COMMUNAUTÉ FRANÇAISE DE BELGIQUE
UNIVERSITÉ DE LIÈGE – GEMBLOUX AGRO-BIO TECH

Function-based Analyses of Bacterial Symbionts Associated with the Brown Alga *Ascophyllum nodosum* and Identification of Novel Bacterial Hydrolytic Enzyme Genes

Marjolaine MARTIN
Essai présenté en vue de l'obtention du grade de docteur en sciences agronomiques et ingénierie biologique

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« *Never underestimate the power of the microbe* » **Jackson W. Foster**

« *Look for the bare necessities*
The simple bare necessities
Forget about your worries and your strife
I mean the bare necessities
Old Mother Nature's recipes
That brings the bare necessities of life »

The Bare Necessities (*"Il en faut peu pour être heureux"*)
The Jungle Book

Marjolaine Martin (2016). Function-based Analyses of Bacterial Symbionts Associated with the Brown Alga *Ascophyllum nodosum* and Identification of Novel Bacterial Hydrolytic Enzyme Genes (PhD Dissertation in English) Gembloux, Belgique, University of Liège, Gembloux Agro-Bio Tech,156 p., 10 tabl., 17 fig.

Abstract

Marine macroalgae are highly colonized by microorganisms, with which they maintain a close relationship characterized by both beneficial and detrimental interactions. Alga-associated bacteria have notably developed a range of enzymes enabling them to colonize the host surface and to use algal biomass as a carbon source. The hydrolytic potential of these bacteria, however, has been investigated almost solely at individual scale. Studies have shown the ability of some seaweed-associated bacterial strains to hydrolyze lipids, algal-cell-wall polysaccharides, and other sugars.

In this work we aimed to investigate the hydrolytic potential of the bacterial microbiota associated with the brown alga *Ascophyllum nodosum.* For this we employed two complementary function-based approaches: functional metagenomics applied to this microbiota and functional analysis of the cultivable fraction thereof.

By functional metagenomics, we identified numerous esterase genes, a beta-glucosidase gene, and an endocellulase gene. The cellulase was purified and biochemically characterized, showing interesting biotechnological features such as halotolerance and activity at low temperature. Furthermore, we assigned tentative origins to the identified genes, thus getting a glimpse of the bacterial taxa associated with the studied alga.

Secondly, we investigated the cultivable surface microbiota associated with three *A. nodosum* samples. More than 300 bacteria were isolated, assigned to a bacterial taxon and screened for algal-polysaccharide-degrading enzymes (agarase, iota-carrageenase, kappa-carrageenase, and alginate lyases). This allowed the identification of several polysaccharolytic isolates, some of them likely to be new strains or novel species, belonging to two classes: the *Flavobacteriia* and the *Gammaproteobacteria*. Subsequently, we constructed and screened two plurigenomic libraries, each produced with the genomes of five representative isolates of each class, and identified several functional genes.

With this work we highlight the presence of *A. nodosum*-associated bacterial taxa likely to entertain a privileged relation with seaweeds and having developed a range of hydrolytic activities assumed to enable them to associate with algae. We also provide information (taxa, abundances, genomic potential) on macroalgal-polysaccharide-degrading bacteria, in which interest has grown over the last ten years.

Marjolaine Martin (2016). Analyses Fonctionnelles des Symbiontes Bactériens Associés à l'Algue Brune *Ascophylum nodosum* et Identification de Nouveaux Gènes Bactériens Codant pour des Enzymes Hydrolytiques (Thèse de doctorat en anglais) Gembloux, Belgique, University of Liège, Gembloux Agro-Bio Tech,156 p., 10 tabl., 17 fig.

Résumé

Les macroalgues marines sont fortement colonisées par des micro-organismes qui leur permettent d'améliorer leurs défenses naturelles, leur croissance et l'absorption d'éléments nutritifs. En contre partie, les algues fournissent aux bactéries un environnement riche en nutriments. Par conséquence, les bactéries associées aux algues ont développé toute une gamme d'enzymes hydrolytiques leur permettant de coloniser ces surfaces et d'utiliser cette biomasse comme source de carbone. Cependant, les activités hydrolytiques de ces bactéries ont principalement été étudiées à l'échelle individuelle. Des études ont démontré la capacité de certaines souches bactériennes isolées en surface d'algues à dégrader des graisses et/ou des polysaccharides.

Dans ce travail, nous nous sommes intéressés à la capacité de la microflore bactérienne associée à l'algue brune *Ascophyllum nodosum*, à synthétiser des enzymes hydrolytiques. Deux approches complémentaires ont été envisagées : d'une part la métagénomique fonctionnelle appliquée à cette microflore, d'autre part une analyse fonctionnelle des bactéries cultivables associées à cette algue. Par métagénomique fonctionnelle, des gènes codant pour plusieurs estérases, une endo-cellulase et une beta-glucosidase ont été identifiés. Le cellulase a été purifiée et caractérisée au niveau biochimique. Halotolérante et active à basse température, elle montre des propriétés biotechnologiques intéressantes. Grace aux différents gènes identifiés, nous avons pu avoir une idée de certains taxa bactériens associés à *A. nodosum*. Ensuite, 324 bactéries cultivables ont été isolées à partir de trois échantillons indépendants d'*A. nodosum*. Ces bactéries ont été assignées à un taxon bactérien et ont été criblées pour des activités hydrolysant des polysaccharides d'algues (alginate, agar, iota- et kappa-carraghénanes). Plusieurs bactéries, au nombre de 78, montraient au moins une des activités criblées et représentaient très probablement des nouvelles souches, voir espèces bactériennes, de la classe des Flavobactéries ou de celle des Gammaprotéobactéries. Dix bactéries ont ensuite été choisies pour construire des banques plurigénomiques, qui ont été criblées pour diverses activités hydrolytiques. Des gènes codant pour une xylanase, une beta-glucosidase, une nouvelle estérase et deux iota-carraghénases ont été identifiés.

En conclusion, ce travail a permis de mettre en évidence la présence de certains taxa bactériens à la surface d'*Ascophyllum nodosum* montrant toute une série d'activités hydrolytiques. Nous avons pu récolter des informations diverses sur ces bactéries qui dégradent des polysaccharides d'algues (sur les taxa présents, leur abondance et leur potentiel génétique), suscitant un intérêt grandissant dans l'exploration du monde marin.

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CONTENTS

LIST OF ABREVIATIONS

AZCL: azurine-cross-linked
BAC : bacterial artificial chromosome
BLAST : basic local alignment search tool
CAZy/CAZyme : carbohydrate active enzyme
CBM : carbohydrate binding module
cDNA: complementary deoxyribonucleic acid
CDS : coding DNA sequence
CMC : carboxymethylcellulose
CTAB: cetyltrimethylammonium bromide
DMSO : dimethylsulfoxide
DNA: deoxyribonucleic acid
DNSA : 3,5-dinitrosalicylic acid
dNTP : deoxynucleotide
EC : *Enzyme Commission*
eDNA : environmental deoxyribonucleic acid
EDTA : ethylenediaminetetraacetic acid
gDNA : genomic deoxyribonucleic acid
GH : glycoside hydrolase
GSO dataset : Global Ocean Survey dataset
HAT : homoserine acetyl transferase
HTH : helix-turn-helix
IPTG : isopropyl β-D-1-thiogalactopyranoside
L6S : L-galactopyranose-6-sulfate
L6S-G : L-galactopyranose-6-sulfate linked to a β-D-galactopyranose
MAPD: macroalgal-polysacharide-degrading
Ni-NTA : nickel-nitrilotriacetic acid
OD : optical density
ORF : open reading frame
PAGE : polyacrylamide gel electrophoresis
PCoA : principal coordinates analyse
PCR : polymerase chain reaction
PEG : polyethylene glycol
PL : polysaccharide lyase
RBS : ribosomal binding site
rRNA : ribosomal ribonucleic acid
SDS : sodium sodecyl sulftae
UV : ultraviolet light
YT : yeast tryptone

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Publications

- Marjolaine Martin, Tristan Barbeyron, Gurvan Michel, Daniel Portetelle, Micheline Vandenbol, **2013**. Functional screening of a metagenomic library from algal biofims. *Communications in Applied Biological Sciences*, Ghent University, 78 (1): 37- 41.

- Marjolaine Martin, Daniel Portetelle, Gurvan Michel, Micheline Vandenbol, **2014**. Microorganisms living on macroalgae: diversity, interactions, and biotechnological applications. *Applied Microbiology and Biotechnology*, 98 (7): 2917–2935 (IF_{2015}: 3.3)

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- Marjolaine Martin, Micheline Vandenbol, **2016**. The hunt for original microbial enzymes: an initiatory review on the construction and functional screening of (meta)genomic libraries. (Revised and resubmitted to *Biotechnologie, Agronomie, Société et Environnement*)

Oral communications

National congress

- « Functional screening of a metagenomic library from algal biofilms », 18th National Symposium of Applied Biological Sciences, UGhent (BE), 8/2/2013 **Best oral presentation in the Biotechnology and Human Health session**

International congress

- "Biotechnological potential of novel bacterial strains isolated on the brown alga *Ascophyllum nodosum*", World Congress and Expo on Applied Microbiology, 18-20/8/2015, Frankfurt (D) **Best Young Research Award for oral presentation**

Poster communications

- « Identification of new microbial enzymes from forest and marine ecosystems by a metagenomic approach. », 14th ISME, Copenhagen, Denmark, 19 – 24/8/2012
- « Microorganisms on algae: An interesting resource of new biomass hydrolyzing enzymes », Bioforum, University of Liège, Belgium, 18/4/2013
- « Biomass hydrolyzing enzymes identified by functional screening of a metagenomic library from algal biofilms », BAGECO12, Ljubjana, Slovenia, 9-13/6/2013
- « Studying the great potential of cultivable bacterial communities associated with the brown alga *Ascophyllum nodosum* », 6th Congress of European Microbiologist (FEMS), Maastricht, Netherlands, 7-11/6/ 2015

Chapter I. General Introduction

1. CONTEXT

Aquatic environments represent more than 70% of the surface of the earth, and marine microorganisms are indispensable to the proper functioning of all ecological processes and biogeochemical cycles that take place in these environments. Furthermore, marine microorganisms live under sometimes extreme conditions (salinity, temperature, access to nutrients and light, pressure, pH, etc.) differing markedly from the living conditions of terrestrial organisms (Dalmaso et al., 2015).

This great diversity of marine ecosystems has led to a considerable abundance, diversification, and adaptation of the microbes that inhabit them, and yet these exceptional microorganisms tend to be insufficiently studied.

In marine ecosystems, microorganisms may live in a planktonic state, i. e. free in the water, or in a sessile state, i. e. associated with surfaces within biofilms. This latter «lifestyle» is the most common, as higher marine organisms offer a nutrient-rich surface allowing a multitude of interactions with symbiotic microorganisms (Pasmore and Costerton, 2003).

Marine macroalgae are notably highly colonized by microorganisms enabling them to improve their natural defences (avoiding colonization by other, parasitic microorganisms), grow better (through morphological modifications), and better absorb nutrients (thanks to (macro)molecule hydrolysis and the synthesis of vitamins and growth factors) (Croft et al., 2005; Dimitrieva et al., 2006; Egan et al., 2013).

In exchange, algal biomass constitutes a good carbon source for the associated heterotrophic bacteria, which have become specialized in the utilization of this biomass and particularly in the degradation and assimilation of algal polysaccharides (Michel and Czjzek, 2013).

Whole seaweed-associated microbiotas have been widely studied for their diversity, the factors influencing them (e.g. Burke et al., 2011; de Oliveira et al., 2012; Fernandes et al., 2012; Lachnit et al., 2011), and their antimicrobial properties (e.g. Lemos et al., 1985; Kanagasabhapathy et al., 2006; Wiese et al., 2009a; Goecke, Labes, et al., 2013). On the other hand, the ability of these bacterial communities to produce hydrolytic enzymes has been studied almost only at the individual scale. Studies have focused on the capacity of some bacterial strains isolated from seaweeds to hydrolyze lipids or sugars (e.g. Kim and Hong, 2012; Mohapatra et al., 2003), and there is growing interest in the particular algal-polysaccharide-degrading activities of such bacteria (e.g. Labourel et al., 2014; Thomas et al., 2013; Yao et al., 2013). The discovery of enzymes acting on seaweed polysaccharides has revealed protein structures that are only distantly related to those of terrestrial polysaccharide-degrading enzymes, and in most cases novel glycoside hydrolase families have been found (e.g. Barbeyron et al., 2000; Flament et al., 2007; Guibet et al., 2007; Rebuffet et al., 2011). To date, only about forty

macroalga-polysaccharide-degrading enzymes isolated from alga-associated bacteria have been characterized at the molecular and biochemical levels. As macroalgal biomass is highly complex (almost ten polysaccharides and as many monosaccharides have been found in brown, green, and red algae) and as the associated bacterial communities are highly diverse, a huge diversity of original hydrolytic enzymes produced by these particular microbiotas remains underexplored.

In this work, we have investigated the microbiota associated with the brown alga *Ascophyllum nodosum* (L.) Le Jolis, found along the coasts of the North Atlantic Ocean. This species is a large (up to 2 m long) and very long-lived brown alga of the *Fucaceae* family (Olsen *et al.*, 2010).

Green and red algae originated 1500 million years ago from a primary endosymbiosis (uptake of a cyanobacterium by a eukaryotic cell), whereas brown algae (Phaeophyceae) emerged much later, approximately 200 million years ago, through a secondary endosymbiosis with a red alga (Popper *et al.*, 2011)(Figure I-1).

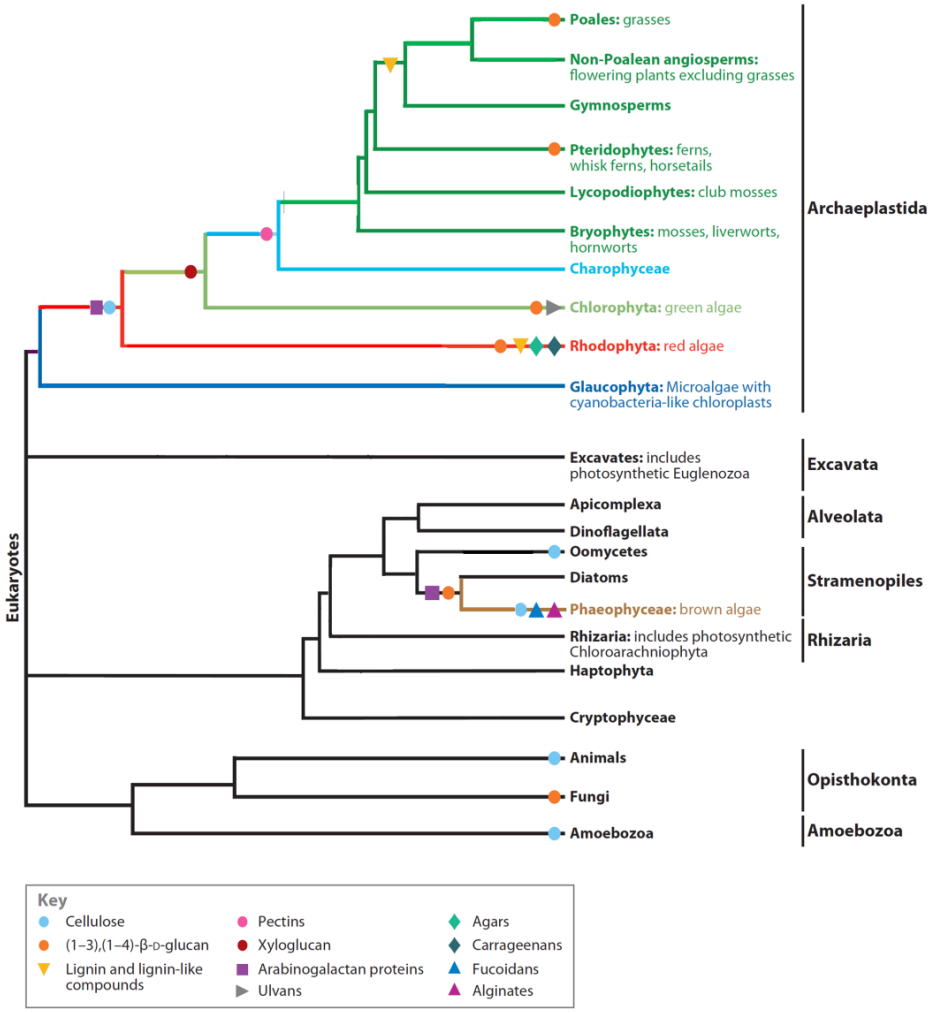

Figure I-1 Simplified eukaryotic phylogenetic tree emphasizing the occurrence of major wall components. (Adapted from Popper *et al.*, 2011).

From this independent evolution of brown algae into complex multicellular structures, brown alga cell walls share polysaccharides with plants (cellulose), animals (sulfated fucans), and even bacteria (alginates) (Michel et al., 2010b; Deniaud-Bouët et al., 2014) (Figure I 2). This original phylogeny and this unique cell-wall composition make brown algae interesting study models.

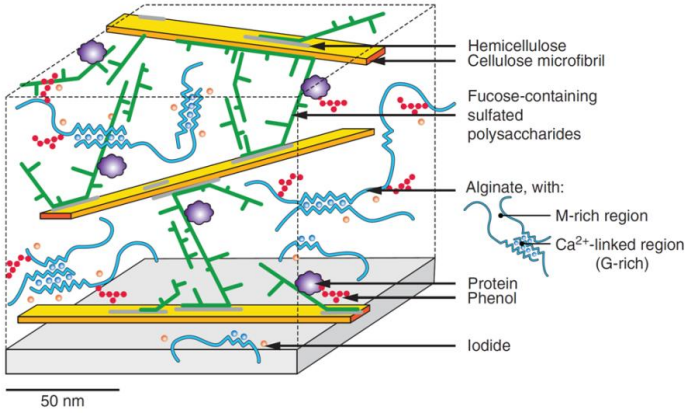

Figure I-2 Cell-wall model for brown algae of the order Fucales. "M-rich region" and "G-rich region" stand for alginate regions that are rich in β-D-mannuronic or α-L-*g*lucuronic acid, respectively (From Deniaud-Bouët *et al.,* 2014).

Prospecting for hydrolytic enzymes and bacteria in brown seaweed surface microbiotas will help to better understand the interactions between algae and their associated bacterial communities by partially answering questions such as "what's happening?" and "who is involved?".

Regarding the chosen alga *A. nodosum*, only two old studies have dealt with its associated microbial population (Chan and McManus, 1969; Cundell et al., 1977), although this ecologically relevant alga should be inhabited by uncommon hydrolytic bacteria (Figure I-3).

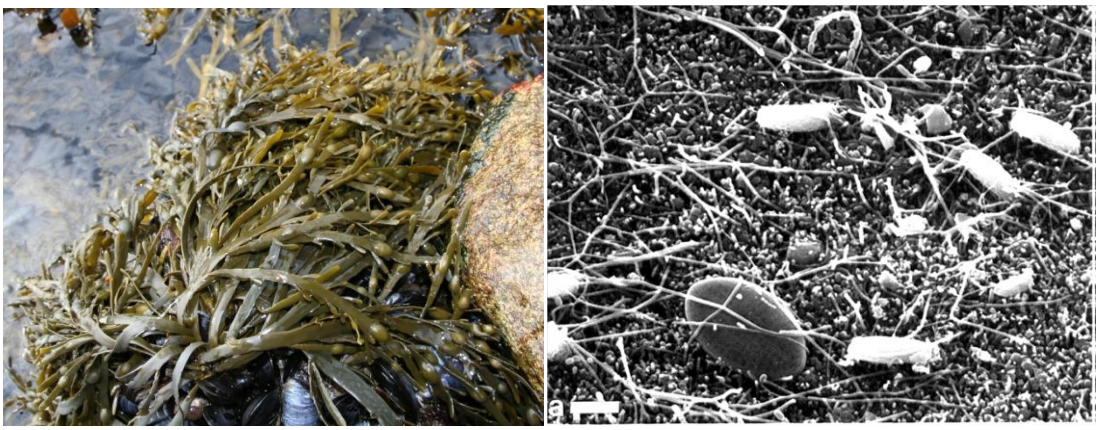

Figure I-3 *Ascophyllum nodosum* (left, Arneoste 2008) and microorganisms at the surface of *Ascophyllum nodosum* examined with an electron microscope (right, adapted from Cundell et al., 1977)

In the course of this PhD work, we collaborated closely with the Marine Glycobiology team of the Station Biologique de Roscoff (FR). My lab, the Microbiology and Genomics Laboratory of Gembloux-Agro-Bio Tech (ULg, BE), has gained expertise in searching for novel enzymes through functional metagenomics and has longstanding experience in pure and molecular microbiology. In the first year of my thesis, I learned to construct and screen metagenomic libraries from environmental samples and to set up diverse screening tests for hydrolytic enzymes.

