Aquimarina* strains. Chitinases of the GH19 family were likewise found for all strains from these three genera (see also **Figure 3.2**). *Vibrio* and *Aliivibrio* species possessed the most versatile genetic machinery for the utilization of chitin and its derivatives according to Pfam-based annotations. Both genera displayed genes encoding

diverse protein domains required for chitin and chitobiose cleavage as well as N-acetylglucosamine utilization (**Figure 3.1**), including numerous chitobiose-specific transport systems and N-acetylglucosamine binding proteins not documented for *Aquimarina*. Interestingly, potential for chitin deacetylation into chitosan could be inferred for all strains (except *Aliivibrio* sp. EL58) due to the presence of polysaccharide deacetylases in their predicted proteomes (**Figure 3.1**). Likewise, using glucosamine-6-phosphate isomerase detection as proxy, Pfam annotations revealed the potential of all strains (except *Kiloniella* sp. EL199) to utilize N-acetyl glucosamine (**Figure 3.1**). Overall, the type and number of protein domains involved in the metabolism of chitin and its derivatives was found to differ in a taxon-dependent manner. While deacetylation and GlcNAc utilization potential were traits common to all genomes, chitin degradation capacities (endo- and exo-) were pronounced features of *Vibrio*, *Aliivibrio* and *Aquimarina* genomes (**Figure 3.1**). Details on all Pfam entries employed to build Figure 1 and their distributions across the examined genomes are provided as Supplementary information (Additional file 1: Table S1). In contrast with Pfam-based annotations, RAST annotations revealed potential exo-chitinase activity for three (instead of one) *Alphaproteobacteria* genomes, showing as well that *Alphaproteobacteria* spp. often carried genes involved in N-acetylglucosamine utilization and transport with specific GlcNAc ABC transporters **(Additional file S3.2, Figure S3.1).**

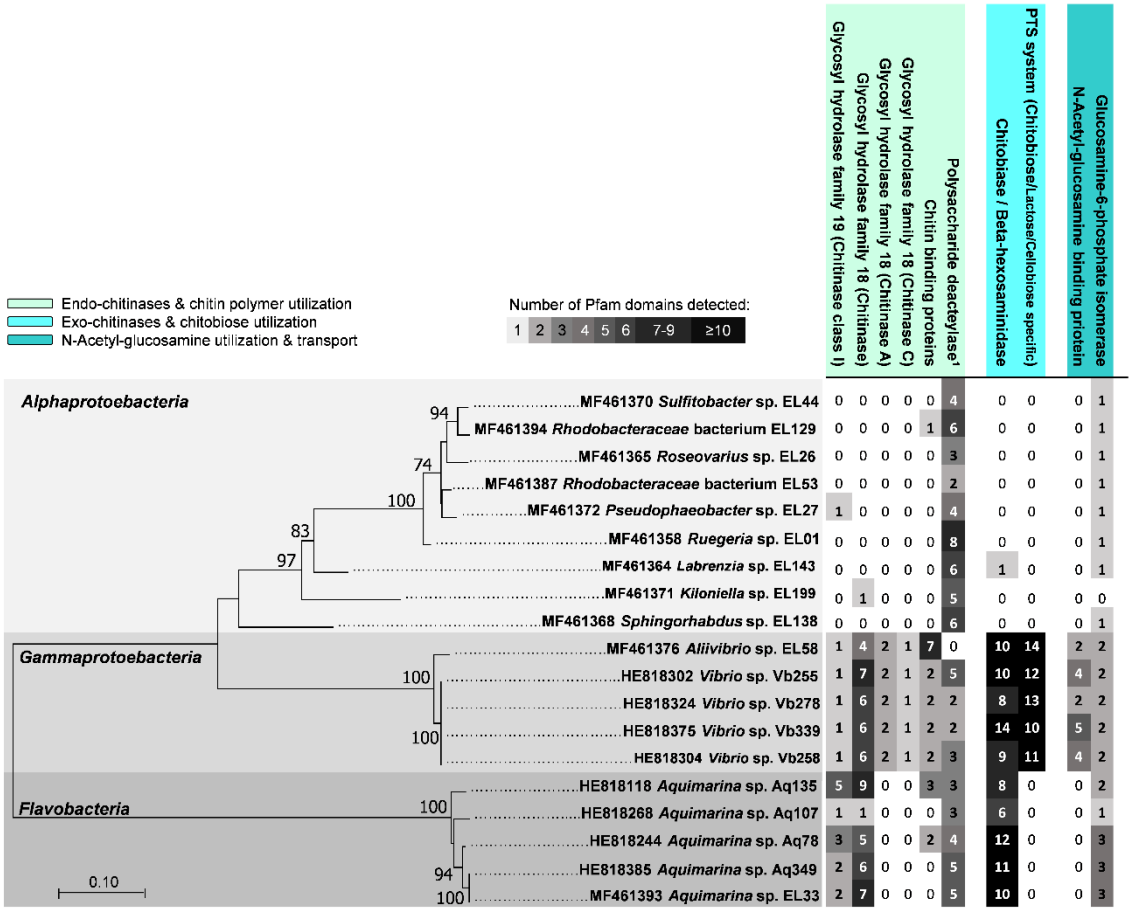


**Figure 3.1** Annotation of chitin and chitin-derivative degradation and utilization genes in cultivated bacterial symbionts of sponges and octocorals. Nineteen genomes available from the panel of 41 strains examined in this study are portrayed, spanning ten formally described and two potentially novel genera across three bacterial classes. The phylogenetic tree on the bottom left is based on a maximum likelihood analysis (Generalised Time Reversible model) of the 16S rRNA gene (16S rRNA gene accession numbers are given next to each strain name). Numbers at nodes represent values based on 300 bootstrap replicates. The table on the right shows Pfam-based annotations for predicted protein domains involved in hydrolysis (endo-chitinases of GH families 18 and 19 – EC 3.2.1.14, chitin-binding proteins) and deacetylation (polysaccharide deacetylases) of the of the large chitin polymer (light green panel), hydrolysis (exo-chitinases, EC 3.2.1.52) of chitin non-reducing ends and chitobiose transport (light blue panel), and N-acetylglucosamine binding and utilization (blue panel), the latter function represented by the presence of glucosamine 6-phosphate isomerases (EC 3.5.99.6). These functional categories have been as well used to estimate the relative abundance of CDSs involved in the breakdown and utilization of chitin and chitin derivatives across the sponge and octocoral metagenome datasets (Figure 3.3). Values in each cell of the table correspond to the number of Pfam domains detected for each functional category in each genome, whereby higher numbers are highlighted in dark-grey shading. The upper left panel shows the molecular structure of chitin and chitosan. It highlights the N-acetyl group characteristic of the chitin polymer, which is cleaved by deacetylases in the process of chitosan formation, and the cleaving sites of endo- and exo-

chitinases along the chitin chain. [1]Corresponds to InterPro database entry IPR002509 (see also Figure 3.3) which describes the metal-dependent deacetylation of O- and N- acetylated polysaccharides such as chitin, peptidoglycan and acetylxylan.

### 3.3.4 Relative abundance of culturable chitin degraders in sponge and octocoral microbiomes

In agreement with previous studies (Hardoim *et al.* 2014; Keller-Costa *et al.* 2017; Karimi *et al.* 2019), 16S rRNA gene-based estimates of relative abundance revealed that the culturable bacterial genera analyzed here correspond to a minor portion of the total microbial metagenome in sponges and octocorals (Additional file 3.1: Table S3.2). This seems particularly true for sponges where such estimates did not surpass the 0.02% threshold, while genera such as *Pseudoalteromonas*, (1.05%), *Shewanella* (0.75%), *Vibrio* (0.42%) and *Aquimarina* (0.34%), all possessing chitinolytic activity, did amount to much higher proportions in the healthy octocoral microbiome. Interestingly, several cultivated taxa displayed increased abundances in necrotic versus healthy octocoral tissue (**Additional file S3.1: Table S3.2**), of which we highlight *Aquimarina* (20-fold increase), *Vibrio* (2-fold), *Ruegeria* (6-fold) and unclassified *Rhodobacteraceae* (7-fold). These trends were corroborated by strain-specific estimates of relative abundance based on genome-metagenome mapping (**Additional file S3.1: Table S3.3**), carried out for all chitinolytic strains with sequenced genomes (**Figure 3.1**). Indeed, both estimates of percent abundance and genome coverage suggest that cultured symbionts correspond to low abundance populations usually more abundant in the octocoral than in the sponge microbiome. Further, sharp increases in genome-wide estimates of abundance were observed for *Aquimarina* and diverse alphaproteobacterial strains in necrotic versus healthy octocoral tissue (**Additional file S3.1: Table S3.3**).

### 3.3.5 Phylogenetic analysis of endo-chitinase-encoding genes

We assessed the phylogenetic diversification of genes encoding for GH18 and GH19 endo-chitinases (EC 3.2.1.14) spanning at least 16 bacterial genera across four phyla, with emphasis on coding sequences identified in the genomes investigated in this study and their closest relatives (**Figure 3.2, Additional file S3.1: Table S3.4**).

The obtained tree topology is consistent with the known heterogeneity of endo-chitinase genes, which contain regions encoding for multiple protein domains - e.g. chitin, cellulose- and carbohydrate-binding, fibronectin III and immunoglobulin domains - arranged in various modes of synteny (Karlsson and Stenlid 2009). Consequently, owing to the low sequence homology between the major clades in the tree, levels of phylogenetic relatedness inferred for such clades must be considered with much caution. Still, at a coarse level of phylogenetic resolution, these major clades (usually presenting bootstrap support > 90%) were found to split the tree into coherent taxonomic or functional categories, discriminating endo-chitinases of *Gammaproteobacteria* (dominated by *Vibrio* spp.), *Flavobacteriia* (dominated by *Aquimarina* spp.) and *Actinobacteria* origins reasonably well (**Figure 3.2**). Furthermore, the affiliation of sequences within major clades was congruent with Pfam-based annotations regarding the detection of GH18 versus GH19 chitinase families, and of group A versus group C GH18 chitinases (in this case, among about half of the *Gammaproteobacteria* sequences). Noticeably, the extent of within-genome diversification of chitinase genes was high for strains of the genera *Aquimarina* and *Vibrio*, with coding sequences from the same genome often spread across distinct clades. This was the case for endo-chitinase gene distributions from e.g. *Vibrio* sp. strains Vb255, Vb258, Vb278 and Vb339 and *Aquimarina* sp. strains Aq135, Aq78 and Aq349 and EL33. Conversely, highly homologous endo-chitinase encoding

sequences from different strains or species of the same genus were as well consistently found across the tree. For example, *Aquimarina* strains EL33, Aq78 and Aq349, which likely represent different species within the genus based on phylogenomic assessments (Silva *et al.* 2019), shared endo-chitinase-encoding genes with > 95% sequence homology (including GH19 CDSs). The same pattern was observed for *Vibrio* sp. strains Vb339, Vb255, Vb258 and Vb278 (all closely related to the species *V. crassostreae*, see e.g. Gonçalves *et al.* 2015), which possess multiple phylogenetically close chitinase coding sequences (including GH19 CDSs). Likewise, *Streptomyces rimosus*, *S. autolyticus* and *S. olivochromogenes* shared highly homologous chitinase-encoding genes (**Figure 3.2**). Some examples of disparate taxa (i.e. belonging to different classes) forming well supported clades incongruent with their expected (16S rRNA gene-based) phylogenies have been found. Pairwise levels of homology between sequences from these taxa were never close to 100%, preventing hypotheses to be made on recent horizontal gene transfer events underlying these patterns. This was the case of the chitinase-encoding genes from *Streptosporangium roseum* DSM 43021, *Streptomyces coelicolor* A3 (both *Actinobacteria*) and *Stenotrophomonas maltophilia* K279a (*Gammaproteobacteria*), which formed a solid cluster (99% bootstrap support) within the larger clade dominated by *Actinobacteria* strains (**Figure 3.2**). There were eight coding sequences from *Aquimarina* strains for which no GH18 or GH19 domains could be annotated, although they shared resemblance with endo-chitinase sequences based on homology searches (Additional file 1: Table S4). These sequences were clustered together into the same phylogenetic clade, suggesting that additional diversity of endo-chitinase domains exist within *Aquimarina* spp. which escapes detection by current Pfam-based annotation.

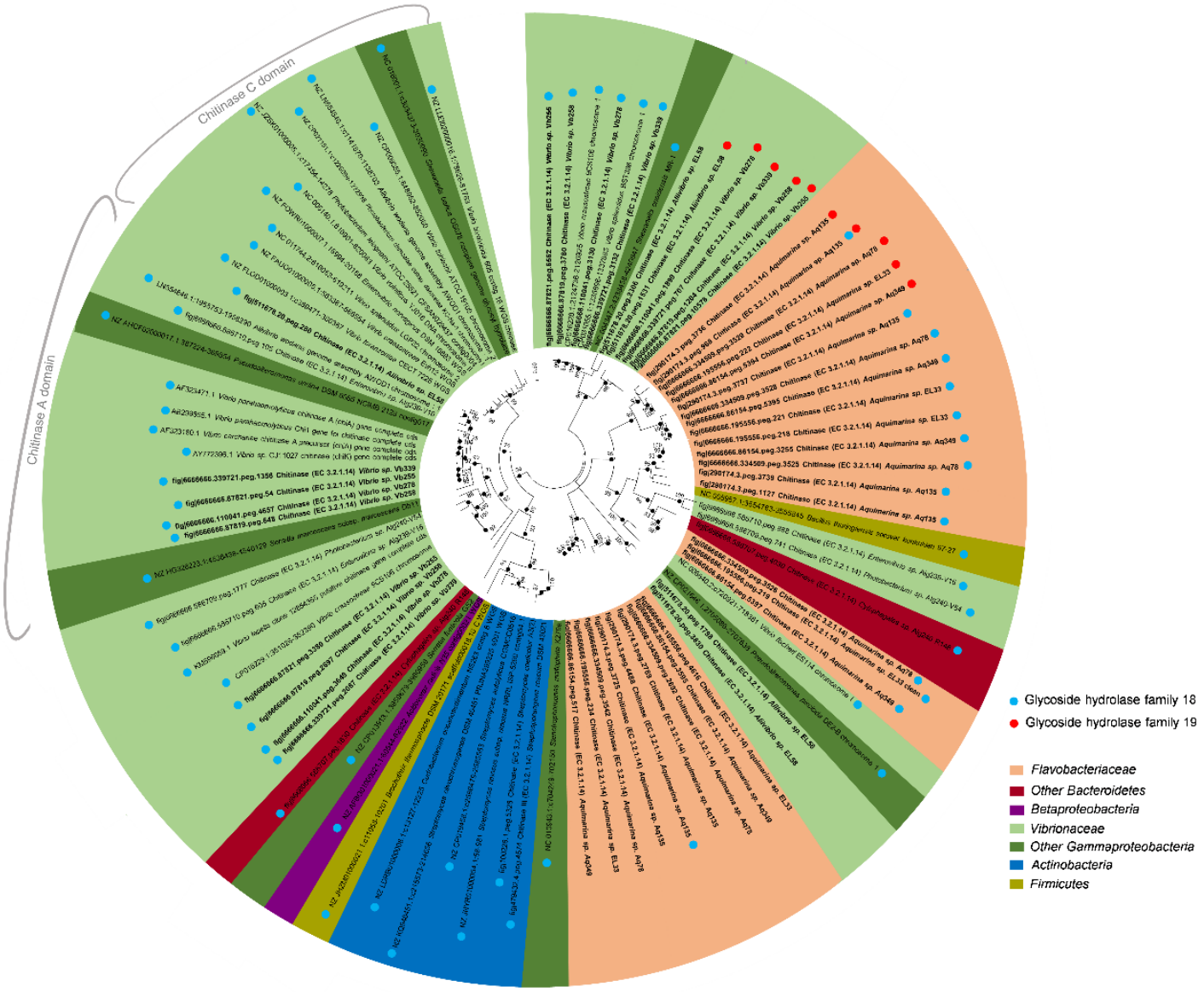
Chitinase C domain
Chitinase A domain
Glycoside hydrolase family 18
Glycoside hydrolase family 19
Flavobacteriaceae
Other Bacteroidetes
Betaproteobacteria
Vibrionaceae
Other Gammaproteobacteria
Actinobacteria
Firmicutes

**Figure 3.2** Phylogeny of full-length endo-chitinase encoding genes from cultivated bacteria. The evolutionary history was inferred using the Maximum Likelihood method based on the Generalised Time Reversible model (GTR). The tree with the highest log likelihood (-69520.19) is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 5.2345)). The tree is drawn to scale, with branch lengths measured in the number of nucleotide substitutions per site. The percentage (≥70%) of trees in which the associated taxa clustered together is shown next to the tree nodes (1000 bootstrap repetitions), with solid and open circles representing ≥ 90% and between 70 and 89% bootstrap support, respectively. Codon positions included were 1st+2nd+3rd+Noncoding, and a partial deletion model with 85% site coverage was employed in tree construction. The analysis involved 90 nucleotide sequences mostly retrieved from fully sequenced bacterial genomes (n = 85), including the 19 genomes examined in detail in this study (Figure 1, Table 1), and span 16 bacterial genera across four phyla. There were a total of 1189 positions in the final dataset. Coding sequences containing GH18 (n = 69) and/or GH19 (n = 10) domains (endo-chitinases - EC 3.2.1.14), as revealed by Pfam annotations, are highlighted with blue and red dots next to tree labels, respectively. Eleven further sequences have been included for which neither GH18 nor GH19 domains could be identified using Pfam-based annotations, but which showed significant levels of homology with endo-chitinase sequences present in NCBI's protein database. Other domains annotated within the analysed coding sequences were as well labelled with coloured dots and are identified on the right panel next to the tree. Trans-membrane and signal peptide domains could be annotated for nearly all analysed coding sequences. For further details on the distribution of protein domains across all sequences and the closest phylogenetic relative to each sequence query, see additional file S3.1: Table S3.4.

### 3.3.6 Relative abundance of chitin metabolism-encoding genes across marine biotopes

We examined whether marine sponge and octocoral microbiomes host genes involved in chitin / COS degradation and N-acetylglucosamine utilization in comparable proportions with those of the environmental surroundings. To this end, a "marine sponge metagenome" (Karimi *et al.* 2017) and an "octocoral metagenome" (Keller-Costa *et al.* 2021) dataset was used to compare, in a cultivation-independent manner, the relative abundance of genomic features involved in breakdown and utilization of chitin and its derivatives (**Figure 3.3**) and the taxonomic composition of endo-chitinase-encoding genes (**Figure 3.4**) across host-associated and free-living biotopes.

For relative abundance analysis of chitin-breakdown related features (**Figure 3.3**), we used InterPro-based annotations of unassembled metagenomic reads (Additional file S3.1: Table S3.5) retrieved with the MGnify v. 2.0 metagenomics pipeline from the European Bioinformatics Institute (EMBL-EBI) (Mitchell *et al.* 2016). For details on the general features of all metagenome samples analysed, including sequencing depth and number of CDSs with function per sample, see **Additional file S3.2**: detailed methodology. The panel of functions portrayed in **Figure 3.3** encapsulates a range of IPR entries detectable across the samples (**Additional file S3.1: Table S3.5**) which collectively serve as proxies for hydrolysis and deacetylation of the chitin polymer, hydrolysis of chitin oligomers (COS), and utilization of the chitin monomer N-acetylglucosamine, also explored in **Figure 3.1** to examine chitin metabolism traits among bacterial cultures.

While the relative abundance of endo-chitinase (EC 3.2.1.14) encoding genes was higher in sediments and seawater than in the *Spongia officinalis* endosymbiotic consortium (**Figure 3.3a**), significant differences were neither observed when the microbiomes of three octocoral species were compared with those of the environmental surroundings (**Figure 3.3f**), nor when the microbiomes of healthy and necrotic octocoral (*Eunicella gazella*) tissues were contrasted (**Figure 3.3k**). When the relative abundances of chitin-binding protein (CBP) encoding genes were considered, we found that seawater microbiomes clearly presented significantly higher proportions than sediment, sponge and octocoral microbiomes (**Figures 3.3b and 3.3g**). The relative abundance of CBP encoding genes dropped considerably in the microbiome of necrotic *E. gazella* tissues in comparison with that of their healthy counterparts yet differences were deemed not statistically significant **(Figure 3.3l**). As for exo-chitinase (EC 3.2.1.52) encoding genes, equivalent abundances were found between sponge-associated and free-living

microbiomes (**Figure 3.3c**), whereas higher relative abundances were registered for free-living microbiomes in comparison with octocoral microbiomes (**Figure 3.3h**).

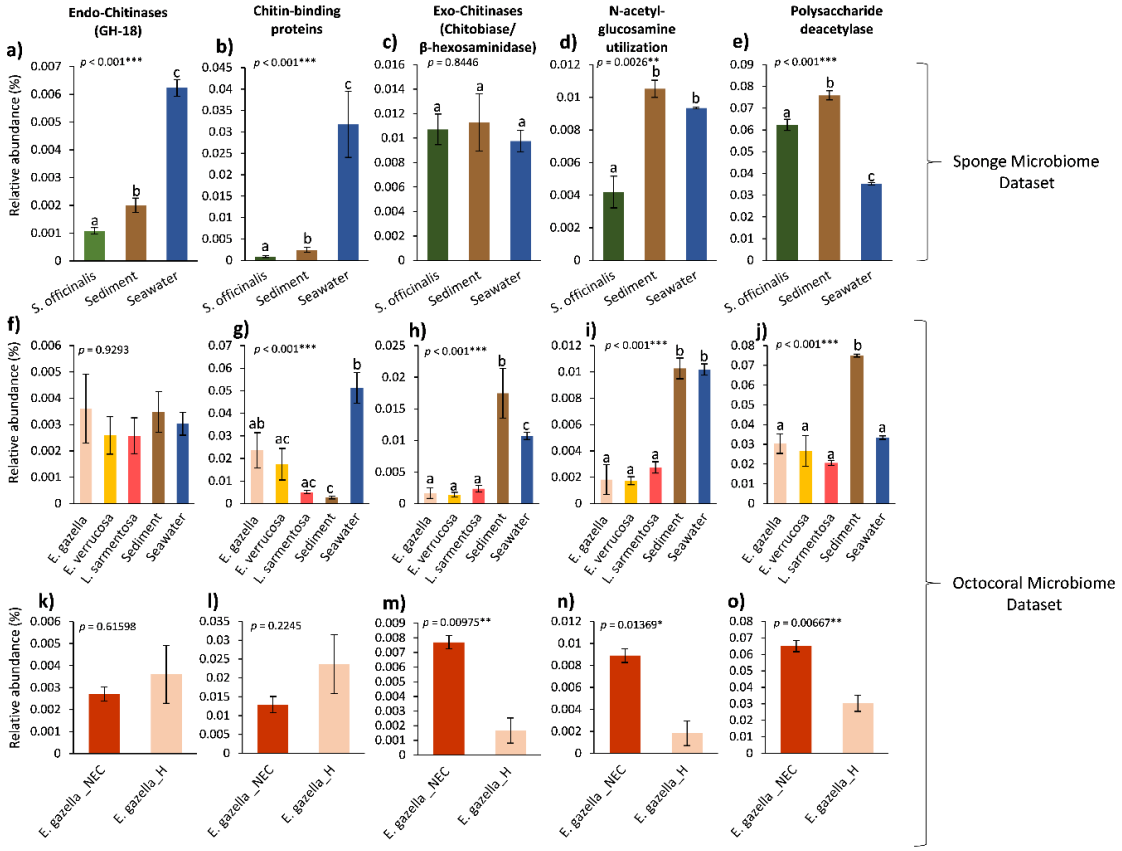


**Figure 3.3 Relative abundance of chitin processing-encoding genes across marine biotopes.** The analysis involved the screening of all microbial metagenomes for InterPro (IPR) database entries corresponding to the presence of GH18 endo-chitinase **(a, f, k)**, chitin-binding protein (**b, g, l**), exo-chitinase (**c, h, m**), N-acetylglucosamine utilization (**d, i, n**), and polysaccharide deacetylase (**e, j, o**) coding sequences (CDSs) (see Additional file 1: Table S5 for details on the InterPro entries used here). The relative abundances (mean ± SE) of IPR entries pertaining to each functional category (calculated as "sum of all CDSs assigned to functional category x / total number CDSs with function") are shown on Y-axes. One-Way ANOVAs, followed by Tukey's post-hoc tests if significant (**a** to **j**) or t-tests (**k** to **o**) were used to test for statistical differences between sample groups. Statistical analyses were performed after Hellinger transformation of the data (square root of relative abundances). Respective *p*-values are given in each panel for general differences among groups and letters (**a** to **j**) or asterisks (**k** to **o**) above error bars indicate significant differences ($p < 0.05$). Panels (**a** to **e**) represent the sponge metagenome dataset (project ID PRJEB11585, Karimi *et al.* 2017) while panels (**f** to **o**) represent the octocoral metagenome dataset (project ID PRJEB13222, Keller-Costa *et al.* 2020). Panels (**f** to **j**) compare the healthy microbiome of octocoral species with those from their environmental vicinities, while panels (**k** to **o**) present relative abundances of chitin degradation-encoding genes in healthy versus necrotic tissue of *Eunicella gazella*.

Higher relative abundance of exo-chitinase encoding genes were also recorded in the microbiomes of necrotic in comparison with healthy *E. gazella* tissue (**Figure 3.3m**). Abundance distributions of N-acetyl-glucosamine utilization genes (**Figure 3.3d, i, n**) were highly congruent with those described for exo-chitinase genes, except for the *S. officinallis* microbiome where N-acetyl-glucosamine utilization genes displayed lower abundance than in seawater and sediments (**Figure 3.3d**). Finally, the frequency distributions of polysaccharide deacetylases (which catalyse the formation of chitosan from chitin) in both the sponge and octocoral metagenome datasets **(Figure 3.3e, j, o**) followed the trends observed for the exo-chitinase (EC 3.2.1.52) encoding genes. The relative abundances of polysaccharide deacetylases were overall higher in comparison with those of endo- or exo-chitinase encoding genes in all the biotopes surveyed.

### 3.3.7 Taxonomic classification of endo-chitinase encoding genes from host-associated and free-living biotopes

Among all features involved in chitin metabolism, this study places focus on the heterogeneity and taxonomy of endo-chitinase encoding genes because of their historical use as indicators of potential chitin degradation across nature's microbiomes (Beier and Bertilsson 2013). To explore the taxonomic composition of chitin-degrading microbiomes across the biotopes studied here, we first fetched potential endo-chitinase encoding gene sequences (EC 3.2.1.14) from the samples using the MG-RAST analysis server (Meyer *et al.* 2008; Keegan, Glass and Meyer 2016) with default parameters. The retrieved sequences were thereafter curated through stringent blastx procedures for the selection of reliable entries to be used in taxonomic profiling. (**Table 3.2;** see Additional file S3.1: Table S3.6 to access each sequence read). The proportion reads identified by MG-RAST which returned chitinase-specific closest hits from bacteria after blastx searches on NCBI

varied considerably across biotopes, ranging from 22.6% in sediment samples of the sponge metagenome dataset to 79.7% in the necrotic tissues of the octocoral *E. gazella* (**Table 3.2**). Taxonomic assignments portrayed here only consider sequence reads which returned chitinase-specific closest hits assigned to the domain Bacteria using NCBI Blastx (**Figure 3.4,** Additional File S3.1, Table S3.7).

**Table 3.2.** Metagenomic reads classified as chitinase-encoding gene sequences with MG-RAST

| **Dataset** | **Sample category** | **# reads MG-RAST[1]** | **# blast[2]** (%) |
|---|---|---|---|
| Octocoral metagenome | *Eunicella gazella* (healthy) | 94 | 68 (72.3%) |
| | *Eunicella gazella* (necrotized) | 59 | 47 (79.7%) |
| | *Eunicella verrucosa* | 1,458 | 795 (54.5%) |
| | *Leptogorgia sarmentosa* | 127 | 100 (79.4%) |
| | Sediments | 401 | 124 (30.9%) |
| | Seawater | 599 | 317 (52.9%) |
| Sponge metagenome | *Spongia officinalis* | 48 | 12 (25.0%) |
| | Sediments | 455 | 103 (22.6%) |
| | Seawater | 257 | 103 (40.1%) |

[1] Number of unassembled metagenomic reads classified by MG-RAST as endo-chitinase (EC 3.1.2.14) coding sequences. [2]Proportion of chitinase reads classified by MG-RAST which were assigned to the domain Bacteria and returned chitinase-specific closest hits after blastx searches on NCBI. Figure 4 shows the taxonomic affiliation of these reads in detail.

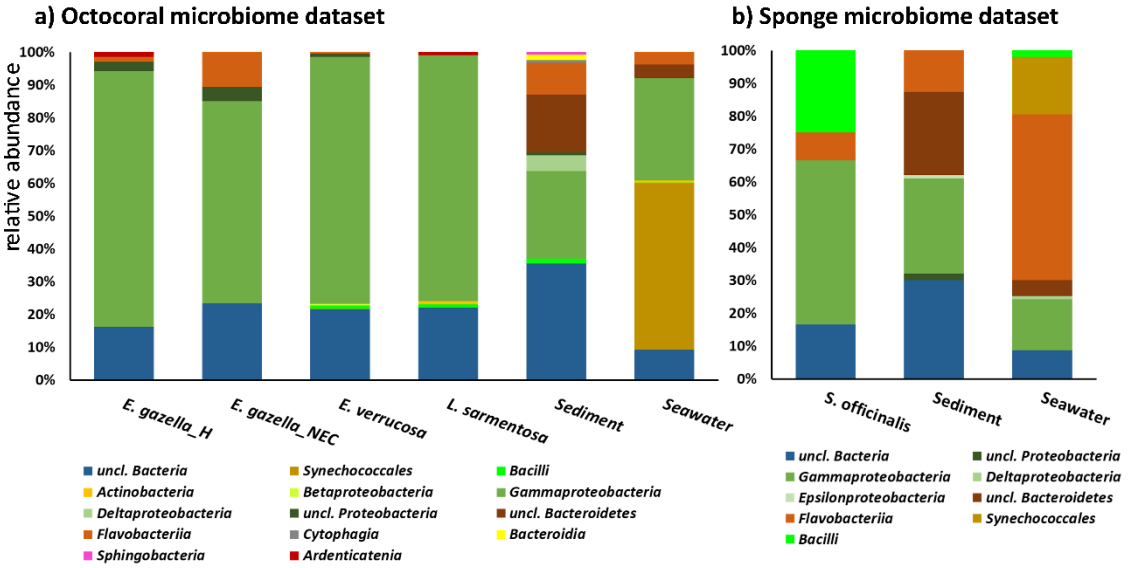


**Figure 3.4** Class-level taxonomic composition of chitinolytic microbial communities across marine biotopes. Microbial metagenome mining and assignment of chitinase-encoding genes were performed on unassembled metagenome samples of seawater, marine sediments, octocorals and marine sponges. Panels (**a** and **b)** represent the octocoral metagenome dataset (project PRJEB13222 Keller-Costa *et al.* 2020) and the sponge metagenome dataset (project PRJEB11585 Karimi *et al.* 2017), respectively. Putative endo-chitinase nucleotide sequences were retrieved from both datasets using the MG-RAST metagenomics analysis platform (Meyer *et al.* 2008) and then subjected to a stringent NCBI blastx search for taxonomic and functional assignments. Only those sequence reads which returned chitinases as closest hits and could be assigned to bacterial taxa after NCBI blastx procedures were used in taxonomic profiling. *E. gazella*_H – healthy *Eunicella gazella* tissue; *E. gazella*_NEC – necrotic *Eunicella gazella* tissue. For genus-level taxonomic profiling of sponge and octocoral samples, see Additional file S3.1: Table S3.7.

For both the octocoral and sponge metagenome datasets, changes in taxonomic composition of chitinolytic communities across host-associated and free-living biotopes were already evident at the class level (**Figure 3.4**), strongly supporting the hypothesis of divergent chitinolytic community structures in these settings. Within both datasets, sediments were found to host the highest number of bacterial classes, followed by seawater (**Figures 3.4a**, **b**). All biotopes housed considerable proportions of bacterial endo-chitinase reads not classifiable at the phylum level (from *c.* 10% in seawater to remarkable 30% in sediments), warranting further bioprospection for chitinolytic

activities / endo-chitinase diversity in these systems. Interestingly, while the seawater microbiome in the octocoral dataset (samples collected at 18m depth close to the summer solstice, 2014) was dominated by *Synechococalles*-derived chitinases, in the sponge metagenome dataset (samples collected at 20 m depth in spring 2014) the dominant reads belonged to *Flavobacteriia*. In agreement with the total taxonomic profiling of healthy octocorals (Keller-Costa *et al.* 2021) (Additional file S3.2: Figure S3.2), chitinolytic communities were found to be conserved, at the class level, across different host species (**Figure 3.4a**), presenting a remarkable dominance of *Gammaproteobacteria*. Also congruent with the total microbiome make-up of octocorals, we observed an enrichment of *Flavobacteriia*-derived chitinases, with consequent reduction in the abundance of *Gammaproteobacteria*-derived chitinases, in necrotic as compared to healthy tissues of the octocoral *E. gazella*. Further divergence between healthy versus necrotic octocoral tissue or octocoral versus sponge chitinolytic assemblages was as well identified, as expected, at lower taxonomic ranks (Additional file S3.1: TableS3.7). For instance, the healthy tissues of all octocoral species had higher proportions of chitinase sequences from unclassifiable *Gammaproteobacteria* (between 33% and 44%) than that observed for necrotic *E. gazella* tissue (20%), again reinforcing trends observed for the total microbiome of octocorals (Additional file S3.2: Figure S3.2). Further, from the pool of *Gammaproteobacteria* endo-chitinase reads that could be further classified into the order *Vibrionales*, higher proportions were found in necrotic (27%) than in healthy *E. gazella* tissues (from 6 to 15%), corroborating our estimates of *Vibrio* relative abundances in these samples (Additional file S3.1: Table S3.2). Moreover, the healthy microbiome of octocorals contained chitinase reads affiliated with other *Gammaproteobacteria* genera such as *Aliivibrio*, *Enterovibrio* and *Shewanella*, and *Pseudoalteromonas*, all of which could be isolated from healthy octocorals and were shown to possess *in-vitro* endo-

chitinase activity (**Table 3.1**). Few endo-chitinase reads belonging to the recently-described genus *Ardenticatena* (class *Ardenticatenia*) (Kawaichi *et al.* 2013) were found in healthy octocorals (Figure 3.4a, Additional file S3.1: Table S3.7). *Gammaproteobacteria* chitinase reads from the sponge microbial metagenome which were classifiable at the genus level affiliated with the genera *Grimontia, Vibrio* and *Microbulbifer*, the latter two also representing chitin-degrading taxa that we could cultivate from marine sponges (**Table 3.1**).

## 3.4 Discussion

Chitin degradation is a keystone process in the oceans. Yet our knowledge of the prevailing microorganisms and metabolic pathways mediating the breakdown of chitin and its derivatives across the highly heterogeneous marine environment is scant. Beier *et al.* (2011) revealed that the structure of endo-chitinase encoding genes within aquatic microbiomes responded significantly to salinity gradients, suggesting that chitinolytic processes, although ubiquitous, are influenced by abiotic factors. In this study, we integrated cultivation-independent and -dependent techniques to approach the ecology of chitin and COS degradation within the microbiomes of foundational sessile marine invertebrates, placing emphasis on the organisms, genes, and enzymes involved in these processes and addressing the hypotheses of divergent chitin catabolism pathways and chitinolytic communities across host-associated and free-living marine biotopes.

### 3.4.1 Chitin degradation capacities revealed for cultivatable symbionts of sponges and octocorals

The observation made in this study that a range of culturable octocoral and marine sponge-associated bacteria possess chitin/COS-degrading abilities allows hypotheses to

be raised on the relevance of chitin/COS breakdown within complex marine symbioses and their role in C and N cycling in marine ecosystems. Such hypotheses are particularly intriguing given the enormous filter- and suspension-feeding capacities of marine sponges and corals, which may lead to high turnover rates of dissolved and particulate organic matter (Gili and Coma 1998). Caution is needed when drawing conclusions on microbiome-driven processes and functions solely based on the activity of cultivated microorganisms. Most of the dominant bacterial symbionts of marine sponges are recalcitrant to cultivation in the laboratory, and cultured representatives have been previously suggested to belong to the "rare biosphere" within these systems (Hardoim *et al.* 2014; Karimi *et al.* 2017). Conversely, higher cultivability has been observed for octocoral-associated bacterial communities as several moderately abundant / dominant bacterial associates of *Eunicella labiata*, except for the canonical coral symbionts belonging to the family *Endozoicomonadaceae* (*Gammaproteobacteria*), could be recently retrieved in culture (Keller-Costa *et al.* 2017). Estimates of relative abundance of our chitin/COS degrading isolates corroborate the trends above, suggesting that, collectively, they may represent a minority portion of the total microbiomes of sponges and octocorals, being particularly less abundant in the former host. For details on chitin degradation by the bacterial taxa analysed in this study, including a thorough assessment of genomics traits involved in chitin metabolism among *Aquimarina* species, see Additional file S3.2: extended discussion.

### 3.4.2 Evidence of substrate cross-feeding among chitin degraders and utilizers in host-associated microbiomes

In highly diverse and complex microbiomes, the release of hydrolysis products by extracellular enzymes can trigger several modes of inter-specific cross-feeding (reviewed

recently by Smith *et al.*, 2019). One such mode, referred to as "substrate cross-feeding", reflects the utilization, by one given organism, of substrates or molecules produced by the metabolism of another organism, being that either organism can still metabolise these products (Smith *et al.* 2019). Regarding chitin degradation, the establishment of interspecific substrate cross-feeding cascades has been considered plausible (Beier and Bertilsson 2013) since some bacteria that grow on GlcNAc (Kaneko and Colwell 1977) or $(GlcNAc)_2$ (Keyhani and Roseman 1997) do not possess enzymes for chitinolytic activity, such as most of the sponge- and octocoral-associated *Alphaproteobacteria* cultivated in this study. Thus, a potential coupling between *Gammaproteobacteria* and *Aquimarina* spp. (or *Flavobacteriia* in general) with *Alphaproteobacteria* in the cycling of chitin could be envisioned where the former two are catabolizing the polymer, while the latter benefit from excess hydrolysis products to further process COS and use GlcNAc residues. The balance between chitin "degraders" and "consumers" has been suggested to influence the chitin destination in a given setting, whereby the former may use GlcNac to produce energy and the latter to build their cell wall (Beier and Bertilsson 2013). Diverse and abundant lineages within these three major bacterial classes are indeed present in marine sponge and octocoral microbiomes (Webster and Taylor 2012; Hardoim *et al.* 2014; Keller-Costa *et al.* 2017; van de Water, Allemand and Ferrier-Pagès 2018) and potential substrate cross-feeding mechanisms between them could lead to continuous turnover of in/ad-host organic carbon and nitrogen, hence affecting the functioning of the microbiomes they belong to and the surrounding ecosystem.

Altogether, potential for chitin and COS degradation, along with utilization of GlcNac derived from these processes, could be identified for diverse, culturable symbionts of these animals. These organisms possess a generalist pattern of occurrence across sediments, seawater, and invertebrate hosts (Esteves *et al.* 2013; Karimi *et al.* 2019; Silva

*et al.* 2019). The diversity in domain architecture and sequence of their endo-chitinases likely equip them with a versatile metabolism fine-tuned to process varied, eventually biotope-dependent forms of chitin microfibrils. Their usually low abundance in marine invertebrates suggests participation of "rare biosphere", transient symbionts in chitin metabolism within these microbiomes, opening questions regarding the chitin-degrading capacities of, and potential substrate cross-feeding among, the pool of more dominant and obligate symbionts which remain uncultured (see below). Previous studies unveiled incongruent *chiA* and 16S rRNA gene tree topologies, suggesting that chitinase-encoding genes are subjected to horizontal gene transfer and duplication events which make them less suitable as phylogenetic markers (Cottrell *et al.* 2000; Hunt *et al.* 2008). Our assessment of full-length endo-chitinase genes is in overall agreement with this perspective, and we provide context to their potential spread within marine invertebrate microbiomes in Additional file S3.2: Extended discussion.

### 3.4.3 Chitin-degrading microbiomes are ubiquitous but possess divergent structures across marine biotopes

Shotgun metagenome sequencing and analyses of unassembled reads from seawater, sediments, octocorals and marine sponges confirmed the presence of endo-chitinases, exo-chitinases and polysaccharide deacetylases in all these microbiomes, favouring the notion of chitin degradation as a ubiquitous process in the oceans (Souza *et al.* 2011). We also found evidence for distinct chitin-degrading microbiomes across biotopes, due to differences in both the proportions of key chitin metabolism traits and composition of chitinolytic taxa identified across host-associated and free-living microbiomes.

Endo-chitinase (EC 3.2.1.14) and chitin-binding protein-encoding genes were clearly less abundant in the sponge associated microbiome as compared to surrounding

environments. Yet, exo-chitinase (EC 3.2.1.52) and polysaccharide deacetylase-encoding genes were of similar or even higher abundance in *S. officinalis* in comparison with seawater and sediment, suggesting that inside the sponge mesohyl, the processing of small oligomers and GlcNac prevails, together with the transformation of chitin to chitosan, rather than the hydrolysis of chitin polymers. This seems to be in contrast with the dynamics predicted for octocorals, where we found equivalent relative abundances of endo-chitinase encoding genes in the microbiomes of healthy octocoral tissue, sediment, and seawater. Further, although the proportion of chitin-binding protein CDSs in healthy octocoral tissue was often lower than that of seawater, in *E. gazella* and *E. verrucosa* specimens they represent about 20-fold increase in comparison with the proportions registered for the sponge microbiome. Conversely, increased abundances of genes involved in the processing of small oligomers, GlcNac utilization, and in the deacetylation of polysaccharides were registered for free-living biotopes and necrotic octocoral tissue compared with healthy octocoral samples.

It is important to note that, in the octocoral metagenome dataset, both the epi- and endo- symbiotic consortium were sampled from octocoral tissue, whereas in the sponge metagenome dataset, only the endo-symbiotic consortium was sampled. Therefore, it cannot be ruled out that the epibiotic microbiome on octocorals and marine sponges may be more fit to hydrolyze the large chitin polymer whereas deep inside the animal tissue, the processing of oligomers is favored. Otherwise, the healthy octocoral microbiome could indeed possess a higher chitin-degrading efficiency, as evidenced by their higher proportions of endo-chitinase and chitin-binding protein-encoding genes. The latter are known to enhance the cells' binding capacity to chitin substrates, enabling more efficient chitin degradation to occur. An explanation for this observation may be based on differential dietary preferences of sponges versus octocorals. Octocorals are suspension-

feeders that capture organic detrital particles, phyto- and zooplankton, including diatoms, protists, and small crustaceans and their larvae (Ribes, Coma and Rossi 2003; van de Water, Allemand and Ferrier-Pagès 2018), which are naturally rich in chitin (Souza *et al.* 2011; Wysokowski *et al.* 2015). A microbial community well-adapted to chitinous food processing and chitin polymer hydrolysis could therefore be beneficial for the octocoral holobiont. The same may be less relevant within the marine sponge mesohyl where the processing of bacterioplankton and dissolved or small particulate organic matter prevails (Yahel *et al.* 2003; Hadas, Shpigel and Ilan 2009). Moreover, it is known that glass sponges (Hexactinellida) and multiple keratose sponge species (Demospongia, Dictyoceratida; the group to which *S. officinalis* belongs) contain endogenous chitin as a structural component in their skeletons (Ehrlich *et al.* 2007b; Wysokowski *et al.* 2015). The presence of a highly active chitinolytic endosymbiotic microbiome may thus be less favoured in the inner sponge mesohyl as it could compromise sponge health and growth (if the sponge structures would become too much of a food source). Future, dedicated analyses of epibiotic microbiomes will be fundamental for a more comprehensive understanding of the chitin and COS degradation potential of the marine sponge holobiont.

In agreement with the notion that chitinolytic communities make-up a small fraction of the total microbiome (Beier and Bertilsson 2013) and with abundance estimates shown in this study for cultivated, chitinolytic bacterial symbionts, relative abundance values for CDSs involved endo- and exo-chitinase activities were considerably low. This outcome also reflects the inherent nature of shotgun sequencing approaches whereby primary metabolism genes common to all community members dominate the data. Despite this limitation, the shotgun, primer-less strategy employed here enabled sufficient data retrieval for the comparative analysis of key functions involved in chitin metabolism.

Although we addressed only the taxonomy of endo-chitinase encoding genes in this study (see below), the exploration of the total chitin-degrading assemblage, through the simultaneous inspection of deacetylase and exo-chitinase encoding genes, holds potential in further revealing the diversity and potential interactive networks mediating the process of chitin across marine settings.

Cottrell *et al.* had previously suggested that the taxonomic composition of cultivated and uncultivated chitin degrading microbiomes from the seawater surface is similar (Cottrell, Moore and Kirchman 1999; Cottrell *et al.* 2000). Our results are in partial agreement with this perspective, since most endo-chitinase metagenomic reads identified in this study affiliate with bacterial taxa well represented by our panel of culturable symbionts. However, the primer-less, cultivation-independent approach employed here also revealed a considerable portion of classifiable and unclassifiable bacterial chitinase reads across all host-associated and free-living biotopes, representative of bacterial clades which apparently evade current cultivation attempts. The most remarkable example of this outcome is the affiliation of most octocoral-derived endo-chitinase reads with unclassified, uncultivated *Gammaproteobacteria* (**Figure 3.4**, see Additional file S3.1, Table S3.7 for details).

In fact, the taxonomic composition of the chitinolytic communities in octocorals seemed to closely follow the overall microbiome composition in these animals where uncultivated *Gammaproteobacteria* – often affiliated with the order *Oceanospirillales*, family *Endozoicomonadaceae* based on 16S rRNA gene assessments (Keller-Costa *et al.* 2017) - largely dominate the healthy octocoral tissue, while in necrotic octocoral tissue *Flavobacteriia,* including *Aquimarina* spp., strongly increase in abundance (Keller-Costa *et al.* 2021). This outcome supports the hypothesis that dysbiosis of the octocoral holobiont involves depletion of thus-far unculturable and unclassifiable, typical coral-

associated *Gammaproteobacteria* which may play an important role as chitin degraders in this system. Indeed, the chitinolytic microbiomes of necrotic octocoral tissues seemed to resemble much more those of seawater and sediments, where exo-chitinase- and polysaccharide deacetylase-encoding genes were more abundant.

As mentioned above, the healthy tissues of all octocoral species had higher proportions of chitinase sequences from unclassifiable *Gammaproteobacteria*, allowing hypotheses to be raised on a potential role for these elusive symbionts in C and N turnover within corals. Ongoing research on metagenome-assembled genomes (MAGs) retrieved from our octocoral specimens supports this hypothesis as endo-chitinase genes were detected on MAGs belonging to the typical coral symbiont family *Endozoicomonadaceae* (Keller-Costa *et al.*, unpublished data). Likewise, the detection of endo-chitinase gene reads belonging to the genus *Ardenticatena* (Kawaichi *et al.* 2013, 2015) suggests an unanticipated, potential participation of *Choloroflexi* symbionts in chitin degradation within animal invertebrates. Strengthening this notion is our own documentation of *chiA* genes on *Chloroflexi* MAGs retrieved from marine sponges (Silva *et al.*, unpublished data) and the recent observation that *Chloroflexi* spp. contribute to chitin degradation in freshwater sediments (Wörner and Pester 2019). Clearly, advanced techniques to link microbial identity and function such as single cell genomics and metagenome-resolved genomics hold great potential to further disentangle the diversity of symbiotic microorganisms involved in chitin degradation and utilization processes, strengthening and validating predictions based on metagenome functional profiling. Future, direct estimates of chitinolytic activity in samples collected in situ (either “holobiont” or host-derived microbial cells) bear promise in solidifying the status of host-associated microbiomes as important chitin degradation settings in the marine environment.

Our data suggest that marine host-associated microbiomes do possess potential for chitin hydrolysis (particularly octocorals) and chitin deacetylation (more pronouncedly in sponges but also in octocorals), leading to the production of COS or chitosan, respectively, and for COS breakdown and utilization (particularly sponges). However, we emphasize that none of the genomic features underlying the functions above were found to be pronouncedly enriched in host-associated microbiomes in comparison with their environmental vicinities. Therefore, the data reported here do not support the notion of sponges and octocorals as "fast processing hubs" of chitin, COS or chitosan when contrasted with free-living microbiomes. Marine sponges and corals are a part of benthic, suspension feeding communities which are known to regulate carbon flux between pelagic and benthic zones and affect the biogeochemical cycling of key nutrients (Gili and Coma 1998; Pita *et al.* 2018). By removing large amounts of particulate or dissolved organic matter from the water column, these holobionts are among the most efficient in up-taking and processing energy in marine ecosystems (Gili and Coma 1998). It seems hence plausible that sponges and octocorals, given the genomic and metagenomic features revealed in this study, are players in elemental turnover through their chitin degradative ability, since chitin presents a significant and critical connection between the carbon and nitrogen cycles in the marine environment (Souza *et al.* 2011). Our study proposes that unique chitin-degrading communities characterize distinct marine biotopes. Thus, a differential capacity to process chitin and its derivatives 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 in some cases.

## 3.5 Conclusion

Our study provides evidence for the existence of biotope-specific chitin-degrading communities in the marine realm. This suggests that differential substrate affinities, polymer versus oligomer uptake and degradation aptitude, and carbon and nitrogen turnover rates dictate multiple processing modes of chitin and chitin-derivatives across distinct micro-niches in the oceans. It is yet to be verified whether such patterns are applicable to a broad range of coral and sponge holobionts and marine environments. Moreover, the multiphasic approach employed in this study enabled us to infer possible substrate cross-feeding patterns among symbionts which may support chitin turnover within sessile marine invertebrates, contributing to the co-existence of chitin and COS degrading bacteria in symbiotic communities. We further highlight *Aquimarina* species as source of putative novel chitinolytic enzymes and break new ground regarding the potential chitin degradation roles of hallmark *Gammaproteobacteria* symbionts of corals and understudied symbionts in the *Choloroflexi* phylum. Future research shall tackle their fundamental properties, hopefully opening new opportunities to further explore marine biomes and understudied microbial clades for biocatalysts of interest in applied-oriented research.

## 3.6 Methods

### 3.6.1 Biological resources and approach

Forty-one bacterial isolates from two previously established culture collections derived from the octocoral *Eunicella labiata* (Keller-Costa *et al.* 2017) and the marine sponges *Sarcotragus spinosulus* and *Ircinia variabilis* (Esteves *et al.* 2013) were used in this study to address the chitin degradation capacities of culturable symbionts of octocorals and marine sponges. The isolates were subjected to chitin degradation and

chitinase activity bioassays, PCR-amplification of chitinase-encoding genes, and Pfam-based annotations to mine for protein domains involved in chitin and COS degradation, chitin deacetylation and GlcNac utilization (when genome sequences were available - **Table 3.1**) underlying chitin degradation ability. To address the relative abundance of the abovementioned functional features mediating chitin consumption and the taxonomic composition of chitinase-encoding genes in seawater, marine sediments, sponges and octocorals, 30 Illumina-sequenced microbial metagenomes representing two different datasets were examined using dedicated *in silico* analyses. These datasets are herein termed (1) the "sponge metagenome dataset", already published elsewhere (Yahel *et al.* 2003; Karimi *et al.* 2017), and the "octocoral metagenome dataset" which is an original contribution of this study (chitin metabolism features) and of a parallel study (unpublished) on the taxonomy and function of the total microbiome (Keller-Costa *et al.* 2021). Briefly, the sponge metagenome dataset consists of four microbiomes sampled from the inner body of four independent *Spongia officinalis* specimens, three independent microbiome samples from seawater, and three independent microbiome samples from sediments (Karimi *et al.* 2017). The octocoral metagenome dataset comprises 13 microbial metagenomes sampled from the tissues of three octocoral species (3x healthy *Eunicella gazella* tissue, 3x necrotic *E. gazella* tissue (same three specimens), 4x healthy *E. verrucosa* and 3x healthy *Leptogorgia sarmentosa*) specimens along with four microbial metagenomes from seawater and three from sediments. All samples, from both datasets, have been collected in the same location off the coast of Algarve, South Portugal ("Pedra da Greta": Lat. 36º 58' 47.2N, Long. 7º 59' 20.8W"). Detailed procedures regarding sampling, metagenome DNA extraction and sequencing, and general features of all metagenome samples from the abovementioned datasets are provided as Supplementary information (Additional file S3.2, Detailed methodology).