During the second year, I worked mostly at the Station Biologique de Roscoff in Brittany (February 2012 till August 2012), within the Marine Glycobiology team. A major focus of this group is the polysaccharides composing macroalgal cell walls and the bacteria-seaweed interactions resulting in the degradation and assimilation of these specific sugars. My work in Roscoff led me to become familiar with algal species and cell wall composition and with marine bacteria and their macroalgal-polysaccharide-degrading enzymes. During my stay, I constructed metagenomic libraries from alga-associated bacteria and learned to devise screening strategies for prospecting algal polysaccharidases.

Throughout the following years of this work, I spent annual short stays in Roscoff to present and discuss my ongoing results, exchange materials, or collect fresh algal samples.

2. AIM OF THE THESIS

The aim of this thesis was to investigate the microbiota associated with the brown alga *Ascophyllum nodosum* and to explore the hydrolytic potential of these bacteria, with special emphasis on the macroalgal-polysaccharide-degrading enzymes they produce.

To reach this goal, we used to two different approaches:

- Functional metagenomics applied to this bacterial population;
- Functional analysis of the cultivable surface microbiota associated with this alga.

3. OUTLINE

This manuscript is a compilation of published scientific papers and is structured as follows:

The work starts with two literature reviews, presented in Chapters II and III:

i. Chapter II reviews the state of the art on **alga-associated microbiotas**, focusing on their immense diversity and density, complex interactions with algae, specific algal polysaccharidases, and biotechnological potential (Chapter II. Alga-associated microbiotas).

ii. The second review explores different functional methods used to identify novel enzymes and focuses on the main decisions that have to be made while **constructing and functionally screening genomic or metagenomic libraries** (Chapter III. Construction and functional screening of (meta)genomic libraries).

The next three chapters describe two approaches used to explore the enzymatic potential of bacteria associated with Ascophyllum nodosum:

i. First, we used **functional metagenomics** to identify novel enzyme genes from the total microflora associated with the brown alga *Ascophyllum nodosum*. Functional metagenomics enabled us to identify several novel esterase-, beta-glucosidase-, and cellulase-encoding genes. We describe the construction and the functional screening of a metagenomic library from the *A. nodosum*-associated microbiota, followed by the purification and characterization of a cold-active and halotolerant endocellulase (Chapter IV. Functional metagenomics).

ii. Next, we looked at the polysaccharolytic potential of the **cultivable surface microbiota** associated with *Ascophyllum nodosum*. We found this subpopulation to be considerably enriched in macroalgal-polysaccharide-degrading bacteria, and we isolated several such bacteria (Chapter V. Cultivable surface microbiota).
Finally, **functional screening of plurigenomic libraries** constructed with some of these polysaccharolytic isolates enabled us to discover an esterase, a beta-glucosidase, a xylanase, and two iota-carrageenases (Chapter VI. Functional screening of plurigenomic libraries).

The work ends with a compilation of the results obtained and a general discussion and conclusion about the bacterial taxa associated with A. nodosum and the identified activities. We propose ways to further improve the function-based techniques used in this work and suggest short- and long-term prospects for continuing this work (Chapter VII. General discussion, Conclusions and Future prospects).

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Chapter II. Alga-associated microbiotas

Outline

As the aim of this thesis work was to explore bacterial symbionts associated with algae and their enzymatic potential, we review in this chapter what is already known about these communities. We discuss the diversity and density of these biofims and further outline interactions between algae and their epibionts leading to the production of particular metabolites. Toward our objectives, we, particularly, review the state of the art of algal-specific polysaccharidases. We end with the potential importance of these microorganisms and their metabolites for further biotechnological applications in diverse industrial fields.

Related publication

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Microorganisms living on macroalgae: Diversity, interactions, and biotechnological applications

Abstract

Marine microorganisms play key roles in every marine ecological process, hence the growing interest in studying their populations and functions. Microbial communities on algae remain underexplored, however, despite their huge biodiversity and the fact that they differ markedly from those living freely in seawater. The study of this microbiota and of its relationships with algal hosts should provide crucial information for ecological investigations on algae and aquatic ecosystems. Furthermore, because these microorganisms interact with algae in multiple, complex ways, they constitute an interesting source of novel bioactive compounds with biotechnological potential, such as dehalogenases, antimicrobials and alga-specific polysaccharidases (e.g. agarases, carrageenases, alginate lyases). Here, to demonstrate the huge potential of alga-associated organisms and their metabolites in developing future biotechnological applications, we first describe the immense diversity and density of these microbial biofilms. We further describe their complex interactions with algae, leading to the production of specific bioactive compounds and hydrolytic enzymes of biotechnological interest. We end with a glance at their potential use in medical and industrial applications.

Keywords

Microbial biofilms, algal symbiosis, glycoside hydrolases, algal polysaccharidases, antimicrobial compounds.

1. INTRODUCTION: SURFACE-ASSOCIATED MARINE MICROORGANISMS

Marine microorganisms live freely in seawater (planktonic microorganisms) or attached to biotic or abiotic surfaces. Microorganisms on a surface commonly form a biofilm, defined as "*an assemblage of microbial cells that is irreversibly associated with a surface and enclosed in a matrix of primarily polysaccharide material*" (Donlan, 2002). This matrix, called the exopolysaccharide layer, allows close spatial proximity, enhancing communication and interactions among bacteria and between bacteria and their host (Kilian *et al.*, 1985; Pasmore and Costerton, 2003; Wilson *et al.*, 2011). In the marine environment, where competition for space and nutrients is intense, the surfaces of marine eukaryotes such as invertebrates and algae offer a nutrient-rich habitat uniquely suited for microbial colonization and biofilm formation (Egan *et al.*, 2008; Goecke *et al.*, 2010). As such surfaces are highly complex and differentiated, marine microbial biofilms should constitute a huge source of diversity, and the bacterial communities forming them should differ considerably in composition from populations of pelagic bacteria. Oddly, although bacteria in marine environments are most often surface-associated, previous investigations have preponderantly focused on the diversity of planktonic microorganisms rather than on microbial epibionts. Yet although investigators are increasingly using both culture-dependent and -independent methods to zoom in on microbial symbionts living on the surfaces of organisms such as corals, sponges, tunicates, and macroalgae (Rohwer *et al.*, 2002; Taylor *et al.*, 2003; Wegley *et al.*, 2007; Wilson *et al.*, 2010; Erwin *et al.*, 2011), the biotechnological potential of these symbionts remains little discussed. In this review we focus on microbial biofilms on marine macroalgae, including their potential importance in developing future biotechnological applications. We discuss the diversity and density of these biofilms and the factors influencing the microbial communities that live on diverse algal species. We further outline interactions between algae and their epibionts leading to the production of metabolites of biotechnological interest. Particularly, we review the state of the art on algal-specific polysaccharidases from seaweed-associated bacteria. Finally we draw attention to the potential importance of these microorganisms and their metabolites, such as secondary bioactive compounds and specific hydrolytic enzymes, for biotechnological applications in diverse industrial fields.

2. DIVERSITY OF MICROORGANISMS ON ALGAE

Microorganisms are very abundant on the surfaces of marine organisms (> $1.1x10^8$ microorganisms/cm²) (Cundell *et al.*, 1977). Although the microorganisms observed and identified on the surfaces of diverse algae include yeasts, fungi, and protists (Cundell *et al.*, 1977; Genilloud *et al.*, 1994; Schaumann and Weide, 1995; Armstrong *et al.*, 2000; Uchida and Murata, 2004), most available reports on alga-associated microbial populations concern bacteria. Therefore this review focuses mainly on alga-associated bacterial communities.

The density of bacteria on algal surfaces has been estimated by cell counts under the microscope (Cundell *et al.*, 1977), by culture-based methods (Mazure and Field, 1980), and by molecular approaches (Armstrong *et al.*, 2000). Mean densities between 10^6 and 10^9 bacteria/cm^2 algal surface have been recorded. There is some controversy regarding the composition of bacterial communities on algae. Bacterioplankton studies have shown most marine bacteria to be gram negative, but recent studies on marine-sediment-associated bacteria have revealed a large proportion of gram-positive bacteria, too (Gontang *et al.*, 2007). Table II-1 shows the most abundantly represented phyla (and classes or orders) of bacteria identified on diverse algal species, with the sampling location and season. Gram-negative bacteria of the phyla *Bacteroidetes* and *Proteobacteria* emerge as the most abundant, having been found on practically all the listed algal species (Table II-1). Although gram-negative bacteria appear to preponderate, gram-positive species are also present. In particular, gram-positive bacteria of the phyla *Actinobacteria* and *Firmicutes* have been observed on most algae (Table II-1). On some species or in a particular season, other bacterial phyla can also be abundant. For instance, Bengtsson et al. found peptidoglycan-less *Planctomycetes* species to dominate the bacterial biofilm on the kelp *Laminaria hyperborea* for long periods of the year (Bengtsson and Øvreås, 2010; Bengtsson *et al.*, 2010, 2013). Ocean surface water shows a phylum distribution quite similar to that of algal surface bacteria, the most abundantly represented phylum being the *Proteobacteria* (particularly the class *Alpha-proteobacteria*) (Morris *et al.*, 2002), followed by the *Bacteroidetes*, *Actinobacteria*, *Planctomycetes*, and *Chloroflexi* (Longford *et al.*, 2007). At the bacterial genus and species levels, however, recent investigations have revealed notable differences in composition between epibacterial communities and the surrounding bacterioplankton communities (Longford *et al.*, 2007; Burke, Thomas, *et al.*, 2011; Lachnit *et al.*, 2011). This suggests that colonization patterns are host-specific and strongly influenced by the seaweed, because of physicochemical constraints, such as cell wall component diversity (Michel *et al.*, 2010b; Popper *et al.*, 2011), and/or active defense mechanisms (Potin *et al.*, 2002; Cosse *et al.*, 2007). Table II-1 further shows that the sampling season and

region and the algal species or phylum can influence community composition. In fact, diverse factors shape the composition of alga-associated bacterial populations:

(i) Recent studies on bacterial biofilm composition have shown it to vary considerably with the **algal phylum** (green, brown, or red algae) and, to a lesser extent, with the **algal species** (Longford *et al.*, 2007; Lachnit *et al.*, 2009). Longford et al. compared the bacterial beta-(between host) and alpha-(within host) diversity of the marine sponge *Cymbastela concentrica* and two co-habiting algae species, the red alga *Delisea pulchra* and the green alga *Ulva australis* (Longford *et al.*, 2007). Between the two algal species, the community patterns were very similar at bacterial phylum level, but at bacterial species level little overlap was observed. Lachnit et al. focused on compositional variability among the bacterial communities associated with diverse species of the three algal phyla *Rhodohpyta*, *Chlorophyta* and *Phaeophyta* (Lachnit *et al.*, 2009). They found that host phylum seems to contribute more than host species to dissimilarity in epibacterial composition, explaining this dissimilarity on the basis of different physico-chemical properties and metabolite compositions and more or less effective defense mechanisms (Potin *et al.*, 2002) and/or attractants (Pasmore and Costerton, 2003). For instance, brown algae produce and secrete large amounts of mannitol (Gravot *et al.*, 2010), a main carbon storage compound (Michel *et al.*, 2010a). This organic exudate was recently shown to affect the formation of biofilms of marine bacteria such as *Pseudolatermonas* spp. 3J6 and D41 and *Zobellia galactanivorans* (Salaün *et al.*, 2012). This latter microorganism, which was isolated from the red alga *Delesseria sanguinea* (T Barbeyron *et al.*, 2001), is a model bacterium for the study of bacteria-seaweed interactions and particularly the bioconversion of algal polysaccharides (Michel and Czjzek, 2013).

(ii) T**he part of the thallus sampled and its age** also influence both the composition and the specificity of the bacterial population. On the brown alga *Laminaria saccharina*, for example, Staufenberger et al. (2008) found a greater bacterial diversity on the old phyloid than on any other part of the alga, explaining it on the basis of tissue age/mechanical stress: this tissue should contain more damaged cells vulnerable to bacterial decomposition, enhancing bacterial colonization. Furthermore, the association appeared most specific (i. e., between-specimen variability was lowest) on the meristem (where new tissue is formed) and cauloid. On all parts of the alga, however, the bacterial communities differed markedly from those of the surrounding seawater. The authors also point out that the composition of the bacterial community present on the substratum-anchored rhizoid is likely to reflect the presence, in the substratum, of other marine organisms with their own surface communities.

(iii) **Seasonal changes** in the composition of alga-associated bacterial populations have also been recorded. Mazure and Field (1980) observed on the brown alga *Laminaria*

saccharina a predominance of mesophilic bacteria in summer, with a switch to a more psychrophilic population in winter. A similar seasonal shift was observed on *Laminaria digitata* (Corre and Prieur, 1990; Salaün, 2009). Furthermore, bacterial abundance can be two to three times greater in summer, likely because the higher temperature favors enhanced microbial metabolism (Rao, 2010). Moreover, Stratil et al. (Stratil *et al.*, 2013) studied the shift in diversity and density of bacterial populations on *F. vesiculosus* when cultured at different temperatures. They found 20% of the bacterial diversity variation between host groups to be due to temperature, but bacterial density was not affected by this factor.

(iv) Rapid changes in bacterial community composition and abundance have also been observed **between healthy and bleached (diseased) algal tissues.** On diseased macroalgae, the density of bacteria and other microfoulers can be as much as 400 times that found on healthy tissues (Weinberger *et al.*, 1994)**.** Furthermore, comparative metagenomics applied to healthy and bleached tissues of *D. pulchra* has evidenced differences in bacterial taxa and functional genes (Fernandes *et al.*, 2012). These shifts have been explained by reduced defenses in stressed thalli (due to high summer temperature), leading to colonization by opportunistic and pathogenic bacteria**.**

(v) Finally, Burke et al. (2011), studying *Ulva australis*, observed **intraspecies differences** in bacterial community composition. They noted similar functional profiles for the communities found on different specimens, suggesting that (functional) genes, rather than bacterial species, may explain the diversity of bacterial epibionts on algae. These intraspecies differences were also observed on *F. vesiculosus* (Stratil *et al.*, 2013). As only 20% of the community variation could be explained by temperature changes, a large proportion of variation between hosts is left unexplained. This strengthens the 'functional profile' theory of Burke et al.

Table II-1 Most represented bacterial phyla (class and order) on diverse algal species

Algae phyla	Algae specie	Sample region	Sample season	Most bacterial represented phylum (and/or class)	Source
Phaeophyta	*Fucus vesiculosus*	Kiel fjord (Western Baltic Sea, Germany)	Winter Summer	*Bacteroidetes, Planctomycetes, Proteobacteria (Alpha and Gamma)* *Bacteroidetes, Cyanobacteria, Proteobacteria (Alpha (Rhodobacterales)), Verrucomicrobia*	(Lachnit *et al.*, 2011)
	Fucus vesiculosus	Kiel fjord (Western Baltic Sea, Germany)	Winter Summer	*Actinobacteria, Bacteroidetes (Flavobacteria), Firmicutes (Bacilli)* *Bacteroidetes (Flavobacteria), Firmicutes (Bacilli), Proteobacteria (Gamma)*	(Goecke, Labes, *et al.*, 2013)
	Dictyota bartayresiana	Island of Curacao (Netherlands Antilles)	(Not specified)	*Bacteroidetes, Cyanobacteria, Proteobacteria*	(Barott *et al.*, 2011)
	Laminaria digitata	Roscoff (France)	(Not specified)	*Actinobacteria, Bacteroidetes, Proteobacteria (Alpha, Gamma)*	(Salaün *et al.*, 2010)
	Laminaria saccharina	Kiel fjord (Western Baltic Sea, Germany)	Winter & Summer	*Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria (Alpha, Beta, Gamma)*	(Wiese *et al.*, 2009a)
	Laminaria hyperborea	Southwest coast of Norway	Summer Winter	*Plantomycetes, Proteobacteria (Alpha, Beta and Gamma), Verrucomicrobia* *Bacteroidetes, Cyanobacteria, Plantomycetes, Proteobacteria (Alpha, Beta and Gamma), Verrucomicrobia*	(Bengtsson and Øvreås, 2010; Bengtsson *et al.*, 2010)
Rodophyta	*Gracileria vermiculophylla*	Kiel fjord (Western Baltic Sea, Germany)	Winter Summer	*Bacteroidetes, Proteobacteria (Alpha (Rhodobacterales and Rhizobiales))* *Bacteroidetes, Proteobacteria (Alpha (Rhodobacterales))*	(Lachnit *et al.*, 2011)
	Jania rubens	Cap Zebib (northern coast of Tunisia)	Summer	*Bacteroidetes, Proteobacteria (Alpha and Gamma)*	(Ismail-Ben Ali *et al.*, 2011)
	Delisea pulchra	Bare Island (Sydney, Australia)	Summer	*Bacteroidetes, Proteobacteria (Alpha), Planctomycetes*	(Fernandes *et al.*, 2012)
	Delesseria sanguinea	Kiel fjord (Western Baltic Sea, Germany)	Winter Summer	*Actinobacteria, Bacteroidetes (Flavobacteria), Firmicutes (Bacilli), Proteobacteria (Gamma)* *Bacteroidetes (Flavobacteria), Firmicutes (Bacilli), Proteobacteria (Gamma)*	(Goecke, Labes, *et al.*, 2013)
Chlorophyta	*Ulva intestinalis*	Kiel fjord (Western Baltic Sea, Germany)	Winter Summer	*Bacteroidetes, Proteobacteria (Alpha (Rhizobiales) and Gamma)* *Bacteroidetes, Proteobacteria (Alpha (Rhodobacterales and Rhizobiales) and Gamma)*	(Lachnit *et al.*, 2011)
	Ulva sp.	Wembury Beach (Devon, UK)	(Not specified)	*Bacteroidetes (Flavobacteria), Proteobacteria (Alpha (Rhodobacterales))*	(Tait *et al.*, 2009)
	Ulva australis	Bare Island (Sydney, Australia)	Winter	*Bacteroidetes, Planctomycetes, Proteobacteria (Alpha and Gamma)*	(Tujula *et al.*, 2010; Burke, Thomas, *et al.*, 2011)
	Bryopsis hypnoides *Bryopsis pennata*	Pacific Mexican coast	Winter	*Bacteroidetes (Flavobacteria and unclassified), Mollicutes (Mycoplasmataceae)* *Bacteroidetes (Flavobacteria)*	(Hollants *et al.*, 2011)
	Caulerpa taxifolia	Mediterranean Tahiti Philippines Australia	(Not specified)	*Cytophaga-Flexibacter-Bacteroides (CFB), Proteobacteria (Alpha and Beta)* *Proteobacteria (Alpha and Delta)* *Proteobacteria (Alpha ,Delta and Gamma)* *Cytophaga-Flexibacter-Bacteroides (CFB), Proteobacteria (Alpha and Beta)*	(Meusnier *et al.*, 2001)

3. ALGA-ASSOCIATED MICROORGANISMS PRODUCE SPECIFIC ENZYMES AND BIOACTIVE COMPOUNDS

Microorganisms on algae, through their complex and numerous interactions with the host, constitute an immense source of bioactive compounds and specific polysaccharidases. Therefore, before discussing the biotechnological potential of algal epibionts and their metabolites, we will have a glance at microorganism-alga interactions and at the biotechnologically useful bioactive compounds and enzymes produced by alga-associated microorganisms.

Seaweed-associated bacteria produce alga-specific polysaccharidases

It is generally assumed that microorganisms benefit from the ready availability of a range of organic carbon sources produced by the host alga. Green, red, and brown algae produce a wide diversity of complex polysaccharides which are essential components of their cell walls (Popper *et al.*, 2011). These polysaccharides constitute a crucial biomass in coastal ecosystems. Interestingly, in contrast to the polysaccharides of terrestrial plants, most algal polysaccharides are non-lignocellulosic and sulfated (Popper *et al.*, 2011). Whereas lignocellulosic biomass consists of cellulose, lignin, and hemicelluloses, macroalgal biomass is much more complex. About ten different polysaccharides (e.g. agars, carrageenans, ulvans) and as many monosaccharides (e.g. glucose, mannose, xylose), are found over the three algal phyla (Jung *et al.*, 2013). Accordingly, alongside common polysaccharidases (e.g. cellulases, beta-glucosidases and amylases), very specific carbohydrate-active enzymes are found in microorganisms living on algae. Here we present the current state of knowledge on these enzymes (see http://www.cazy.org/, Cantarel et al., 2009), focusing solely on those characterized at both the molecular and biochemical levels, and particularly on those whose 3D structure has been determined (Table II-2).