### 3.6.2 Bacterial strains

Of the 41 marine bacterial strains screened for chitinolytic activities in this study, 24 were retrieved from the octocoral *Eunicella labiata* by Keller-Costa *et al.* (2017), while 17 were obtained from Irciniidae sponges by Esteves *et al.* (2013) (**Table 3.1**). Each isolate represents a unique phylotype / genotype in its corresponding source study and makes part of an in-house collection of microbial symbionts. Isolates are available upon request. Prior to chitinolytic activity assays, all strains were re-activated from glycerol stocks and grown in half-strength Marine Broth (MB 1:2; ROTH Navarra, Spain) made with 1:1 v/v $dH_2O$ : artificial seawater (for composition, see Esteves *et al.* 2013).

### 3.6.3 Chitin degradation activity screening

Chitin degradation by the target isolates was tested with a Petri dish assay on colloidal chitin (CC) agar medium prepared with sterile artificial seawater. Eight replicates per isolate were used in the bioassays. After inoculation, CC plates were incubated at RT for 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 (see legend to **Table 3.1** for details). For specifics on CC preparation, medium composition, and inoculation procedures, see Additional file S3.2: Detailed methodology.

### 3.6.4 Endo- and exo-chitinase activity assays

Chitinolytic enzyme activity was determined fluorometrically for the 41 strains studied using the chitinase assay kit (CS0980) from Sigma-Aldrich/Merck, following the

manufacturer's instructions, and a multi-mode microplate reader (Filter Max F5, Molecular Devices). For specifics on sample preparation prior to endo-chitinase and exo-chitinase activity assays, please see Additional file 2: Detailed methodology. In brief, enzymatic activities were measured as the release of 4-methylumbelliferone (4-MU) from various 4-MU labelled substrates. Exo-chitinase (EC 3.2.1.52) activities were detected using the substrates 4-methylumbelliferyl N-acetyl-β-D-glucosaminide and 4-methylumbelliferyl N,N′-diacetyl-β-D-chitobioside hydrate 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 4-methylumbelliferyl β-D-N,N′,N″-triacetylchitotriose as substrate (release of GlcNAc trimers). All assays were performed at substrate concentrations of 0.5 mg/mL and sample volumes of 10 µL. Further details on physical-chemical parameters used in the assays and registration of results are provided in Additional file S3.2: detailed methodology.

### 3.6.5 PCR amplification of *chiA* gene fragments

PCR amplification of *chiA* gene fragments - targeting "group A" glycoside hydrolase family 18 endo-chitinases (EC 3.2.1.14), based on amino acid sequences of this catalytic domain (Suzuki *et al.* 1999), was carried out on genomic DNA of each strain analysed in this study. The primer pair *chiA*_F2 / *chiA*_R2 (*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') was employed, which generates amplicons of approximately 240 bp (Hobel *et al.* 2005b). Details on thermal cycling, Sanger sequencing and phylogenetic inference of the *chiA* sequences obtained are provided in Additional file S3.2: detailed methodology; Figure S3.3.

### 3.6.6 Genome-wide assessment of chitin metabolism traits in bacterial isolates from sponges and octocorals

Sixteen of the 19 bacterial genomes (available from the panel of 41 strains) investigated in this study for chitin / COS breakdown and utilization features have been published elsewhere (for octocoral-derived bacterial genomes see Keller-Costa *et al.*, 2016; Raimundo *et al*., 2018; Rodrigues *et al.*, 2018; Silva *et al.*, 2018); for marine sponge-derived bacterial genomes see Díez-Vives *et al.*, 2018; Gonçalves *et al.*, 2015), while the three *Vibrio* sp. genomes Vb255, Vb258 and Vb339 are original to this study. Genomic DNA was extracted using the Wizard genomic DNA purification kit (Promega, Madison, USA) from a pure, culture freshly grown in MB 1:2 and was sequenced on an Illumina MiSeq platform , as described elsewhere (Gonçalves *et al.* 2015; Karimi *et al.* 2017). The sequence reads were assembled *de novo* into contigs with the NGen DNA assembly software by DNAStar, Inc and the contigs underwent taxonomic identification and quality checks as described in Karimi *et al.*, 2019. Coding sequence predictions were performed with the Rapid Annotation using Subsystem Technology (RAST) prokaryotic genome annotation server, version 2.0 (Overbeek *et al.* 2014). Amino acid fasta files obtained from RAST were used as input data for protein families (Pfam)-based annotations using the WebMGA server (Wu *et al.* 2011) as explained in detail by Silva *et al.* (2019). We mined the data for Pfam entries underlying endo- and exo-chitnase activities, chitin deacetylation into chitosan (polysaccharide deacetylases), transport of chitin oligosaccharides and N-acetylglucosamine utilization (**Figure 3.1**; see Additional file S3.1: Table S3.1 for a complete list of the Pfam entries used).

### 3.6.7 Phylogenetic analysis of chitinase encoding genes in bacterial strains

We used the Rapid Annotation Using Subsystem Technology (RAST) v2.0 server (http://rast.nmpdr.org) (Aziz *et al.* 2008) to identify full endo-chitinase (EC 3.2.1.14) gene sequences from the 19 bacterial symbionts examined here for which whole genomes are available (Gonçalves *et al.* 2015; Keller-Costa *et al.* 2016; Díez-Vives *et al.* 2018; Raimundo *et al.* 2018; Rodrigues *et al.* 2018; Silva *et al.* 2018) (**Table 3.1, Figure 3.1**). This resulted in 96 predicted full-length endo-chitinase CDSs annotated by RAST across the 19 genomes. These CDSs were then subjected to translation followed by Pfam annotations using the EMBOSS Transeq (https://www.ebi.ac.uk/Tools/st/emboss_transeq) and hmmscan (https://www.ebi.ac.uk/Tools/hmmer/search/hmmscan) algorithms of EMBL-EBI. Forty-seven of the 96 CDSs were found to encode for either a GH18 or GH19 endo-chitinase domain according to Pfam annotations. These were selected for tree construction along with further 11 CDSs from our genomes which presented high levels of homology with endo-chitinase sequences present in NCBI's protein database. Closest and moderately close endo-chitinase gene relatives (n = 32) to the above-mentioned sequences were included in the analysis, totalling 90 full endo-chitinase gene sequences spanning four bacterial phyla (*Proteobacteria*, *Bacteroidetes*, *Actinobacteria* and *Firmicutes*) and 16 formally described genera.

The phylogenetic tree was constructed using the MEGAX software package (Kumar, Stecher and Tamura 2016). Chitinase sequences were aligned using ClustalW, after which the most suitable evolutionary model for each dataset was inferred. The Generalised Time Reversible model (GTR) was considered the best fit in both cases and was used for phylogenetic inference with the Maximum Likelihood method. Tests of phylogeny consisted of 1,000 bootstrap repetitions. Initial tree(s) for the heuristic search were

obtained automatically by applying Neighbour-Joining 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. Trees were drawn to scale, with branch lengths measured in the number of substitutions per site (Kumar *et al*. 2016).

### 3.6.8 Relative abundance of chitin metabolism coding sequences across host-associated and free-living microbiomes

To determine whether the relative abundance of genes involved in chitin / COS degradation and GlcNAc utilization (the same functional categories and enzymes as addressed in **Figure 3.1**) differed between biotopes, we explored InterPro (IPR) functional annotations obtained from unassembled reads (101 bp) of the marine sponge (Karimi *et al.* 2017) and octocoral metagenome (Keller-Costa *et al.* 2021) datasets using the European Bioinformatics Institute (EMBL-EBI) metagenomics analysis pipeline MGnify (Mitchell *et al.* 2016). Shortly, within MGnify, reads are subjected to coding sequences (CDSs) prediction using FragGeneScan (Rho, Tang and Ye 2010). The InterProScan procedure is then employed for functional annotation of CDSs against the latest release of the IPR database, which integrates several protein sequence databases such as Pfam, TIGRFams and PANTHER. Contingency IPR versus sample tables were retrieved for each dataset and examined for IPR entries involved in key chitin metabolism processes: chitin and chitin-oligosaccharide hydrolysis, using relative abundances of endo-chitinase (EC 3.2.1.14) and exo-chitinase (EC 3.2.1.52) encoding genes as proxies; chitin binding ability, using the relative abundance of chitin-binding proteins as proxies; potential chitin deacetylation, using the relative abundances of polysaccharide deacetylases as proxies, and N-acetylglucosamine utilization, using the relative

abundance of glucosamine-6-phosphate isomerase (EC 3.5.99.6) encoding genes as proxies (see Additional file S3.1: Table S3.5 for details on IPR entries used). Individual IPR entries related to each of the chitin metabolism functions named above were compiled and summed together to represent the abundance of each inspected function in the corresponding metagenome. To normalize the data, the absolute numbers of CDSs assigned to IPR entries were subjected to Hellinger transformation (i.e. calculation of relative abundance values followed by square root transformation of relative abundances). Thereafter, mean Hellinger-transformed abundance values and standard errors were calculated for each analysed function in each biotope and statistical analyses were carried out. Normality was confirmed using the Shapiro-Wilk-Test. For the analysis of the sponge and the octocoral metagenome datasets encompassing all healthy octocoral species plus sediment and seawater samples, One-Way ANOVA was used followed by a Tukey's post-hoc test if significant. For the analysis of microbial metagenomes from healthy versus necrotic *Eunicella gazella* tissue, a Student's t-test was used.

### 3.6.9 Taxonomic classification of chitinase-encoding genes from microbial metagenomes

To examine the taxonomic composition and structure of chitinolytic microbiomes across the studied biotopes in a cultivation-independent manner, we used the Meta-Genome Rapid Annotation using Subsystems Technology server (MG-RAST) v3.0 (Meyer *et al.* 2008) with default search parameters. This tool was used to mine for endo-chitinase protein sequences (EC 3.2.1.14) from unassembled reads across all samples in both datasets. Briefly, default MG-RAST procedures comprise gene calling with FragGeneScan (Rho *et al.* 2010) and translation of predicted CDSs into proteins with clustering set at 90% homology using the Uclust algorithm (Edgar 2010). Translated reads

are then annotated using the best-hit annotation tool against the M5NR database (Wilke *et al.* 2012). The stringency of the BLAST parameter is a maximum e-value of 1e-5, a minimum sequence identity of 60%, and a minimum alignment length of 15 aa for the predicted proteins. All sequence entries from each of the 30 surveyed metagenomes (10 from the sponge metagenome and 20 from the octocoral metagenome dataset) assigned as endo-chitinases (EC 3.2.1.14) by MG-RAST under the abovementioned parameters were downloaded. Within each dataset, endo-chitinase reads from replicate samples of the same biotope were pooled and then blasted against the NCBI protein sequence database using the blastx algorithm with an e-value cut-off of 10. The resulting alignment files were analysed using the MEGAN6 software package (Huson *et al.* 2016) to obtain taxonomic assignments. During this analysis we found that not all the sequences that were classified as endo-chitinases (EC 3.2.1.14) by MG-RAST also had necessarily an endo-chitinase sequence as their closest blastx hit (Table 2). Therefore, taxonomic assignments shown in this study considered only those reads identified by MG-RAST that also had an endo-chitinase sequence as their closest blastx hit and that could as well be taxonomically assigned to the Domain Bacteria.

In this study, inference of gene relative abundances (**Figure 3.3**) and taxonomic assignments (Figure 4) were performed using unassembled reads to make best use of the total sequencing effort employed in the generation of the sponge and octocoral metagenome datasets. This way, we could integrate sediment samples in the comparative scheme, since metagenome assemblies of this biotope are usually poor due to its extremely high microbial diversity – resulting in few and short contigs and usage of less than 5% of the generated reads (Karimi *et al.* 2017). Preliminary analyses revealed, moreover, that taxonomic and functional assignments of assembled and unassembled metagenomes were highly congruent for seawater, octocoral (Keller-Costa *et al.* 2021)

and sponge (Karimi *et al.* 2017) samples, thus supporting our choice to assess unassembled reads as a suitable and robust means to achieve the goals established in this study.

### 3.7 Supplementary information

High resolution figures and supplementary information pertaining to this chapter can be found online at:

https://drive.google.com/drive/folders/16MraFJqb0KiDAtLFMuDTx7FT-xpTPD0w?usp=sharing

**Additional file S3.1: Table S3.1.** Protein family (Pfam) entries involved in chitin and chito-oligosaccharides degradation and N-acetylglucosamine utilization used in Figure 1. **Table S3.2.** 16S rRNA gene-based estimates of relative abundance of culturable bacterial genera in the microbiomes of octocorals and sponges. **Table S3.3.** Genome-based estimates of relative abundance for culturable, chitinolytic symbionts of sponges and corals in their respective microbiomes. **Table S3.4.** Features of 90 (nearly) fulllength coding sequences displaying endo-chitinase catalytic domains (from glycoside hydrolase families 18 and 19, Pfam-based annotations) used for phylogenetic inferences in Figure 2. **Table S3.5.** InterPro (IPR) abundance of coding sequences involved in chitin and chito-oligosaccharides degradation and N-acetyl-glucosamine utilization in (a) the marine sponge metagenome dataset (project accession number: PRJEB11585) and (b) the octocoral metagenome dataset (project accession number: PRJEB13222), retrieved using the MGNify (EMBL-EBI) pipeline. **Table S3.6.** Endo-chitinase (EC 3.2.1.14) nucleotide sequences retrieved from (a) the marine sponge (*Spongia officinalis*) metagenome dataset (project accession number: PRJEB11585) and (b) the octocoral metagenome dataset (project accession number: PRJEB13222), using the MG-RAST metagenomics analysis

server (version 4.0.3). **Table S3.7.** Genus level taxonomy of endo-chitinase gene reads in the microbial metagenomes of sponges and corals. **Additional file S3.2:** Detailed Methodology, Extended Results and Discussion. **Supplementary Figure S3.1.** RAST annotation of chitin and chitin-derivative degradation and utilization genes in cultivated bacterial symbionts of sponges and octocorals. For details on strains and phylogenetic tree, see legend to Fig. 1. The table on the right side shows chitin degradation (including both hydrolysis and deacetylation processes) and N-acetylglucosamine transport and utilization encoding genes detected on each bacterial genome using RAST-based classification, in contrast with Pfam annotations show in Fig. 1. Values in each cell correspond to the respective coding sequence (CDS) numbers present in each genome, whereby higher CDS numbers are highlighted in dark-gray shading. Entries highlighted in bold represent chitin processing functions examined across the sponge and octocoral metagenome datasets (Fig. 3), while the phylogeny, diversity and taxonomic composition of endo-chitinase encoding genes (EC 3.2.14) are examined in Figs. 2 and 4. For each functional entry, enzyme commission (EC) numbers and specific terminology are given in brackets, when appropriate. 1 Chitinases that hydrolyse chitin oligosaccharides -(GlcNAc)4 to (GlcNAc)2 and (GlcNAc)5,6 to (GlcNAc)2 and (GlcNAc)3 but are inactive toward chitin (UniProtKB P96156). 2 Corresponds to InterPro database entry IPR002509 (see also Fig. 3) which describes the metal-dependent deacetylation of O- and N-acetylated polysaccharides such as chitin, peptidoglycan and acetylxylan. **Supplementary Figure S3.2.** Class-level prokaryotic community profiles of healthy (EG_H) and diseased (EG_N) *Eunicella gazella* tissue, healthy *Eunicella verrucosa* (EV01-EV04) and *Leptogorgia sarmentosa* (LS06-LS08) specimens as well as seawater (SW01-SW04) and sediment samples (SD01- SD03). Taxonomic assignments are based on 16S rRNA gene reads retrieved from unassembled metagenomes using the MGnify

metagenomics pipeline version 2.0 (EMBL-EBI) for the octocoral metagenome dataset (project PRJEB13222). Relative abundances are displayed for taxa representing more than 1% of the total dataset reads. Taxa with abundances below 1% across the data are collectively labelled as "rare classes". **Supplementary Figure S3.3.** Maximum Likelihood phylogenetic tree of chiA gene sequences amplified from bacterial isolates. Sequences were obtained for eight marine sponge and 11 octocoral-derived bacterial isolates through PCR amplification from their respective genomic DNA. The evolutionary history was inferred using the General Time Reversible model. The tree with the highest log likelihood (-892.58) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches (1,000 bootstrap replicates). A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 3.1129)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 19.83% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 19 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 164 positions in the final dataset.

## Declarations

### Ethics approval and consent to participate

This article does neither contain any studies with human participants nor vertebrate animals or cephalopods performed by any of the authors. This study was exempt from ethical approval procedures according to the current Portuguese legislation. All procedures involving animals were in accordance with the ethical standards of the institution (Centre of Marine Sciences (CCMAR), Faro, Portugal) at which the sponge

and octocoral samples were processed. This study did not involve endangered or protected sponge and octocoral species (according to the IUCN red list of threatened species, 02/11/2019: http://www.iucnredlist.org/searc). The sampling of sponges and octocorals did not occur within privately owned or protected areas. Sampling procedures were minimally intrusive and preserved sponges and octocoral colonies at the field site.

**Consent for publication**

Not applicable.

**Availability of data and material**

The 16S rRNA gene sequences of marine sponge and octocoral bacterial isolates were deposited at NCBI GenBank under the accession numbers HE818111–HE818389 (Esteves *et al.* 2013) and MF461358-MF461394 (Keller-Costa *et al.* 2017). *ChiA* gene sequences of sponge and octocoral bacterial isolates were also deposited at NCBI GenBank under the accession numbers MK570943-MK570958 and MK675637-MK675639. Full genome sequences of sponge and octocoral bacterial isolates were deposited in public databases by Raimundo *et al.* (2018) (bacterial genomes retrieved from *Eunicella labiata*); Díez-Vives *et al.*, 2018 (*Aquimarina* genomes from marine sponges); and Gonçalves *et al.* (2015) (*Vibrio* sp. Vb278), and accession numbers are provided in **Table 1**. The genome sequence of *Vibrio* sp. Vb339 has been submitted in this study to the European Nucleotide Archive/European Molecular Biology Laboratory (ENA/EMBL) under the assembly accession number GCA_902751245.1. Shotgun microbial metagenome sequences examined here were deposited at the European Nucleotide Archive (ENA/EMBL): the octocoral dataset (20 metagenomes Keller-Costa *et al.*, 2021) under the study accession number PRJEB13222 and the sample accession

numbers SAMEA3913358 to SAMEA3913367, and the sponge dataset (10 metagenomes Karimi *et al.*, 2017) under the study accession number PRJEB11585 and the sample accession numbers SAMEA3642063 to SAMEA3642072.

## Competing interests

The authors declare that they have no competing interests.


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## Authors' contributions

RC and TK-C conceived and designed the study. RC and AL-L provided reagents and materials. IR, RS, LM, SMV and TK-C performed laboratory experiments. IR, RS, TK-C and RC analysed data and prepared figures and tables. IR and RC wrote the first manuscript draft. TK-C and RC wrote the final manuscript draft. All authors revised and commented on the manuscript and approved the submitted version.

## Conflicts of Interest

The authors declare no conflict of interest. The sponsors had no role in the design, execution, interpretation, or writing of the study.

# Chapter 4

## Functional profiling of metagenome-assembled genomes suggests an unanticipated role for *Rhodothermia* symbionts in chitin and polysaccharide metabolism within marine sponges

Contents of this chapter are being prepared for submission as an original research article to the journal *Science Advances*.

**Functional profiling of metagenome-assembled genomes suggests an unanticipated role for *Rhodothermia* symbionts in chitin and polysaccharide metabolism within marine sponges**


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**Keywords**

Chitinases, GH18, MAGs, Porifera*, Spongia agaricina*, *Spongia officinalis*

## 4.1 Abstract

Marine sponges (phylum Porifera) are widely distributed across benthic ecosystems and populated by diverse and dense bacterial communities. Although a general role in polysaccharide degradation has been proposed for several lineages of marine sponge symbionts, little is known about the capacity of this symbiotic consortium to degrade and utilize chitin, the most abundant polysaccharide in the ocean. This study employs genome-resolved metagenomics to delineate the community of potential chitin degraders and utilizers within the marine sponge microbiome. Twelve microbial metagenomes from *Spongia agaricina*, *Spongia officinalis,* and seawater were each assembled and binned into 137 metagenome-assembled genomes (MAGs) which were subsequently classified into 18 prokaryotic classes across 13 phyla. The capacity to degrade the large chitin polymer via hydrolysis is likely a bottleneck function within the sponge symbiotic consortium, as endo-chitinase domains were encoded by 12 of 137 MAGs only (8.76%), classified into the classes *Anaerolineae*, *Dehalococcoidia*, *Gammaproteobacteria*, *Rhodothermia*, *Bacteroidia,* and WGA-4E (phylum *Candidatus* Poribacteria). The potential for chitin hydrolysis into dimers and monomers, degradation via deacetylation into chitosan, and utilization of the chitin monomer N-acetylglucosamine was frequent among symbionts, being annotated on 47 (33.58%), 107 (78.10%) and 73 MAGs (53.28%), respectively. Remarkably, all MAGs classified in the order *Rhodothermales* (class *Rhodothermia*, phylum *Rhodothermota*; n = 6) possessed all chitin degradation and utilization domains examined here, suggesting an unanticipated role of these symbionts in chitin and general polysaccharide metabolism within marine sponges. Phylogenetic analysis of GH18 endo-chitinase amino acid sequences across 11 bacterial classes revealed a major and robust phylogenetic clade composed exclusively by endo-chitinase domains from sponge-specific, uncultured symbionts of the order *Rhodothermales*. This

indicates chitin hydrolysis is an adaptive feature of these symbionts to living in the *in-spongia* milieu. Phylogenomic inference spanning 52 genomes across all families helped circumscribe the *Rhodothermales* order further by uncovering three sponge-specific, deeply branching clades formed exclusively by uncultured symbionts of sponges. Each of these clades likely represents a new family, expanding our view of *Rhodothermales* diversification, host range, and biogeographical occurrence within marine sponges. Our findings open new avenues to the study of *Rhodothermota* taxonomy and suggest *Rhodothermales* symbionts as keystone members of the marine sponge holobiont owing to their versatile carbohydrate metabolism.

### 4.2 Introduction

Sponges (phylum Porifera) are widely distributed across aquatic environments in habitats that vary from warm tropical reefs to the cold deep sea, freshwater lakes, and streams (van Soest *et al.*, 2012). Diverse and usually abundant bacterial communities live in the extracellular mesohyl matrix of marine sponges (Taylor *et al.*, 2007; Webster and Taylor, 2012). These communities encompass a wide phylogenetic breadth of taxa with more than 50 microbial phyla and candidate phyla reported to date from marine sponges (Webster and Thomas, 2016). Although several prokaryotic phyla and symbionts may be common to multiple sponge hosts, at the species level the structure of sponge-associated microbial communities is often specific to the host species (Hardoim *et al.* 2012; Thomas *et al.* 2016). Yet it may vary over time and space in response to diverse factors such as host-derived nutrients, immune response of the host species, pH, and other physical chemical parameters (Taylor *et al.* 2007; Thomas *et al.* 2016).

Owing to the high diversity and complexity of the sponge-associated microbiome, it is challenging, if not impractical, to study most of the ecological functions of sponge-

enriched bacteria, as well as their potential benefits to the host organism, using solely cultivation-dependent methods (Kamke *et al.*, 2014; Webster and Thomas, 2016). Although cultivation attempts have been instrumental in improving our understanding of the range of occurrence, physiology, and, most notably, natural product biosynthetic potential of diverse sponge-associated bacterial taxa (Dieterich *et al.*, 2022; Esteves *et al.*, 2013; Helfrich *et al.*, 2019; Karimi *et al.*, 2019; O' Halloran *et al.*, 2011), only a small portion of the sponge-associated prokaryotic community can be cultivated in the laboratory. Indeed, Hardoim *et al.* (2014) estimated that between only 10 and 14% of the bacterial community associated with the keratose sponges *Ircinia variabilis* and *Sarcotragus spinosulus* might be amenable to cultivation in the laboratory. However, cultured symbionts usually correspond to low abundance species within the marine sponge microbiome (Hardoim *et al.*, 2014; Karimi *et al.*, 2019). Therefore, molecular-based, cultivation-independent studies relying on the analysis of host-derived metagenomic DNA samples are fundamental to a comprehensive understanding of the diversity and function of these highly complex symbiotic communities, particularly of the most dominant, so-far uncultured marine sponge symbionts (Webster *et al.*, 2010; Webster and Thomas, 2016). Collectively, these studies suggest that sponge-associated microbial communities play fundamental roles in polysaccharide breakdown, host-microbe nutrient exchange, and cross-feeding involving carbon, nitrogen, and sulfur cycling, removal of host-derived metabolic by-products, and vitamin provision (Bayer *et al.*, 2008; Hentschel *et al.*, 2012; Karimi *et al.*, 2017; Lackner *et al.*, 2017; Taylor *et al.*, 2007; Thomas *et al.*, 2016). In addition, symbionts participate in natural product biosynthesis with putative functions suggested in host defence or microbe-microbe antagonistic interactions (Piel *et al.*, 2004; Wilson *et al.*, 2014).

Advances in the field of metagenomics and bioinformatics now enable the (re)construction of bacterial genomes from metagenomic samples without the need of cultivation and have opened a new era to the study of uncultivated prokaryotes using functional and comparative genomics approaches (Albertsen *et al.*, 2013). The field of marine microbiology is greatly benefiting from these novel methodologies (Paoli *et al.*, 2022; Robbins *et al.*, 2019), with recent studies revealing several novel functions possibly played by different sponge symbionts. In terms of nutrient metabolism, these functions include sulfur oxidation by *Gammaproteobacteria* (Tian *et al.*, 2014), taurine metabolism and utilization by *Alphaproteobacteria* (Karimi *et al.*, 2018) and many other symbiotic lineages (Engelberts *et al.*, 2020), carnitine and sulfated hydrocarbon utilization (Slaby *et al.*, 2017), and versatile polysaccharide assimilation profiles of symbionts belonging to the *Poribacteria* and *Chloroflexi* phyla, for instance (Bayer *et al.*, 2018; Kamke *et al.*, 2013, 2014). Altogether, MAG-based studies of the marine sponge microbiome suggest that symbionts possess a versatile carbohydrate metabolism, with hypotheses being raised on their potential role in recycling the extracellular polysaccharide matrix of their host, either for *Poribacteria* (Podell *et al.*, 2018), *Chloroflexi* (Bayer *et al.*, 2018) or *Gammaproteobacteria* (Cai *et al.*, 2021), among other lineages.