Carrageenases

Carrageenans and agars are sulfated galactans. They are the main cell wall components of red macroalgae (Popper et al, 2011). Carrageeenases are currently divided into three classes according to the number of sulfate substituents per disaccharide repeating unit which are specifically recognized: kappa- (1 sulfate, EC 3.2.1.83), iota- (2 sulfates, EC 3.2.1.157) and lambda-carrageenases (3 sulfates, EC 3.2.1.-). All these enzymes cleave β-1,4 glycosidic bonds in carrageenans.

Kappa-carrageenase genes have been cloned from several *Pseudoalteromonas* species (Barbeyron *et al.*, 1994; G.-L. Liu *et al.*, 2011; Kobayashi *et al.*, 2012), from *Zobellia* species (Barbeyron *et al.*, 1998; Z. Liu *et al.*, 2013), and from *Cellulophaga lytica* strain

N5-2 (Yao *et al.*, 2013). The corresponding enzymes belong to glycoside hydrolase family 16 (GH16) (Barbeyron *et al.*, 1994). The kappa-carrageenase of *P. carrageenovora* adopts a β jelly-roll fold and displays a tunnel active site (Figure II-1A). These features suggest that this enzyme has an endo-processive mode of action (Michel, Chantalat, Duee, *et al.*, 2001), and this prediction has been biochemically confirmed (Lemoine *et al.*, 2009).

The first cloned iota-carrageenase genes originated from the marine bacterium *Alteromonas fortis* and from *Z. galactanivorans*, and their products defined the GH82 family (Barbeyron *et al.*, 2000). Additional iota-carrageenase genes have been cloned from *Cellulophaga* sp. QY3, a flavobacterium isolated from the red alga *Grateloupia livida* (Ma *et al.*, 2013),and from *Microbulbifer thermotolerans* JAMB-A94T, a deep-sea bacterium (Hatada *et al.*, 2011). The iota-carrageenase CgiA of *A. fortis* adopts a right-handed β-helix fold with two additional domains (A and B) in the C-terminal region (Michel, Chantalat, Fanchon, *et al.*, 2001). Upon substrate binding, the (α/β)-fold domain A shifts towards the β-helix cleft, forming a tunnel that encloses the iota-carrageenan chain (Figure II-1B), thus explaining the highly processive character of CgiA (Michel *et al.*, 2003). A mechanistic study has demonstrated that CgiA is chloride ion dependent and that its catalytic residues are Glu245 and Asp247 (Rebuffet *et al.*, 2010).

Lambda-carrageenases constitute a new GH family, unrelated to kappa- and iota-carrageenases (Guibet *et al.*, 2007). Only two genes have been cloned so far, one from the seaweed-associated bacterium *P. carrageenovora* (Guibet *et al.*, 2007) and one from the deep-sea bacterium *Pseudoalteromonas* sp. strain CL19 (Ohta and Hatada, 2006). The products of these genes are highly similar (98% sequence identity), explaining why no CAZY family number has yet been attributed (Cantarel *et al.*, 2009). These large enzymes (~105 kDa) feature a low-complexity linker connecting two independent modules, an N-terminal domain predicted to fold as a β-propeller and a C-terminal domain of unknown function (Guibet *et al.*, 2007).

Agarases

Agarases are divided into two classes, alpha-agarases (EC 3.2.1.158) and beta-agarases (EC 3.2.1.81), which respectively hydrolyze α-1,3 and β-1,4 linkages between neutral agarose motifs in agar chains. The first alpha-agarase activity was purified and characterized from *Alteromonas agarlyticus* twenty years ago (Potin *et al.*, 1993). The gene was later cloned, revealing a large enzyme (154 kDa) with a complex modular architecture including five calcium-binding thrombospondin type 3 repeats, three family-6 carbohydrate-binding modules (CBM6s), and a C-terminal catalytic module defining a novel GH family (GH96) (Flament *et al.*, 2007). Bioinformatic studies suggest that the CBM6s specifically bind agars and were acquired from modular GH16 beta-agarases (Michel *et al.*, 2009). A highly similar alpha-agarase (72% sequence identity) has also

been cloned from *Thalassomonas* sp. JAMB-A33, a strain isolated from marine sediment (Hatada *et al.*, 2006).

Beta-agarases are found in four unrelated CAZY families: GH16, GH50, GH86, and GH118 (Cantarel *et al.*, 2009). The first beta-agarases to be both structurally and biochemically characterized were the GH16 beta-agarases *Zg*AgaA and *Zg*AgaB of *Z. galactanivorans* (Allouch *et al.*, 2003; Jam *et al.*, 2005). *Zg*AgaA is an extracellular monomeric enzyme with a GH16 module appended to a putative CBM and a PorSS secretion domain, while *Zg*AgaB is a dimeric lipoprotein anchored to the outer membrane (Jam *et al.*, 2005). In both enzymes, the GH16 module displays a β jelly-roll fold with an open catalytic groove (Allouch *et al.*, 2003). Two agar-binding sites have been identified in the structure of *Zg*AgaA$_{GH16}$ complexed with oligo-agars: one in the active site cleft and one at the external surface of the protein, explaining the high agar-fiber-degrading efficiency of this enzyme (Allouch *et al.*, 2004). The crystal structure of a third beta-agarase from *Z. galactanivorans* has been solved recently. ZgAgaD has a longer catalytic groove with 8 subsites (Figure II-1C) and is specific for unsubstituted agarose motifs (Hehemann, Correc, *et al.*, 2012). Numerous GH16 beta-agarases have been cloned from bacteria isolated from seawater or marine sediments, but relatively few from seaweed-associated bacteria (Schroeder, 2003; Oh *et al.*, 2010; Yang *et al.*, 2011; Kim and Hong, 2012).

The first GH50 beta-agarase was cloned from *Vibrio* sp. JTO107, isolated from seawater in Japan (Sugano *et al.*, 1993). So far, however, no GH50 gene has been cloned from an alga-associated microorganism. The first structure of a GH50 beta-agarase was determined last year: Aga50D from *Saccharophagus degradans* (Pluvinage *et al.*, 2013). This bacterium was isolated from a halotolerant land plant in a salt marsh, and is thus not a genuine marine microorganism (Andrykovitch and Marx, 1988). Aga50D features two domains, a $(\beta/\alpha)_8$-barrel connected to a small β-sandwich domain reminiscent of a CBM (Figure II-1D). The putative catalytic residues (Glu534 and Glu695) are located in an active site with a tunnel topology, in keeping with the exo-lytic mode of action of this beta-agarase (Pluvinage *et al.*, 2013).

One of the first characterized beta-agarases (AgrA) was purified from *Pseudoalteromonas atlantica* Tc6, a gammaproteobacterium isolated in Canada from the red alga *Rhodymedia palmata* (Yaphe, 1957). Its gene remained an orphan sequence for a long time (Belas, 1989), before defining the GH86 family (Cantarel *et al.*, 2009). No other GH86 beta-agarase has been characterized from alga-associated bacteria.

The GH118 family includes only 8 sequences from marine bacteria, and none of them was isolated from a seaweed-associated bacterium. The first GH118 beta-agarase was cloned from *Vibrio* sp. PO-303 (Dong *et al.*, 2006). The beta-agarase of *Pseudoalteromonas* sp. CY24 has also been extensively characterized, revealing a large binding site with 12 subsites. This GH118 enzyme proceeds according to a mechanism of

inversion of the anomeric configuration (Ma *et al.*, 2007), in contrast to GH16 beta-agarases, which act via a retaining mechanism (Jam *et al.*, 2005). The families GH50 and GH86 are also predicted to encompass retaining enzymes (Pluvinage *et al.*, 2013). Currently there is no GH86 or GH118 beta-agarase of known 3D structure, although a note mentions the crystallization of a beta-agarase from *Pseudoalteromonas* sp. CY24 (Ren *et al.*, 2010).

Porphyranases

Porphyran is the usual name of the agar extracted from red algae of the genus *Porphyra*. The porphyran backbone is composed of ~30% agarose repetition moieties (LA-G), the remaining moieties being essentially L-galactopyranose-6-sulfate (L6S) linked via an α-1,3 bond to a beta-D-galactopyranose (G) residue. A porphyran repetition moiety (L6S-G) is linked via a β-1,4 linkage to either another porphyran moiety or to an agarose moiety (Correc *et al.*, 2011). Such a hybrid structure is usual for agars, and the number of porphyran motifs varies according to the red algal species (Popper *et al.*, 2011). Recently, a new class of enzymes has been discovered in the genome of *Z. galactanivorans*: β-porphyranases, which specifically hydrolyze the β-1,4 linkage between porphyran motifs in agars. These enzymes define a new subfamily within the GH16 family. The crystal structures of *Zg*PorA (Figure II-1E) and *Zg*PorB reveal a porphyran binding mode involving conserved basic amino acids (Hehemann et al, 2010). The fine differences in substrate specificity between the β-agarases and β-porphyranases of *Z. galactanivorans* have been further studied, and a comprehensive model for this complex agarolytic system has been proposed (Hehemann, Correc, *et al.*, 2012). Fascinatingly, β-porphyranase genes from algal epibionts have been found in human gut bacteria isolated from Japanese individuals, suggesting that edible seaweeds with their associated marine bacteria were the route through which the gut bacteria acquired these novel polysaccharidases (Hehemann *et al.*, 2010). This hypothesis is strengthened by the experimental demonstration that the Japanese gut bacterium *Bacteroides plebeius* can grow on porphyran (Hehemann, Kelly, *et al.*, 2012). Moreover, the putative glycoside hydrolases BpGH16B and BpGH86A have been characterized as active β-porphyranases. The structure of BpGH86A in a complex with an oligo-porphyran has also been solved (Figure II-1F), revealing a TIM barrel domain with an extended substrate-binding cleft and two accessory β-sandwich domains (Hehemann, Kelly, *et al.*, 2012). Thus, GH86 enzymes constitute a polyspecific family including both β-agarases and β-porphyranases.

Table II-2 Census of the algal-specific polysaccharidases from seaweed-associated bacteria**.** Only enzymes characterized at the molecular and biochemical level have been considered. Some bacterial sequences from other environments have been added when they constitute representative enzymatic activities or 3D structure.

Protein	CAZy	Bacterial species	Associated algal species (or isolation habitat)*	Genbank	PDB	References
Carrageenases						
κ-carrageenase *Pc*CgkA	GH16	*Pseudoaltermonas* carrageenovora ATCC 43555	seawater	CAA50624.1	1DYP	(Barbeyron et al. 1994 Michel et al. 2001a)
κ-carrageenase	GH16	*Pseudoalteromonas porphyrae* LL1	Decayed seaweed	ADD92366.1	-	(Liu et al. 2011)
κ-carrageenase *Zg*CgkA	GH16	*Zobellia galactanivorans* Dsij	Delesseria sanguinea (R)	CAZ94309.1	-	(Barbeyron et al. 1998)
κ-carrageenase	GH16	*Zobellia sp*. ZM-2	Decayed seaweed	AGS43006.1	-	(Liu et al. 2013)
ι-carrageenase *Af*CgiA	GH82	*Alteromonas fortis* ATCC 43554	Seawater	CAC07801.1	1H80 1KTW 3LMW	(Barbeyron et al. 2000 Michel et al. 2001b Michel et al. 2003 Rebuffet et al. 2010)
ι-carrageenase *Zg*CgiA1	GH82	*Zobellia galactanivorans* Dsij	*Delesseria sanguinea* (R)	CAC07822.1	-	(Barbeyron et al. 2000)
ι-carrageenase *Zg*CgiA2	GH82	*Zobellia galactanivorans* Dsij	*Delesseria sanguinea* (R)	CAZ96312.1	-	(Rebuffet et al. 2010)
ι-carrageenase *Zg*CgiA3	GH82	*Zobellia galactanivorans* Dsij	*Delesseria sanguinea* (R)	CBW46642.1	-	(Rebuffet et al. 2010)
ι-carrageenase CgiA	GH82	*Cellulophaga sp*. QY3	*Grateloupia livida* (R)	AEV89930.1	-	(Ma et al. 2013)
ι-carrageenase CgiB	GH82	*Cellulophaga sp*. QY3	*Grateloupia livida* (R)	AGN70890.1	-	(Ma et al. 2013)
λ-carrageenase *Pc*CglA	GHnc	*Pseudoaltermonas carrageenovora* ATCC 43555	seawater	CAL37005.1	-	(Guibet et al. 2007)
λ-carrageenase CglA	GHnc	*Pseudoalteromonas sp*. CL19	Deep-sea sediment	BAF35571.1	-	(Ohta et al. 2006)
Agarases						
α-agarase *Aa*AgaA	GH96	"*Alteromonas agarlytica*" DSM 12513	Seawater	AAF26838.1	-	(Potin et al. 1993) Flament et al. 2007)
α-agarase Aga33	GH96	*Thalassomonas agarivorans* JAMB A33	Deep-sea sediment	BAF44076.1	-	(Hatada et al. 2006)
β-agarase *Zg*AgaA	GH16	*Zobellia galactanivorans* Dsij	*Delesseria sanguinea* (R)	CAZ98338.1	1O4Y 1URX	(Allouch et al. 2003 Allouch et al. 2004 Jam et al. 2005)
β-agarase *Zg*AgaB	GH16	*Zobellia* galactanivorans Dsij	*Delesseria sanguinea* (R)	AAF21821.1	1O4Z 4ATF	(Allouch et al. 2003 Jam et al. 2005 Hehemann et al. 2012a)
β-agarase *Zg*AgaD	GH16	*Zobellia galactanivorans* Dsij	*Delesseria sanguinea* (R)	CAZ98378.1	4ASM	(Hehemann et al. 2012a)
β-agarase *Aa*gA	GH16	*Pseudoalteromonas gracilis* B9	*Gracilaria gracilis* (R)	AAF03246.1	-	(Schroeder et al. 2003)
β-agarase	GH16	*Pseudoalteromonas sp*. AG4	*Chondrus crispus* (R)	ADD60418.1	-	(Oh et al. 2010)
β-agarase AgaYT	GH16	*Flammeovirga yaeyamensis* YT	*Gracilaria tenuistipitata* (R)	AEK80424.1	-	(Yang et al. 2011)
β-agarase AgaG1	GH16	*Alteromonas sp*. GNUM-1	*Sargassum serratifolium* (B)	AGW43026.1	-	(Kim et al. 2012)
Exo-β-agarase AgaD	GH50	*Saccharophagus degradans* 2-40	*Spartina alterniflora* (salt marsh plant)	ABD81904.1	4BQ2 4BQ3 4BQ4 4BQ5	(Pluvinage et al. 2013a)
β-agarase II (AgrA)	GH86	*Pseudoalteromonas atlantica* T6c	*Palmaria palmata* (R)	ABG40858.1	-	(Belas 1986)
β-agarase AgaC	GH118	*Vibrio sp*. PO-303	*seawater*	BAF03590.1	-	(Dong et al. 2006)
β-agarase AgaB	GH118	*Pseudoalteromonas sp*. CY24	*seawater*	AAQ56237.1	-	(Ma et al. 2007)
Porphyranases						

β-porphyranase ZgPorA	GH16	*Zobellia galactanivorans* Dsij	*Delesseria sanguinea* (R)	CBM41182.1	3ILF 4ATE	(Hehemann et al. 2010)
β-porphyranase ZgPorB	GH16	*Zobellia galactanivorans* Dsij	*Delesseria sanguinea* (R)	CAZ95074.1	3JUU	(Hehemann et al. 2010)
β-porphyranase BpGH16B	GH16	*Bacteroides plebeius* DSM 17135	Japanese gut microbiota	EDY95423.1	4AWD	(Hehemann et al. 2013)
β-porphyranase BpGH86A	GH86	*Bacteroides plebeius* DSM 17135	Japanese gut microbiota	EDY95427.1	4AW7	(Hehemann et al. 2013)
α-1,3-(3,6-anhydro)-L-galactosidases						
ZgAhgA	GH117	*Zobellia galactanivorans* Dsij	*Delesseria sanguinea* (R)	CBM41465.1	3P2N	(Rebuffet et al. 2011)
SdNABH	GH117	*Saccharophagus degradans* 2-40	*Spartina alterniflora* (salt marsh plant)	ABD81917.1	3R4Y 3R4Z	(Ha et al. 2011)
BpGH117	GH117	*Bacteroides plebeius* DSM 17135	Japanese gut microbiota	EDY95405.1	4AK5 4AK6 4AK7	(Hehemann et al. 2012b)
Laminarinases						
Algal laminarin-specific β-glucanase *Zg*LamA	GH16	*Zobellia galactanivorans* Dsij	*Delesseria sanguinea* (R)	CAZ96583.1	4BOW 4BPZ 4BQ1	(Labourel et al. 2014)
Fucoidanases						
Fucoidanase *Mf*FcnA	GH107	*Marineflexile fucanivorans* SW5	Alginate-extraction factory	CAI47003.1	-	(Colin et al. 2006)
Alginate lyases						
Alginate lyase *Zg*AlyA4	PL6	*Zobellia galactanivorans* Dsij	*Delesseria sanguinea* (R)	CAZ98265.1	-	(Thomas et al. 2012)
Endo-guluronate lyase *Zg*AlyA1	PL7	*Zobellia galactanivorans* Dsij	*Delesseria sanguinea* (R)	CAZ95239.1	3ZPY	(Thomas et al. 2012) (Thomas et al. 2013)
Exo-alginate lyase *Zg*AlyA5	PL7	*Zobellia galactanivorans* Dsij	*Delesseria sanguinea* (R)	CAZ98266.1	4BE3	(Thomas et al. 2012) (Thomas et al. 2013)
Poly-mannuronate lyase AlxM	PL7	*Photobacterium sp*. ATCC 43367	*Sargassum fluitans* (B)	CAA49630.1	-	(Mallisard et al. 1993)
Alginate lyase Aly1	PL7	*Streptomyces sp*. ALG-5	green seaweed (G)	AAP47162.1	-	(Kim et al 2009)
Alginate lyase AlyVI	PL7	*Vibrio sp*. QY101	*Laminaria sp*. (B)	AAP45155.1	-	(Han et al. 2004)
Alginate lyase *Zg*AlyA7	PL14	*Zobellia galactanivorans* Dsij	*Delesseria sanguinea* (R)	CAZ98462.1	-	(Thomas et al. 2012)
Poly-MG alginate lyase alyPEEC	PL18	*Pseudoalteromonas elyakovii* IAM14594	*Laminaria sp*. (B)	AAD16034.1	-	(Sawabe et al. 2001)
Alginate lyase Aly-SJ02	PL18	*Pseudoalteromonas sp*. SM0524	Kelp (B)	ACB87607.1	-	(Li et al. 2011)
Ulvan lyases						
Ulvan lyase	PLnc	*Persicivirga ulvanivorans* PLR	Feces of *Aplysia* punctata (sea hare) feed with *Ulva sp*. (G)	AEN28574.1	-	(Nyvall-Collen et al. 2011)

*B= Brown alga, G= Green alga, R= Red alga

α-1,3-(3,6-Anhydro)-L-galactosidases

Z. galactanivorans has also been pivotal in the discovery of a third class of enzymes involved in the catabolism of agars: the hypothetical protein Zg4663, distantly related to GH43 enzymes, has emerged as a specific α-1,3-galactosidase catalyzing the removal of 3,6-anhydro-L-galactose residues from the non-reducing ends of oligo-agars released by β-agarases, hence the name α-1,3-(3,6-anhydro)-L-galactosidase (*Zg*AhgA, also known as α-1,3-L-neoagarooligosaccharide hydrolase). It defines a new family of glycoside hydrolases, the GH117 family (Rebuffet *et al.*, 2011). AhgA features a helix-turn-helix (HTH) domain connected to a five-bladed β-propeller domain and forms a dimer by swapping of the HTH domain (Figure II-1G). The putative catalytic residues, partially conserved with GH43 enzymes, are located at the bottom of the funnel-like active site. The mechanism of *Zg*AhgA is cation dependent, and a zinc ion has been identified in the active site, with an unusual coordination sphere occupied by water molecules. The amino acids binding these water molecules (and thus indirectly this cation) are strictly conserved with the GH117 family(Rebuffet *et al.*, 2011). Two homologs of *Zg*AhgA have been characterized more recently, *Sd*NABH from *S. degradans* and *Bp*GH117 from *B. plebeius*. While no cation was found in the structure of *Sd*NABH (Ha *et al.*, 2011), *Bp*GH117 features a magnesium ion at the position conserved with *Zg*AhgA (Hehemann, Smyth, *et al.*, 2012), suggesting a degree of plasticity for this cation-binding site. The structure of an inactive mutant of *Bp*GH117 has also been determined, in a complex with neoagarobiose, identifying key residues for substrate recognition and catalysis. A mutagenesis approach has confirmed the involvement of five residues in catalysis: Asp90, Asp245, and Glu303 (conserved in family GH43), Glu167 (involved in the cation-binding site), and His302 (Hehemann, Smyth, *et al.*, 2012).