Despite evidence for profuse carbohydrate catabolic potential among sponge symbionts, the capacity of the marine sponge microbiome to degrade chitin - the most abundant biopolymer in the oceans (Paulsen *et al.*, 2016) – has been rarely addressed. Following recent evidence for specific chitinolytic communities inhabiting different marine biotopes such as corals, sponges, sediments, and seawater (Raimundo *et al.*, 2021) (Chapter III), in this study we examine metagenome-assembled genomes from marine sponge symbionts to determine the chitin degradation and utilization potential within the marine sponge microbiome. We screened 137 MAGs retrieved from the high microbial

abundance Dictyoceratida sponges, *Spongia officinalis* and *Spongia agaricina*, as well as from seawater, for the presence of genes coding for enzymes involved in chitin degradation (e.g., endo- and exo-chitinases and deacetylases), and N-acetylglucosamine utilization. We delineate for the first time the genetic coding potential for chitin turnover within the sponge symbiotic consortium in a cultivation-independent manner, shedding light on the putative roles of elusive, sponge-specific *Rhodothermales* symbionts in chitin degradation and global carbon and nitrogen cycling.

## 4.3 Materials and Methods

### 4.3.1 Sampling

In the present study, 12 shotgun-sequenced microbial metagenomes were assembled and subjected to binning and screening of MAGs for genes involved in the breakdown and utilization of chitin and its derivatives. These microbial metagenome samples represent three herein denoted biotopes: the marine sponge species *Spongia agaricina* and *Spongia officinalis*, and seawater (**Table 4.1**).

Sampling of *Spongia officinalis* specimens (*c.* 10 g of each specimen excised *in situ*, n = 4) and seawater (2 L, n = 3) took place in May 2014 by SCUBA diving at 20 m depth off the coast of Pedra da Greta (36◦58047.2N;7◦ 59020.8W), Algarve, southern Portugal, as described in detail by Karimi *et al.* (2017). Seawater samples were taken about 1 m above the sampled sponge specimens. Water pH was 8.13, temperature 18 ºC, and salinity 36.40 psu (Karimi *et al.*, 2017). Sampling of *Spongia agaricina* specimens (*c.* 50-100 g of each specimen excised *in situ*, n=5, this study) took place in the same location, in September 2016. Water temperature was 20.1 ºC and salinity 36.20 psu. Underwater and sample transportation procedures followed the methodology described in Hardoim *et al.* (2012) for both sampling events.

**Table 4.1** General features of the microbial metagenomes in this study

| Metagenome sample information | | Unassembled metagenomes | | Assembled metagenomes (this study) | | | | |
|---|---|---|---|---|---|---|---|---|
| **Sample** | **Origin** | **Total length (bp)** | **GC (%)** | **Total assembly length (bp)** | **GC (%)** | **# of contigs** | **Longest Contig (bp)** | **N50** |
| SA261g | *Spongia agaricina* | 3506291558 | 61.97 | 150804239 | 62.25 | 23704 | 770687 | 22482 |
| SA262g | *Spongia agaricina* | 3347794480 | 64.97 | 131006944 | 62.48 | 33265 | 1223308 | 7646 |
| SA263g | *Spongia agaricina* | 3042823566 | 63.20 | 168532645 | 62.77 | 39903 | 662553 | 9144 |
| SA264g | *Spongia agaricina* | 2585781800 | 62.53 | 139886992 | 61.98 | 32413 | 516346 | 8707 |
| SA265g | *Spongia agaricina* | 2667585740 | 63.50 | 84476135 | 61.28 | 14218 | 641839 | 29644 |
| SO230 | *Spongia officinalis* | 1497796468 | 58.35 | 104258254 | 58.76 | 24500 | 886490 | 7645 |
| SO231 | *Spongia officinalis* | 1481908360 | 59.93 | 89039836 | 60.45 | 21656 | 409025 | 8700 |
| SO232 | *Spongia officinalis* | 1353369902 | 58.26 | 85769473 | 59.72 | 20336 | 353621 | 8413 |
| SO233 | *Spongia officinalis* | 1596767984 | 59.89 | 94598118 | 60.63 | 22935 | 449938 | 7988 |
| SW01 | Seawater | 1397684460 | 42.66 | 50354948 | 41.7 | 11578 | 235233 | 8882 |
| SW02 | Seawater | 1460400612 | 43.27 | 46619841 | 41.69 | 11274 | 209627 | 8727 |
| SW03 | Seawater | 1450710066 | 44.05 | 40280313 | 41.49 | 9164 | 284090 | 10797 |

### 4.3.2 Microbial metagenome DNA extraction and sequencing

Microbial metagenome DNA extraction from *Spongia officinalis* (Karimi *et al.*, 2017) and *Spongia agaricina* (this study) samples followed the general procedures described by Hardoim *et al.* (2014), with slight modifications. Briefly, this protocol relies on the retrieval of microbial cell suspensions (MCSs) from each sponge specimen using maceration in calcium/magnesium free artificial seawater (CMFASW, Garson *et al.*, 1998) with mortar and pestle. While 2.5 g (fresh weight) of the internal body each *S. officinalis* specimen were macerated in 22.5 mL of CMFASW by Karimi *et al.* (2017) to produce sponge derived MCSs, in this study five g of each *S. agaricina* specimen were macerated in 45 mL of CMFASW for the same purpose. A two-step differential centrifugation procedure of the resulting suspensions was then carried out to obtain microbial cell pellets from the samples, which were then subjected to metagenomic DNA

extraction. First, microbial cell suspensions were well mixed by vortexing in a 50 mL sterile polypropylene tube and centrifuged thereafter for two min at 500 *g*. The resulting supernatant was then transferred to a new, 50-mL polypropylene tube and centrifuged for 30 min at 10,000 *g*. The obtained microbial cell pellets were kept at -80 ºC until metagenomic DNA extraction as detailed below. Seawater samples collected by Karimi *et al.*, (2017) and analysed in this study were filtered through 0.2-µm nitrocellulose filters (Millipore, Billerica, MA, USA) with the aid of a vacuum pump. The filters were then kept at -80 ºC until metagenomic DNA extraction as detailed below.

Metagenome DNA extraction from sponge-derived microbial cell pellets and nitrocellulose filters containing seawater microbes was performed with the UltraClean® Soil DNA isolation kit (MO BIO, Carlsbad, CA, United States) following the manufacturer's instructions. Prior to metagenome DNA extraction, microbial cell pellets of each sponge sample were well resuspended in the kit's Bead Solution Tube, while nitrocellulose filters containing seawater microbes were aseptically cut into small pieces to facilitate mixing and cell lysis. Metagenomic DNA yields were quantified for each sample with the Qubit dsDNA HS Assay Kit (Life Technologies Qubit 2.0®), and DNA quality and integrity were analysed on a NanoDrop® spectrophotometer (Thermo Scientific) and by electrophoresis in a 1.2 % agarose gel, respectively. Next generation sequencing of metagenome DNA samples was carried out on an Illumina Hiseq 2500 apparatus at Mr. DNA (Shallowater, TX, USA). DNA libraries were prepared for sequencing using the Nextera DNA Sample preparation kit (Illumina) and sequenced paired-end for 200 cycles with a sequence depth calibrated for c. 15 million 101-bp reads per sample (*Spongia officinalis* and seawater, Karimi *et al.*, 2017) and 20 million 101-bp per sample (*Spongia agaricina*, this study).

### 4.3.3 Metagenome data processing and assembly

To label the microbial metagenome samples used for the construction of MAGs in this study, different codes were attributed for the five samples from *S. agaricina* (SA261g-SA265g), for the four samples from *S. officinalis* (SO230-SO233) and for the three samples from seawater (SW01-SW03).

Metagenomic data handling and processing, from quality-filtering of raw reads to MAGs construction, were achieved using a suite of bioinformatic tools available on The United States Department of Energy Systems Biology Knowledgebase (KBase - http://www.kbase.us; Arkin *et al.* 2018). Default settings were used all the bioinformatic tools employed unless stated otherwise. First, raw metagenome reads were quality checked using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), which was also used to retrieve basic metrics for the unassembled metagenomes (**Table 4.1**). Quality-filtering of raw sequence reads was performed with Trimmomatic v1.2.14 (Bolger, Lohse and Usadel 2014). Metagenome reads were then assembled using the MetaSPAdes module 3.13.0 (Nurk *et al.* 2017), with a minimum contig length of 1000 bp. A quality report was generated using QUAST - Quality Assessment Tool for Genome Assemblies (Gurevich *et al.* 2013), which allowed visualization of the number of contigs generated in each assembly, and the GC (%) content of each metagenome, among other metrics (**Table 4.1**).

### 4.3.4 Metagenome binning

Binning of metagenome assemblies into MAGs was performed using the MetaBAT2 Contig Binning tool v1.7 (Kang *et al.*, 2019). Briefly, MetaBAT2 uses nucleotide composition information and source strain abundance (measured by depth-of-coverage by aligning the reads to the contigs) in MAGs construction (Kang *et al.*, 2019). Assembled

metagenomes and their corresponding, unassembled, paired-end reads were provided as input, and binning of each metagenome was carried out after selection of 2500 bp as minimum contig length. Thereafter, CheckM v1.0.18 (Parks *et al.*, 2015) was used to estimate the completeness and contamination rates of the generated MAGs. Based on the contamination and completeness estimates delivered by CheckM, a quality score (QS) was calculated according with the formula: QS = (completeness – 5*contamination). Only MAGs possessing QS>50 were selected for further analysis. Among these, MAGs possessing >90% completeness and <5% contamination estimates were considered "high quality" MAGs, whereas MAGs presenting 70-89% completeness and/or 5-10% contamination estimates were regarded as "medium quality".

### 4.3.5 Taxonomy and Functional annotation of MAGs

Taxonomic classification and functional annotations were performed for all MAGs obtained in this study which passed the above-mentioned QS threshold (n=137). Taxonomic analysis was carried out using GTDB-Tk v0.1.4 (Chaumeil *et al.*, 2019), while genome annotations were conducted using the in-house Melange pipeline (https://github.com/sandragodinhosilva/melange). Melange is a user-friendly CLI tool that first performs a gene calling step using Prokka v1.14.6 (Seemann 2014) with default settings to predict open reading frames (ORFs) and translates the gene sequences into amino acid sequences. It then executes a multi parallel genome annotation workflow, encompassing the Pfam (Finn *et al.* 2014), COG (Tatusov *et al.* 2000), KEGG orthologs (Kanehisa *et al.* 2023) and CAZymes (Cantarel *et al.* 2009) databases. In this study, Pfam and CAZymes annotations were employed in a comparative analysis of all MAGs to obtain their "total" (Pfam annotations) and "carbohydrate-degrading" (CAZymes) functional profiles. Both annotation schemes were used as input data in multivariate

statistics using Principal Coordinates Analysis (PCoA). Bray-Curtis dissimilarities between the MAGs were then calculated based on their functional profiles using the square root of absolute abundances of Pfam and CAZyme entries per MAG as input data, and PCoA was run using PAST v3.25 (Hammer *et al.*, 2001). Permutational Analysis of Variance (PERMANOVA) was thereafter performed to determine whether functional profiles of MAGs belonging to different prokaryotic classes were statistically different.

Besides comparing the functional profiles of all MAGs using Pfam and CAZymes annotations in a comprehensive fashion, we further screened the Pfam annotations for an individualized inspection of protein domains involved in the breakdown and utilization of chitin and its derivatives across all MAGs. This analysis encompassed the search for Pfam categories related with endo-chitinase, exo-chitinase, polysaccharide/chitin deacetylase and N-acetylglucosamine utilization domains across all MAGs obtained in this study. For the sake of simplicity, we opted for using Pfam annotations in such specific assessments owing to the general congruency between both Pfam and CAZymes annotation schemes regarding the presence of endo-chitinase and exo-chitinase domains across MAGs. Furthermore, because the Pfam database is a large collection of protein families, each represented by multiple sequence alignments, ancillary features and functions involved in the degradation and utilization of chitin and its derivatives, such as chitin binding proteins and N-acetylglucosamine utilization features, could as well be explored in our specific assessment of each MAG. The Pfam functions that contributed the most to the dissimilarity between MAGs at the class level (following GTDB-tk taxonomic assignments) were further examined using similarity percentage analysis (SIMPER) in PAST v 3.25. SIMPER results and Pfam annotation outcomes were inspected for the presence of typical and/or enriched symbiosis features among those MAGs and bacterial classes that were found to possess endo-chitinase encoding genes.

The symbiosis functional features that were assessed encompassed those involved in host-symbiont establishment and persistence (such as eukaryotic-like repeat proteins – ELPs), anti-viral defense (CRISPR-Cas proteins, restriction endonucleases), sulfated polysaccharide degradation (sulfatases), and genome rearrangement potential (presence of transposases), which are known to be enriched in microbial metagenomes of marine sponges (Fan *et al.*, 2012; Karimi *et al.*, 2017). Other specific functional features were also explored based on their differential distribution among MAGs possessing endo-chitinase encoding genes (see Results and Discussion).

Because all the sponge-derived MAGs classified in the *Rhodothermales* order (class *Rhodothermia*) possessed endo-chitinase encoding genes, a custom statistical analysis was performed to reveal enriched genomic features of these symbionts in comparison with the remaining lineages/MAGs uncovered in this study. To this end, one-side Welch's t-tests for unequal variances were performed on STAMP v.2.1.3 (Parks *et al.* 2014) to uncover Pfam entries significantly enriched on *Rhodothermales* MAGs in comparison with (i) all other sponge-derived MAGs, (ii) all seawater-derived MAGs and (iii) all other MAGs in general. Moreover, to delve into the phylogenetic uniqueness of putative chitinolytic *Rhodothermales* spp., dedicated phylogenetic assessments of endo-chitinase-encoding genes and of whole-genome diversification within the order *Rhodothermales* were performed and are delineated in detail below.

### 4.3.6 Phylogenetic analysis of endo-chitinase amino acid sequences

As a result of our screening for endo-chitinase protein domains across all MAGs using Pfam annotations, a customized phylogenetic analysis of endo-chitinase amino acid sequences predicted from MAGs classified into the *Rhodothermales* order (phylum *Rhodothermia*) and relatives in the *Bacteroidota* phylum was carried out. First, Melange

was used to retrieve the specific amino acid sequences underlying each endo-chitinase domain (Pfam annotation) found on sponge-derived MAGs classified as *Rhodothermales.* Other endo-chitinase encoding sequences from MAGs classified into the *Gammaproteobacteria* class and the *Flavobacteriales* order, retrieved from seawater samples, were included in the analysis. The retrieved sequences were then used as queries in blastp searches against related sequences available on NCBI's protein database (https://blast.ncbi.nlm.nih.gov/). Amino acid sequences with the highest homology to our queries were selected and used in a phylogenetic assessment of endo-chitinases from *Rhodothermales* and their closest relatives on blastp, encompassing diverse bacterial taxa in a total of 49 distinct sequences.

To obtain a more detailed view on the presence and organization of protein domains on each of the abovementioned endo-chitinase amino acid sequences, the hmmscan algorithm of EMBL-EBI (https://www.ebi.ac.uk/Tools/hmmer/search/hmmscan) was used for protein homology searches using profile hidden Markov models (HMMs). All sequences coded for a glycoside hydrolase family 18 (GH18) endo-chitinase domain and were selected for phylogenetic tree construction using the MEGAX software package (Kumar *et al.*, 2016). The sequences were aligned using ClustalW (Thompson, Higgins and Gibson 1994), after which the most suitable evolutionary model was inferred within MEGAX. The Jones-Taylor-Thornton model (JTT) (Whelan and Goldman 2001) was considered the best fit and was used for phylogenetic inference with the Maximum Likelihood method. Tests of phylogeny consisted of 1000 bootstrap repetitions. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances and then selecting the topology with superior log likelihood value (Kumar *et al.*, 2016). Graphical editing of the tree was performed using ITOL v4 – Interactive Tree of Life (Letunic and Bork 2019).

### 4.3.7 Whole-genome assessment of relatedness among *Rhodothermales* species

To explore the relatedness of the six marine, sponge-derived MAGs classified as *Rhodothermales* with other genome sequences belonging to the same order, a whole genome comparative approach was employed that enables fast tree building from multiple genome sequences in a computationally tractable fashion. Whole genome tree construction was performed using 52 *Rhodothermales* genomes including – in addition to our MAGs - 42 genomes publicly available in the assembly database of NCBI (stand: 25$^{th}$ of July 2022), three genomes from *Rhodotermus* strains (family *Rhodothermaceae*), and the so-called Bin80 genome, a MAG retrieved from the marine sponge *A. aerophoba* that represents an uncultured lineage in the GTDB-tk taxonomy to which our MAGs are related (Table 2). The tree was constructed using MashTree v1.2.0 (Katz *et al.*, 2019), a tool that calculates the pairwise distance matrix between genome summaries composed of the most frequent kmers excised from the genomes. Briefly, kmers are created and sorted with the MinHash algorithm v2.2 (Ondov *et al.*, 2016) and this information is then used to build a genome-wide tree using the Neighbor-Joining method with the QuickTree tool v2.5 (Howe *et al.*, 2002). In this process, bootstrapping with 1000 repetitions was performed, with Mashtree being run with a random seed in each repetition. Graphical visualization and editing of the tree were performed using Interactive Tree of Life (iTOL) v4 (Letunic and Bork, 2019).

In addition to tree construction, we used the FastANI algorithm (Goris *et al.*, 2007; Jain *et al.*, 2017) available on Kbase to compute whole-genome average nucleotide identity (ANI%) values in a pairwise fashion for a selection of 34 MAGs of interest. Briefly, these MAGs were chosen based on statistically robust clusters formed after tree building, representing genomes of different *Rhodothermales* families and/or deeply

branching sponge-specific clusters formed exclusively by uncultivated bacteria (i.e., MAGs).

## 4.4 Results and Discussion

### 4.4.1 Basic metagenome features

Despite originating from different sampling events, all microbial metagenomes analysed in this study went through the same data processing and analysis pipeline, and their basic features are shown in **Table 4.1**. The number of assembled contigs ranged from 9164 in seawater sample SW03 to 40195 in one of the *S. agaricina* specimens (SA263g). The same pattern was observed for the total length of the metagenomes, with SW03 being the shortest (40,280,313 bp) and SA263g the largest one (170,729,704 bp). The largest contig was also assembled from *S. agaricina* (SA262g) and the shortest from seawater (SW02). These results are consistent with the higher sequencing depth of the *S. agaricina* metagenomes, leading to longer assemblies and contigs, as well as higher N50 metrics in comparison with *S. officinalis* metagenomes. *Spongia* metagenome assemblies had GC content values ranging from 58 to 62% while seawater metagenome assemblies displayed values around 41% (**Table 4.1**). Such sharp differences in GC content suggest contrasting microbial community structures in sponge and seawater biotopes, consistent with the recent literature and knowledge of the structure of symbiotic communities in *Spongia officinalis* and marine sponges at large (Karimi *et al.*, 2017; Thomas *et al.*, 2016). Remarkably, we even depicted a trend for different GC content among microbial metagenomes from different sponge species, with slightly higher values observed for *S. agaricina* in comparison with *S. officinalis* metagenomes. This difference was more pronounced when comparing the GC content of unassembled metagenomes, which

ranged from about 62 to 65% in the former and about 58 to 60% in the latter sponge species.

### 4.4.2 Quality assessment and classification of MAGs

From the 12 assembled metagenomes, a total of 298 bins was obtained. After applying the quality score threshold (QS>50%) used in this study to select for good quality MAGs based on completeness and contamination estimates, 137 bins were selected as good- to high-quality MAGs and used in further analyses. Of these 137 MAGs, 81 were obtained from *S. agaricina* metagenomes, 39 from *S. officinalis* metagenomes and 17 from seawater metagenomes, and an overview of the quality parameters pertaining to these MAGs is shown in Supplementary Table S1. Ninety-three MAGs presented completeness estimates higher than 90% and contamination estimates lower than 5% and were considered high-quality MAGs in this study. Of these, 59 MAGs came from *S. agaricina*, 20 from *S. officinalis,* and 14 from seawater metagenomes (**Table S4.1**). Seventeen MAGs were estimated to have no contamination (0%), of which 11 MAGs originated from *S. agaricina*, 4 from *S. officinalis,* and 2 from seawater.

Figure 1 provides an overview of the phylum- (**Figure 4.1A**) and class-level (**Figure 4.1B**) taxonomy of MAGs retrieved from *S. agaricina*, *S. officinalis* and seawater biotopes, indicating the proportion of MAGs assigned to different taxa at each taxonomic level. The taxonomic classification of each MAG assembled in this study across all hierarchical ranks is shown in Supplementary **Table S4.2**. Of the 137 MAGs examined, three MAGs were classified as *Archaea* and 134 as *Bacteria* (**Table S4.1**). All the *Archaea* MAGs were obtained from *S. agaricina* samples and belonged to the genus *Nitrosopumilus* in the phylum *Nitrososphaerota* (formerly *Thaumarchaeota*, **Table S4.2**).

Totals of 13 prokaryotic phyla and 18 classes were registered from the analysed MAGs. MAGs retrieved from both *S. agaricina* and *S. officinalis* species were classified into eleven different phyla (**Figure 4.1A**), consistent with the notion of a high richness of prokaryotic phyla being usually found in association with high microbial abundance sponges (Thomas *et al.*, 2016; Webster *et al.*, 2010). In contrast, MAGs belonging to two phyla only (*Bacteroidota* and *Proteobacteria*) were obtained from seawater metagenomes, which is consistent with the dominance of these taxa in bacterioplankton communities at the study site (Karimi *et al.*, 2017). Independently of the origin of MAGs, the most represented phylum in the dataset was *Proteobacteria*, which accounted for a total number of 31 MAGs. However, besides *Proteobacteria,* the phyla identified more often among the MAGs originating from marine sponges were *Actinobacteriota* and *Chloroflexota*, followed by *Acidobacteriota* and *Poribacteria.* All of these are consistently present in both sponge species (**Figure 4.1A**). We also noted the presence of MAGs from the *Rhodothermota* phylum (formerly *Bacteroidota*) in both sponge species, yet MAGs belonging to this phylum were more frequently obtained from *S. officinalis* than from *S. agaricina* specimens. Conversely, MAGs belonging to the phylum *Chloroflexota* were assembled more often from *S. agaricina* than from *S. officinalis* specimens (Figure 1A). Altogether, the taxonomic composition of sponge-derived MAGs observed in this study strengthens findings made using 16S rRNA gene amplicon sequencing on the structure and taxonomy of prokaryotic communities associated with keratose sponges (e.g., *Ircinia*, *Sarcotragus* and *Spongia*) of the order Dictyoceratida in the northeast Atlantic (Hardoim *et al.*, 2012, 2014; Hardoim and Costa, 2014; Karimi *et al.*, 2017). These findings are also in line with our current notion of the phylum-level

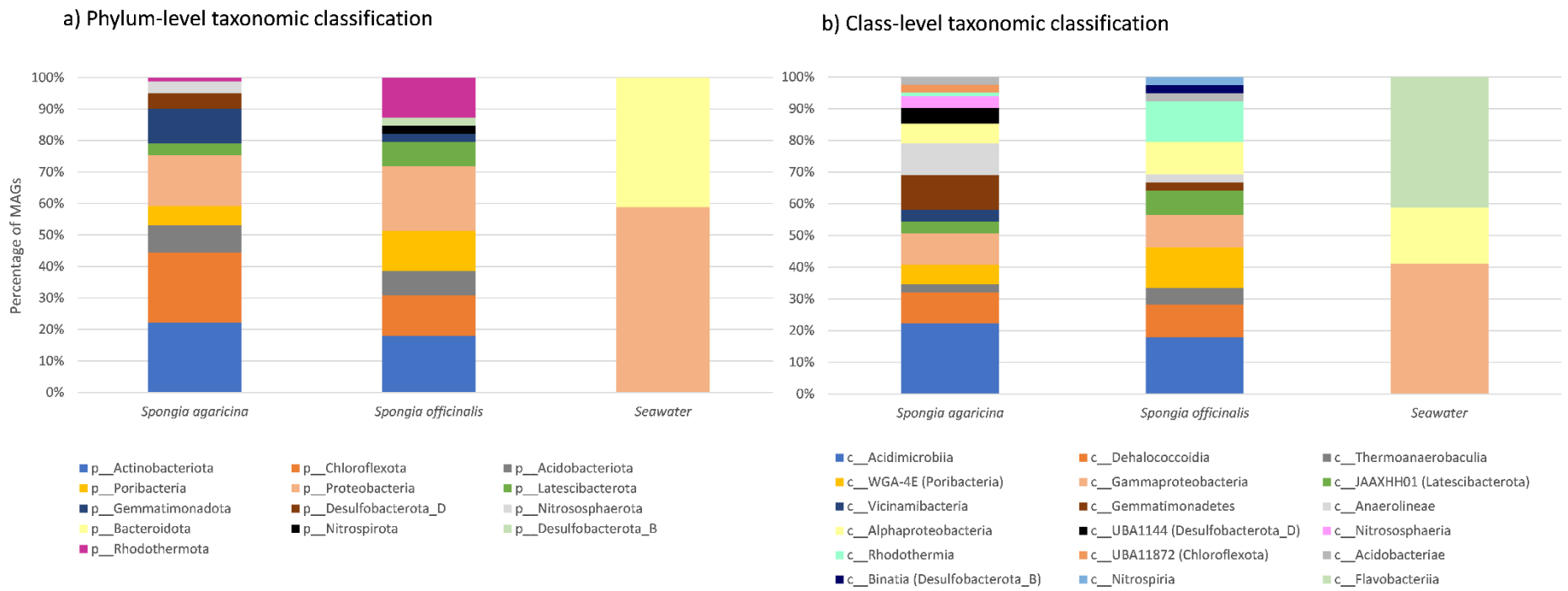


**Figure 4.1 Taxonomic composition of the 137 MAGs retrieved from *Spongia agaricina*, *Spongia officinalis* and seawater at (a) phylum- and (b) class-level.** The classification was performed using GTDB-tk on MAGs obtained from microbial metagenome samples assembled with metaSPADES. Only MAGs that passed the quality thresholds employed in this study were analysed. The Y-axis shows the proportion of the total number of MAGs assigned to each phylum (a) and class (b) per biotope. For taxonomic classification of each MAG assembled in this study across all hierarchical ranks, see supplementary Table S4.2.

taxonomic composition of prokaryotic communities in high microbial abundance sponges, usually characterized by a dominance of *Proteobacteria*, *Actinobacteriota*, *Choloroflexota*, *Acidobacteriota* and *Poribacteria* phyla.