Alginate lyases

Alginate is a polymer of D-mannuronate and of its C5-epimer L-guluronate. It is an expolysaccharide in some bacteria and also the main cell wall compound of brown algae (Popper *et al.*, 2011). Interestingly, genomic analysis has provided evidence that the common ancestor of brown algae acquired the alginate biosynthesis pathway from actinobacteria (Michel *et al.*, 2010b). This highlights the importance of associated bacteria in the evolution of macroalgae. Alginate lyases (EC 4.2.2.3) are the key enzymes in alginate degradation and remodeling, to be found in seven polysaccharide lyase families: PL5, PL6, PL7, PL14, PL15, PL17, and PL18. Despite the importance of algal alginate as renewable biomass, most of the characterized alginate lyases originate from alginate-producing bacteria and from terrestrial bacteria feeding on bacterial alginate (Cantarel *et al.*, 2009). Only five genes have been cloned from seaweed-associated bacteria: three PL7 genes (Malissard *et al.*, 1993; Han *et al.*, 2004; Kim *et al.*,

2009) and two PL18 genes (Sawabe *et al.*, 2001; Li *et al.*, 2011). Knowledge in this field has recently advanced with the characterization of the alginolytic system of *Z. galactanivorans* (Thomas *et al.*, 2012). This flavobacterium possesses seven alginate lyase genes (two PL6, three PL7, one PL14, and one PL17 gene) and a PL15 gene of uncertain specificity. Five of these genes are organized in clusters: a small cluster (*alyA4*, *alyA5*, *alyA6*) and a large cluster including *alyA2*, *alyA3*, and numerous carbohydrate-related genes predicted to be involved in alginate uptake and assimilation and in transcriptional regulation. These clusters have been shown to be genuine operons induced by alginate. *Zg*AlyA1, *Zg*AlyA4, *Zg*AlyA5, and *Zg*AlyA7 have been overexpressed in *Escherichia coli* and confirmed to be active alginate lyases. *Zg*2622 and *Zg*2614 are, respectively, a dehydrogenase and a kinase, further converting the terminal unsaturated monosaccharides released by alginate lyases to 2-keto-3-deoxy-6-phosphogluconate (Thomas *et al.*, 2012). An in-depth study has demonstrated that *Zg*AlyA1 (PL7) is an endolytic guluronate lyase (EC 4.2.2.11), and *Zg*AlyA5 (PL7) cleaves unsaturated units, α-L-guluronate, or β-D-manuronate residues at the nonreducing ends of oligo-alginates in an exolytic fashion (EC 4.2.2.-). Despite a common jelly-roll fold, these striking differences in mode of action are due to different active site topologies: an open cleft in *Zg*AlyA1 (Figure II-1H), whereas *Zg*AlyA5 displays a pocket topology due to the presence of additional loops partially obstructing the catalytic groove (Figure II-1I). Lastly, in contrast to PL7 alginate lyases from terrestrial bacteria, both enzymes proceed according to a calcium-dependent mechanism, suggesting an exquisite adaptation to their natural substrate in the context of brown algal cell walls (Thomas *et al.*, 2013).

Fucoidanases

Fucoidans are sulfated polysaccharides containing α-L-fucose residues and present in the cell wall of brown algae. They encompass a continuous spectrum of highly ramified polysaccharides, ranging from high-uronic-acid, low-sulfate polymers with significant proportions of D-xylose, D-galactose, and D-mannose to highly sulfated homofucan molecules (Popper et al, 2011). Only one fucanolytic gene has been cloned to date: the fucoidanase *fncA* from *Mariniflexile fucanivorans* SW5 (Colin *et al.*, 2006). This marine flavobacterium was isolated from a water-treatment facility that recycles the effluent from an algal alginate extraction plant (Descamps *et al.*, 2006; Barbeyron, L'Haridon, *et al.*, 2008). FcnA encompasses an N-terminal catalytic module (~400 residues), three immunoglobulin-like modules, and a PorSS secretion module. A recombinant protein including the N-terminal module and the immunoglobulin-like modules has been overexpressed in *E. coli*, purified, and shown to retain the same activity as the wild-type enzyme. This fucoidanase releases as end products a tetrasaccharide and a hexasaccharide, and cleaves the α-1,4 glycosidic bonds between L-fucose-2,3-disulfate-α-1,3-L-fucose-2-sulfate repeating units. The N-terminal catalytic module displays ~25%

identity to two patented fucoidanases from the bacterial strain SN-1009, and together these three proteins define a novel family of glycoside hydrolases, family GH107 (Colin *et al.*, 2006).

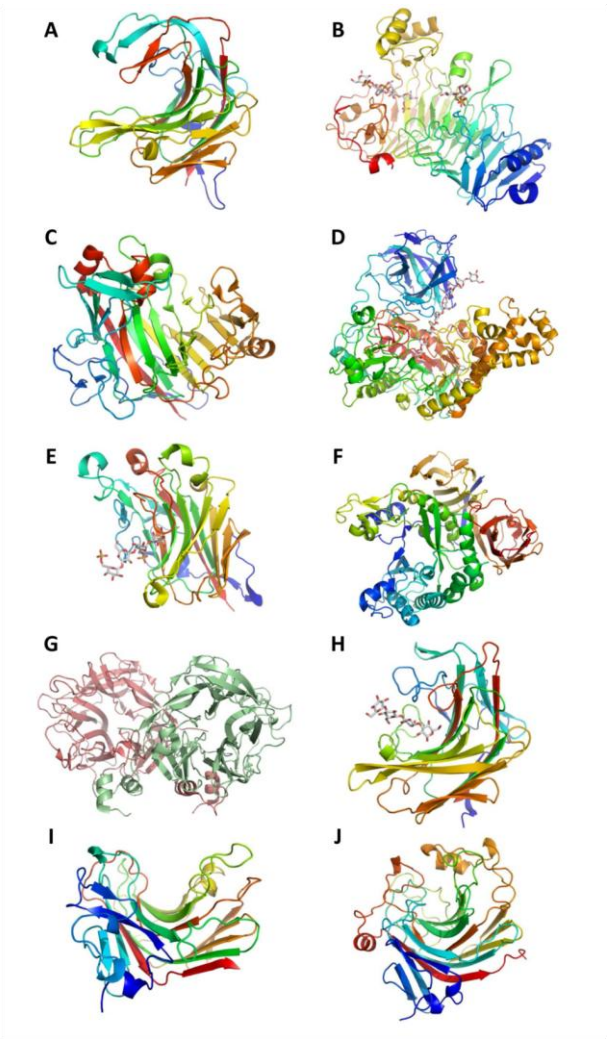

Figure II-1 Representative crystal structures of algal specific-polysaccharidases. Structure of the GH16 kappa-carrageenase from Pseudoalteromonas carrageenovora (A, PDB 1DYP), of the GH82 iota-carrageenase from Alteromonas fortis in complex with oligo-iota-carrageenans (B, PDB 1KTW), of the GH16 beta-agarase ZgAgaD from Zobellia galactanivorans (C, PDB 4ASM), of the GH50 exo-beta-agarase Aga50D from Saccharophagus degradans in complex with an oligo-agar (D, PDB 4BQ5), of the GH16 beta-porphyranase ZgPorA from Z. galactanivorans in complex with an oligo-porphyran (E, PDB 3ILF), of the GH86 beta-porphyranase BpGH86A from Bacteroides plebius (F, PDB 4AW7), of the GH117 α-1,3-(3,6-anhydro)-L-galactosidase ZgAhgA from Z. galactanivorans (G, PDB 3P2N), of the GH16 laminarinase ZgLamA from Z. galactanivorans in complex with an oligo-laminarin (H, PDB 4BOW), of the PL7 endo-guluronate lyase ZgAlyA1 from Z. galactanivorans (I, PDB 3ZPY), and the PL7 exo alginate lyase ZgAlyA5 from Z. galactanivorans (J, PDB 3ZPY). The β-strands and the α-helices are represented by arrows and ribbons, respectively. The oligosaccharides are displayed with a stick representation. With the exception of ZgAhgA, all the structures are colored with a rainbow spectrum from the N- (blue) to the C-terminus (red). Chains A and B of ZgAhgA are colored in pink and green, respectively. This figure was prepared with the program Pymol.

Laminarinases

Laminarin, the storage polysaccharide of brown algae, is a small vacuolar beta-1,3-glucan containing ~25 glucosyl residues and some occasional β-1,6-linked branches. It includes two series, the minor G-series, containing only glucose residues, and the more abundant M-series, displaying a D-mannitol residue at the reducing end (Read *et al.*, 1996). The unique presence of mannitol in laminarin is also explained by the horizontal gene transfer event involving the common ancestor of brown algae and an ancestral actinobacterium (Michel *et al.*, 2010a). Laminarinases (EC 3.2.1.6 and 3.2.1.39) are found in several GH families (GH16, GH17, GH55, GH64, GH81, and GH128). Numerous beta-1,3-glucanases of terrestrial bacteria have been characterized in the context of the degradation of cell-wall beta-1,3-glucans of fungi, oomycetes, and land plants. Amazingly, however, among all the characterized beta-1,3-glucanases reported in the CAZY database (Cantarel *et al.*, 2009), only one laminarinase gene has been cloned from a seaweed-associated bacterium: the GH16 laminarinase *Zg*LamA of *Z. galactanivorans* (Labourel *et al.*, 2014). The 3D structure of *Zg*LamA$_{GH16}$ and of two enzyme-substrate complexes, one with laminaritetraose and one with a trisaccharide of 1,3-1,4-β-D-glucan, have been determined this year. Compared to other GH16 laminarinases, ZgLamA$_{GH16}$ contains a unique additional loop which gives a bent shape to the active-site cleft of the enzyme. This particular topology is perfectly adapted to the U-shaped conformation of laminarin chains in solution, and thus explains the predominant specificity of ZgLamAGH16 for this substrate (Labourel *et al.*, 2014).

Ulvan lyases

Ulvans are the main cell-wall components of green algae of the genus *Ulva* (Popper *et al.*, 2011). These complex sulfated polysaccharides are composed mainly of sulfated L-rhamnose, D-glucuronic acid and its C5-epimer L-iduronic acid, and a minor fraction of D-xylose (Lahaye and Robic, 2007). The first described ulvanolytic bacterium was isolated at a "green tide" site in the Saint-Brieuc Bay (Brittany). This bacterium was not further characterized, but a semi-purified enzyme was shown to cleave the β-(1,4) linkage between L-rhamnose-3-sulfate (Rha3S) and D-glucuronic acid (GlcA), releasing an oligosaccharide with an unsaturated uronic acid at the non-reducing end. This enzyme was thus a polysaccharide lyase, referred to as an ulvan lyase (Lahaye *et al.*, 1997). The only ulvan lyase gene to have been cloned was obtained from *Persicivirga ulvanivorans* (Nyvall Collén *et al.*, 2011) a flavobacterium isolated from the faeces of the mollusc *Aplysia punctata* having fed on *Ulva* sp. (Barbeyron *et al.*, 2011). This enzyme is endolytic and cleaves the glycosidic bond between the sulfated rhamnose and a glucuronic or iduronic acid. The sequence of this ulvan lyase has no similarity to known

proteins (Nyvall Collén *et al.*, 2011) and is currently an unclassified polysaccharide lyase in the CAZY database.

Microorganisms enhance algal defense, growth, and nutrient uptake

An increasing number of reviews discuss the beneficial contribution of microorganisms to algae, and notably their role in improving algal defense and nutrient uptake and in stimulating algal morphology and algal spore germination (Goecke *et al.*, 2010; Barott *et al.*, 2011; Harder *et al.*, 2012; Egan *et al.*, 2013). Alga-associated bacteria contribute to algal defense by producing antimicrobial and antifouling compounds (Wilson *et al.*, 2011). Table II-3 shows diverse algal species on which bacteria with antimicrobial activities have been identified. Some 27% of isolated strains, on the average, show antimicrobial/antibacterial activity. The percentage is much lower for planktonic strains isolated from seawater (only 7% show antimicrobial activity) and even lower in terrestrial samples (Penesyan *et al.*, 2009). The most represented bacterial genera are *Bacillus, Pseudoalteromonas, Pseudomonas,* and *Streptomyces.* Gram-positive *Bacillus* and *Streptomyces* and gram-negative *Pseudomonas* and *Pseudoalteromonas* are genera known for their ability to produce bioactive compounds (Bhatnagar and Kim, 2010).

Prokaryotes have also been observed to synthesize necessary vitamins (Croft *et al.*, 2005, 2006) and growth factors (Dimitrieva *et al.*, 2006; Tsavkelova *et al.*, 2006) and to improve algal growth by making these compounds accessible in sufficient amount.

Lastly, microbial epibionts produce common hydrolytic enzymes that improve algal nutrient uptake and development. A bacterial strain isolated from the red alga *Sargassum serratifolium*, for example, was shown to contain, in addition to agarase activities, diverse other hydrolytic activities such as amylase, alkaline phosphatase, esterase and lipase (C14), β-galactosidase, and urease activities (Kim and Hong, 2012). In other bacterial strains also found on *Sargassum sp.*, amylase, carboxymethylcellulase, and protease activities were found (Mohapatra *et al.*, 2003). An alkaline serine protease with potential use in the laundry industry was found in *Bacillus megaterium* RRM2, isolated from a red alga species (Rajkumar *et al.*, 2011). Furthermore, bacterial enzymes such as lipases and esterases (Rajkumar *et al.*, 2011), cellulases (Gibbs *et al.*, 1992; Dong *et al.*, 2010; Fu *et al.*, 2010), proteases (Cristóbal *et al.*, 2011; Yang *et al.*, 2013), amylases (Both *et al.*, 1993; Liu *et al.*, 2012), laccases (Ge *et al.*, 2011; Fang *et al.*, 2012), and beta-glucosidases (Cristóbal *et al.*, 2009; Mai *et al.*, 2013), distantly related to terrestrial ones and displaying original biochemistry, are increasingly being isolated from the marine environment.

All these interesting bioactive compounds and enzymes produced by microorganisms in interaction with algae might predictably be very useful in diverse medical and industrial applications, as described in the next section.

Table II-3 Percentages and genera of bacterial strains exhibiting antimicrobial activities, isolated on seaweeds.

Algae phyla	Algae species	Isolated strains with antimicrobial activities (total isolated strains)	Bacteria genera identified with antimicrobial activities	Source
Phaeophyta	*Laminaria saccharina*	49% (210)	*Bacillus, Glaciecola, Kopriimonas, Mesorhizobium, Pseudoalteromonas, , Streptomyces,*	(Wiese *et al.*, 2009b)
	Pelvetia canaliculata *Fucus ceranoides*	7 % (55) 13 % (45)	*Alteromonas, Pseudomonas*	(Lemos *et al.*, 1985)
	Fucus vesiculosus.	53% (69)	*Bacillus, Parracoccus, Pseudomonas, Streptomyces*	(Goecke, Labes, *et al.*, 2013)
	Sargassum serratifolium *Sargassum fusiforme* *Sargassum filicinum* *Padina arborescens* *Undaria pinnatifida* *Petalonia fascia* *Colpomenia sinuosa* *Scytosiphon lomentaria* *Ecklonia cava*	20% (116)	*Bacillus*	(Kanagasabhapathy *et al.*, 2006)
Rodophyta	*Delesseria sanguinea*	51% (97)	*Algoriphagus, Microbacterium, Paenibacillus, Pseudoalteromonas, Streptomyces, Zobellia*	(Goecke, Labes, *et al.*, 2013)
	Jania rubens	36 % (19)	*Aquamarina, Bacillus, Paracoccus, Pseudoalteromonas, Pseudomonas*	(Ismail-Ben Ali *et al.*, 2011)
	Pachymeniopsis lauceola *Plocamium telfairiae* *Gelidium amansii* *Chondrus oncellatus* *Grateloupia filicina* *Ceramium kondoi* *Lomentaria catenata* *Schizymenia dubyi* *Porphyra yezoensis*	33% (92)	*Bacillus, Microbacterium, Psychrobacter, Vibrio*	(Kanagasabhapathy *et al.*, 2008)
	Delisea pulchra	12% (325)	*Micrococcus, Phaeobacter, Pseudoaltermonas, Rhodobacteraceae, Roseobacter, Ruegeri, Schwenalla, Vibrio*	(Penesyan *et al.*, 2009)
Chlorophyta	*Ulva australis*	12% (325)	*Bacillus, Flavobacteriaceae, Phaeobacter, Photobacterium , Roseobacter*	(Penesyan *et al.*, 2009)
	Enteromorpha intestinalis *Enteromorpha compressa* *Ulva lactuca*	34 % (46) 18% (33) 13 % (45)	*Alteromonas, Pseudomonas*	(Lemos *et al.*, 1985)

4. INTEREST OF MACROALGA-ASSOCIATED MICROORGANISMS IN BIOTECHNOLOGICAL APPLICATIONS

Medical and pharmaceutical applications

Microbial pathogens are becoming increasingly resistant to antibiotics, making some human infections untreatable. Hence, **new antimicrobial compounds** of natural origin, specifically targeting certain pathogens, are urgently needed. The marine environment is increasingly explored for such compounds. Although marine macroorganisms, including algae, are known to produce many interesting antimicrobial, antifungal, and potentially therapeutic compounds (Engel *et al.*, 2002; Steinberg and de Nys, 2002; Kubanek *et al.*, 2003; Mayer and Gustafson, 2003; Takamatsu *et al.*, 2003; Paul and Puglisi, 2004), ensuring a continued supply of eukaryotic compounds seems quite impossible. Producing such compounds would require growing macroorganisms in large quantity, and this would require much time and space (Dobretsov *et al.*, 2006). Furthermore, the chemical synthesis of complex eukaryotic compounds is difficult. Therefore as microorganisms on algae release many bioactive compounds that prevent extensive colonization by other microorganisms, larvae, or algae, they could represent an interesting source of new antimicrobials (Table II-3), easily exploitable as they produce compounds faster in large quantity and are easier to culture. Moreover, marine microorganisms seem extremely productive of **secondary metabolites**: in addition to antimicrobial metabolites, they have been found to produce antitumor, anticancer, cytotoxic, and photoprotective compounds (Bhatnagar and Kim, 2010). For example, it has recently been shown that phloroglucinol, a precursor of brown algal phlorotannins used in medicine to treat abdominal pain (Chassany *et al.*, 2007), is synthesized by a polyketide synthase acquired through horizontal gene transfer (HGT) from an ancestral actinobacterium (Meslet-Cladiere *et al.*, 2013). This highlights the importance of bacterial epibionts both in algal evolution and as a source of interesting bioactive compounds (Meslet-Cladiere et al, 2013). Lastly, algal-polysaccharide-degrading enzymes have a wide range of medical and pharmaceutical applications because they produce remarkable biologically **active oligosaccharides** with properties useful in maintaining human health, such as anticoagulant (Pereira *et al.*, 1999; Pushpamali *et al.*, 2008), anti-inflammatory (Berteau and Mulloy, 2003), antioxidant (Hatada *et al.*, 2006; Jiao *et al.*, 2012), or immunostimulating activity (Bhattacharyya *et al.*, 2010). Furthermore, oligosaccharides derived from ulvans, agars, carrageenans, alginates, and other less known algal polysaccharides are explored for their potential use as prebiotics favoring gut health in humans and animals (O'Sullivan *et al.*, 2010). To obtain active oligosaccharides

with the desired properties, enzymatic production with specific algal-polysaccharide-degrading enzymes is required. As microorganisms on algae are main producers of such specific enzymes, they represent a great source of them.

Production of biofuels

The need to preserve fossil fuels has prompted increasing efforts to produce biofuels. Initial efforts focused on producing biofuels from plant biomass. Unfortunately, using this biomass requires complex extraction methods due to the presence of recalcitrant polysaccharides such as lignocellulose. Furthermore, to obtain plant biomass one needs land for cultivation, in competition with human and animal food. Therefore, non-lignocellulosic macroalgal biomass, requiring no land for cultivation and possessing a high carbohydrate content, seems an interesting alternative for biofuel production. Promising results have been obtained in studies aiming to produce bioethanol from brown algae (Wargacki *et al.*, 2012; Enquist-Newman *et al.*, 2013) or red algae (H. T. Kim *et al.*, 2012). All of these studies used microbial enzymes, directly or indirectly, to degrade specific algal polysaccharides. For example, Wargacki et al. (2012) used *Escherichia coli* strains transformed with DNA encoding enzymes involved in alginate transport and metabolism, in combination with an extracellular depolymerization system, to metabolize alginate and synthesize ethanol. Kim et al. (2012) used several microbial agarases to saccharify agarose to monosugars for further fermentation to ethanol. Lastly, the very recent study of Enquist-Newman et al. (2013) used bacterial alginate and mannitol catabolism genes in *Saccharomyces cerevisiae* to metabolize alginate monomers (4-deoxy-L-erythro-5-hexoseulose uronates) and mannitol from brown seaweeds, for further fermentation of sugar to ethanol. These recent promising works demonstrate the advantage of identifying algal polysaccharide-degrading enzymes and the encoding genes for the production of green energy.

Industrial applications

The diversity of non-lignocellulosic, sulfated poly- and monosaccharides makes algal hydrocarbons interesting for diverse industrial and biotechnological applications (Table II-4). The most used and studied alga-specific polysaccharides are agars and carrageenans (red algae), ulvans (green algae), alginates, laminarin, and sulfated fucoidans (brown algae) (Popper *et al.*, 2011). **Polysaccharide biotechnology** uses enzymes or enzyme systems to convert carbohydrate polymers to added-value new polysaccharides (De Ruiter and Rudolph, 1997) and thus requires hydrolytic enzymes such as agarases, carrageenases, alginate lyases, fucoidanases, porphyranases, and sulfatases to modify useful algal polysaccharides, improve their structures, and enhance their functionalities. Moreover, enzymatic hydrolysis is increasingly viewed as a promising alternative to current chemical extraction methods (Gavrilescu and Chisti, 2005).

Therefore, industrialists also seek hydrolytic enzymes with commonly exploited activities, e.g. proteases, cellulases, amylases, beta-glucosidases and laccases, but with original properties making them suitable for new applications. To date, most enzymes used in industry have been isolated from microorganisms living in terrestrial environments, mainly soils. Marine microorganisms, being exposed to extreme temperature, pressure, salinity, and nutrient availability conditions, should provide new enzymes with original biochemistry and characteristics (Kennedy *et al.*, 2011). Lastly, alga-associated microbial communities respond to their exposure to alga-derived metabolites by producing a range of **specific compounds**, potentially of biotechnological interest. For example, various industries use halogenated compounds that become a hazard when they end up in the environment. Many studies therefore focus on optimizing their biodegradation by microbial dehalogenases (Swanson, 1999). Alga-associated microorganisms constitute a potential source of dehalogenases, as they appear to resist the halogenated metabolites that algae produce as a defense mechanism (Potin *et al.*, 1999).

Table II-4 Industrial applications using algal polysaccharides.

Industrial domain	Industrial use	Algal polysaccharides	Source
Pharmacy	Laxative	Agars	(McHugh, 2003; Li *et al.*, 2008; Bixler and Porse, 2010; Jiao *et al.*, 2011; Jung *et al.*, 2013)
	Toothpaste	Carrageenans	
	Therapeutic peptides	Carrageenans, Fucoidans	
	Pharamaceutical tablet desintegrant	Alginates	
	Medical fiber	Alginates	
	Wound dressing	Alginates	
	Controlled release of medical drugs and other chemicals	Alginates	
	Dietary food	Alginates, Carrageenans	
Food industry	Gelling properties	Agars, Carrageenans	(McHugh, 2003)
	Stabilizer	Agars, Alginates	
	Thickener	Agars, Alginates	
	Meat substitute	Agars	
	Wine clarification	Agars	
	Prevent pulp precipitation in fruit juices	Alginates	
	Conservation of frozen fish	Alginates	
Laboratory	Bacterial growth	Agar	(McHugh, 2003)
Phytopharmacie	Activate signal pathway in plants and enhance their immune system	Alginates, Carrageenans, Fucans, Laminarin, Ulvan	(Vera *et al.*, 2011)
Paper industry	Smooth paper	Alginates	(McHugh, 2003)
Textile industry	thickeners for the paste containing the dye	Alginates	(McHugh, 2003)
Biofuel	Biogas, bioethanol, biobutanol	Alginate, Laminarin, Mannitol	(Wargacki *et al.*, 2012; Jung *et al.*, 2013)
Other industry	Welding rod	Alginates	(McHugh, 2003)
	Immobilized biocatalyst	Alginates	

5. PROSPECTS FOR EXPLOITING ALGAL EPIBIONTS

As shown in the first part of this review, microorganisms living on algae are highly diverse but underexplored. The composition of alga-associated microbial communities varies, for example, according to the alga phylum and species, the season, and the age of the thalli. Furthermore, as these microorganisms constantly metabolize algal products, they produce numerous specific enzymes and secondary metabolites. From their immense diversity and their constant activity stems their great potential as a source of novel and original enzymes and metabolites. Furthermore, specific hydrolytic enzymes with novel biochemistry are increasingly sought for biotechnological applications in biomass and biofuel production, medicine, and wide-ranging industrial applications. Algal polysaccharidases identified to date (such as agarases, carrageenases, and alginate lyases) display very specific structures and biochemistry, related only distantly to those of known terrestrial glycoside hydrolases. This highlights their huge potential for new and original biotechnological uses and the importance of investigating these interesting enzymes.

Most published investigations on algal epibionts and their metabolites have relied on cultivation methods. To our knowledge, indeed, all specific enzymes isolated from algal epibionts have been obtained from cultivable microbial strains. Some high-throughput screens of algal microbial communities have been performed, but functional metagenomics has not been used to identify new microbial enzymes and metabolites. Functional metagenomics and techniques such as high-throughput sequencing are powerful means of gaining knowledge on the microorganisms composing these underexplored communities and of identifying novel metabolites and specific enzymes produced by alga-associated microbes.

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Conflicts of Interest

The authors declare no conflict of interest.

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Chapter III. Construction and functional screening of (meta)genomic libraries

Outline

The previous chapter describes the numerous interactions between alga-associated bacteria and their host and states that these bacteria are obviously able to produce common and macroalgal-specific polysaccharidases. In order to investigate which functional analyzing methods could be the most appropriated to explore this microflore for its particular enzymes, this second review-chapter briefly discuss common practices used to identify novel enzymes from environmental samples and focus on the construction and functional screening of genomic or metagenomic libraries.

Related publication

The hunt for original microbial enzymes: an initiatory review on the construction and functional screening of (meta)genomic libraries

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Marjolaine Martin, Micheline Vandenbol

The hunt for original microbial enzymes: an initiatory review on the construction and functional screening of (meta)genomic libraries.

Abstract

Introduction. Discovering novel enzymes is of interest in both applied and basic science. Microbial enzymes, which are incredibly diverse and easy to produce, are increasingly sought by diverse approaches.

Literature. This review first distinguishes culture-based from culture-independent methods, detailing within each group the advantages and drawbacks of sequence- and function-based methods. It then discusses the main factors affecting the success of endeavors to identify novel enzymes through construction and functional screening of genomic or metagenomic libraries: the sampled environment, how DNA is extracted and processed, the vector used (plasmid, cosmid, fosmid, BAC, or shuttle vector), and the host cell chosen from the available prokaryotic and eukaryotic ones. Conclusion. Library construction and screening can be tricky and requires expertise. Combining different strategies, such as working with cultivable and non-cultivable organisms, using sequence- and function-based approaches, or performing multihost screenings, is probably the best way to identify novel and diverse enzymes from an environmental sample.

Keywords

Functional genomics, functional metagenomics, cultivable, non-cultivable, alternative host, enzymes, microorganisms, vectors.

1. INTRODUCTION

In both applied and basic science, there is currently great interest in identifying and producing novel enzymes and biocatalysts. On the one hand, this could contribute to develop green industrial applications and white biotechnologies (Gavrilescu and Chisti, 2005), while on the other hand, the discovery of novel enzyme genes and functions can help us understand specific ecosystems (Ufarte et al., 2015). Furthermore, the study of original enzymes with novel three-dimensional structures or catalytic mechanisms can shed light on the complex relationships between protein structure and function (Ufarte et al., 2015).

Microorganisms are the greatest and most studied source of enzymes, mainly because they are easy to manipulate and to produce in large scales. In addition, their enzymes are biochemically diverse and have broad range of activities facing variation in environmental parameters as pH, temperature, salinity, etc (Adrio and Demain, 2014). To discover novel microbial enzymes, diverse types of functional analysis can be applied either to microorganisms themselves or to microbial genomes. In this review, we highlight the different ways in which DNA libraries screening can lead to identify novel genes, enzymes, protein families, and functions. We first briefly place the different techniques used for this purpose in their respective contexts, distinguishing culture-based from culture-independent methods. We then discuss the factors liable to limit the output of these approaches: the sampled environment, the chosen vector and DNA insert size range, the paucity of available host cells, and certain crucial or optional steps performed during functional screening. The chart on Figure 1 provides an outline of these methods. For complementary information on the various topics broached, readers can refer to the most recent reviews cited throughout this publication.

2. TRADITIONAL AND CURRENT TECHNIQUES

Towards the end of the 19th century, researchers discovered that certain natural proteins, for which the term “enzyme” was coined, act as biocatalysts. They also became aware of the potential use of enzymatic catalysis to replace chemical catalysis, and set out to develop such applications, using either whole cultivable cells or (partially) purified preparations of natural enzymes. Efforts then focused on finding or creating enzymes with improved features. In the 1990s, directed evolution emerged as a novel means of improving known enzymes. It involves generating from a microbe producing a protein of interest a library of mutants by random approaches and then screen the library for specific and better activity, selectivity, and/or stability (Cobb et al., 2013). It now includes a package of traditional and modern mutation strategies for improving or altering the activity of known biocatalysts (for recent reviews, see Denard et al., 2015;

Packer and Liu, 2015). Another milestone was the advent of metagenomics, the culture-independent genomics of entire microbial consortia present in environmental samples. Metagenomics was first used to assess bacterial diversity through phylogenetic analysis of 16S rRNA sequences and to answer the question "who is in there?". It rapidly gained a more functional dimension, with attempts to answer more difficult questions: "what are they doing?" or "what can they do?" (Handelsman, 2004).

Microorganisms in an environmental sample include a small minority of cultivable ones and a huge majority of not-yet-cultivable microorganisms. Approaches to identify novel enzymes from each of these groups are described below.

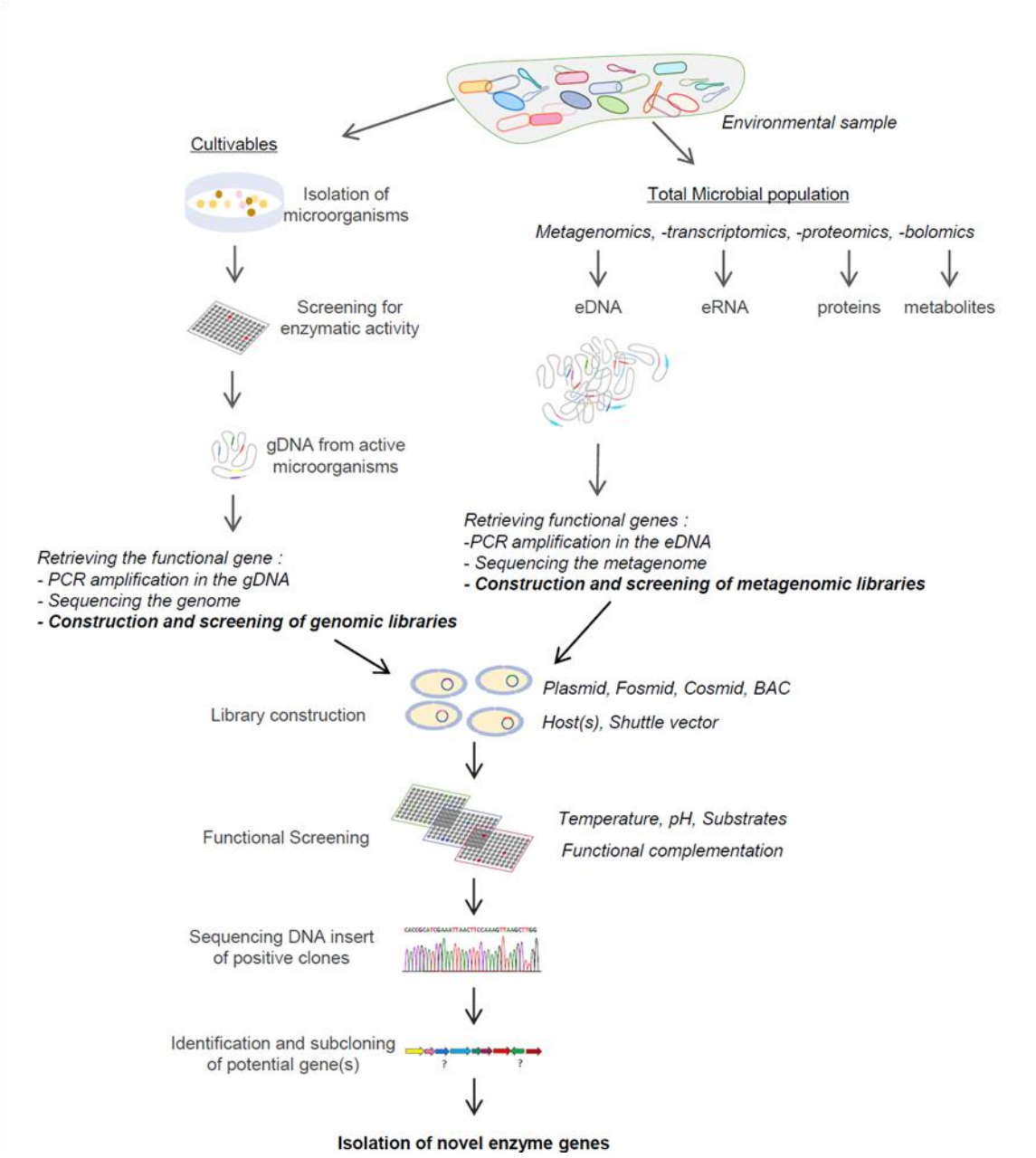

Figure III-1 Representation of steps leading to the identification of novel enzymes through the construction and functional screening of (meta)genomic libraries.

Culture-dependent or independent approaches

Enzymes have long been recovered from cultivable microorganisms exhibiting specific activities. Microorganisms isolated from an environmental sample are screened in liquid or solid medium for activities of interest. Active natural isolates can then be used directly in bioreactors, either to produce the enzyme or to catalyze an industrial reaction (Roberts et al., 1995). However, optimizing the process can require countless adjustments, diverse side reactions might dominate or interfere with the substrate, and a product or co-solvent might disrupt the enzyme (Roberts et al., 1995). An alternative approach is to clone the enzyme-encoding gene into a well-known host cell whose behavior can be controlled. To retrieve the gene of interest sequence-based and function-based approaches can be used (discussed below). In contrast, culture-independent approaches (working with non-cultivable organisms or total microflorae) always involve, as a first step, extraction of nucleic acids or gene products. According to what is extracted from an environmental sample, i.e. total microbial DNA, RNA, proteins, or metabolites, researchers speak of metagenomics, metatranscriptomics (Warnecke and Hess, 2009), metaproteomics (Schofield and Sherman, 2013), or metabolomics (Prosser et al., 2014). At the time of sampling, the last three disciplines mentioned retrieve only gene transcripts or produced proteins or metabolites. They are mainly used to understand functional interactions and discover novel metabolic pathways. Here we will focus solely on metagenomics. This discipline also includes sequence-based and function-based approaches (discussed below).

Sequence-based approaches

To retrieve a gene encoding for an enzyme of interest in genomic DNA (gDNA) or environmental DNA (eDNA), one can either amplify it by Polymerase Chain Reaction (PCR), using primers designed from sequence motifs found in similar enzymes, or identify it by sequencing the entire microbial gDNA or eDNA (shot-gun or DNA library sequencing) and comparing its sequences against genomic databases. This last method was unthinkable before, as Sanger sequencing was costly and very time consuming. Fortunately, sequencing has become less expensive in recent years, and results are now rapidly obtained thanks to second (e.g. 454, MiSeq Illumina, Ion torrent) and third (e.g. PacBio) generation sequencing methods (reviewed in Bleidorn, 2015; Faure and Joly, 2015; Rhoads and Au, 2015). Yet even though processing of sequence data has been simplified more and more by progress in bioinformatics, it can be difficult or time consuming to choose a specific enzyme in the immensity of generated data, to predict the characteristics of identified putative enzymes or whether a protein will be produced easily in cultivable host cells for further analysis. These sequence-based approaches are possible only if the enzymes sought are closely related to known ones; they cannot lead to the discovery of completely novel enzymes or enzyme families. Finally, this type of

approach can also yield false hits, due to the numerous wrong annotations found in non-curated databases. Sequence-based methods are therefore used mostly to explore the microbial diversity of an environment on the basis of 16S or 18S rRNA gene sequences or to understand the gene arrangement in a microbial genome.

Function-based approaches

The gDNA from a microorganism of interest or the eDNA from an studied environment might be used to construct (meta)genomic libraries in a well-known cultivable host cell, and then screening these libraries for clones displaying the sought enzymatic activity. Functional (meta)genomics, which relies solely on gene function rather than sequence similarities, has a considerable advantage when applied to novel bacterial taxa (strains, species or genera) or unknown bacteria, since it has a high probability of yielding genes encoding novel enzymes. In addition, it is possible to screen for specific enzymatic characteristics by varying the screening conditions (e.g. temperature, pH and substrate concentration). Lastly, if an enzyme-encoding gene is recovered by activity screening, the protein should be readily produced in the well-known host used for library construction. Functional (meta)genomics has already led to the discovery of extraordinary novel biocatalysts from all around the world and to assigning numerous "hypothetical proteins" in databases (Ferrer et al., 2016). Nevertheless, the screening is very fastidious, particularly when applied to metagenomes (many clones have to be screened to cover a majority of the genes present in an environmental sample). The screening yields are generally low, given the multiple constraints (such as heterologous expression in the chosen host cell, substrate affinity or a missing co-factor) (Ekkers et al., 2012a), even more with functional metagenomics because no selection of "active" microorganisms is done upstream from library construction and screening (which can be realized while working with cultivable microorganisms). Therefore functional metagenomics is recommended for work on low-density populations of microbes that are hard to grow. Robotized high-throughput screening may also considerably enhance the number of screened clones and inevitably the number of positive hits. Function-based approaches on cultivable microorganisms can yield enzymes closely related to those of other cultivable organisms. To maximize the yield and the novelty of the resulting discoveries, it is therefore advisable to exploit underexplored environments and/or to develop novel growth media, conditions and/or techniques of isolation (Highlander, 2014; Kamagata, 2015). Over the past decade, innovations have emerged in the culture and isolation of microorganisms (reviewed in Pham and Kim, 2012). Cycling cultures implies cyclical varying culture and growth conditions (Dorofeev et al., 2014). Culture in micro wells with phenotypic microarrays are used to screen for and identify optimal growth conditions (Borglin et al., 2012). In situ techniques are also developed to enhance interaction with the environment and the other microorganisms living in it (e.g. Jung et

al., 2014; Steinert et al., 2014). The use of novel isolation media will lead to the identification of unknown bacterial taxa and hence to the discovery of exciting novel enzymes.

3. SAMPLED ENVIRONMENTS

Microorganisms are found in every single environment on earth and must obviously produce enzymes enabling them to survive wherever they live (Yarza et al., 2014). Therefore, the functional analysis of each microbial niche should contribute to the knowledge on how ecosystems work and lead to identifying original functional genes and enzymes. The choice of an environment to be prospected will depend on the type of enzyme one seeks and on the desired features of the identified biocatalysts. All environments are not equal in the manner they should be explored and in the diversity and novelty of the findings they will yield.