Similar patterns were observed when class-level taxonomic assignments were performed, with 15 out of 18 classes represented among *S. agaricina*-derived MAGs and 13 classes among *S. officinalis*-derived MAGs, while only three bacterial classes were recorded for the MAGs obtained from seawater (**Figure 4.1B**). MAGs belonging to the *Acidimicrobiia* (*Actinobacteriota*) class (n=25) were the most frequent across the entire dataset, accounting for 18 and seven MAGs retrieved from *S. agaricina* and *S. officinalis* specimens, respectively. Most of the *Chloroflexota* MAGs could be assigned to the classes *Dehalococcoidia* and *Anaerolineae*. From *S. agaricina*, equal numbers of *Dehalococcoidia* and *Anaerolineae* MAGs were obtained, while *Dehalococcoidia* MAGs were assembled from *S. officinalis* more often (**Figure 4.1B**). Notably, all MAGs classified into the class *Rhodothermia* (phylum *Rhodothermota*, formerly *Bacteroidota*) were obtained from sponge samples (**Figure 4.1B**).

The taxonomic assignments obtained in this study strongly suggest a high degree of phylogenetic novelty among the MAGs. Indeed, from the 137 MAGs under study only 29 and nine could be classified at family and genus levels, respectively, while 14 MAGs were classified to the species level (**Table S4.2**). Because MAGs are inherently akin to represent the most dominant organisms from each biotope, that is, those that possess higher coverage values in metagenome assemblies, these results indicate that the prevailing symbionts of *S. agaricina* and *S. officinalis* represent thus far "unculturable" microorganisms which currently lack a formal taxonomy. Dedicated, genome-wide comparative analyses coupling phylogenomic assessments to whole-genome digital hybridization and average nucleotide / amino acid identity estimates hold promise in

establishing a novel, cultivation-independent taxonomy to properly catalogue the phylogeny and diversity of uncultured symbionts of marine sponges that would also enable a connection between (novel) taxonomy and functions to be made. On this note, Keller-Costa *et al.* (2022) have recently proposed a new genus in the *Endozoicomonadaceae* family (*Gammaproteobacteria*), denoted *Candidatus* Gorgonimonas, solely based on *in silico* genome-wide approaches to describe a novel, presumably chitinolytic, bacterial taxon of specific and widespread occurrence in association with octocorals.

Finally, the here reconstructed genomes of sponge symbionts displayed a wide range of GC content values that were specific to the phyla they belong to (**Table S4.2**). For instance, the GC content of widely known sponge-specific or sponge-enriched symbiotic lineages ranged from around 40% for *Poribacteria* MAGs to above 60% for *Actinobacteriota*, *Acidobacteriota*, *Chloroflexota* and several *Alphaproteobacteria* MAGs, in line with findings made elsewhere (Karimi *et al.*, 2018; Slaby *et al.*, 2017).

### 4.4.3 Pfam-based annotation of endo- and exo-chitinase protein domains

A full analysis of the 137 MAGs for the presence of endo- and exo-chitinase, polysaccharide deacetylase, and N-acetylglucosamine utilization encoding genes, using the Pfam annotation database, is shown in Supplementary **Table S4.3**. Twelve out of the 137 MAGs (8.76%) were found to have endo-chitinase domains (See **Table S4.3** for specifics on the Pfam domains searched): nine out of 120 MAGs obtained from marine sponges (7.5%) and three out of 17 MAGs obtained from seawater (17.6%). These MAGs were classified into six bacterial classes, namely *Anaerolineae* and *Dehalococcoidia* from the *Chloroflexota* phylum, *Gammaproteobacteria* (*Proteobacteria*), *Rhodothermia* (*Rhodothermota*), *Bacteroidia* (*Bacteroidota*) and WGA-4E (*Poribacteria*). The highest

number of endo-chitinase domains (n=3) was found on one single MAG belonging to the *Dehalococcoidia* class (SA265g_bin11). The MAGs belonging to the *Rhodothermia*, *Anaerolineae*, *Gammaproteobacteria* and *Flavobacteriia* classes presented one endo-chitinase domain assigned to the glycosyl hydrolase family 18 of bacterial chitinases (PF00704), while the *Poribacteria* and *Dehalococcoidia* MAGs were found to possess chitinase A, N-terminal domains (PF06483), indicating different substrate affinities among these taxa. Noticeably, a GH18 domain was found on all the *Rhodothermia*/*Rhodothermales* MAGs identified in this study (**Table 4.2, Table S4.3**), suggesting a consistent role of these symbionts in chitin processing within the sponge holobiont. Overall, these results indicate that only a small portion of the total marine sponge microbiome can degrade the large chitin polymer by means of hydrolysis (endo-chitinolytic activity). In contrast, genes coding for exo-chitinases, which are involved in the hydrolysis of smaller chitin oligosaccharides (COS) and dimers of N-acetylglucosamine, were found in a broad range of marine sponge symbionts, being present on 47 MAGs from 13 bacterial classes, namely *Acidimicrobiia*, *Alphaproteobacteria*, *Anaerolineae*, *Bacteroidia*, *Binatia* (phylum *Desulfobacterota B*), *Dehalococcoidia*, *Gammaproteobacteria*, *Gemmatimonadetes*, JAAXHH01 (phylum *Latescibacterota*), *Rhodothermia*, *Thermoanaerobaculia*, *Vicinamibacteria*, and WGA-4E (*Poribacteria*). Collectively, MAGs with exo-chitinases represented 33.58% of the total number of MAGs analysed, and 33.33% were assembled from marine sponges only. The highest number of exo-chitinase domains (8) were presented on one single MAG belonging to the *Bacteroidia* class (SW03_bin07), but a high number of domains could also be found on several *Poribacteria* and *Rhodothermia* MAGs. Moreover, all the MAGs belonging to the *Rhodothermia*, *Binatia* and *Thermoanaerobaculia* classes presented exo-chitinase domains, and only one MAG from both WGA-4E, JAAXHH01, *Bacteroidia,*

and *Anaerolineae* did not present any exo-chitinase domain. This shows that exo-chitinase domains are widespread over the above-mentioned taxa. The presence of exo-chitinase domains in all *Rhodothermia* MAGs, in addition to the endo-chitinase domains, further supports the notion that these symbionts play a consistent role in chitin degradation within the marine sponge holobiont. Moreover, these findings also suggest that even if only a few symbionts present chitinolytic activity a large part of the community can utilize chitin degradation products. This observation is consistent with the hypothesis that chitin degradation and utilization cascades may promote the coexistence of diverse marine sponge symbionts and play a fundamental role in carbon and nitrogen cycling in benthic ecosystems (Raimundo *et al.*, 2021). Another pattern depicted in our analysis reveals that MAGs which present endo-chitinase domains also possess exo-chitinase domains. This is true for 10 out of the 12 MAGs with endo-chitinase domains with the exceptions being one MAG belonging to class *Gammaproteobacteria* (SW02_bin04) and another belonging to class *Dehalococcoidia* (SA265g_bin11). These results support the hypothesis that both enzymes can act together to enhance the efficiency of the degradation process. In contrast, members of the class *Thermoanaerobaculia* (phylum *Acidobacteria*) only presented a coding potential for exo-chitinolytic domains with no genes found on those MAGs possibly encoding endo-chitinases and the other domains involved in chitin metabolism. Thus, *Thermoanaerobaculia* and other symbionts possessing exo-chitinases only are either dependent on chitin degradation products derived from the activity of chitinolytic symbionts, in line with the substrate cross-feeding hypothesis raised by Raimundo *et al.* (2021), may specialize on remnants of chitin degradation / COS present in the water column which are pumped into the mesohyl matrix through sponge filtering activity or, alternatively, rely on the availability of N-acetylglucosamine disaccharides deriving from the degradation of peptidoglycans in the sponge mesohyl. Finally, despite

recent studies finding novel chitinases in *Archaea* species (Chen *et al.*, 2019; Nishitani *et al.*, 2018), we did not find genes encoding any chitinolytic domains on the three archaeal MAGs classified as *Nitrosopumilus*, which are otherwise known for their ammonium oxidation capacities and likely roles in the removal of metabolic waste by-products (ammonia) within the marine sponge holobiont (Bayer *et al.*, 2008; Hardoim and Costa, 2014).

**Table 4.2** Taxonomic classification of the 12 metagenome-assembled genomes (MAGs) found to possess endo-chitinase encoding genes.

| Bin_ID | Sample description | GTDB-tk Taxonomy [1] Phylum | GTDB-tk Taxonomy[1] Class | GTDB-tk Taxonomy[1] Order | GTDB-tk Taxonomy[1] Family | GTDB-tk Taxonomy[1] Genus | GTDB Taxonomy[1] Species | Endo-chitinase[2] | exo-chitinase[3] | deacetylase[4] | N-acetylglucosamine utilization[5] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| SA261g_bin14 | *Spongia agaricina* | p__Poribacteria | c__WGA-4E | o__WGA-4E | f__WGA-3G | g__WGA-3G | s__WGA-3G sp000406005 | 1 | 3 | 3 | 2 |
| SA261g_bin23 | *Spongia agaricina* | p__Chloroflexota | c__Anaerolineae | o__SBR1031 | f__A4b | g__UBA6055 | s__ | 1 | 2 | 2 | 0 |
| SA262g_bin15 | *Spongia agaricina* | p__Bacteroidota | c__Rhodothermia | o__Rhodothermales | f__Bin80 | g__ | s__ | 1 | 5 | 1 | 2 |
| SA265g_bin11 | *Spongia agaricina* | p__Chloroflexota | c__Dehalococcoidia | o__UBA3495 | f__UBA3495 | g__Bin87 | s__ | 3 | 0 | 9 | 0 |
| SO230_bin03 | *Spongia officinalis* | p__Bacteroidota | c__Rhodothermia | o__Rhodothermales | f__Bin80 | g__ | s__ | 1 | 3 | 2 | 2 |
| SO231_bin11 | *Spongia officinalis* | p__Bacteroidota | c__Rhodothermia | o__Rhodothermales | f__Bin80 | g__ | s__ | 1 | 3 | 1 | 1 |
| SO231_bin15 | *Spongia officinalis* | p__Bacteroidota | c__Rhodothermia | o__Rhodothermales | f__Bin80 | g__Bin80 | s__ | 1 | 3 | 1 | 2 |
| SO232_bin18 | *Spongia officinalis* | p__Bacteroidota | c__Rhodothermia | o__Rhodothermales | f__Bin80 | g__Bin80 | s__ | 1 | 3 | 1 | 2 |
| SO233_bin05 | *Spongia officinalis* | p__Bacteroidota | c__Rhodothermia | o__Rhodothermales | f__Bin80 | g__ | s__ | 1 | 3 | 1 | 2 |
| SW02_bin04 | Seawater | p__Proteobacteria | c__Gammaproteobacteria | o__SAR86 | f__D2472 | g__SCGC-AAA076-P13 | s__ | 1 | 0 | 2 | 1 |
| SW02_bin07 | Seawater | p__Bacteroidota | c__Flavobacteriia | o__Flavobacteriales | f__Flavobacteriaceae | g__SCGC-AAA160-P02 | s__ | 1 | 4 | 1 | 1 |
| SW03_bin07 | Seawater | p__Bacteroidota | c__Flavobacteriia | o__Flavobacteriales | f__Flavobacteriaceae | g__MAG-121220-bin8 | s__MAG-121220-bin8 sp002700465 | 2 | 8 | 0 | 2 |

Values display Protein Family (Pfam) functional annotation results for the presence of endo-chitinases and associated enzymes involved in chitin metabolism. Highlighted in blue are sponge-derived MAGs classified into the order *Rhodothermales*, class *Rhodothermia*, phylum *Bacteroidota*.

[1] Taxonomic classification was obtained using GTDB-tk v1.5.0 with reference data of release 06-RS202 (https://gtdb.ecogenomic.org/) and then manually curated to comply with the List of Prokaryotic names Standing in Nomenclature (LPSN; https://www.bacterio.net/).

[2] Sum of the protein domains assigned to Pfam entries PF00182.20, PF00704.29, PF08329.11 and PF06483.11
[3] Sum of the of the protein domains assigned to Pfam entries PF03174.14, PF13290.7, PF03173.13, PF02838.16, PF00728.23, PF14845.6, PF03174.14, PF13290.7 and PF07555.14
[4] Sum of the of the protein domains assigned to Pfam entries PF01522.22 and PF04748.14
[5] Protein domains assigned to Pfam entry PF01182.21

### 4.4.4 Functional ordination of MAGs

Multivariate ordination of MAGs based on their functional profiles by means of Principal Coordinates Analysis (PCoA) was carried out using two annotation schemes, namely Pfams (**Figure 4.2a,c**) and CAZymes (**Figure 4.2b,d**). Ordination diagrams presented in this study provide an overview of the "total" functional profiles (Pfam-based annotations) and carbohydrate catabolism profiles (CAZymes-based annotations) of all MAGs (**Figure 4.2a,b**) and only sponge-derived MAGs from bacterial classes possessing at least one MAG encoding one endo-chitinase domain (**Figure 4.2c,d**). Overall, both Pfam- and CAZymes-based ordinations suggest a good match between taxonomy and function, with a well delineated phylogenetic signal underlying significant variation in functional profiles (**Figure 4.2a,b**), as ascertained by One-Way PERMANOVA ($p<0.0001$ for differences between classes). Pairwise PERMANOVA results confirmed significant differences in functional profiles between MAGs from different bacterial classes for nearly all pair combinations, the most notable exceptions being bacterial classes represented by only one MAG (e.g., *Binatia* and *Nitrospiria*), which could not be distinguished from other classes owing to the lack of replicate genomes. When ordinations were performed to compare classes containing at least one sponge-derived MAG with an endo-chitinase encoding gene (Figure 2c,d), a clear pattern was observed whereby members from the classes *Rhodothermia*, *Anaerolineae*, *Dehalococcoidia*, *Flavobacteriia*, and WGA-4E were well separated in the ordination space (**Figure 4.2c,d**), corroborating their significantly different functional profiles (pairwise PERMANOVA p values < 0.003). MAGs of the *Rhodothermia* class clustered tightly together in the ordination space, suggesting a high degree of functional conservation within *Rhodothermia* symbionts of *Spongia* hosts. In contrast, MAGs of the candidate class WGA-4E (phylum *Poribacteria*) were split into two functional groups, most notably

according to Pfam annotations (**Figure 4.2c**). Regarding the *Chloroflexota* classes, a large degree of functional variation was found for MAGs belonging to the class *Dehalococcoidia* following both annotation schemes, while *Anaerolinae* MAGs displayed a comparatively higher degree of functional conservation though they were not as tightly clustered as *Rhodothermia* MAGs (**Figure 4.2c,d**). **Figure 4.2** also illustrates, via colour coding, the highly consistent pattern of co-occurrence of endo- and exo-chitinase encoding genes on *Rhodothermia* MAGs (highlighted in purple).

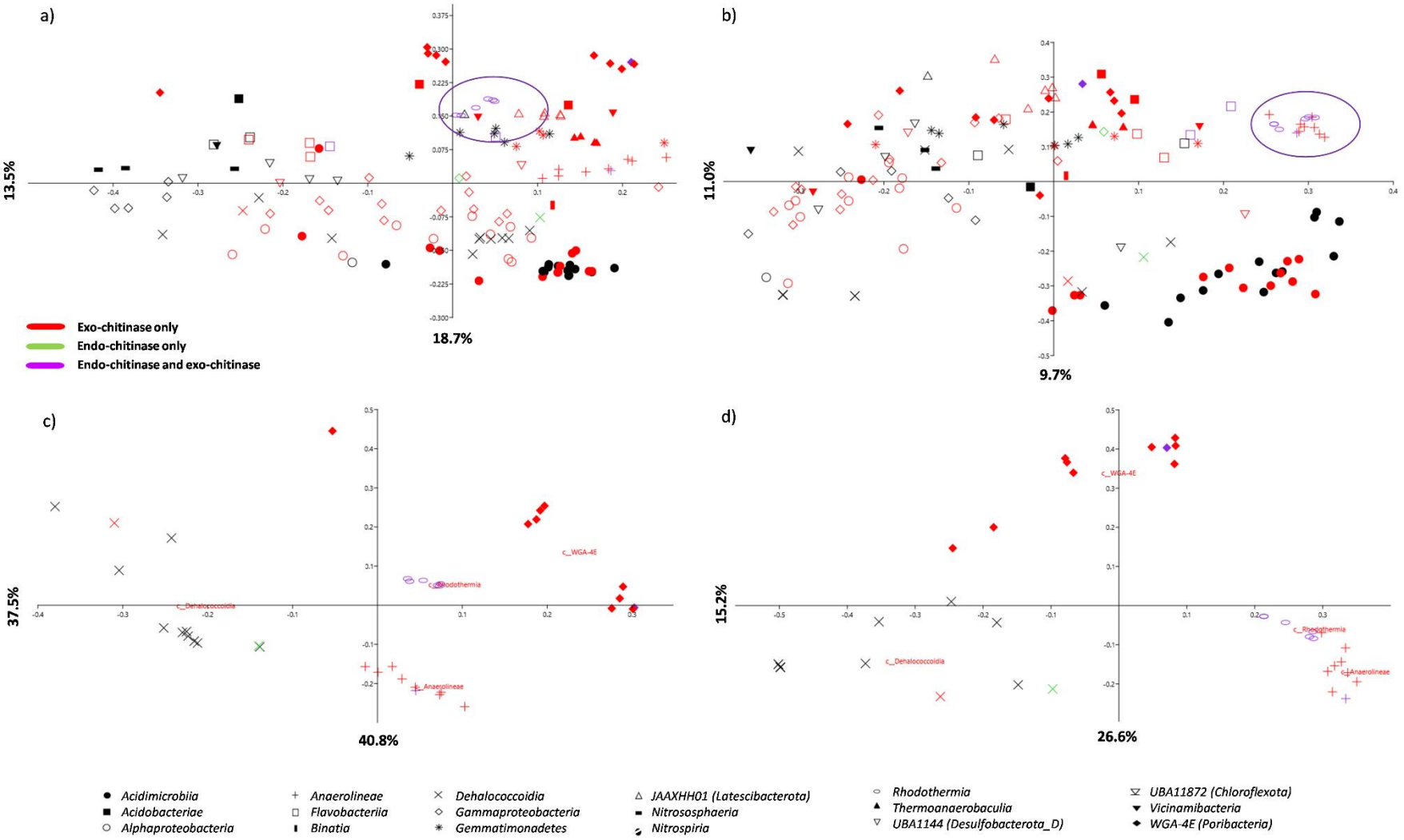
a)
b)
c)
d)
13.5%
18.7%
11.0%
9.7%
37.5%
40.8%
15.2%
26.6%
Exo-chitinase only
Endo-chitinase only
Endo-chitinase and exo-chitinase
c__WGA-4E
c__Rhodothermia
c__Dehalococcoidia
c__Anaerolineae
Acidimicrobiia
Acidobacteriae
Alphaproteobacteria
Anaerolineae
Flavobacteriia
Binatia
Dehalococcoidia
Gammaproteobacteria
Gemmatimonadetes
JAAXHH01 (Latescibacterota)
Nitrososphaeria
Nitrospiria
Rhodothermia
Thermoanaerobaculia
UBA1144 (Desulfobacterota_D)
UBA11872 (Chloroflexota)
Vicinamibacteria
WGA-4E (Poribacteria)

**Figure 4.2** Ordination analysis of MAG functional profiles based on Pfam and CAZymes annotations. The functional profiles of the MAGs were ordinated by means of Principal Coordinates Analysis (PCoA) with different annotation schemes. Figure 2a refers to functional profiles based on Pfam annotations for all the MAGs under study while Figure 2c presents the Pfam-based ordination of sponge-derived MAGs belonging to the classes that possess at least one MAG with endo-chitinase domains. Likewise, Figures 2b and 2d ordinate MAGs in the same fashion as described above, respectively, based on CAZymes annotations. Values on the diagrams' axes (principal coordinates) represent the percent of the total variation in the data explained by principal coordinates 1 (x-axis) and 2 (y-axis). The centroid position of each class is shown in the ordination diagram, with MAGs belonging to the class *Rhodothermia* encapsulated by a purple ellipse. For all graphs a-d, we colour-highlight MAGs possessing endo- and/or exo-chitinase encoding genes. MAGs presenting a coding potential for both endo and exo-chitinase domains are highlighted in purple, MAGs presenting endo-chitinase domains only are highlighted in green, and MAGs presenting exo-chitinase domains only are highlighted in red. MAGs for which neither endo- nor exo-chitinase encoding genes were found are shown in black

### 4.4.5 Other domains involved in chitin degradation processes

In addition to the endo- and exo-chitinase domains, **Tables 4.2, S4.2** and **S4.3** show results for the presence of polysaccharide deacetylase and N-acetylglucosamine utilization domains in the MAGs according to the Pfam annotations. Although these domains are not directly involved in the degradation of the chitin polymer into chitin oligosaccharides, they may still have an important role in polysaccharide turnover and chitin metabolism, either in the formation of chitosan or in the utilization of the chitin monomer N-acetylglucosamine. Congruent with their more generalist roles in carbon (besides chitin) metabolism, those domains were found to be putatively encoded by 107 MAGs in the case of polysaccharide deacetylases, and 73 MAGs in the case of N-acetylglucosamine utilization domains, representing 78.10% and 53.28% of the MAGs analysed, respectively (Table S3). The presence of deacetylase-encoding genes was slightly higher when considering only MAGs assembled from marine sponges, increasing

to 80.83%, whereas a similar value was observed for N-acetylglucosamine utilization in all MAGs (53.33% of the sponge-derived MAGs). Deacetylase domains were present in MAGs from 15 different classes, almost all the classes found in the community, except for *Thermoanaerobaculia*, *Nitrososphaeria* and *Nitrospiria* (**Table S4.3**). These results suggest that polysaccharide deacetylation is widespread across the marine sponge microbiome and can be a common process involved in chitin transformation within the animal host, contrasting with the narrower diversity spectrum within the endo-chitinolytic symbiotic consortium. We have noted previously that genes coding for polysaccharide deacetylases are enriched in the microbial metagenome of *S. officinalis* in comparison with seawater (Raimundo *et al.*, 2021). The observation that diverse symbiont lineages may perform polysaccharide deacetylation is consistent with the prevalence of this metabolic feature in the *Spongia*-associated microbiome. Moreover, N-acetylglucosamine utilization domains were found to be encoded by MAGs from 13 different classes, namely *Acidimicrobiia*, *Alphaproteobacteria*, *Anaerolineae*, *Bacteroidia*, *Binatia* (phylum *Desulfobacterota B*), *Dehalococcoidia*, *Gammaproteobacteria*, *Gemmatimonadetes*, JAAXHH01 (phylum *Latescibacterota*), *Nitrospiria*, *Rhodothermia*, UBA11872 (phylum *Chloroflexota*) and WGA-4E (phylum *Poribacteria*). Finally, all the MAGs classified as *Rhodothermia* possessed both the deacetylase and the N-acetylglucosamine utilization domains, suggesting that members of this class may, in fact, play a central and decisive role in chitin and polysaccharide metabolism in the sponge holobiont.

### 4.4.6 Phylogenetic analysis of endo-chitinase amino acid sequences

The phylogenetic diversification of GH18 endo-chitinases was assessed using amino acid sequences translated from GH18 encoding genes present on MAGs and bacterial

genomes from several bacterial classes. The sequences were selected with emphasis on coding sequences identified on the selected MAGs and their closest relatives (**Figure 4.3**, Supplementary **Table S4.4**). Particularly, we focused on the sponge-associated MAGs belonging to the order *Rhodothermales*, owing to the consistent pattern of occurrence of endo-chitinase encoding genes across these genomes. We further added the endo-chitinase sequences from our seawater-derived MAGs to determine their phylogenetic relationships with sponge-specific, *Rhodothermales* sequences.

The most conspicuous feature of the obtained tree topology was the formation of a major phylogenetic cluster, presenting 99% bootstrap support, composed exclusively of 13 endo-chitinase protein sequences from sponge-derived MAGs of the order *Rhodothermales*, which are placed apart from sequences of all MAGs belonging to the other classes included in the analysis, among which the *Flavobacteriia*, *Bacteroidia*, *Cytophagia*, and *Chitinophagia* classes of the *Bacteroidota* phylum (**Figure 4.3**, Cluster I). Therefore, Cluster I in **Figure 4.3** encompasses endo-chitinase protein sequences found exclusively among so-far unculturable, sponge-specific symbionts classified in the order *Rhodothermales*. Three subclusters were formed within cluster I, and endo-chitinase sequences retrieved from *Rhodothermales* MAGs in this study fell into two such subclusters, one including sequences from MAGs SO231 bin15 and SO232 bin18 and the other containing sequences from MAGs SA262g bin15, SO230 bin03, SO231 bin11 and SO233 bin05, indicating that two endo-chitinase phylotypes were found among the *Rhodothermales* symbionts (MAGs) identified in this study. The third subcluster contains the endo-chitinase sequence of bin80, assembled by Slaby *et al.* (2017) from the marine sponge *Aplysina aerophoba*, along with other three endo-chitinase sequences from sponge-derived MAGs. A high degree of sequence homology was observed within each subcluster of cluster I. Interestingly, Bin80 serves as the representative of a putative, so-

far undescribed family in the *Rhodothermales* order according with the current GTDB-tk taxonomy, with our *Rhodothermales* MAGs being affiliated with this lineage following the taxonomic assignment performed in this study (**Table 4.2**). Although endo-chitinase sequences may not serve as accurate phylogenetic markers owing to their proneness to horizontal gene transfer events or duplication and loss (Raimundo *et al.*, 2021, see more below), the close homology among endo-chitinase sequences from cluster I and their relatedness to the endo-chitinase sequence of bin80 supports the notion that this cluster encapsulates novel endo-chitinases so-far only found among *Rhodothermales* symbionts of marine sponges.