Soils and oceans have been intensely investigated for their microbial diversity. However marine waters have been much less studied by functional analysis. Nevertheless, the microbial diversity of soils and oceans is so immense that these resources still remain undersampled, and their potential as sources of new enzyme discoveries seems infinite. Microbial hotspots and/or hot moments, described respectively by ecologists as spots and short periods of time showing disproportionately high reaction rates relative to the surrounding matrix or to adjacent longer time periods (Kuzyakov and Blagodatskaya, 2015; De Monte et al., 2013), could help in choosing the particular habit to be explored in these vast environments and the moment of the sampling.

Exploring extreme environments has also led to identifying original biocatalysts with unusual characteristics: so-called microbial extremozymes (Raddadi et al., 2015). Recent reviews focus on how to improve screening conditions and yields in the case of samples from cold (Vester et al., 2015) or saline environments (de Lourdes Moreno et al., 2013; Raval et al., 2013), using culture-dependent and -independent methods. Bioprospection for enzymes of other extremophilic microbes, such as piezophiles from deep-sea sediments (Kato, 2012) or (halo)alkaliphiles (Borkar, 2015), is still in its infancy, because of the very specific culturing and screening conditions it requires. Nevertheless, such microorganisms should have huge biotechnological potential.

In the last decade, functional studies have also focused on gut microbiota, biofilms, and symbionts. Reviews on the subject include for example one devoted to microbes inhabiting the human gut (Walker et al., 2014), one on insect symbionts (Berasategui et al., 2015), one on rumen microbes (Morgavi et al., 2013), and one on algal biofilms (Martin et al., 2014). Interactions between microorganisms and their host are generally intense, and sites where symbiosis occurs are rich in enzymes. Microorganisms living in tight, specialized symbiosis with a host or with other microbes tend not to grow well in

culture and should therefore be best suited for functional metagenomics (Handelsman, 2004).

Finally, naturally or artificially enriched environments (Kamagata, 2015), such as copper-enriched (Riquelme et al., 1997) and oil-fed soils (Narihiro et al., 2014), can also be explored for novel enzyme types with important ecological or industrial applications.

The good news is that a practically infinite number of environments remain to be tapped for novel enzymes. Even among the environments that have been studied by metagenomics over the last 20 years, it seems that only 11% have been studied with this goal in mind (Ferrer et al., 2016). Functional analysis of samples taken from as yet unexplored habitats is bound to yield original and exceptional microbial biocatalysts.

4. DNA EXTRACTION AND PROCESSING

Once the environment is chosen, it is necessary to culture microbial cells, screen them for activity, and extract gDNA or to directly extract eDNA (culture-independent approach). The quality of the extracted DNA might be checked on a agarose gel and the its quantity and its purity by spectophotometry (e.g. with the NanoDropTM spectrophotometers). The extracted gDNA or eDNA should not be degraded and be as pure as possible. If the DNA is degraded, its quantity and the average insert size will be affected, and if contaminants (e.g. humic acids coextracted from soil samples (Zhou et al., 1996), host DNA from alga-associated bacteria (Burke et al., 2009), or residual chemicals from the extraction method) remain, it will be hard to achieve enzymatic DNA restriction and ligation or the libraries will be biased. DNA could be purifiy and size-selected on agarose gel or by ethanol or PEG/NaCl precipitation (He et al., 2013). If eDNA is recovered, the extraction method yield must be high, to not preferentially retain or eliminate some taxa and, thus, to avoid diversity bias (Thomas et al., 2012).

When the quality of the extracted DNA has been checked, the DNA is digested with restriction enzymes to the desire insert-size (see below small- and large-insert libraries) and to obtain compatible ends for further cloning. Then, the purified fragments are cloned into cloning vectors by enzymatic DNA ligation for introduction into host cells. The restriction enzyme is chosen mainly according to the type of ligation envisaged (blunt or sticky ends), whether and where the extracted DNA is methylated (some restriction enzymes are sensitive to dam, dcm, or CpG methylation), and the desired DNA insert size. Two types of libraries can be constructed: small- and large-insert libraries (reviewed in Kakirde et al., 2010).

Small-insert libraries contain DNA fragments smaller than 20 kb inserted into plasmids. These vectors have high copy numbers and strong vector-borne promoters, thus favoring higher enzyme production and better activity detection. Small DNA fragments are easily manipulated, ligated into vectors, and introduced into host cells, but working with

plasmids is fastidious, as they cover only small fragments of DNA the screening to find positive clones requires a large number of clones to be analyzed.

Large-insert libraries are technically harder to construct but have the advantage of providing more information on the phylogenetic affiliation of the DNA insert and the identified functional genes. Furthermore, large inserts favor the identification of enzymes encoded by genes in large clusters or operons and whose synthesis depends on constitutional promoters upstream from the genes of interest. On the other hand, a larger insert is more likely to have a transcription terminator before the gene of interest, and thus to display early transcription termination (Gabor et al., 2004). To prevent this, adequate vectors and host strains have been developed by genetic engineering (Terrón-González et al., 2013). Cosmids and fosmids can accommodate DNA inserts 25 to 50 kb in size, and bigger ones (up to 300 kb) can be cloned into bacterial artificial chromosomes (BACs). Cosmids are artificially constructed vectors containing the Cos site, which permits packaging of DNA into phage lambda for transfection of E.coli. BACs, designed to introduce large DNA inserts into E.coli, are based on the single-copy F plasmid of this bacterium. The inserted DNA is present in low copy number and is thus more stable (Shizuya et al., 1992; Wanga et al., 2014). Fosmids are cosmid-based vectors containing the replication origin of the E. coli F plasmid as well. They thus combine the stability-favoring properties of BACs with easier manipulation (Rodriguez-Valera, 2014). Kits are now available for easy cloning of DNA into fosmids/BACs and even for increasing the copy number of the insert-bearing vector in E. coli. Examples include the cloning kits CopyRight® v2.0 Fosmid (Lucigen, USA), CopyControl™ BAC, and CopyControl™ Fosmid Library (Epicentre, USA).

When choosing a cloning vector one should also consider the host cell to be used for library construction and screening. If one intends to use different hosts, it could be best to use a shuttle vector or a broad-host-range vector containing more than one replication origin, suitable for expression in various hosts (Aakvik et al., 2009; Martinez et al., 2004).

5. HOST CELLS FOR LIBRARY CONSTRUCTION AND/OR SCREENING

Heterologous expression is a major challenge in functional screening of (meta)genomic libraries. The transformed host cell must be able to express the foreign DNA and ensure proper folding of the resulting protein(s), and this is not easily achieved. Promoter, terminator, and ribosome binding sites can be added to cloning vectors, and expression can be predicted by bioinformatics (Gabor et al., 2004), but some factors affecting transcription, translation, or the state of a protein in the host cell can be problematic and impossible to control. For example, rare codons unrecognized by the

host cell can lead to bad translation, production of truncated polypeptides or formation of inclusion bodies after translation, resulting in insoluble and inactive proteins.

Host-cell characteristics for functional (meta)genomics

Host cells for constructing DNA libraries are not easy to find, because they must meet many requirements. (i) Being transformable (i.e. having natural competence) is not enough; they should have high transformation yields. When constructing libraries, numerous unique recombinant plasmids must be introduced. (ii) Microbial cells don't easily accept and express foreign DNA. The host cells should thus be genetically accessible and modifiable. They generally contain mutations affecting the production of enzymes liable to affect good heterologous expression, such as DNAses, proteases, or recombinases. Expression of foreign genes might be further enhanced by introducing genes encoding heterologous sigma factors (recognizing heterologous promoters) into the host genome (Gaida et al., 2015). (iii) Transformed host cells should be easily detected. The sensitivity of bacteria to some specific antibiotic is generally used. If the cloning vector contains a resistance gene for this antibiotic (selection marker), only transformed cells are able to grow on a medium containing the antibiotic. Yeast transformants can be selected by functional complementation of an auxotrophic marker. For example, if a gene required for uracil production is disrupted in the host cell and if the cloning vector carries the functional gene, transformants can be recognized on the basis of their ability to grow on uracil-free medium. (iv) A good host should also show no activity on the screening medium. Ideally, it should show as few enzymatic activities as possible to make it a good host for functional screening. Host cells can also be deprived by mutation of certain vital activities (e.g. DNA polymerase) to allow isolation of enzyme genes by functional complementation (Simon et al., 2009).

Prokaryotic hosts

The most widely used bacterial host is the model bacterium Escherichia coli. This Gram-negative host is commonly used for library construction, because of its amenability to genetic engineering, its high transformation efficiency, and the availability of numerous genetic tools created for it. Several chemically competent or electro-competent E. coli strains are commercially available as well as efficient laboratory protocols to prepare competent E. coli cells. Although libraries are almost always constructed in E.coli, they can be screened in other bacteria if shuttle vectors are used. Examples of other bacterial species that have been used in (meta)genomic library screens include the proteobacterium Pseudomonas putida and it psychrophilic variant P. antartica, the thermophile Thermus thermophilus, and the Gram-positive bacteria Bacillus subtilis and Streptomyces lividans, (reviewed in Liebl et al., 2014; Leis et al., 2013; Taupp et al., 2011). It can be assumed that close phylogenetic relationship between the expression

host and the organism from which the foreign DNA derives should favor heterologous expression, and the efficiency of multi-host screenings in the identification of enzymes or molecules has indeed frequently been demonstrated. Despite the advantages of using different hosts, one should bear in mind that it always requires specific molecular tools (see host characteristic above).

Eukaryotic hosts

Microbial eukaryotes can also be used as screening hosts. Yeasts such as Saccharomyces cerevisiae (whose genetics is well known and for which many genetic tools are available) and Pichia pastoris (with which excellent protein production yields are achieved) are widely used for their numerous advantages in high-level heterologous expression of genes encoding for enzymes (Liu et al., 2013). Such organisms combine the advantages of unicellular cells (easy to grow and manipulate genetically) with those of eukaryotic cells (better protein processing than in prokaryotes, allowing post-translational modifications and glycosylation) (Gündüz Ergün and Çalık, 2015; Porro et al., 2005). A eukaryotic host should thus be the best choice for expressing genes from eukaryotic microbes. Yet as eukaryotic genomic DNA contains numerous introns, splicing of heterologous DNA could be problematic for the host. This explains why the use of cDNA libraries (obtained from RNA) is recommended for screening in eukaryotes (Kellner et al., 2011). The biggest limitations of using yeasts in functional screening could be poor recognition of heterologous promoters (especially if they are bacterial), low transformation yields (the libraries are then constructed in E.coli and screened in yeast), and the multiple enzymatic activities displayed by yeasts (the host should be mutated in all genes encoding enzymes of interest).

6. FUNCTIONAL SCREENING OF LIBRARIES

Almost 7000 enzyme types are currently listed in the BRENDA database (Chang et al., 2015). They are classified in six classes: oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases. As it is impossible to cover all the existing screening tests developed to date, next we show the most important steps in functional screening.

Usually, **isolated host-cell colonies** containing plasmids with unique DNA inserts are recovered in 96-well plates (and stored at -80°C in glycerol) for further screening. Otherwise, the colonies can be pooled in liquid culture, which is less fastidious at the outset but which can lead to generating biased libraries (some clones becoming dominant over or toxic towards others) and makes recovery of positive clones more laborious (as multiple copies of each clone will be present in the pool). **How many clones** one should screen depends on the size of the screened genome (easily estimated for gDNA but less obvious for eDNA and depending of the number of species present in the environmental

sample), the DNA insert size range or the sizes and expression patterns of the genes sought (expression might depend on a vector promoter and/or RBS region and on the average distance between start codon and terminator) (for a review, see Gabor, 2004). According to Gabor et al (2004), the number of clones screened should exceed 10^7, which is seldom the case (it is generally around 10^4-10^6), as generating and screening such huge libraries is probably too fastidious.

Hydrolytic activities are generally assayed by growing the clones on agar plates or in well plates with liquid screening medium, and then detecting specific phenotypic traits. A color change occurring around the colony or in the well (directly or after addition of a second substrate), a clear halo, degradation of the medium, and fluorescence are the major visual observations used to detect an active clone. Such screens are easy to perform and do not require specific or high-technology material (unless colony-picking robots or microplate readers are used to speed up the screening). As the sensitivity of these phenotypic detection methods is usually low, they are used mostly when the aim is to scan the functional potential of a library (i.e. to scan for a broad range of enzymes), rather than to find a specific type of enzyme. One should bear in mind that a positive clone might appear negative because of inappropriate screening conditions, an inappropriate substrate, or because the enzyme is not been secreted (the phenotype may then appear later as a result of cell lysis).

It is recommended to vary the **screening conditions**, to enhance the screening yield. Plates can easily be placed at different temperatures, for example, preferentially after overnight growth at the optimal growth temperature of the host. As enzymes might "prefer" some kind of substrates, prospection can be carried out on a broad range of natural, modified, and fully synthetic substrates (Leis et al., 2013). Well-known cofactors of the searched enzyme type can be added to the screening medium. Although varying the screening conditions may be time consuming, it can save time later by providing knowledge for the selection of clones with particular properties and/or for further characterization of the enzymes responsible for detected activities.

To avoid the problem of **non-secretion**, one can mix cell lysates (obtained by enzymatic, physical, or chemical cell lysis) or permeablilized cells (obtained by treatment with a gentle detergent) with the screening substrate to enhance sensitivity (Taupp et al., 2011). Before cell lysis or permeabilization, the clones can be grown with the substrate of the enzyme sought, to enhance induction of a constitutional promoter of the gene of interest on the DNA insert. Likewise, UV- and heat-inducible vectors causing cell lysis have been developed to enhance extracellular activities (Li et al., 2007; Xu et al., 2006).

As stated above, functional screening can also be done with a mutant host cell impaired in a vital enzymatic activity. The advantage of heterologous complementation is that the

host cell is equipped to produce a protein having the same function, and that only clones producing the enzyme sought are viable (Ekkers et al., 2012b). This method is very sensitive, but it is applied mostly to the identification of metabolites, as few vital enzymes are sought.

7. CONCLUSION

The construction of libraries and their functional screening require experience and expertise. Choosing which environment to sample and which enzymes to seek is already a daunting task, given the immense diversity of both environments and enzymes. Once these decisions are made, success is likely to depend on other choices: the host cell used, the DNA insert size range, the targeted microorganisms, among others. In fact, there is no single perfect way to obtain high yields and to discover novel enzymes. In most cases, functional (meta)genomics with adequate adjustments should lead to the identification of novel enzymes. The best way to obtain a wide diversity of novel enzymes is probably to combine different strategies, such as working with cultivable and non-cultivable organisms, using sequence- and function-based approaches, performing multi-host screenings, and constructing libraries in both plasmids and fosmids.

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Chapter IV. Functional metagenomics

Outline

So far, enzymes from algal epibionts have been mainly obtained from cutlivable bacterial strains and algal biofilms have never been prospected for hydolytic enzymes by functional metagenomics. Towards our objective, we thus decided to work, as a first approach, with the whole bacterial population associated with the brown alga *Ascophyllum nodosum*, (which includes a small minority of cultivable and a huge majority of not-yet-cultivable microorganisms). Therefore, we set up the construction of a metagenomic library from algal biofilm and screeneed it, in a first instance and as a proof of feasibility for hydrolytic enzymes with established activity test. This resulted in the identification of several novel enzyme genes, including an interesting cold-active and halotolerant endo-cellulase which was purified and partially characterized.

Related publication

Identification and characterization of a halotolerant, cold-active marine endo-β-1,4-glucanase by using functional metagenomics of seaweed-associated microbiota

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Identification and characterization of a halotolerant, cold-active marine endo-β-1,4-glucanase by using functional metagenomics of seaweed-associated microbiota

Abstract

A metagenomic library was constructed from microorganisms associated with the brown alga *Ascophyllum nodosum*. Functional screening of this library revealed 13 novel putative esterase loci and two glycoside hydrolase loci. Sequence and gene cluster analysis showed the wide diversity of the identified enzymes and gave an idea of the microbial populations present during the sample collection period. Lastly, an endo-β-1,4-glucanase having less than 50% identity to sequences of known cellulases was purified and partially characterized, showing activity at low temperature and after prolonged incubation in concentrated salt solutions.

Keywords

Microbial enzymes, *Ascophyllum nodosum,* metagenomics, plates tests, biomass hydrolysis, cellulase, beta-glucosidase, esterase, functional screening, GH5

1. INTRODUCTION

Previous surveys have revealed that less than 1% of existing microorganisms can be studied by traditional culturing methods. This leaves most microorganisms and their by-products unknown and unexploited (Amann *et al.*, 1990; Pace, 1997) and explains why metagenomics, a culture-independent approach using total microbial genomes from environment samples, has met with great success over the past decade (Handelsman, 2004; Sleator *et al.*, 2008). Sequence-based metagenomics has already provided information about the composition, organization, functioning, and hierarchy of microbial communities in particular habitats (Edwards *et al.*, 2010). On the other hand, functional genomics applied to metagenomic libraries from diverse environments has led to the discovery of many new enzymes and bioactive compounds and to substantial enrichment of genome databases. To date, most of the enzymes brought to light through metagenomics have been derived from soil (Feng *et al.*, 2007; Sharma *et al.*, 2010; Geng *et al.*, 2012; Biver and Vandenbol, 2013) and gut (Tasse *et al.*, 2010; Mattéotti *et al.*, 2011; Wang *et al.*, 2011; Ferrer *et al.*, 2012) samples.

Marine microorganisms represent great candidate sources of original biocatalysts, as they are exposed to extreme conditions of temperature, pressure, salinity, nutrient availability, etc. Hence, functional screening of marine microbial populations should yield new enzymes with specific and unique physiological and biochemical properties, produced by organisms far different from those usually described in terrestrial environments (Kennedy *et al.*, 2011). New enzymes have already been identified in marine metagenomic libraries from seawater samples (Lee *et al.*, 2006; D.-G. Lee *et al.*, 2007) and from microorganisms in symbiosis with marine organisms such as sponges and corals (Sim *et al.*, 2012). To our knowledge, however, nobody has yet performed functional screening of metagenomic libraries from algal microbial communities. As algal microbial biofilms are in constant interaction with algal biomass, they represent an interesting source of enzymes and other active compounds (Martin, Portetelle, *et al.*, 2014). Sequence-based studies have already revealed the importance and functions of microbial communities living on the surfaces of algae, showing tight interdependence between algae and their biofilms (Fisher *et al.*, 1998; Burke, Steinberg, *et al.*, 2011; Lachnit *et al.*, 2011). Furthermore, many genes coding for enzymes involved in hydrolyzing algal biomass, such as cellulases, xylanases, α-amylases, and specific algal polysaccharidases (e.g. agarases, carrageenases, and alginate lyases) have been identified from cultivable marine bacteria (Debnath *et al.*, 2007; Michel and Czjzek, 2013). Only a few such enzymes have been found again in marine metagenomes by functional screening. For example, no cellulase gene has yet been identified by marine metagenomics, although cellulase genes have been identified in the genomes of cultivable marine bacteria such as *Caldocellum saccharolyticum* (Gibbs *et al.*, 1992),

Paenibacillus sp. BME14 (Fu *et al.*, 2010), *Martelella mediterranea* (Dong *et al.*, 2010), and *Marinobacter sp*. MS1032 (S. Shanmughapriya *et al.*, 2010). Cellulases are widely used in biotechnological applications (e. g. in the pulp and paper, textile, and food industries) and their capacity to convert cellulosic biomass is increasingly exploited in sustainable applications and bioprocesses (Kuhad *et al.*, 2011). Although several cellulases are already in use, there is an increasing demand for cellulases with better or special industrial properties (Duan and Feng, 2010). Here we describe the construction and functional screening of a metagenomic library from microorganisms living on the annual brown alga *Ascophyllum nodosum*. The library was screened for particular hydrolytic enzymes. The screen has yielded genes encoding new esterases belonging to diverse lipolytic enzyme families, a novel beta-glucosidase, and a novel cellulase. These discoveries highlight the utility of seeking original biocatalysts in algal biofilm populations. We have purified and characterized the identified new cellulase. To our knowledge, no functional screening of a metagenomic library from alga-associated microbial biofilms has been realized before, and no cellulase has already been identified by functional metagenomics in a marine environment.