We further highlight other three robust phylogenetic clusters in **Figure 4.3** (Clusters II, III and IV), all of which have 100% bootstrapping support, whose internal topologies are more consistent with the known evolutionary trajectory of endo-chitinases, in which sequences from taxonomically disparate taxa eventually present high levels of homology, suggestive of horizontal gene transfer events (Raimundo *et al.*, 2021). Clusters II and IV are dominated by endo-chitinases from the *Flavobateriia* class, in which few endo-chitinases from the classes *Gammaproteobacteria* and *Cytophagia* (Cluster II) and from the classes *Alphaproteobacteria*, *Bacteroidia* and *Chitinophagia* (Cluster IV) are observed to present homology with *Flavobacteriia* endo-chitinases. A subcluster within Cluster IV, however, was composed exclusively by *Flavobacteriia* endo-chitinases (Figure 3). Cluster III, in turn, is a somewhat smaller cluster composed by four endo-chitinase sequences, three of which from the *Deltaproteobacteria* class and one from the *Myxococcia* class (phylum *Myxococcota*). Here, endo-chitinase sequences from *Cystobacter fuscus* and *Melittangium boletus*, particularly, were found to be closely related with one another (**Figure 4.3**).

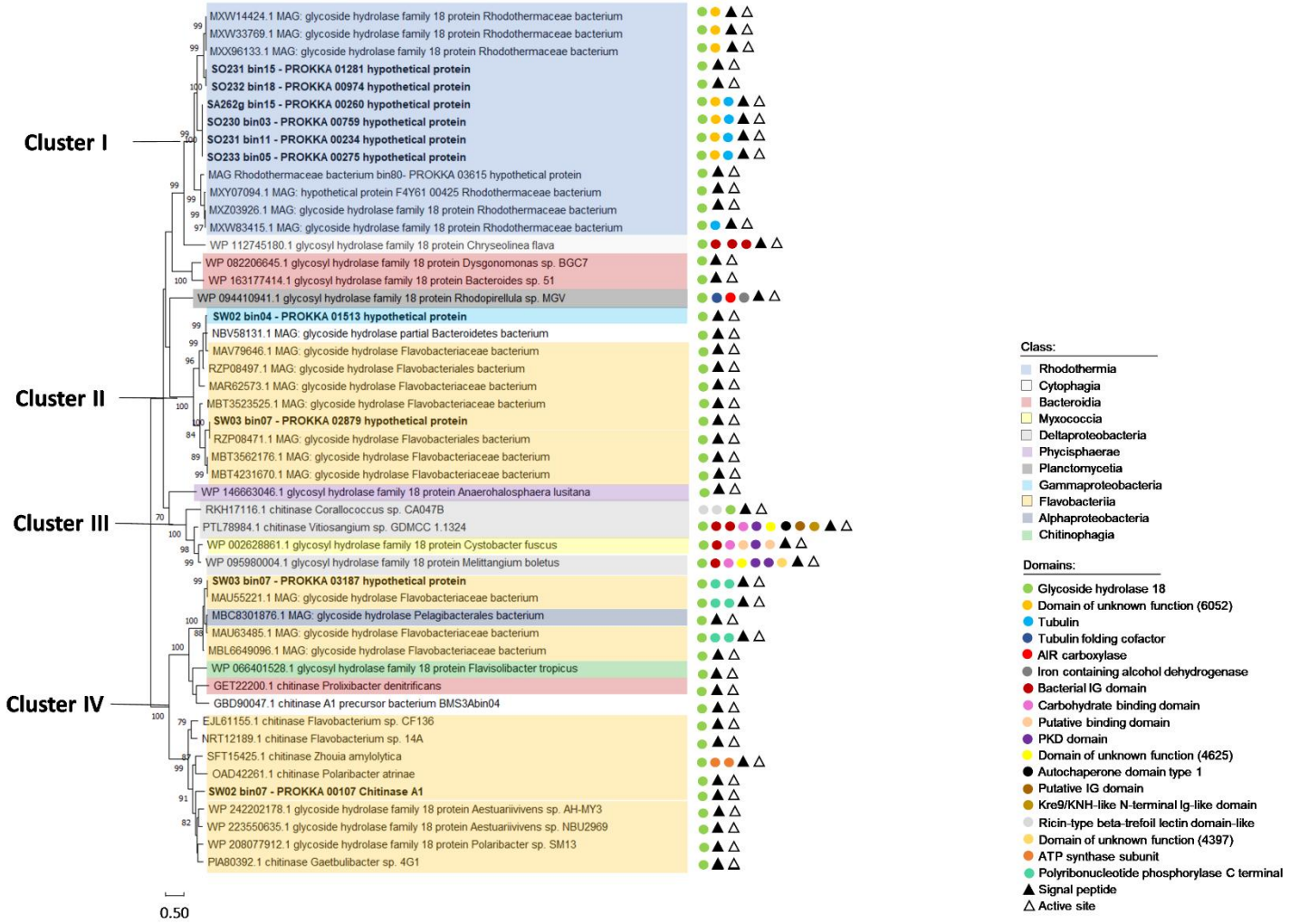
Cluster I
Cluster II
Cluster III
Cluster IV
MXW14424.1 MAG: glycoside hydrolase family 18 protein Rhodothermaceae bacterium
MXW33769.1 MAG: glycoside hydrolase family 18 protein Rhodothermaceae bacterium
MXX96133.1 MAG: glycoside hydrolase family 18 protein Rhodothermaceae bacterium
SO231 bin15 - PROKKA 01281 hypothetical protein
SO232 bin18 - PROKKA 00974 hypothetical protein
SA262g bin15 - PROKKA 00260 hypothetical protein
SO230 bin03 - PROKKA 00759 hypothetical protein
SO231 bin11 - PROKKA 00234 hypothetical protein
SO233 bin05 - PROKKA 00275 hypothetical protein
MAG Rhodothermaceae bacterium bin80- PROKKA 03615 hypothetical protein
MXY07094.1 MAG: hypothetical protein F4Y61 00425 Rhodothermaceae bacterium
MXZ03926.1 MAG: glycoside hydrolase family 18 protein Rhodothermaceae bacterium
MXW83415.1 MAG: glycoside hydrolase family 18 protein Rhodothermaceae bacterium
WP 112745180.1 glycosyl hydrolase family 18 protein Chryseolinea flava
WP 082206645.1 glycosyl hydrolase family 18 protein Dysgonomonas sp. BGC7
WP 163177414.1 glycosyl hydrolase family 18 protein Bacteroides sp. 51
WP 094410941.1 glycosyl hydrolase family 18 protein Rhodopirellula sp. MGV
SW02 bin04 - PROKKA 01513 hypothetical protein
NBV58131.1 MAG: glycoside hydrolase partial Bacteroidetes bacterium
MAV79646.1 MAG: glycoside hydrolase Flavobacteriaceae bacterium
RZP08497.1 MAG: glycoside hydrolase Flavobacteriales bacterium
MAR62573.1 MAG: glycoside hydrolase Flavobacteriaceae bacterium
MBT3523525.1 MAG: glycoside hydrolase Flavobacteriaceae bacterium
SW03 bin07 - PROKKA 02879 hypothetical protein
RZP08471.1 MAG: glycoside hydrolase Flavobacteriales bacterium
MBT3562176.1 MAG: glycoside hydrolase Flavobacteriaceae bacterium
MBT4231670.1 MAG: glycoside hydrolase Flavobacteriaceae bacterium
WP 146663046.1 glycosyl hydrolase family 18 protein Anaerohalosphaera lusitana
RKH17116.1 chitinase Corallococcus sp. CA047B
PTL78984.1 chitinase Vitiosangium sp. GDMCC 1.1324
WP 002628861.1 glycosyl hydrolase family 18 protein Cytobacter fuscus
WP 095980004.1 glycosyl hydrolase family 18 protein Melittangium boletus
SW03 bin07 - PROKKA 03187 hypothetical protein
MAU55221.1 MAG: glycoside hydrolase Flavobacteriaceae bacterium
MBC8301876.1 MAG: glycoside hydrolase Pelagibacterales bacterium
MAU63485.1 MAG: glycoside hydrolase Flavobacteriaceae bacterium
MBL6649096.1 MAG: glycoside hydrolase Flavobacteriaceae bacterium
WP 066401528.1 glycosyl hydrolase family 18 protein Flavisolibacter tropicus
GET22200.1 chitinase Prolixibacter denitrificans
GBD90047.1 chitinase A1 precursor bacterium BMS3Abin04
EJL61155.1 chitinase Flavobacterium sp. CF136
NRT12189.1 chitinase Flavobacterium sp. 14A
SFT15425.1 chitinase Zhouia amylolytica
OAD42261.1 chitinase Polaribacter atrinae
SW02 bin07 - PROKKA 00107 Chitinase A1
WP 242202178.1 glycoside hydrolase family 18 protein Aestuariivivens sp. AH-MY3
WP 223550635.1 glycoside hydrolase family 18 protein Aestuariivivens sp. NBU2969
WP 208077912.1 glycoside hydrolase family 18 protein Polaribacter sp. SM13
PIA80392.1 chitinase Gaetbulibacter sp. 4G1
0.50
Class:
Rhodothermia
Cytophagia
Bacteroidia
Myxococcia
Deltaproteobacteria
Phycisphaerae
Planctomycetia
Gammaproteobacteria
Flavobacteriia
Alphaproteobacteria
Chitinophagia
Domains:
Glycoside hydrolase 18
Domain of unknown function (6052)
Tubulin
Tubulin folding cofactor
AIR carboxylase
Iron containing alcohol dehydrogenase
Bacterial IG domain
Carbohydrate binding domain
Putative binding domain
PKD domain
Domain of unknown function (4625)
Autochaperone domain type 1
Putative IG domain
Kre9/KNH-like N-terminal Ig-like domain
Ricin-type beta-trefoil lectin domain-like
Domain of unknown function (4397)
ATP synthase subunit
Polyribonucleotide phosphorylase C terminal
Signal peptide
Active site

**Figure 4.3** Phylogenetic analysis of endo-chitinase amino acid sequences from MAGs obtained in this study and their closest relatives. GH18 endo-chitinase sequences were assessed spanning amino acid sequences translated from GH18 encoding genes present on MAGs and bacterial isolates' genomes. These sequences were selected with emphasis on coding sequences identified on the MAGs investigated in this study and their closest relatives, identified by blastp. For more information on sequences ID and homology, see Supplementary Table S4. The tree with the highest log likelihood is shown. The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. This analysis involved 49 amino acid sequences, from (at least) 11 taxonomic classes. Different classes are highlighted in different colors, according to the caption. Sequences that were not possible to determine the class are presented with no color background. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. Other protein domains identified on each sequence entry using hmmerscan are indicated by symbols next to the sequences. Note the presence of a signal peptide and an active site domain in each sequence.

In general, however, highly homologous endo-chitinase sequences from strains or species of the same class are consistently found across the tree, as was the case of the species *Zhouia amylolytica* and *Polaribacter atrinae*.

We further note that several endo-chitinase encoding sequences presented quite complex protein domain profiles. This is in agreement with previous observations of endo-chitinases usually displaying multiple protein domains arranged in several modes of synteny (Karlsson and Stenlid, 2009; Raimundo *et al.*, 2021) and related with various functions – e.g., carbohydrate- and putative-binding, tubulin, bacterial IG, autochaperone type I, ATP synthase and polyribonucleotide phosphorylase terminal domains, among others – some of them even unknown (**Figure 4.3**). Noticeably, the extent of within-class domain diversification of endo-chitinase sequences was relatively high for representatives of the *Deltaproteobacteria* class (Cluster III), with a maximum of nine domains per sequence. Conversely, protein domain diversification within endo-chitinase sequences was much lower among *Flavobacteriia* MAGs, with most of the sequences presenting only the GH18 domain. Finally, one major outcome of the individual

annotation of each GH18 coding sequence using hmmscan was the presence of one signal peptide and one active site domain across all sequences inspected, indicating protein functionality owing to the possibility of cross-membrane transport and the presence of a substrate binding site, respectively.

### 4.4.7 Adaptive features of potentially chitinolytic symbionts of marine sponges

To better understand the adaptive features of putative chitinolytic symbionts to life in association with sponges, a more detailed screening of the Pfam annotation database was conducted for the 12 MAGs presenting endo-chitinase encoding genes in this study, with focus on selected functions known to play a role in the establishment and persistence of sponge-prokaryote interactions, along with a few features observed to be characteristic of the *Rhodothermales* MAGs (**Table 4.3**, Supplementary **Figures S4.1-S4.3**). The selected functions are involved in numerous processes regulating host-microbe interactions such as host colonization and persistence (e.g., ELPs, TonB-dependent receptors), antiviral and mobile genetic elements defense mechanisms (CRISPR-Cas systems, endonucleases, toxin-antitoxin systems), cell metabolism and nutrition (sulfatases, cellulases, chondroitinases, heparinases), adherence to surfaces (Type IV secretion systems), genome rearrangement or genetic exchange (e.g., transposases). For a comprehensive analysis of these functions, several Pfam entries were considered and summed to obtain the final values detailed in **Table 4.3**. Following the Welch's t-tests for unequal variance performed in this study, 63, 56 and 55 Pfam features were significantly enriched in *Rhodothermales* MAGs in comparison with (i) the remaining sponge-derived MAGs, (ii) seawater derived MAGs and (iii) all other MAGs (**Figures S4.1-S4.3**). Among these, we highlight GH18 (endo-chitinase), GH20 (exo-chitinase), chondroitinase, heparinase, transposase and several carbohydrate binding module domains, along with leucine rich

repeat proteins, as typically enriched features of *Rhodothermales* symbionts of sponges with statistical support. Their frequency distributions are displayed in **Table 4.3** along with other distinctive *Rhodothermales* features uncovered using SIMPER. These features are discussed in more detail below.

**Table 4.3** Functional features enriched in marine sponge symbionts (MAGs) possessing endochitinase encoding genes in comparison with their planktonic counterparts.

| **Functional features** | **MAGs from marine sponges** | | | | | | | | | **MAGs from seawater** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | SA261gbin014_Poribacteria | SA261gbin023_Anaerolinea | SA265gbin011_Dehalococcoidia | SA262gbin015_Rhodothermia | SO230bin003_Rhodothermia | SO231bin011_Rhodothermia | SO231bin015_Rhodothermia | SO232bin018_Rhodothermia | SO233bin005_Rhodothermia | SW02bin004_Gammaproteobacteria | SW02bin007_Flavobacteriales | SW03bin007_Flavobacteriales |
| Tetratricopeptide repeats | 868 | 182 | 116 | 260 | 253 | 236 | 146 | 154 | 254 | 60 | 94 | 117 |
| Ankyrin repeats | 30 | 10 | 0 | 15 | 15 | 10 | 0 | 0 | 15 | 0 | 0 | 0 |
| WD40 repeats | 321 | 32 | 5 | 14 | 21 | 10 | 12 | 12 | 11 | 3 | 2 | 2 |
| Leucine-rich repeats | 3 | 0 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 |
| Type IV TA System | 61 | 63 | 31 | 26 | 27 | 34 | 36 | 32 | 29 | 14 | 14 | 23 |
| CRISPR-Cas proteins | 4 | 0 | 0 | 5 | 5 | 5 | 11 | 11 | 5 | 0 | 0 | 0 |
| Restriction endonucleases | 77 | 46 | 53 | 22 | 16 | 23 | 34 | 35 | 28 | 7 | 13 | 19 |
| Carboxypeptidase regulation | 95 | 9 | 16 | 180 | 174 | 147 | 145 | 144 | 181 | 40 | 72 | 124 |
| Sulfatases/Arylsulfatases | 76 | 35 | 1 | 41 | 40 | 45 | 26 | 26 | 42 | 20 | 4 | 44 |
| Cellulases | 0 | 4 | 0 | 4 | 4 | 1 | 1 | 1 | 4 | 1 | 0 | 4 |
| TonB-dependent receptors | 32 | 0 | 1 | 89 | 80 | 69 | 66 | 66 | 84 | 55 | 34 | 66 |
| Type IV secretion system | 2 | 1 | 2 | 6 | 8 | 9 | 8 | 8 | 9 | 7 | 2 | 3 |
| Transposases | 40 | 7 | 16 | 63 | 36 | 39 | 42 | 32 | 40 | 3 | 2 | 1 |
| Chondroitinase | 0 | 0 | 0 | 2 | 2 | 1 | 2 | 2 | 3 | 0 | 0 | 0 |
| Heparinase | 1 | 0 | 0 | 2 | 2 | 1 | 2 | 2 | 1 | 0 | 0 | 0 |

Values display Protein Family (Pfam) functional annotation results for the presence of protein domains related with the selected features. Within each feature, values correspond to the sum of protein domains assigned to multiple Pfam entries involved in that feature.

Compared with the seawater-derived, endo-chitinase-carrying MAGs of the *Gammaproteobacteria* and *Flavobacteriia* orders found in this study, we observed that the sponge-derived, endo-chitinase-containing MAGs, regardless of their taxonomic affiliation, were usually enriched for many of the abovementioned symbiosis features (**Table 4.3**). This is the case of several ELPs such as tetratricopeptide repeats (profusely enriched in the *Poribacteria* MAG), ankyrin repeats (except for two *Rhodothermia* MAGs and one *Dehalococcoidia* MAG) and WD40 repeats. ELPs are known to be involved in patterns of host-microbe recognition and evasion of bacterial symbionts from phagocytosis by the sponge host (Nguyen *et al.*, 2014; Reynolds and Thomas, 2016). Particularly, the abundance of tetratricopeptide and WD40 repeats among our chitinolytic sponge symbionts is coherent with the enrichment of these repeats in microbial metagenomes of sponges in the order Dictyoceratida, namely *Spongia officinalis* (Karimi *et al.*, 2017) and *Ircinia ramosa* (Engelberts *et al.*, 2020), and the reported dominance of *Poribacteria* and *Rhodothermales* symbionts in these species.

Likewise, type IV Toxin-antitoxin (TA) and clustered regularly interspaced short palindromic repeat-associated protein (CRISPR-Cas) systems are, along with restriction endonucleases, altogether more abundant among endo-chitinase encoding MAGs retrieved from sponges than from seawater (**Table 4.3**). Particularly the presence of CRISPR-Cas systems was observed to be a pronounced feature of the *Rhodothermia* symbionts within this select group of MAGs (**Table 4.3**). These metabolic features are suggested to be important factors underpinning microbe-sponge symbiosis by presumably allowing the symbionts to colonize the host and persist by avoiding viral infection and eventual microbiome dysbiosis through external acquisition of other mobile genetic elements such as plasmids (Robbins *et al.*, 2021).

Finally, we observed a consistent pattern of occurrence and enriched abundance of Type IV secretion (possibly involved in cell adherence and host colonization processes), TonB-dependent receptors (which coordinate outer membrane transport processes - Fujita *et al.*, 2019 - and may play a role in host invasion), transposase, cellulase, chondroitinase and heparinase encoding genes across sponge-derived *Rhodothermia* MAGs. Specifically, the finding of the latter three coding features in *Rhodothermia* MAGs, together with a large abundance of sulfatase-encoding genes, enable novel insights into the ecophysiology of these organisms and their participation in the utilization of compounds present in the extracellular matrix of sponges. Intriguingly, cellulases may be used by these symbionts to further degrade chitosan into monomers of glucose (Xia, Liu and Liu 2008). Alternatively, bacterial symbionts of marine sponges may use cellulases to degrade the cell walls of algae and microalgae, or remnants thereof, which reach the internal sponge body via filter-feeding activities of the animal (Gili and Coma 1998). Heparinases and chondroitinases are known for their role in cell metabolism by delivering disaccharide and oligosaccharide products (including disaccharide chains of N-acetylglucosamine) through the degradation of the glycosaminoglycans (GAGs) heparin and heparan sulfate (Boyce and Walsh, 2022) and chondroitin sulfate (Tkalec *et al.*, 2000), respectively. GAGs are highly complex sulfated polysaccharides with diverse biological functions (Zhang *et al.*, 2010) known to abound in the extracellular matrix of sponges forming glycoprotein complexes (Fernàndez-Busquets and Burger, 2003; Kamke *et al.*, 2013). Heparin-like sulfated polysaccharides and chondroitin sulfates have been documented as structural components of marine sponges (Marques *et al.*, 2016; Vázquez *et al.*, 2013). The arsenal of sulfatases, heparinases and chondroitinases encoded by *Rhodothermia* symbionts of marine sponges hints at a pivotal role of these organisms in the utilization of complex carbohydrate sources and peptidoglycans that make up the

sponge mesohyl, raising intriguing questions for further investigations regarding possible animal-microbe cross-feeding and the positioning of this symbiosis along the parasitic-mutualistic continuum.

Enrichment of specific glycolytic enzymes (GH families) among marine sponge symbionts was observed by Robbins *et al.*, (2021) in a recent analysis of thousands of MAGs from coral reef marine sponges as well as in previous studies dedicated to the analysis of specific sponge-enriched lineages such as members of the *Poribacteria* and *Chloroflexota* phyla (e.g., Astudillo-García *et al.*, 2017; Bayer *et al.*, 2018; Kamke *et al.*, 2013), reflecting specific functional guilds capable of aiding in the degradation of reef dissolved organic matter. Congruent with these findings, we here describe the coding potential and putative ecophysiology of a notably elusive sponge-specific symbiont clade to reveal a remarkable potential of these organisms to perform polysaccharide degradation within marine sponges and, by extension, a presumably key role in carbon, nitrogen, and sulfur cycling in benthic ecosystems.

The distribution of genes encoding ELPs, polysaccharide degrading enzymes, restriction endonucleases, and CRISPRs across distantly related taxa suggests that they either derive from a common ancestor or that they were acquired by lateral gene transfer events; the latter potentially mediated by mobile genetic elements, known to be enriched in sponge-associated microbial communities (Pita *et al.*, 2018). In fact, many of the enriched genes are known to be laterally transferred between microbial lineages, suggesting that this mechanism is important for conferring a selective advantage to specific sponge-associated bacteria (Robbins *et al.*, 2021).

### 4.4.8 Whole genome-based classification of novel, putative chitinolytic *Rhodothermales* species associated with keratose marine sponges

Whole-genome sequence analysis of the *Rhodothermales* order comprised 52 genomes, including the six sponge-derived MAGs retrieved in this study (one MAG originating from *S. agaricina* - SA262bin015 - and five MAGs from *S. oficinallis* - SO230bin03, SO231bin011, SO231bin015, SO232bin18 and SO233bin05). The remainder of genome sequences (n = 46) were publicly available isolate-derived genomes (n = 10) representing all formally described families (*Rhodothermaceae*, *Rubricoccaceae*, *Salinibacteraceae* and *Salisaetaceae*) and genera within the *Rhodothermales* order, in addition to MAGs (n = 36) from diverse sources such as sponges, corals, seawater, and sediments (**Figure 4.4**). Many of these latter MAGs were found to be unclassifiable at the family level and fell into clusters represented exclusively by uncultured bacteria.

Most of the isolate-derived genome taxa within the *Rhodothermales* order were confined to two major clusters containing only cultured representatives except for the *Longibacter salinarum* strain that was placed in-between large MAG-specific clusters (**Figure 4.4**). Indeed, in only five out of 206 pairwise combinations did the isolate genomes and MAGs display ANI values higher than 75% (Supplementary **Table S4.5**), supporting their separation on the whole-genome cladogram (**Figure 4.4**). Additionally, the cladogram topology revealed three well delineated sponge-specific clusters (SSC), identified as clusters I, II and III in Figure 4. The first cluster (SSC I) was formed exclusively by MAGs identified in this study, being supported by bootstrap analysis, and separated from the coral- and sediment-specific MAG clusters that formed their sister group. ANI values obtained for the genomes present in SSC I were very high and around 99% in general, ranging from 98.3124% between SA262gbin015 and SO231bin011 to 99.8831% between SO233bin05 and SO230bin03. These values are well above the 95%

ANI threshold used to categorize prokaryotic strains as members of the same species (Barco *et al.*, 2020; Jain *et al.*, 2017). Therefore, MAGs from SSC I comprise strains of the same species and likely represent, as well, a novel genus and family in the *Rhodothermales* order, since these MAGs did not share ANI values with other genomes larger than the boundaries suggested by Zhang *et al.* (2014) for two strains in the same genus (ANIs > 83.6%) or for two strains in the same family (ANIs > 78.9%). Notably, the highest ANI values obtained between MAGs in SSC I and any other genome in the tree were 76.66% and 77.11%, observed for two of our MAGs in this cluster and the genome sequence ASM1227120v1_JAAXGT010000191.1_MAG_*Rhodothermales*_bacterium derived from the coral host *Porites lutea*, in line with the tree topology shown in **Figure 4.4**. Interestingly, the patterns of relatedness between SSC I genomes and the coral- and sediment-derived genome clusters that branched out of SSC I suggest a process of adaptive radiation of specific, closely related *Rhodothermales* bacteria into different marine biotopes. Based on these findings, we here propose a novel candidate taxon denoted family *Candidatus* Spongiachitinolyticaceae to refer to the provenance (specific to sponges of the genus *Spongia*) and the coding potential for chitin hydrolysis revealed for symbionts belonging to SSC I (**Figure 4.4**).

The second sponge-specific, exclusively uncultivated, *Rhodothermales* cluster observed in our tree (SSC II, **Figure 4.4**) did not contain any of the *S. officinalis* nor *S. agaricina*-derived MAGs assembled in our study. Instead, MAGs present in this cluster derive from the sponge hosts *Ircinia* sp., *Ircinia campana*, and *Ircina felix* collected at several geographical locations over the Mesoamerican Barrier Reef (Caribbean Sea). As observed for SSC I, pairs composed by two genome sequences from SSC II presented high ANI values varying from 94.42% to 97.90% (**Table S4.5**), all of them similar to or above the accepted threshold for classification into the same species (95%). Conversely,

only one MAG sequence from SSC II was found to display ANI higher than 75% with one sequence outside SSC II itself, that of *Rhodothermus marinus* DSM_4252 (75.8969% ANI, **Table S4.5**). Altogether, these data suggest that MAGs in SSC II may as well represent a new family in the *Rhodothermales* order following the current thresholds for genus and family delineations based on ANI values (Zhang *et al.*, 2014). Intriguingly, MAGs from SSC II were assembled from specimens belonging to the genus *Ircinia* (family Ircniidae) of the Dictyoceratida order of keratose sponges, the same of the sponge species analysed in our study (genus *Spongia*, family Spongiidae). Therefore, in this study we propose a second, novel candidate family termed *Candidatus* Irciniachitinolyticaceae highlighting the provenance (so-far specific to sponges of the genus *Ircinia*) and potential for chitin hydrolysis revealed for symbionts belonging to SSC II (**Figure 4.4**).

Finally, the SSC III group was composed of several MAGs including two MAGs from *S. oficinallis* obtained in this study in addition to many MAGs deriving from the same *Ircinidae* hosts as in SSC II except for the presence of bin80, which derives from the marine sponge *Aplysina aerophoba*. We observed the formation of three different sub-clusters within SSC III (**Figure 4.4**) which corroborated ANI estimates very well (**Table S4.5**). While the first subcluster contained the genome sequences of bin80 from *Aplysina aerophoba* (Adriatic Sea) and of one *Ircinia*-derived MAG (Caribbean Sea) the other two subclusters each contain MAGs deriving exclusively from the same host genus, namely *Spongia* and *Ircinia*, respectively. Pairs of MAGs belonging to the *Ircinia*-specific subcluster presented 94-97% ANI, while pairs composed by MAGs in the *S. officinalis*-specific subcluster presented around 99% ANI. However, ANI values ranging from 76-79% were obtained for genome pairs composed by genome sequences belonging to any of the different subclusters described above. These values and the tree topology obtained for SSC III revealed more genome heterogeneity within this cluster in comparison with

SSCs I and II. More genomes/data are needed to better address hypotheses favoring the existence of host species-specific *Rhodothermales* clades within SSC III and whether which subcluster within it may be truly representative of different families.

In summary, our genome-wide assessments consistently revealed the existence of diverse clusters of so-far unculturable *Rhodothermales* symbionts of marine sponges likely representing novel genera and even families within this bacterial order. They further suggest that *Rhodothermales* symbionts of Dictyoceratida sponges may possess a global distribution and are diversified into distinct lineages resulting from independent host- and geographical-driven evolutionary trajectories. The trends acquired thus far are indicative of genome-wide diversification within uncultured *Rhodothermales* bacteria possibly responding to environmental and host-associated life histories (sediments vs. corals vs. sponges, and *Ircinia* vs. *Spongia* genome clusters). However, the extent to which *Rhodothermales* symbionts diversify and evolve in response to their environmental/geographical context and their host species requires thorough biogeographical assessments, including a larger number of *Rhodothermales* genomes from multiple hosts and biogeographical backgrounds, remaining an intriguing research question for the future. These discoveries open new avenues to the study of *Rhodothermota* taxonomy and the roles played by such elusive bacteria in the marine environment. With respect to chitin degradation capacities in particular, future genome mining should further inspect the extent to which this function is conserved among symbionts of marine sponges (as suggested by **Figure 4.3**) and other relevant multicellular hosts such as corals and algae, allowing researchers to better evaluate the contribution of *Rhodothermales* symbionts to host homeostasis and biogeochemical cycling across benthic ecosystems at large.

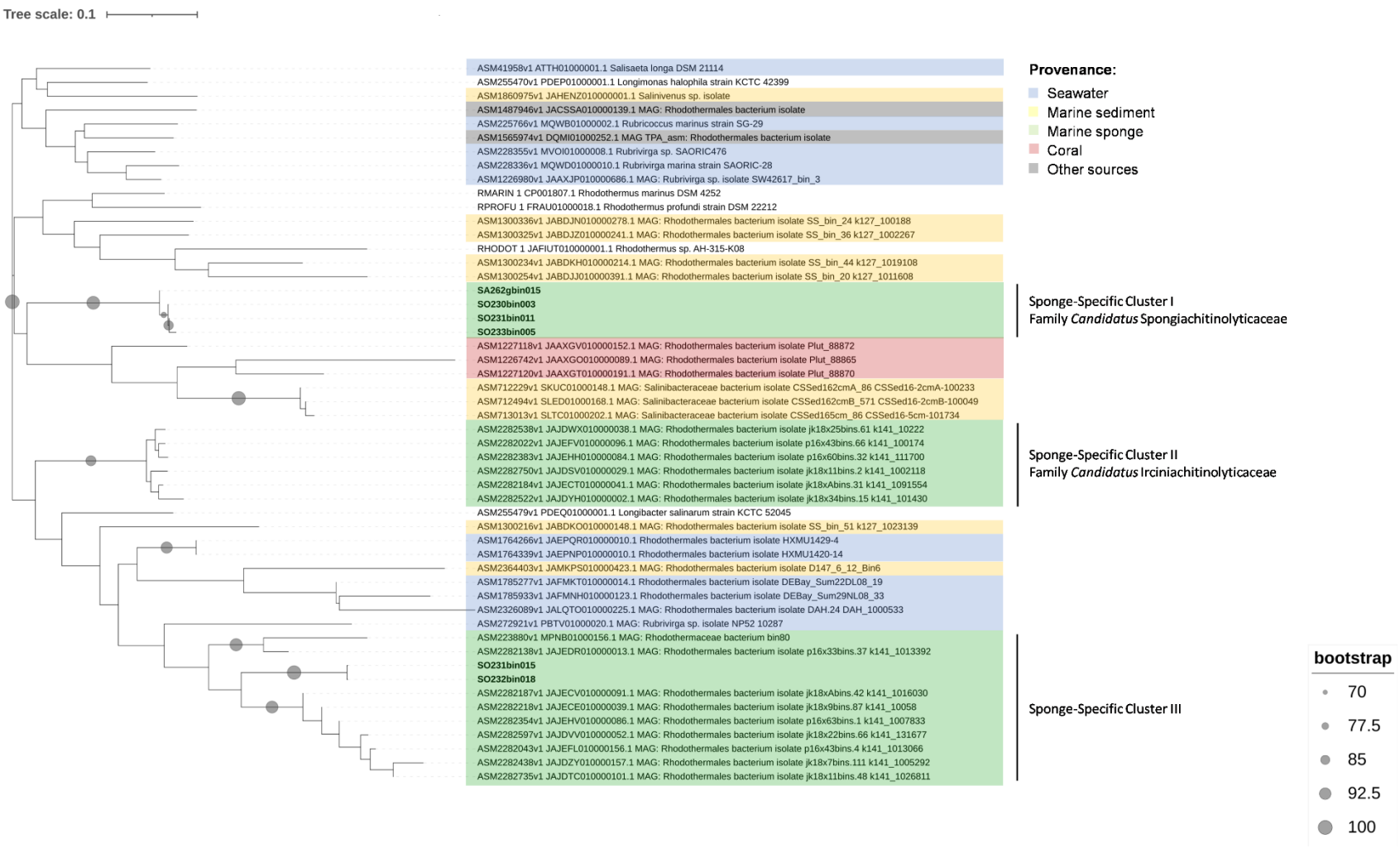
Tree scale: 0.1
ASM41958v1 ATTH01000001.1 Salisaeta longa DSM 21114
ASM255470v1 PDEP01000001.1 Longimonas halophila strain KCTC 42399
ASM1860975v1 JAHENZ010000001.1 Salinivenus sp. isolate
ASM1487946v1 JACSSA010000139.1 MAG: Rhodothermales bacterium isolate
ASM225766v1 MQWB01000002.1 Rubricoccus marinus strain SG-29
ASM1565974v1 DQMI01000252.1 MAG TPA_asm: Rhodothermales bacterium isolate
ASM228355v1 MVOI01000008.1 Rubrivirga sp. SAORIC476
ASM228336v1 MQWD01000010.1 Rubrivirga marina strain SAORIC-28
ASM1226980v1 JAAXJP010000686.1 MAG: Rubrivirga sp. isolate SW42617_bin_3
RMARIN 1 CP001807.1 Rhodothermus marinus DSM 4252
RPROFU 1 FRAU01000018.1 Rhodothermus profundi strain DSM 22212
ASM1300336v1 JABDJN010000278.1 MAG: Rhodothermales bacterium isolate SS_bin_24 k127_100188
ASM1300325v1 JABDJZ010000241.1 MAG: Rhodothermales bacterium isolate SS_bin_36 k127_1002267
RHODOT 1 JAFIUT010000001.1 Rhodothermus sp. AH-315-K08
ASM1300234v1 JABDKH010000214.1 MAG: Rhodothermales bacterium isolate SS_bin_44 k127_1019108
ASM1300254v1 JABDJJ010000391.1 MAG: Rhodothermales bacterium isolate SS_bin_20 k127_1011608
SA262gbin015
SO230bin003
SO231bin011
SO233bin005
ASM1227118v1 JAAXGV010000152.1 MAG: Rhodothermales bacterium isolate Plut_88872
ASM1226742v1 JAAXGO010000089.1 MAG: Rhodothermales bacterium isolate Plut_88865
ASM1227120v1 JAAXGT010000191.1 MAG: Rhodothermales bacterium isolate Plut_88870
ASM712229v1 SKUC01000148.1 MAG: Salinibacteraceae bacterium isolate CSSed162cmA_86 CSSed16-2cmA-100233
ASM712494v1 SLED01000168.1 MAG: Salinibacteraceae bacterium isolate CSSed162cmB_571 CSSed16-2cmB-100049
ASM713013v1 SLTC01000202.1 MAG: Salinibacteraceae bacterium isolate CSSed165cm_86 CSSed16-5cm-101734
ASM2282538v1 JAJDWX010000038.1 MAG: Rhodothermales bacterium isolate jk18x25bins.61 k141_10222
ASM2282022v1 JAJEFV010000096.1 MAG: Rhodothermales bacterium isolate p16x43bins.66 k141_100174
ASM2282383v1 JAJEHH010000084.1 MAG: Rhodothermales bacterium isolate p16x60bins.32 k141_111700
ASM2282750v1 JAJDSV010000029.1 MAG: Rhodothermales bacterium isolate jk18x11bins.2 k141_1002118
ASM2282184v1 JAJECT010000041.1 MAG: Rhodothermales bacterium isolate jk18xAbins.31 k141_1091554
ASM2282522v1 JAJDYH010000002.1 MAG: Rhodothermales bacterium isolate jk18x34bins.15 k141_101430
ASM255479v1 PDEQ01000001.1 Longibacter salinarum strain KCTC 52045
ASM1300216v1 JABDKO010000148.1 MAG: Rhodothermales bacterium isolate SS_bin_51 k127_1023139
ASM1764266v1 JAEPQR010000010.1 Rhodothermales bacterium isolate HXMU1429-4
ASM1764339v1 JAEPNP010000010.1 Rhodothermales bacterium isolate HXMU1420-14
ASM2364403v1 JAMKPS010000423.1 MAG: Rhodothermales bacterium isolate D147_6_12_Bin6
ASM1785277v1 JAFMKT010000014.1 Rhodothermales bacterium isolate DEBay_Sum22DL08_19
ASM1785933v1 JAFMNH010000123.1 Rhodothermales bacterium isolate DEBay_Sum29NL08_33
ASM2326089v1 JALQTO010000225.1 MAG: Rhodothermales bacterium isolate DAH.24 DAH_1000533
ASM272921v1 PBTV01000020.1 MAG: Rubrivirga sp. isolate NP52 10287
ASM223880v1 MPNB01000156.1 MAG: Rhodothermaceae bacterium bin80
ASM2282138v1 JAJEDR010000013.1 MAG: Rhodothermales bacterium isolate p16x33bins.37 k141_1013392
SO231bin015
SO232bin018
ASM2282187v1 JAJECV010000091.1 MAG: Rhodothermales bacterium isolate jk18xAbins.42 k141_1016030
ASM2282218v1 JAJECE010000039.1 MAG: Rhodothermales bacterium isolate jk18x9bins.87 k141_10058
ASM2282354v1 JAJEHV010000086.1 MAG: Rhodothermales bacterium isolate p16x63bins.1 k141_1007833
ASM2282597v1 JAJDVV010000052.1 MAG: Rhodothermales bacterium isolate jk18x22bins.66 k141_131677
ASM2282043v1 JAJEFL010000156.1 MAG: Rhodothermales bacterium isolate p16x43bins.4 k141_1013066
ASM2282438v1 JAJDZY010000157.1 MAG: Rhodothermales bacterium isolate jk18x7bins.111 k141_1005292
ASM2282735v1 JAJDTC010000101.1 MAG: Rhodothermales bacterium isolate jk18x11bins.48 k141_1026811
Provenance:
Seawater
Marine sediment
Marine sponge
Coral
Other sources
Sponge-Specific Cluster I
Family Candidatus Spongiachitinolyticaceae
Sponge-Specific Cluster II
Family Candidatus Irciniachitinolyticaceae
Sponge-Specific Cluster III
bootstrap
70
77.5
85
92.5
100

**Figure 4.4** Whole-genome cladogram of the *Rhodothermales* order. The cladogram was built with Mashtree v1.2.0 and is based on the pairwise distance between genome sketches composed by the top k-mers, created and sorted with the min-hash algorithm and constructed with the neighbour-joining method. The tree is drawn to scale and represents the k-mer-based similarities of 52 *Rhodothermales* genome sequences including representatives of all its formally accepted families. Grey dots on the cladologram nodes refer to bootstrap support of >70%, and larger dots indicate increasing bootstrap support from 70 to 100%. The six *Rhodothermales* MAGs obtained in this study are highlighted in bold, and a "MAG" acronym was used to label all other genome sequences assembled from metagenomes present in the cladogram. The remainder of the sequences, which do not present any specific designation along with the strain identification, represent isolate genomes. Besides our MAGs, all other *Rhodothermales* genomes (n=46) were publicly available on NCBI. Assembly accession numbers are given with strain identification. *Rhodothermales* genomes derived from the microbiomes of different sources, including marine sponges (highlighted in green), corals (highlighted in coral), marine sediments (highlighted in yellow) and other sources such as waste-water or hydrothermal plumes (highlighted in grey) are colour-coded. For the genomes whose provenance was not possible to determine, no color background was attributed.

## 4.5 Conclusions

Although the marine sponge microbiome cannot be seen as an isolated entity separated from its environmental vicinities, it seems to be capable of fully processing chitin and reutilizing the chitin degradation derivatives within the community itself. Indeed, all the enzymes required to degrade chitin to N-acetylglucosamine and to utilize N-acetylglucosamine further in cell metabolism are present within the sponge symbiotic consortium. Marine biotopes and transitional ecotones are supposedly strongly interconnected by exchanges and fluxes of energy, matter, and organisms. Various forms of chitin-derived products, involving COS of diverse molecular weights, fibrillar arrangements and contexts, as well as chitosan types of various degrees of deacetylation, may be laterally acquired by the sponge host via filtration. Such inter-environmental connectivity, where countless products and nutrients can reach the community through host-driven pumping activity, may function as an important source of energy for marine sponge symbionts which lack the ability to degrade the large chitin polymer via hydrolysis but can utilize COS and N-acetylglucosamine. Notwithstanding, possible substrate cross-

feeding cascades dictating the fate of chitin and chitin derivatives within the holobiont are not to be ruled out, and may co-occur with lateral acquisition of smaller chitin derivatives to feed the diverse community of symbionts capable of utilizing these substrates. Furthermore, our findings indicate that endo-chitinolytic activity within the marine sponge microbiome is a bottleneck function involving a narrow range of symbiont taxa, while several other members can perform more generalist functions pertaining to chitin metabolism such as exo-chitinolytic activity, deacetylation, and N-acetylglucosamine utilization. Our study suggests that members of the *Rhodothermales* order in the *Rhodothermia* class are the most consistent chitin degraders within the community. The extent to which this specialized function – that is, endo-chitinolytic activity - makes *Rhodothermales* symbionts keystone species within the marine sponge microbiome remains to be elucidated, and will largely depend on how integrated chitin substrate cross-feeding cascades are among symbionts, and on the importance of chitin hydrolysis as a pivotal metabolic pathway in the recycling of chitin within the sponge host. In fact, chitin has been identified as a major structural component of the skeleton of various sponge species (Ehrlich *et al.*, 2018; Ehrlich *et al.*, 2007a; Ehrlich *et al.*, 2007b). Versatile polysaccharide degraders such as *Poribacteria* and *Chloroflexi* have been suggested to play a role in recycling organic compounds in the extracellular mesohyl matrix of marine sponges (Bayer *et al.*, 2018; Kamke *et al.*, 2013), and analogous roles may be proposed for the utilization of chitin by bacterial symbionts within these animals. Further, cross-feeding cascades possibly maintained by endo-chitinolytic bacteria or, alternatively, fueled by lateral acquisition of chitin derivatives, can be even larger and more complex if chitosan, the deacetylation product of chitin, is taken into account. Chitosan may be further degraded by other enzymes such as cellulases for energy acquisition via the utilization of glucose monomers, and cellulase-encoding genes were

also found to be present in some MAGs analyzed in this study, most consistently among *Rhodothermales* and *Anaerolineae* symbionts.

Taken together, our results suggest an unanticipated role of *Rhodothermales* symbionts in chitin and general polysaccharide metabolism within sponges. Evidence for this role is supported by the consistent observation of endo-chitinase, exo-chitinase, polysaccharide deacetylase and N-acetylglucosamine utilization encoding genes across all *Rhodothermales* MAGs uncovered in this study, which was unmatched by any other group of sponge symbionts examined, including those also possessing some potential for endo-chitinolytic activity. Several symbiotic features known to be widespread across quintessential marine sponge symbionts from many taxonomic groups were recorded in this study for all *Rhodothermales* MAGs, along with a few additional specific functions particularly enriched in these symbionts in comparison with other classes, hinting at possible niche differentiation processes underpinning the evolution of a sponge-enriched clade which to date has been regarded as possessing a rather non-specialized metabolism within the complex marine sponge microbiome (Slaby *et al.*, 2017). It has been argued that functional characterization of symbiont communities inhabiting marine sponges has been restricted to just a few lineages of interest, potentially biasing our view of sponge symbiosis and the microbes which can play a role in these relationships (Robbins *et al.*, 2021). It remains to be understood whether the endo-chitinolytic potential uncovered here is a conserved feature of *Rhodothermia* symbionts across a large diversity of sponge species. Future, synthetic biology surveys aiming at expressing chitinases encoded by *Rhodothermia*, *Chloroflexi,* and *Poribacteria* symbionts hold promise in shedding light on their structures, functionality, and substrate affinities. Likewise, meta-transcriptomic and stable isotope probing analyses may help strengthen the hypotheses raised here and

elsewhere on the potential for chitin degradation and utilization by the symbiotic consortium of sponges and corals (Keller-Costa *et al.*, 2022; Raimundo *et al.*, 2021).

### 4.6 Supplementary Information

High resolution figures and supplementary information pertaining to this chapter can be found online at:

https://drive.google.com/drive/folders/16MraFJqb0KiDAtLFMuDTx7FT-xpTPD0w?usp=sharing

**Figure S4.1.** Protein family (Pfam) features (*N*=63) significantly enriched (*q*-value < 0.05) in the *Spongia*-derived *Rhodothermales* MAGs (*N*= 6, orange), compared with all other MAGs obtained from *Spongia* spp. (*N*=114, green) in this dataset. A one-sided, Welch's t-test for unequal variances was performed with the STAMP (Statistical Analysis of Metagenomic Profiles) v.2.1.3 software. Multiple test correction was performed using the Benjamini-Hochberg correction (FDR). To further limit the displayed number of significant entries, an effect size filter was also applied, setting the "ratio of proportions" to 10.00. **Figure S4.2.** Protein family (Pfam) features (*N*=56) significantly enriched (*q*-value < 0.05) in the *Spongia*-derived *Rhodothermales* MAGs (*N*= 6, orange), compared with all MAGs obtained from Seawater samples (*N*=17, blue) in this dataset. A one-sided, Welch's t-test for unequal variances was performed with the STAMP (Statistical Analysis of Metagenomic Profiles) v.2.1.3 software. Multiple test correction was performed using the Benjamini-Hochberg correction (FDR). To further limit the displayed number of significant entries, an effect size filter was also applied, setting the "ratio of proportions" to 10.00. **Figure S4.3.** Protein family (Pfam) features (*N*=55) significantly enriched (*q*-value < 0.05) in the *Spongia*-derived *Rhodothermales* MAGs (*N*= 6, orange), compared with all other MAGs (*N*=131, purple) obtained in this dataset. A one-sided, Welch's t-test

for unequal variances was performed with the STAMP (Statistical Analysis of Metagenomic Profiles) v.2.1.3 software. Multiple test correction was performed using the Benjamini-Hochberg correction (FDR). To further limit the displayed number of significant entries, an effect size filter was also applied, setting the "ratio of proportions" to 10.00. **Table S4.1**. Overview of the number and quality of MAGs obtained from sponge- and seawater-derived microbial metagenome samples. **Table S4.2**. General features of the 137 metagenome-assembled genomes (MAGs) obtained from sponge- and seawater-derived microbial metagenomes and analysed in this study, including Pfam annotations for functions related with chitin metabolism. **Table S4.3**. Annotation of Pfam entries related with endo-chitinase, exo-chitinase, deacetylase and N-acetylglucosamine utilization domains across all MAGs assembled in this study. **Table S4.4**. Top-five endo-chitinase amino acid sequences present in public databases closest to endo-chitinase sequences retrieved from the MAGs analysed in this study. **Table S4.5**. Average nucleotide identity estimates (ANI) for pairs of MAGs Rhodothermales MAGs subjected to phylogenomics assessments.

**Data availability**

Microbial Metagenome data from *Spongia agaricina* were deposited in the European Nucleotide Archive (ENA) under the study accession number PRJEB35088. *S. officinalis* and seawater metagenome data are available under PRJEB11585. SRA accession numbers are provided in Table 1.

**Author contributions**

R.C. designed the study and administered the project. R.C., J.M.S.G. and C.J.C. provided resources. R.C., C.J.C., and T.K.C. supervised the work. R.S. processed and

analysed the data, and prepared tables and figures. R.S. wrote the first manuscript draft. R.S. and R.C. revised the first draft. All authors edited and revised the manuscript and approved its final version.

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**Ethics approval and consent to participate**

This article does neither contain any studies with human participants nor vertebrate animals or cephalopods performed by any of the authors. This study was exempt from ethical approval procedures according to the current Portuguese legislation. All procedures involving animals were in accordance with the ethical standards of the institution (Centre of Marine Sciences (CCMAR), Faro, Portugal) at which the sponge samples were processed. This study did not involve endangered or protected sponge species (according to the IUCN red list of threatened species, 02/11/2019:

http://www.iucnredlist.org/searc). The sampling of sponges did not occur within privately owned or protected areas. Sampling procedures were minimally intrusive and preserved sponges at the field site.

**Conflict of Interest**

The authors declare no conflict of interest.

# Chapter 5

## General Discussion and Conclusions

## General Discussion and Conclusions

In this thesis, a polyphasic approach was employed to examine bacterial communities of marine sponges and octocorals involved in chitin degradation and in the utilization of chitin-derived products. This approach combined cultivation-dependent and independent methods and proved to be useful in providing complementary and novel information regarding chitinoclastic processes in marine ecosystems that would not be possible to retrieve with only one type of methodology.

The cultivation-dependent strategy, described in Chapter 3, revealed bacterial strains classified as *Vibrio* and *Aquimarina* as the main culturable degraders of chitin, while culturable *Alphaproteobacteria* strains were identified as consumers of smaller oligomers and N-acetylglucosamine, enabling hypotheses to be raised on possible substrate cross-feeding within these animal-associated communities.

Cultivation-independent methods (employed in chapters 3 and 4) revealed that other symbiont taxa may also engage in chitin degradation within these communities. Such taxa fundamentally represent so-far uncultivated, novel bacterial lineages not yet classified at species, genus, family and, often times, even order levels in the classes *Anaerolineae* and *Dehalococcoidia* (phylum *Chloroflexota,* formerly *Chloroflexi*), WGA-4E (phylum *Poribacteria*), *Rhodothermia* (phylum *Rhodothermota*, formerly *Bacteroidota*/*Bacteroidetes*), *Flavobacteriia* (phylum *Bacteroidota*, formerly *Bacteroidetes*) *Gammaproteobacteria* (phylum *Pseudomonadota*, formerly *Proteobacteria*) and *Actinobacteria* (phylum *Actinomycetota*, formerly *Actinobacteria*). Moreover, consistent with the cultivation-dependent methods compelling evidence was found that the chitin-utilizing consortium of symbionts of octocorals and sponges is "hierarchically" arranged so that a few symbionts acts to hydrolyse the large chitin

polymer, while a large number of other symbionts can degrade smaller COS, deacetylate chitin into chitosan, and utilize N-acetylglucosamine. This is in line with the hypothesis of a large substrate cross-feeding cascade that would, ultimately, contribute to shape the structure of the octocoral and marine sponge microbiomes relying on chitin breakdown as a key metabolic process (Chapter 3). Indeed, the sponge matrix itself could function as a main substrate provider fuelling this cascade, as chitin is now known to be a structural component of several sponge species (Ehrlich *et al.*, 2018 and refs. therein). Concurrent with a "self-sustaining", microbial-based substrate cross-feeding hypothesis relying on the processing of structural chitin present in the animal host is the view of the marine sponge holobiont as an open system. Under this perspective, presented more thoroughly in Chapter 4, chitin and chitin derivatives are found in a range of lengths and degrees of deacetylation continuously reaching the sponge microbiome through filtering activity, thus help maintaining a large community of chitin utilizers within this community independently of the degradation of chitin as a structural element of sponges. Also, the degradation of glucosaminoglycans (GAGs), present in structural proteoglycans within the sponge mesohyl, through the joint activities of sulfatases, chondroitinases, and heparinases seem to function as an important, potential source of N-acetylglucosamine disaccharides that can drive exo-chitinase and N-acetylglucosamine utilization in the sponge holobiont independently of direct degradation of the chitin polymer. Particularly, potential endo-chitinase, chondroitinase and heparinase activities were revealed in chapter 4 to constitute specific and enriched traits, respectively, of *Rhodothermales* symbionts of sponges. Along with their consistent coding potential underlying sulfatase, exo-chitinase, deacetylase and N-acetylglucosaminidase activities, *Rhodothermales* symbionts are suggested to may act as formidable polysaccharide degraders within the

sponge symbiotic consortium, possibly possessing a key role in the turnover of chitin and cycling of carbon and nitrogen in benthic settings (**Figure 5.1**).