2. MATERIALS AND METHODS

Environmental Sample Collection

Several plants (~ 100g) of *Ascophyllum nodosum* were collected from the foreshore (48° 43' 36.07" SE, -3° 59' 22.96") in Roscoff (Brittany, France), at the start of February 2012. Samples were collected in triplicate and put in sterile plastic bags. The algae were rinsed three times with sterile seawater to remove loosely associated microorganisms and cut into smaller sections. Washed and cut algae were freeze-dried at -80°C in new sterile plastic bags until DNA extraction.

Microbial Genomic DNA Extraction

Bacterial DNA was extracted by swabbing the algal surfaces with sterile cotton tips followed by classic DNA extraction (Lachnit *et al.*, 2009). Six cotton tips were used per 25 g of algae. Microbial DNA was extracted from the cotton tips with 6.75 ml lysis buffer (100 mM Tris-HCl; 0.5 M EDTA; 100 mM sodium phosphate; 5 M NaCl; 10% CTAB) and incubated with 50 µl proteinase K (10 mg/ml) at 37°C. After one hour, 750 µl of 20% SDS was added and the cotton tips were incubated for 2 h at 65°C, with manual inversion every 20 minutes. After centrifugation, 1 volume of chloroform-isoamyl alcohol (v:v, 24:1) was added to the supernatant and the mixture centrifuged. The aqueous phase was recovered and DNA was precipitated with 0.6 vol isopropanol for one hour at room temperature. DNA was recovered by centrifugation (30 min – 10.000g) at 4°C, washed with 70% ethanol, and the pellet was air dried and resuspended in 100 µl Tris-

EDTA. The DNA extracted from triplicate samples was pooled for DNA library construction. The quantity of DNA was measured with a Qubit® (Invitrogen) and its quality was checked on a 0.8% agarose gel.

Library Construction and functional screening

The pool of DNA obtained from triplicate samples was partially cut with DpnII (New Engeland Biolabs). A PEG/NaCl size-selective precipitation was done according to Biver et al. (Biver and Vandenbol, 2013) in order to remove small DNA inserts. An equal volume of 6.5% PEG/0.4M NaCl was added to the restriction fragments (final DNA concentration: 20 ng/µl) and the mixture was incubated for 2 h at RT. The DNA was purified by migration through a 1% low-melting-point agarose gel (Promega) and DNA inserts between 1.5 and 7 kb were recovered by AgarACE digestion (Promega). The inserts were ligated into the BamHI (Roche) linearized and dephosphorylated (Roche) shuttle vector YEp356 (Novagen) with T4 DNA ligase (Roche). Electrompetent Electromax *E. coli* cells (Life Technologies) were finally transformed with the ligation product. The average DNA insert size was estimated by isolation and purification of 20 randomly chosen plasmids. The transformants were pooled and the metagenomic library was plated and screened for lipolytic activity on 3% tribuytrin 2xYT agar plates containing 100 µg/ml ampicillin. Beta-glucosidases were sought on 2xYT agar containing 0.5% esculin (Sigma—Aldrich) and 0.1% ammonium iron (III) citrate (Sigma—Aldrich) (Mattéotti *et al.*, 2011). Cellulolytic activity was detected by plating the library on 2xYT agar plates containing 1 g/l insoluble synthetic AZCL-HE-cellulose (Megazyme) and 100µg/ml ampicillin. A blue halo was observed around colonies exhibiting cellulase activity. The plasmids of positive clones were extracted and used for activity confirmation in DH5α *E. coli* subcloning cells (Life Technologies). Insert sizes were estimated with restriction enzymes cutting at both extremities of the DNA insert (*HindIII* and *EcoRI*, Roche).

DNA sequencing and sequence analysis

DNA inserts of positive clones were sequenced at GATC Biotech (Germany). Sequence similarity searches were carried out with the NCBI BlastP program. Phylogenetic trees were produced by the neighbor-joining method with MEGA6.06. Signal peptides were predicted with the SignalP 4.1 server (Petersen *et al.*, 2011). All putative proteins corresponding to the identified ORFs in the inserts were analyzed with BlastP against the GenBank non-redundant database and Swissprot (curated database). The putative esterases were also analyzed with Pfam (Finn *et al.*, 2014).

Overexpression and purification of the CellMM5.1 cellulase

The coding sequence of the *Cell5.1_3* gene was amplified, without its putative peptide signal, with Platinum *Pfx* DNA Polymerase, 0.5x PCRx Enhancer Solution (Invitrogen), 5'-gggaattcCATATGCAAACCTCTGACAGCTATGTTCCGC -3' as forward primer, and 5'-cgcGGATCCTCATCA**GTGGTGGTGGTGGTGGTG**GCGCCCAAGCAAGGCGT -3' as reverse primer. The restriction sites (NdeI, BamHI) used for subcloning are underlined and the histidine tag sequence appears in bold. PCR products were cloned into the pET30b(+) vector (Novagen) and the resulting plasmids were introduced into *E. coli* Rosetta 2 (DE3) cells (Novagen). The cellulolytic activity of the obtained transformants was evaluated by spotting 5 µl overnight culture (grown at 37°C in liquid 2xYT medium, 30 µg/ml chloramphenicol and 30 µg/ml kanamycin) onto solid 2xYT medium supplemented with 10 µM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Fermentas), 1g/l AZCL-HE-cellulose (Megazyme), and appropriate concentrations of antibiotics (30 µg/ml chloramphenicol and 30 µg/ml kanamycin). The bacteria were allowed to grow at 37°C for 3 days. To purify the recombinant cellulase, an overnight culture of transformed *E. coli* Rosetta cells was diluted to OD_{600nm}=0.005 in a 1l shake flask containing 200 ml 2xYT medium and the corresponding antibiotics. The cultures were grown at 37°C under shaking at 160 rpm until the OD_{600nm} reached about 0.4. Overproduction was induced by addition of 1 µM IPTG followed by incubation at 16°C for ~20 h. The cells were harvested by centrifugation and lysed at 37°C for 30 min in 17 ml lysis buffer (20 mM Tris-HCl buffer pH 8 containing 0.3 M NaCl, 1 ml/l Triton X-100, and 5 mg/ml lysozyme) according to Biver et al (2013). After sonication on ice (4 cycles of 5 s, 100 W, Sonifier 250, Branson), the lysate was centrifuged at 10,000g for 20 min (4°C) to separate insoluble (I0) from soluble (S0) proteins. To solubilize the recombinant cellulase found mostly in the resulting pellet (I0), the latter was incubated at 4°C for 4 h in 5 ml solubilization buffer (20 mM Tris-HCl pH 8, 0.5 M NaCl, 20 ml/l Triton X-100, 2 M urea). The partly resuspended fraction was then centrifuged again to collect the soluble fraction (S1) now containing a sufficient amount of His-tagged cellulase, which was next purified by Ni-NTA affinity chromatography (Qiagen). The soluble fraction was diluted 10 times in 20 mM Tris-HCl pH 8 and 0.5 M NaCl to reduce the urea and Triton X-100 concentrations to 0.2 M and 2 ml/l, respectively, and then incubated overnight at 4°C with 200 µl Ni-NTA agarose pre-equilibrated with binding buffer (20 mM Tris-HCl pH 8, 0.5 M NaCl, 2 ml/l Triton X-100, 0.2 M urea). The resin was recovered by centrifugation at 800 g for 5 min and washed twice with 1 ml washing buffer 1 (20 mM Tris-HCl pH 7.5, 0.5 M NaCl, 1 ml/l Triton X-100, 10 mM imidazole) and twice with 1 ml washing buffer 2 (20 mM Tris-HCl pH 7.5, 0.5 M NaCl, 1 ml/l Triton X-100, 20 mM imidazole) before elution with 2 ml of 20 mM Tris-HCl pH 7.5, 0.5 M NaCl, 250 mM imidazole. After analysis by SDS-PAGE, this

first eluate (E1) was repurified by Ni-NTA chromatography (E2) and then dialyzed at 4°C against 50 mM sodium phosphate pH 6.8 containing 50 mM NaCl.

Cell5.1_3 cellulase activity characterization

Enzymatic activity was quantified by measuring the quantity of generated reducing sugars with the 3',5'-dinitrosalicylic acid reagent (DNSA, Sigma) (Miller, 1959). The enzymatic assay was performed with 1% (w/v) carboxymethylcellulose (CMC) (Ultra low viscosity, Fluka), 50 mM phosphate buffer pH 7 for 10 minutes at 40°C in a total volume of 390 µl, to which 10 µl suitably diluted enzyme was added. Then an equal volume (400 µl) of DNSA was added and the mixture heated for 10 minutes at 99°C. After cooling on ice, the absorbance was read at 540 nm (Multiskan Go, ThermoScientific). The concentration of reducing sugar was determined against a glucose standard. Cell5.1_3 activity was measured in µmoles of glucose released per minute. All assays were performed in triplicate. The temperature range tested was from 0°C to 70°C. The tested pH ranges were from pH 4 to pH 6 (in sodium acetate buffer), from pH 6 to pH 8 (in sodium phosphate buffer), from pH 8 to pH 9 (in Tris/HCl buffer), and from pH 8 to pH 10 (in glycine/NaOH buffer). The temperature and pH stability were estimated, respectively, by incubating the enzyme for 1 hour at the appropriate temperature or for 24 h at the appropriate pH (in the appropriate buffer). Residual activities were then measured under standard conditions. Halotolerance was determined by measuring the residual activity after 1.5, 5, and 24 h of incubation in 3 M KCl or 4 M NaCl. Substrate specificity was determined by replacing CMC in the standard assay with 1% (w/v) lichenan (1-3,1-4-β-D-glucan, Megazyme), laminarin (Sigma), xyloglucan (Megazyme), mannan (1,4-β-D-mannan, Megazyme), or crystalline cellulose (Avicel, Sigma). For the insoluble substrates mannan and Avicel, the reaction time was extended to 5 h at room temperature, with automatic inversion. Effects of metal ions on the activity of the Cell5.1_3 cellulase were determined under standard conditions with the metal ion of interest at 1 mM final concentration. Effects of the chemicals EDTA (10 mM), SDS (1%), ethanol (10%), Triton (5%), glycerol (15%), and DMSO (15%) were also tested under standard conditions. Kinetic parameters (K_m *and* k_{cat}) were determined from a Lineweaver-Burk plot obtained by varying the concentration of CMC (0.5 – 4%) in assays performed under standard conditions.

Nucleotide sequence accession numbers

The DNA insert sequences of *LipMM5.1*, *LipMM5.3*, *LipMM5.5*, *LipMM5.7*, *LipMM5.8*, *LipMM5.11*, *LipMM5.12*, *LipMM5.13*, *LipMM5.14*, *LipMM5.15*, *LipMM5.19*, *CellMM5.1,* and *BglucMM5.1* have been submitted to GenBank under accession numbers KF726988, KF726989, KF726990, KF726991, KF726992, KF726993, KF726994, KF726995,

KF726996, KF726997, KF726998, KF726999, and KJ499810, respectively (see also Table IV-1).

3. RESULTS

DNA extraction, library construction, and functional screening

Bacterial DNA was first extracted according to Burke et al. (Burke *et al.*, 2009) with a tri-enzymatic cleaner (ANIOS PlaII, France). As this procedure resulted in co-extraction of polyphenols, present in brown algal cell walls (Popper *et al.*, 2011), the DNA could not be purified (data not shown). Therefore, a method described by Lachnit et al. (Lachnit *et al.*, 2009), consisting of swabbing the algal surfaces with sterile cotton tips followed by classic DNA extraction, was tried. DNA quality was checked by migration through an agarose gel. High-molecular-weight DNA, without any visible degradation smear, was observed (data not shown). An average of 0.11 µg DNA per gram of *Ascophyllum nodosum* was recovered with this method. As 75 g of algae were swabbed, approximately 8 µg of microbial DNA was recovered and used to construct the metagenomic library. The DNA was restricted and small DNA fragments were removed by PEG/NaCl precipitation followed by agarose purification. DNA fragments with a size between 3 and 10 kb were ligated into the YEp356 cloning vector and the resulting recombinant plasmids were inserted into electrocompetent DH10b *E. coli* cells. The generated metagenomic library was plated, resulting in approximately 40,000 clones (180 Mb) with an average DNA insert size of 4.5 kb (estimated on 20 randomly chosen recombinant plasmids). The recombinant clones were pooled and screening was done by plating the library several times on 2xYT agar plates containing tributyrin, esculetin, or AZCL-HE-cellulose (for detection of lipase (clear halo), beta-glucosidase (brown precipitate), or cellulase (blue halo) activity, respectively). The screens yielded respectively 20, 1, and 1 candidate(s). Activity was confirmed by extraction of their recombinant plasmids and transformation of DH5α *E. coli* cells. The novel recombinant clones were then plated again on selective media: from the 20 candidates showing lipase activity, 11 plasmids were found to confer lipolytic activity (*LipMM5.1, LipMM5.3, LipMM5.5, LipMM5.7, LipMM5.8, LipMM5.11, LipMM5.12, LipMM5.13, LipMM5.14, LipMM5.15, LipMM5.19*), and the plasmids assumed to carry a cellulase gene (*CellMM5.1*) or a beta-glucosidase gene (*BglucMM5.1*) accordingly conferred the corresponding activity.

Table IV-1 ORFs on the esterase-, cellulase-, and beta-glucosidase-encoding plasmid sequences showing sequence identities to known esterases and glycoside hydrolases.

Activity	Candidate name	Gene name	ORF size (amino acids)	Best hit against GenBank (Source organisms)	Sequence Identity(%)	Family assignment [a]	Accession number
Carboxyesterase	***LipMM5.1***	*Lip5.1_5*	295	Lipase/esterase [uncultured bacterium] ACZ16567	58	Est IV	KF726988
	LipMM5.3	*Lip5.3_3*	314	Alpha/beta hydrolase [*Hirschia baltica* ATCC 49814] YP_003058522	41	Est IV	KF726989
	LipMM5.5	*Lip5.5_4*	328	Alpha/beta hydrolase [*Maricaulis maris*] YP_757536.1	36	Est IV	KF726990
	LipMM5.7	*Lip5.7_2*	414	Lysophospholipase [*Burkholderia sp.* KJ006] YP_006332790	33	/	KF726991
	LipMM5.8	*Lip5.8_1*	237	Hydrolase GDSL [*Ponticaulis koreensis*] WP_006609299.1	46	Est II	KF726992
		Lip5.8_2	221	ABC Transporter [*Erythrobacter litoralis* HTCC2594] YP_458689.1	57	/	
		Lip5.8_3	844	Putative ABC-type transport system involved in lysophospholipase L1 biosynthesis, permease component [*Caulobacter vibrioides*] WP_004617736.1	41	/	
	LipMM5.11	*Lip5.11_3*	378	Penicillin-binding protein, beta-lactamase class C [*Caulobacter vibrioides*] WP_004621811	51	Est VIII	KF726993
		Lip5.11_4	153	Thioesterase superfamily protein [*Caulobacter sp.* K31] YP_001686513	49	/	
		Lip5.11_5	138	Thioesterase superfamily protein [*Parvibaculum lavamentivorans* DS-1] YP_001411720.1	52	/	
	LipMM5.12	*Lip5.12_2*	288	Esterase [uncultured bacterium] ACF49126.1	38	/	KF726994
	LipMM5.13	*Lip5.13_1*	332	Alpha/beta hydrolase [*Pseudomonas pseudoalcaligenes*] WP_004422344.1	34	Est V	KF726995
		Lip5.13_3	348	Lipase/esterase [uncultured sludge bacterium] ADC79145.1	47	Est IV	
	LipMM5.14	*Lip5.14_3*	314	Alpha/beta hydrolase [*Hirschia baltica* ATCC 49814] WP_017932623.1	41	Est IV	KF726996
	LipMM5.15	*Lip5.15_3*	383	Esterase [*Glaciecola nitratireducens* FR1064] YP_004870252.1	57	Est VIII	KF726997
	LipMM5.19	*Lip5.19_3*	283	Esterase [*Erythrobacter litoralis* HTCC2594] YP_458833.1	48	Est IV	KF726998
Beta-glucosidase	***BglucMM5.1***	*Bgluc5.1_1*	749[b]	1,4-beta-D-glucan glucohydrolase [*Xanthomonas campestris*] WP_017932269	56	GH3	KJ499810
		Bgluc5.1_2	244	G-D-S-L family lipolytic protein [*Hirschia baltica* ATCC 49814] YP_003059731.1	37	Est II	
		Bgluc5.1_4	521 [b]	Xylulokinase [*Novosphingobium sp*. PP1Y] YP_004534688	60	/	
Cellulase	***CellMM5.1***	*Cell5.1_3*	359	Glycoside hydrolase family protein (*Hirschia baltica* ATCC 49814) YP_003060354.1	42	GH5	KF726999

[a] / , not assigned to esterase or GH families
[b] Incomplete ORF

DNA Sequence analysis

The DNA inserts of the positive clones were sequenced. ORFs on the DNA inserts were sought with ORF Finder. On the DNA insert of each positive candidate, 3 to 7 ORFs were indentified. Each ORF gene product was analyzed with BlastP against the GenBank non-redundant database and Swissprot (a curated database). The putative esterases were also analyzed with Pfam (Finn *et al.*, 2014) (Supplementary Data, DATASET S1).

In each cluster, we identified at least one ORF whose predicted product shows sequence similarity to the amino acid sequence of a known esterase, beta-glucosidase, or cellulase (Table IV-1). Putative esterase genes were assigned to four carboxyesterase families by amino acid sequence alignment (data not shown). Lip5.8_1 shows sequence similarity to members of bacterial esterase family II (Arpigny and Jaeger, 1999; Hausmann and Jaeger, 2010). Lip5.1_5, Lip5.3_3, Lip5.5_4, Lip5.13_3, Lip5.14_3, and Lip5.19_3 belong to family IV, Lip5.13_1 to family V, and Lip5.11_3 and Lip5.15_3 to family VIII. The gene products of *Lip5.7_2* and *Lip5.12_2* could not be assigned to any esterase family described by Arpigny (Arpigny and Jaeger, 1999) nor to one of the novel families EstA (Chu *et al.*, 2008) and LipG (Lee *et al.*, 2006). However, these gene products could be assigned to the Pfam alpha/beta-hydrolase family (subfamilies 6 and 5, respectively), which contains numerous esterases. In fact, the esterase families IV and V correspond to alpha/beta-hydrolase subfamilies 3 and 6, respectively (Finn *et al.*, 2014).

On the DNA insert of the beta-glucosidase-positive clone, the following were identified: an incomplete ORF encoding a putative glycoside hydrolase (GH), an incomplete ORF encoding a putative xylulokinase, and a complete ORF encoding a putative esterase (GDSL). The incomplete glycoside hydrolase gene (*Bgluc5.1_1*) was probably responsible for the beta-glucosidase activity and was assigned to family GH3 of the CAZyme database (Cantarel *et al.*, 2009). The esterase gene (*Bgluc5.1_2*) encodes a putative GDSL lipolytic protein assigned to family II by amino acid sequence alignment (Arpigny and Jaeger, 1999; Hausmann and Jaeger, 2010).

On the *CellMM5.1* candidate, we identified an ORF (*Cell5.1_3*) of 359 amino acids showing 42% sequence identity to a glycoside hydrolase of *Hirschia baltica* ATCC 49814 (GenBank: YP_003060354.1). A GH5-family protein signature was also identified. Phylogenetic analysis showed that the enzymes most similar to the Cell5.1_3 putative cellulase are subfamily-GH5_25 enzymes of proteobacteria such as *Hirschia baltica*, *Caulobacter sp*., and *Ramlibacter sp.* (Aspeborg *et al.*, 2012) (Figure IV-1). A signal peptide was also predicted. As no other cellulase has been identified to date by functional metagenomics in a marine

environment, we chose to purify and characterize the putative cellulase gene (*Cell5.1_3*) product.

It is difficult to attribute a specific biological function to the different gene clusters on the basis of gene annotations, especially for the clusters containing esterases. In contrast, curated gene annotations give insights into the taxonomic affiliation of the *Ascophyllum*-associated bacteria, at least at the level of the class or order. In most of the clusters, the different genes have a consistent taxonomic affiliation (based on the bacterial origin of the closest homologous proteins). The *LipMM5.1* insert is an exception: its affiliation is ambiguous, with homologous genes found among the *Firmicutes*, *Actinobacteria,* and *Gammaproteobacteria*. The *LipMM5.7* and *LipMM5.13* inserts seem to come from representatives of the *Betaproteobacteria* and *Gammaproteobacteria*, respectively. The remaining inserts originate from *Alphaproteobacteria* members, mainly of the order *Rhodobacterales*. Interestingly, several inserts seem to belong to bacteria close to *Robiginitomaculum antarcticum*, a marine *Rhodobacterales* species isolated from Antarctic seawater (K. Lee *et al.*, 2007).