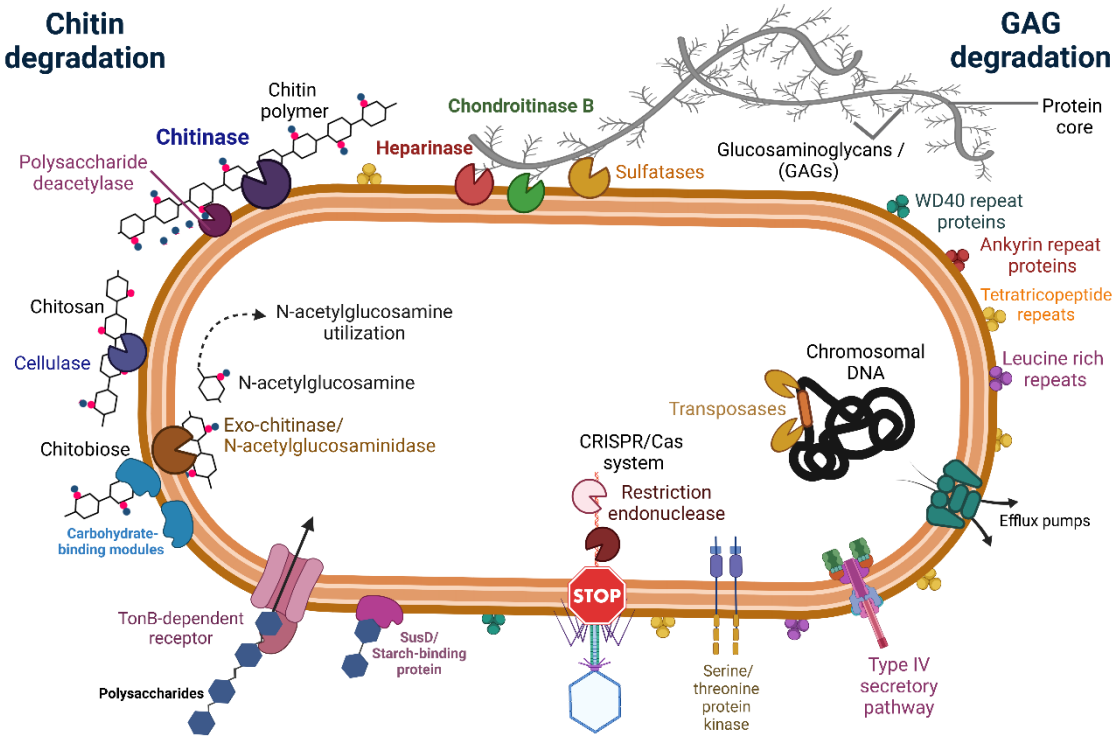


**Figure 5.1** Schematic drawing of a *Rhodothermales* cell emphasizing adaptive strategies to symbiotic living with marine sponges, including enriched traits involved in chitin and peptidoglycan metabolism detailed in Chapter 4.

A major result of the genome-resolved metagenomics quest for putative chitinolytic symbionts of marine invertebrates employed in this thesis, was the identification of novel, so-far unculturable and sponge-specific lineages in the *Rhodothermales* order. These symbionts are potentially able to degrade chitin due to the presence of GH18-encoding genes on their genomes and of a signal peptide in the corresponding amino-acid sequences, suggestive of protein translocation to the outer membrane. This is indicative of a likely keystone role for *Rhodothermales* symbionts of marine sponges, which have

been otherwise regarded as elusive organisms whose functions had been believed to be non-specific and/or of a more generalist nature (Slaby *et al.* 2017) (**Figure 5.1**). Although it is true that chitin degradation is undertaken by diverse marine microorganisms across several biotopes, including seawater, sediments, and a range of host-associated settings (Douglas *et al.* 2007; Beier and Bertilsson 2013; Wieczorek, Hetz and Kolb 2014; Wörner and Pester 2019), chitinolytic assemblages usually constitute a minor portion of the entire microbiome in any given environment (Brzezinska *et al.* 2014). In this thesis, so-far uncultured *Gammaproteobacteria* symbionts of octocorals along with *Poribacteria*, *Chloroflexota* and – particularly – *Rhodothermales* symbionts of marine sponges are revealed as part of a keystone, selected microbial guild that may dictate the degradation of the large chitin polymer in marine invertebrates. Future research developing from these findings must harness the here predicted chitin metabolism using innovative approaches, as suggested below.

### 5.1 An explanation for the presence of chitin degradation processes within sessile, filter-feeding marine invertebrates

As it was previously discussed in this thesis, marine microbiomes are in general potential hotspots for chitin degradation since this substrate is highly available in these environments and, therefore, it is likely that a portion of the microbial community possesses the molecular machinery to successfully execute the required pathways involved in chitin catabolism. This seems likely for the microbiomes of corals and marine sponges due to the suspension-feeding (octocorals) or filter-feeding (sponges) nature of these organisms. Although the feeding mechanisms of sponges and octocorals are different, both are almost constantly exposed to the presence of chitin. This can result not only from the chitin polymer or COS that may be simply taken-up from the water column

by the hosts to be processed, but also from the chitin as a constituent of the organisms they feed on. Octocorals, for instance are known to rely on heterotrophic feeding on particulate organic matter (POM), phyto- and zooplankton, including diatoms, protists, invertebrate eggs, copepods and other small crustaceans and their larvae (Ribes, Coma and Rossi 2003; Coma *et al.* 2015; van de Water, Allemand and Ferrier-Pagès 2018) which they capture with the tentacles of their polyps. Indeed, the Mediterranean gorgonian coral *Eunicella singularis* (same genus as studied in chapter 3) is known to heavily prey on zooplankton between 40 and 920 μm in size (Coma *et al.* 2015). Zooplankton is naturally chitin enriched (Souza *et al.* 2011; Wysokowski *et al.* 2015), implying that adaptive strategies involved in chitinous food processing and chitin polymer hydrolysis may constitute a selective advantage within the microbial communities of the octocoral holobiont. Marine sponges, on the other hand, daily filter hundreds to thousands of liters of seawater through their bodies. Myriad tiny pores (ostia) draw in seawater which is later pushed out again by larger pores (oscula). While seawater passes through the channels and chambers inside the sponge, bacterioplankton, small particulate organic matter (POM), and dissolved organic matter (DOM) are taken up as food by the sponge cells (Yahel *et al.* 2003; Hadas, Shpigel and Ilan 2009; Bart *et al.* 2021). Moreover, to this date several sponge species have already been documented to contain endogenous chitin as a structural component in their skeletons (Ehrlich *et al.* 2007a; Wysokowski *et al.* 2015). Along with lateral acquisition of chitin and COS via filtration, the potential utilization of structural chitin by the diverse microbial consortium of marine sponges may constitute another relevant route for chitin metabolism within these animals.

The hypotheses raised above need to be well supported with extensive empirical evidence and, thereafter, experimental validation. In this regard, cultivation-independent approaches were extremely important in shedding light on a wider diversity of chitin

degraders and COS utilizers in the examined symbiotic communities, as the dominant bacterial symbionts in both sponges and octocorals are often difficult to isolate and cultivate in the laboratory, while some of the cultured bacteria have been suggested to represent rare species within the community (Hardoim and Costa 2014b; Karimi *et al.* 2019). The findings obtained by these approaches in the present study are coherent with evidence found in recent studies either on versatile carbohydrate degradation capacities within the marine sponge microbiome or on true chitin degradation potential of uncultivated bacteria in other environmental settings. For instance, Robbins *et al.* (2021) found an enrichment of glycosyl hydrolases on *Poribacteria* and *Chloroflexota* (including the class *Anaerolineae*), among others, highlighting them as part of a set of polysaccharide-degraders within the marine sponge microbiome, yet a precise role for chitin degradation by a portion of the community has not been addressed. In line with previous findings for versatile polysaccharide degradation capacities in the marine sponge microbiome (Kamke *et al.* 2014; Bayer *et al.* 2018; Robbins *et al.* 2021), this thesis uncover compelling evidence for chitin degradation potential for a select cohort of sponge symbionts (*Chloroflexota, Poribacteria*, *Rhodothermia*) along with a vast diversity of sponge-associated taxa possessing the ability – as inferred from MAG annotations - of utilizing smaller COS, N-acetylglusamine and transforming chitin into chitosan via deacetylation.

*Chloroflexota* species were found to be more represented in communities colonizing beads presenting a substrate of chitin than beads with cellulose as a substrate in the anoxic and sulfide-rich waters of the Black Sea (Suominen *et al.* 2021). Ara *et al.* (2020) also characterized and discussed the presence of a set of glycosyl hydrolases from a genome of *Rhodothermus marinus* (order *Rhodothermales*), an organism known to possess a GH18 endo-chitinase (Hobel *et al.* 2005a). Finally, *Chloroflexales* and *Rhodothermales*

were identified by Rezgui *et al.* (2021) as two out five bacterial orders represented in soil after the addition of pea residues to the samples, which together with more chitin and carbon sources available, were linked with a higher rate of carbon mineralization in the first days of incubation. Based on this evidence from the terrestrial environment, an important role of *Chloroflexales* and *Rhodothermales* in carbon mineralization may be infered. The evidence obtained in this study that marine species belonging to *Chloroflexota* and *Rhodothermales* are potential chitin degraders is further strengthening this concept, since chitin is a known source for the carbon and nitrogen cycles in the oceans. Members of the phylum *Bacteroidota* are also well-known key players in mediating the global cycling of carbon in the ocean, because of their role as primary degraders of microalgal polysaccharides, for instance during phytoplankton blooms. This is the case of *Formosa* spp. (Unfried *et al.* 2018) and *Polaribacter* spp. (Avci *et al.* 2020). In line with this perspective, this thesis reveals that members of the *Bacteroidetes/Chlorobi* super group such as *Aquimarina* (phylum *Bacteroidota*) and *Rhodothermales* [now placed in a newly created phylum, called *Rhodothermota* (Oren and Garrity 2021)] are likely involved in chitin degradation and turnover in sessile marine invertebrates. These microbial associates are posit to play a significant role in global carbon and nitrogen cycling in benthic ecosystems congruent with the recognized roles of other *Bacteroidota* taxa within planktonic communities. Nevertheless, it remains to be determined to which extend the above-mentioned taxa are directly benefitting the animal host with their chitin-degrading capacities, and whether it promotes a mutualistic interaction, and interkingdom cross-feeding based on chitin-derived C and N transfer within the sponge holobiont. Experimental, *in-vivo* validation in this field of research is utterly challenging and hence still extremely scarce yet it is indispensable for stepping up from genomics-guided concepts to empirical evidence. Few, breaking studies have started

to use stable isotope probing (Campana *et al.* 2021) and nanoscale secondary ion mass spectrometry (nanoSIMS) (Achlatis *et al.* 2019; Rix *et al.* 2020) to assess, quantify, and visualize DOM assimilation, and carbon and nitrogen transfer by the sponge host cells and associated symbionts. Recent incubation experiments with the aquarium-kept marine sponge *Plakortis angulospiculatus* and labelled ($^{13}C$) DOM proved that bacterial strains belonging to the phyla *PAUC34f, Poribacteria,* and *Chloroflexota* are indeed active consumers of DOM filtered by the sponge and are capable of organic matter degradation through heterotrophic carbon metabolism (Campana *et al.* 2021).

### 5.2 Integrative approaches permit a holistic view of chitin degradation within sessile marine invertebrates

In this thesis, three different approaches were employed to examine the chitinolytic capacity in the studied microbiomes: 1) cultivation-dependent assessment of chitinolytic communities (Chapter 3); 2) cultivation-independent exploration of chitinolytic communities using unassembled metagenomes (Chapter 3) and 3) cultivation-independent investigation of chitinolytic communities using assembled metagenomes and *a posteriori* construction of MAGs. This multidimensional approach led to considerable differences in the outcomes observed, especially when cultivation-dependent and -independent strategies are compared. Such distinct perspectives do not represent incongruencies but instead complement each other, allowing us to glean an integrative picture of chitin degradation and transformation potential from these microbiomes. To illustrate this and to better compare the different approaches, in this subsection the presence and diversity of chitinolytic bacteria and endo-chitinases in sponges and corals is discussed, with emphasis on the former since the marine sponge microbiome was subjected to the three methodological approaches mentioned above. One obvious

difference lies in the distinct taxonomic inventories of the bacterial communities identified using each of the methodologies. As previously mentioned, the bacteria analysed by cultivation-dependent assays represent low abundance members of marine sponges and octocoral communities (Chapter 3). The dominant members of these communities are usually unculturable or thus-far uncultivated (Hardoim *et al.* 2014; Keller-Costa *et al.* 2017; Karimi *et al.* 2018) and their existence is known primarily due to metagenomics and DNA sequencing-based studies. Therefore, by using cultivation the study is most likely assessing the chitinolytic potential of rare "biosphere" members that are oftentimes not detected using amplicon sequencing methods nor using MAGs. This is congruent with the evidence of *Vibrio* and *Aquimarina* strains being among the most promising chitinolytic bacteria in Chapter 3 but not being present on the MAGs reconstructed and classified in Chapter 4. A recent meta-study of the 'Sponge Microbiome Project' (SMP) dataset examined the relative abundances of 95 Operational Taxonomic Units (OTUs) from the *Aquimarina* genus across 3413 marine samples from 81 sponge species, seawater sediment and other biotopes. Indeed, the study found that all *Aquimarina* OTUs had a mean relative abundance below the defined rarity threshold of 0.1% (Silva *et al.* 2022). Additionally, other chitinolytic bacterial taxa revealed in cultivation-dependent assays, such as *Pseudoalteromonas*, *Microbulbifer*, and *Shewanella* were as well not observed as dominant chitinolytic taxa in marine sponges using mining of unassembled metagenome reads for the presence of endo-chitinase encoding genes (Chapter 3). Like *Vibrio* and *Aquimarina*, no MAGs classified into these culturable taxa could as well be constructed from the metagenome reads examined in this thesis (Chapter 4), reinforcing the notion that these microorganisms, although possessing interesting chitin degradation attributes, are not abundant in the sponge symbiotic consortium. The reconstruction of MAGs in Chapter 4 was important to obtain greater

insight into the chitinolytic potential of the marine sponge microbiome in addition to the analysis of the unassembled reads (Chapter 3). In the latter analysis the full extent of information retrieved by the sequencing procedure can in principle be explored, including some reads from low abundance taxa that could not be assembled into larger contigs or MAGs. However, since unassembled reads are usually very short sequences (101 bp in this thesis), the exclusive analysis of those reads would in general present lower phylogenetic resolution in comparison with the analysis of long gene sequences and, therefore, functional assignments of unassembled reads should be performed and interpreted with caution. In contrast, a whole genome MAG-dependent approach allows a direct coupling of taxonomy and gene function of the most dominant community members, since nearly full genome sequences can be analysed and annotation of genes of interest (endo-chitinase, exo-chitinase, deacetylase encoding genes, etc.) can be as well greatly facilitated owing to the longer gene sequences available. On this note, it was observed in Chapter 3 that some of the *S. officinalis*-derived unassembled reads which were classified as endo-chitinases from *Gammaproteobacteria* were assigned to the genera *Vibrio* and *Microbulbifer*. Although these chitinolytic genera could be retrieved in culture (Chapter 3), none of the MAGs obtained from *Spongia officinalis* and *Spongia agaricina* in Chapter 4 were identified as *Vibrio* or *Microbulbifer*. It is posited that such reads were not present in sufficient amounts in the sequenced microbial metagenomes to enable MAGs belonging to these taxa to be assembled. In contrast with some of the major differences observed for the marine sponge microbiome as noted above, the analysis of octocoral-derived unassembled reads (Chapter 3) and MAGs (Keller-Costa *et al.* 2022b) seemed to be, in general, more congruent in the description of the chitinolytic assemblage associated with these animals. Indeed, while in Chapter 3 the dominance of endo-chitinase encoding reads from thus-far unclassified *Gammaproteobacteria* in healthy

octocoral tissue was emphasized, the analysis of MAGs obtained from these metagenomes by Keller-Costa *et al.* (2022) revealed two species belonging to a novel genus in the *Endozoicomonadaceae* family (order *Oceanospirillales*, class *Gammaproteobacteria*), termed *Candidatus* Gorgonimonas eunicellae and Gorgonimonas leptogorgiae, as the dominant chitinolytic members of the microbiome of healthy octocorals. Likewise, Keller-Costa *et al.* (2022) were able to construct a MAG classified as *Aquimarina* (family *Flavobacteriaceae*, order *Flavobacteriales*) from necrotic octocoral tissue samples, in accordance with the detection of unassembled reads classified into the *Flavobacteriia* class in Chapter 3. Congruently, an earlier study by Keller-Costa *et al.* (2021) found an *Aquimarina* OTU enriched in necrotic octocoral tissue, even suggesting it could be an indicator of dysbiosis. As mentioned earlier, *Aquimarina* spp. show in general low abundance distributions across diverse marine biotopes, however, they can increase their abundance under specific, favorable conditions such as a diseased or compromised host. Certain *Aquimarina* species are indeed emerging pathogens of algae (Lin *et al.* 2012; Kumar *et al.* 2016) and crustaceans (Midorikawa *et al.* 2020; Ooi *et al.* 2020). Their efficient chitin-degrading ability, together with a rich repertoire of peptidases (Silva *et al.*, unpublished data) and a high capacity to synthesize cytotoxic compounds (Silva *et al.* 2022) makes them formidable opportunists.

It should also be noted that results and trends obtained with both unassembled metagenomes and/or MAGs always depend on the databases and annotation methodologies employed. This means that in addition to the differences between the assembled and unassembled reads themselves, differences resulting from the analytical pipeline, including the databases employed for the annotation of metagenome and MAGs also apply. This is the case of this thesis, where MG-RAST was used to annotate and analyse the unassembled reads with homology-based approaches (blastx) to detect

possible endo-chitinase sequences in the data while Pfam and CAZyme annotations were used to analyse the MAGs.

Taken together, the use of multiple approaches to examine the chitin degradation potential of the microbiomes of sessile marine invertebrates enables a comprehensive understanding of the high- and low- abundant taxa possibly involved in chitin turnover within these animals. Nevertheless, it is evident that the genome-resolved metagenomics approach employed in Chapter 4 was instrumental to more accurately delineate the cohort of dominant symbionts putatively capable of hydrolysing the large chitin polymer. Moreover this methods was also able to identify species of *Rhodothermales* symbionts of sponges as major players in this process. In this regard, the MAGs approach was useful not only because it sheds light on the functional potential of so-far unculturable microbes as noted above, but also because it allows the retrieval of full-length gene sequences which can be accurately annotated and subjected to phylogenetic analysis and deep phylogenomic assessments of unculturable lineages (see Figures 3 and 4 of Chapter 4). This facilitates the discovery of novel taxa and their coding potential and allows for a greater understanding of niche partitioning and the roles played by different community members in a given setting. However, it is so far not possible to obtain a complete view of all the chitin degraders in the community using this approach, because only the most dominant organisms can be assembled into MAGs. One possible strategy to overcome this limitation is to apply more sequencing power to describe these communities, which may become more feasible as the throughput of next generation sequencing technologies increases throughout the years. Also, the use of long-read sequencing technologies, such as e.g., PacBio and Nanopore, may facilitate the processes of metagenome assembly and binning of MAGs in future research projects dedicated to the study of the microbiomes of sessile marine invertebrates.

### 5.3 Looking ahead: harnessing the metabolism of chitinolytic symbionts of marine sponges and corals

As described in Chapters 1 and 2, the properties of chitinases and chitin-derived products, such as COS and chitosan, may serve many biotechnological applications across multiple fields, from the agriculture and food to medical and pharmaceutical sectors (Patel and Goyal 2017; Yadav *et al.* 2019). In this thesis, the microbiomes of marine sponges and octocorals are revealed as distinctive biological hubs for chitin breakdown in the ocean, increasing the understanding of chitin utilization and turnover in benthic ecosystems and opening new avenues for the exploration of novel chitinolytic enzymes in bioprocessing and biotechnology. Tailored enzymatic processes based on natural chitinases are indeed more sustainable than industrial procedures employed in chitin degradation that rely on hazardous chemicals (Hamed, Özogul and Regenstein 2016). Few chitinases, mainly from terrestrial sources are currently commercially available. These include chitinases from the soil fungus *Trichoderma viride* (Sigma-Aldrich/MERCK), and from Gram-positive bacteria such as *Streptomyces griseus* (Sigma-Aldrich/MERCK) and *Streptomyces violaceus* (BOC Sciences). These terrestrial chitinases often have their maximum activity at high(er) temperatures and a simultaneously low(er) pH. For example, the optimum temperature for chitinolytic activity of *T. viride* was found to be 50 ºC and optimal pH was 5 (Ekundayo, Ekundayo and Bamidele 2016). Owing to the unique properties of ocean ecosystems and seawater (e.g., higher pH ~8.1 and higher salinity (35ppt)), marine chitinases may possess distinct biochemical characteristics, such as wider pH and ionic strength optima and/or ample temperature regimes (Beygmoradi *et al.* 2018) and could thus represent an important niche in the bioeconomy sector. The chitinolytic fungus *Beauveria bassiana*, isolated

from marine sediment, showed, for example, maximum enzyme production at pH 9.2 (Suresh and Chandrasekaran 1999). Beygmoradi *et al.*, 2018 reported that the pH optima of marine bacterial chitinases vary according to the isolation source, for example from 5.5 in *Paenibacillus barengoltzii* to 8 in *Alteromonas* sp., while temperature optima ranged from 20ºC in *Pseudoalteromonas* sp. to.50ºC in *Alteromonas* sp. Moreover, the endo-chitinase of the psychrophilic bacterium *Pseudoalteromonas* sp. DL-6 isolated marine sediments, showed very high catalytic activity even at 4 °C, while exhibiting maximal activity at pH 8.0 and 20 °C (Wang *et al.* 2014). This natural range of physical-chemical optima may constitute an asset in the future exploration of marine-derived chitinases for biotechnological purposes given that the spectrum of commercially available chitinases is still quite limited. The use of "cold-active" chitinases may allow milder processing conditions and a reduction of operating temperatures, leading to energy conservation and hence a lesser impact on the environment.

The studies conducted for this thesis show the potential for biotecnological exploitation of the microbiomes of sessile marine invertebrates. Yet much research is still needed from discovery and characterization to possible application and implementation of bacterial chitinases in small and medium-scale bioprocesses. In this regard, the finding of *Aquimarina* species as a novel source of putative novel chitinolytic enzymes should be further investigated with future expression, purification, and biochemical characterization of their endo-chitinases. Additionally, to explore the potential of the unculturable bacterial symbionts with chitinolytic capacity, synthetic biology approaches may be applied, as they hold promise in expressing novel endo-chitinases in heterologous hosts. Endo-chitinase genes now known from the MAGs can be synthesized *in vitro*, inserted into appropriate plasmid vectors, and culturable bacteria such as *Escherichia coli* may be transformed with those constructs to express the desired proteins. The proteins can then

be purified and thereafter their enzymatic activity can be studied in detail in the laboratory to characterize substrate preference, temperature and pH optima, salt tolerance and resistance to heavy metals, among others. On this note, the Microbial Ecology and Evolution Research Group at BSRG (iBB) is currently developing innovative, synthetic biology methodology to enable the heterologous expression of endo-chitinases coded by the uncultivated symbionts of octocorals *Candidatus* Gorgonimonas eunicellae and Gorgonimonas leptogorgiae, representative species of a novel genus in the *Endozoicomonadaceae* family as noted above (Keller-Costa *et al.* 2022b). The procedures established in the framework of this ongoing RandD project can be applied in the future to enable the expression and biochemical characterization of other chitinases from uncultivated bacterial lineages, such as the *Rhodothermales* symbionts of sponges discovered in this thesis, as well as the chitinases possibly produced by *Chloroflexota* and *Poribacteria* symbionts of sponges. The successful achievement of the proposed tasks for this project may lead to similar experiments with other groups of symbionts, creating an endo-chitinase "expression factory" exploring synthetic biology approaches to study novel chitinases from hard-to-cultivate and uncultivated bacteria across multiple environments.

# Chapter 6

## Publications and Communications

### 6.1 Scientific publications

**Silva, R.**, Silva, S.G., Gonçalves, J., Cox, C.J., Keller-Costa, T., Costa, R. Functional profiling of metagenome-assembled genomes suggests an unanticipated role for *Rhodothermia* (*Bacteroidota*) symbionts in chitin and polysaccharide metabolism within marine sponges, in prep to be submitted to *Science Advances*

**Silva, R.**, Keller-Costa, T., Cox, C.J. and Costa, R. Chitin degradation in a diverse microbial world: from metabolic pathways to organismal interactions and biotechnological applications, *Submitted to FEMS Microbiology Ecology*

Raimundo, I.*, **Silva, R.***, Meunier, L., Valente, S.M, Lago-Lestón, A., Keller-Costa, T., and Costa, R. (2021) Functional metagenomics reveals differential chitin degradation and utilization features across free-living and host-associated marine microbiomes. *Microbiome* 9:43 (DOI: 10.1186/s40168-020-00970-2). *Authors contributed equally to this the work.

### 6.2 Conferences and Outreach

#### 6.2.1 Oral Presentations

**Silva, R.** (Bio)plastics sea: How can the marine bacteria save the day? Maritech2019, 3-5 December 2019, Lisbon, Portugal (Invited Speaker).

**Silva, R.***, Pereira, C., Raimundo, I., Keller-Costa, T., Costa, R. Multi-omics and laboratory-assisted evaluation of the chitin-degrading capacities of gorgonian coral-associated bacteria. Microbiotec'17, 7-9 December, 2017, Porto, Portugal. *Presenting author

### 6.2.2 Poster Presentations

**Silva, R.*,** Raimundo, I., Meunier, L., Valente, S.M., Lago-Lestón, A., Keller-Costa, T. and Costa, R. Functional metagenomics reveals differential chitin degradation and utilization features across free-living and host-associated marine microbiomes, 1st Microbiome PT Summit, 4 Feb 2021, virtual. *Video Poster.*

**Silva, R.***, Raimundo, I., Meunier, L., Keller-Costa, T., Costa, R. Chitin-degrading capacities of sponge and octocoral microbiomes. 15th Symposium on Bacterial Genetics and Ecology (BAGECO), 26-30 May, 2019, Lisbon, Portugal.

Keller-Costa, T.*, **Silva, R.**, Raimundo, I., Meunier, L., Costa, R. Multi-omics and laboratory-assisted evaluation of chitin-degrading capacities in sponge and coral microbiomes. 17th International Symposium on Microbial Ecology (ISME), 12-17 August, 2018, Leipzig, Germany.

### 6.2.3 Outreach Events

**Silva, R.** Mar de (bio)plásticos: como podem as bactérias marinhas salvar o dia? 60 minutos ciência, 19 September 2019, Lisbon, Portugal (Invited Speaker)

Participant of TV show "Grande Reportagem – Plástico nosso de cada dia", 20 June, 2019, SIC, Portugal.

Participant, Exhibitor and/or Speaker at the International Microorganism Day (IMD) 2018, 2019 and 2020 Instituto Superior Técnico, Lisbon, Portugal (17.09.)

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