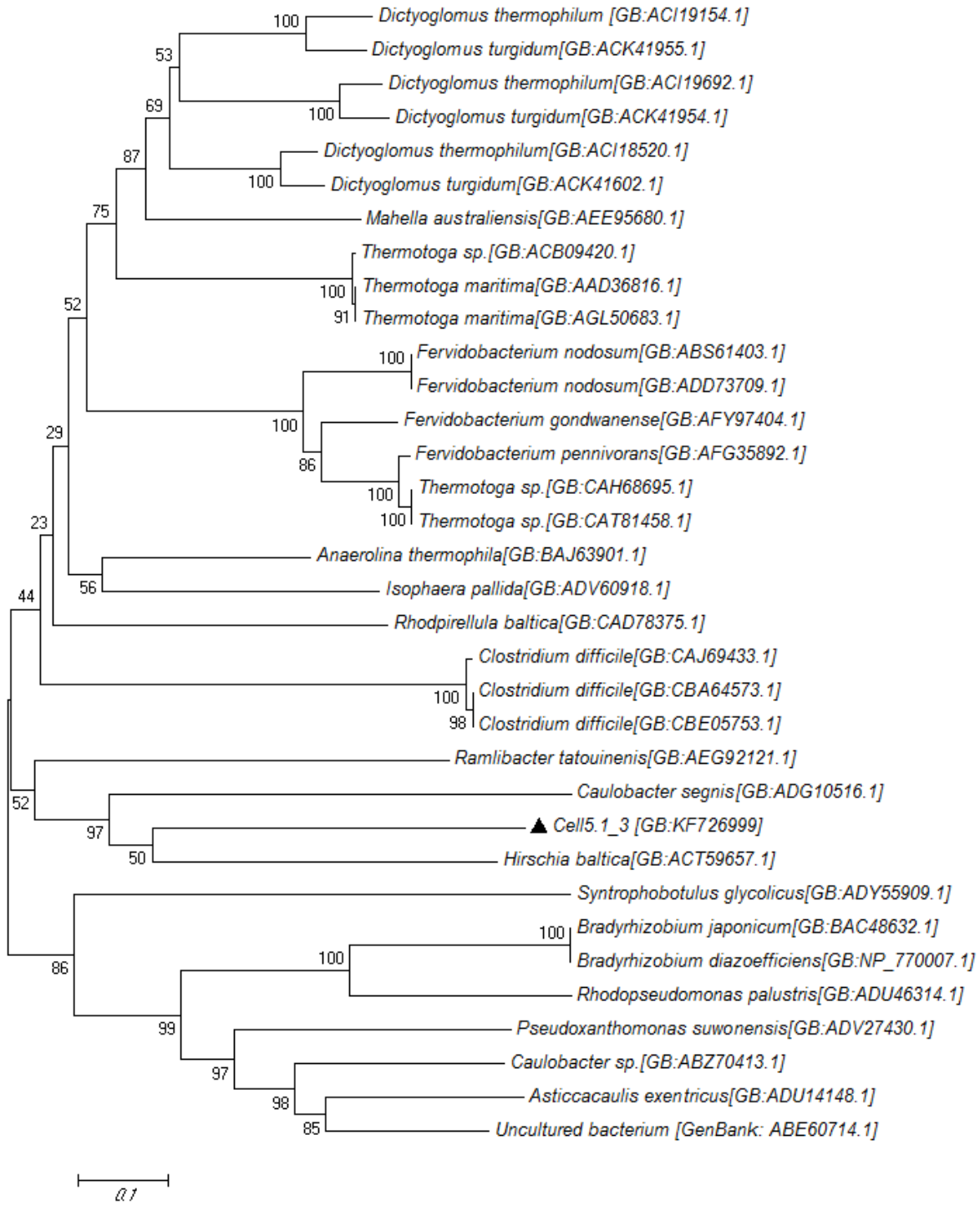

Figure IV-1 Neighbor-joining phylogenetic tree of subfamily GH5_25 and classification of the cellulase gene *Cell5.1_3.* Bootstrap values higher than 50% are indicated at branch points (1,000 replicates). Reference sequences from the GenBank database are added.

Cellulase purification and activity characterization

The recombinant Cell5.1_3 cellulase, without its signal peptide, was produced in *E. coli* Rosetta 2 (DE3) from the pET30b vector and purified by Ni-chromatography. SDS-PAGE of the eluted fractions showed a clear band between 30 and 40 kDa (Figure IV-2), in accordance with the predicted molecular mass (38 kDa). The activity of the Cell5.1_3 enzyme was tested by the DNSA method on CMC. Its optimal temperature was found to be about 40°C, although the enzyme proved unstable after a 1-hour incubation at this temperature (Figure IV-3A). At 40°C, the optimal pH was pH 7, and the cellulase appeared stable over a wide pH range when pre-incubated for 24 h at 4°C in different buffers (Figure IV-3C,D). The kinetic parameters determined for the reaction with CMC, at optimal pH and temperature, were K_m = 8.37 µM and k_{cat} = 10.35 s^{-1}. Halotolerance was also observed: the activity of the enzyme remained unchanged, under standard conditions, after a 24-hour pre-incubation with 4 M NaCl or 3 M KCl (Figure IV-3B). The specificity of the enzyme was tested in the presence of different substrates (Table IV-2). The Cell5.1_3 cellulase failed to hydrolyze the insoluble substrates crystalline cellulose (Avicel) and mannan, even when incubated for 5 h at 20°C. Among the soluble substrates tested, it showed no activity against xyloglucan, low activity against laminarin, and higher activity against CMC and lichenan (β-1,3;1,4-glucan). The activity of the enzyme was enhanced by Mn^{2+} and Cd^{2+} but clearly inhibited by Cu^{2+}, Zn^{2+}, and Fe^{2+} (Figure IV-3E). Triton X-100 was found to increase the activity of the enzyme to 350%, while the other tested additives were found to reduce (EDTA, glycerol) or totally inhibit (SDS, EtOH, DMSO) its activity (Figure IV-3E).

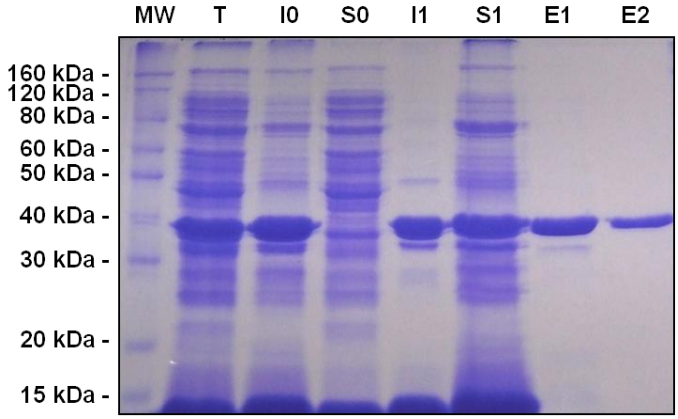

Figure IV-2 SDS-PAGE analysis of the recombinant cellulase. **MW**: Molecular weight, **T**: Total extract. **I0** and **S0** show the insoluble and soluble fractions obtained by sonication, followed by centrifugation, of *E. coli* cells overexpressing the cellulase gene *Cell5.1_3*. As the GH was mostly present in the insoluble fraction (I0), we resuspended it for 4 h in a solubilization buffer and centrifuged it, aiming to obtain a higher, and sufficient, amount of our cellulase in the soluble fraction (S1). However, a high amount of our cellulase was still present in the newly obtained insoluble fraction (I1). This fraction was then purified twice by chromatography (E1 and E2)

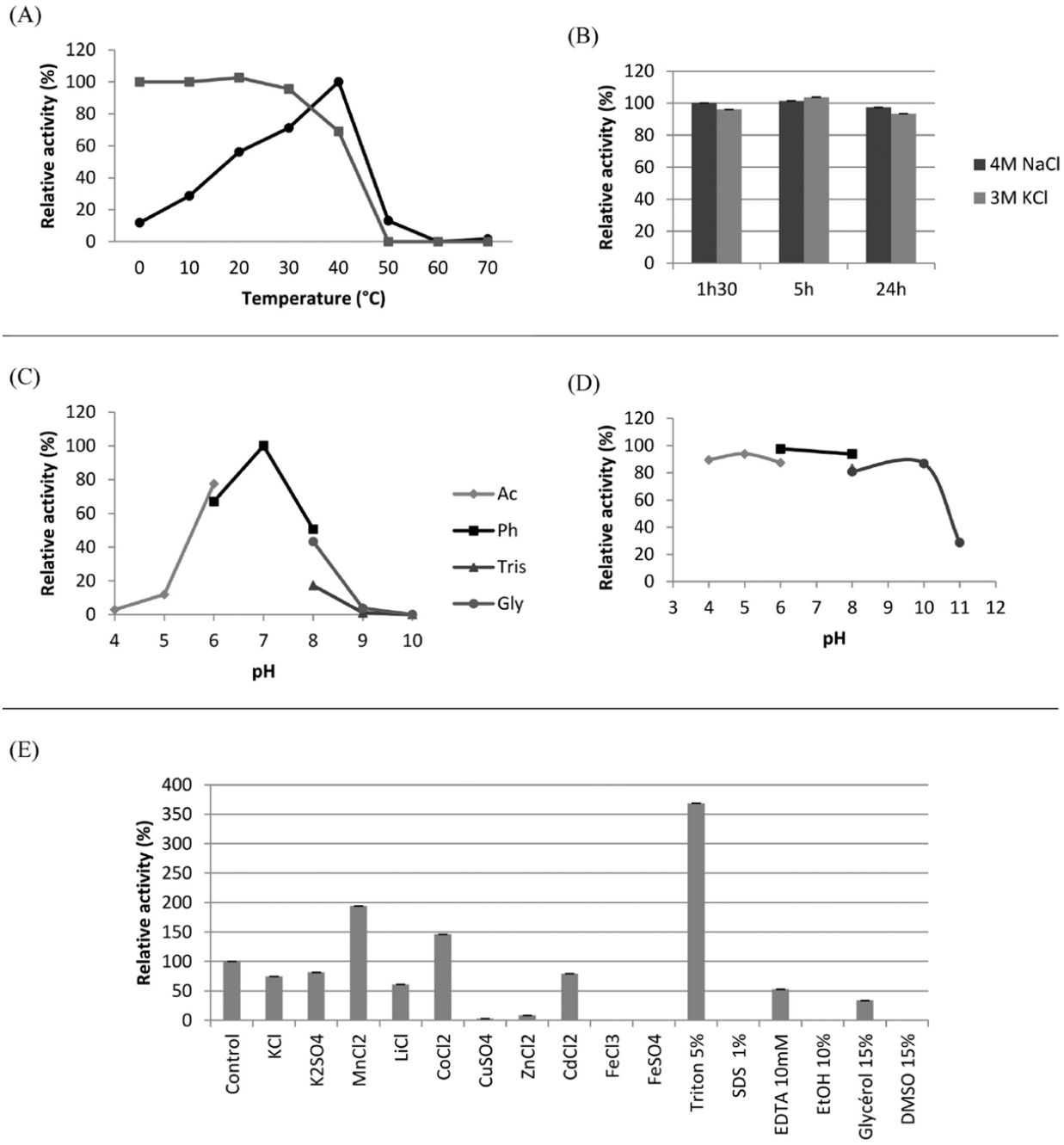

Figure IV-3 Characterization of the activity of Cell5.1_3 **(A)** Influence of temperature on Cell5.1_3 activity (●) and thermostability after one hour incubation at indicated temperature (■) **(B)** Halotolerance of Cell5.1_3 **(C)** Influence of pH on Cell5.1_3 activity **(D)** Residual activity of Cell5.1_3 after a 24-hour incubation at the indicated pH, (Ac : acetate Buffer, Ph: phosphate buffer, Tris : Tris-HCl buffer, Gly : glycine-NaOH buffer) **(E)** Effects of ions and other additives on the activity of Cell5.1_3.

Table IV-2 Substrate specificity of Cell5.1_3

Substrates	Main linkage type	Specific activity[a] (U/mg)
Soluble substrates		
Carboxymethylcellulose	ß-1,4-glucan	10.22 ± 0.002
Lichenan	ß-1,3/1,4-glucan	12.61 ± 0.008
Laminarin	ß-1,3/1,6-glucan	5.08 ± 0.004
Xyloglucan	ß-1,4-xyloglucan	UD
Insoluble substrates [b]		
Mannan	ß-1,4-mannan	UD
Avicel	ß-1,4-glucan	UD

UD, Undetectable
[a] One unit of enzyme activity (U) corresponds to 1 µmole of glucose released in 1 min. Measurements were performed in triplicate and standard deviations were calculated.
[b] For these substrates, the reaction time was extended to 5 h at 20°C, with automatic inversion.

4. DISCUSSION

Microorganisms on algae, in the context of their numerous interactions with the host, are known to produce diverse hydrolytic enzymes. Furthermore, the complexity of the marine environment in which these microorganisms live leads to the production of specific enzymes with original biochemistry (Martin, Portetelle, *et al.*, 2014). Here we have constructed a metagenomic library from the genomic DNA of the bacterial communities associated in February 2012 to the brown alga *Ascophyllum nodosum* (*Fucales* order) and have screened it for diverse hydrolytic enzymes. From this library we have identified a range of new esterase genes and two new glycoside hydrolase genes, thus demonstrating the pertinence of seeking new enzymes in such microbial communities by functional metagenomics. All these hydrolase genes can most likely be assigned to the phylum *Proteobacteria* and essentially to the class *Alphaproteobacteria*, and a few to the classes *Betaproteobacteria* and *Gammaproteobacteria*. This is consistent with previous findings on the bacterial community associated in January 2007 with another *Fucales* species, *Fucus vesiculosus*, as this bacterial community appeared dominated by *Alphaproteobacteria* and *Gammaproteobacteria* species (Lachnit *et al.*, 2011). Nonetheless, *Bacteroidetes* and *Planctomycetes* species are also relatively abundant on brown algal surfaces (Lachnit *et al.*, 2011; Bengtsson *et al.*, 2013), and one can be surprised that our functional metagenomic screen yielded no genes identified as originating from these phyla. An explanation might be that our *E. coli* expression strain (*Gammaproteobacteria*) does not readily recognize gene promoters of *Bacteroidetes* and *Planctomycetes* species.

Esterases are the category of enzymes most frequently isolated by functional metagenomics. Here we have identified 13 esterase-encoding genes by screening a relatively low number of Mb: approximately 180 Mb, which makes on the average 1 esterase per 14 Mb screened. This is much higher than in other metagenomic studies (see review of Steele et al. (Steele *et al.*, 2009)). The 13 esterase genes identified here can be assigned to four esterase families. Six of them (*Lip5.1_5, Lip5.3_3, Lip5.5_4, Lip5.13_3, Lip5.14_3, Lip5.19_3*) have been assigned to family IV. This esterase family appears highly represented in marine metagenomes, as out of 34 esterases identified in 8 screens of marine metagenomes, 27 have been classified as family-IV esterases (Chu *et al.*, 2008; Jeon *et al.*, 2009, 2012; Hu *et al.*, 2010; Okamura *et al.*, 2010; Fu *et al.*, 2011; Jiang *et al.*, 2012; Oh *et al.*, 2012). We have assigned one of the esterase genes identified here (*Lip5.13_1*) to esterase family V, which appears to count relatively few members. Esterases of this family have been found in microorganisms displaying very different growth-temperature ranges, such as *Sulfolobus* (thermophilic archaea), *Psychrobacter* (psychrophiles), and *Moraxella* (mesophiles) species (Arpigny and Jaeger, 1999; Hausmann and Jaeger, 2010). Only two esterases of marine origin (FJ483459, FJ483468), identified in a metagenomic library from marine sediments, have been assigned to this family previously (Hu *et al.*, 2010). The Lip5.11_3 and Lip5.15_3 esterases have been classified as family-VIII esterases. Members of family VIII show no typical α/β-hydrolase fold but are very similar to β-lactamases. Furthermore, they have a typical molecular weight around 42 kDa (Hausmann and Jaeger, 2010). The predicted Lip5.11_3 esterase shows sequence identity to β-lactamases and has an estimated molecular weight of 41.4 kDa. The predicted Lip5.15 esterase has an estimated molecular weight of 41.5 kDa. To our knowledge, no marine-metagenome-derived esterase has previously been assigned to this family. We have identified conserved domains of the SGNH superfamily (family-II esterases) in the esterase gene (*Bgluc5.1_2*) found on the DNA insert of the beta-glucosidase candidate *BglucMM5.1* and in the esterase gene (*Lip5.8_1*) of *LipMM5.8*. Only one marine-metagenome-derived esterase (AB432912), identified in a sponge-associated bacterial metagenome (Okamura *et al.*, 2010), has been classified previously as a member of this family. SGNH hydrolases are a subgroup of the GDSL family II esterases. GDSL esterases have thioesterase, protease, lysophospholipase, and arylesterase activities (Akoh *et al.*, 2004). In the BLAST results for both Bgluc5.1_2 and Lip5.8_1, we found sequence identities to thioesterases and lysophospholipases.

Finally, the two thioesterase genes identified on *LipMM5.11* (*Lip5.11_4 and _5*) seem to be in an operon. The first gene (*Lip5.11_4*) has sequence identity to a thioesterase of *Caulobacter sp.* K31 (Caul_4896, YP_001686513.1), located with another thioesterase in a 3'-5' oriented operon (Caul_4895, YP_001686512.1). The other thioesterase gene

(*Lip5.11_5*) has sequence identity to a thioesterase gene of *Parvibaculum lavamentivorans* DS-1 (Plav_0440, YP_001411720.1), which is also in a 3'-5' oriented operon with another thioesterase (Plav_0441, YP_001411721.1). The thioesterases and carboxylesterases constitute separate enzyme groups, although a few proteins classified as carboxylesterases also show thioesterase activity. Despite careful annotation of the esterase-containing gene clusters, it is difficult to predict the exact biological function of these esterases on the basis of the genomic context.

Because (i) we have found thirteen genes encoding esterases, (ii) we have assigned these genes to four esterase families, and (iii) most of the predicted protein sequences are less than 50% identical to known esterases, we conclude that this library constructed from algal biofilms can be considered rich and diverse in novel microbial genes and enzymes.

As compared to esterase genes, few cellulase genes have been identified by functional metagenomics. To our knowledge no cellulase has been identified by metagenomics in a marine environment before. All of the cellulases identified by analysis of soil (Voget *et al.*, 2006; Soo-Jin *et al.*, 2008; Pang *et al.*, 2009; Nacke *et al.*, 2012) or rumen (Bao *et al.*, 2011; Rashamuse *et al.*, 2013) metagenomes display more than 50% sequence identity to known cellulases, in contrast to our cellulase, which is only 42% identical to the closest known cellulase. Hence, our cellulase appears new and only distantly related to known cellulases. We have assigned the Cell5.1_3 cellulase to endo-ß-1,4-glucanase family GH5, subfamily 25. Although most cellulases identified by metagenomics have been classified as GH5-family enzymes (Voget *et al.*, 2006; Bao *et al.*, 2011; J. Liu *et al.*, 2011; Geng *et al.*, 2012; Rashamuse *et al.*, 2013), only one of them (ABE60714.1), identified in a pulp sediment metagenome from a paper mill effluent, belongs to subfamily 25 (Y. Xu *et al.*, 2006). The other members of this subfamily were identified in cultivable microorganisms such as *Caulobacter, Clostridium, Dictyoglomus, Fervidobacterium,* and *Thermotoga* species isolated from diverse environments (CAZyme database, www.cazy.org). Only two subfamily-25 enzymes have been characterized so far: *Tm_Cel5A* (Q9X273) of *Thermotoga maritima* (Chhabra *et al.*, 2002; Pereira *et al.*, 2010) and *FnCel5A* (A7HNC0) of *Fervidobacterium nodosum* (Wang *et al.*, 2010). Both of these appear thermostable. Cell5.1_3, in contrast, shows low thermostability: when pre-incubated for 1 hour at various temperatures, it began to show decreased CMC-hydrolyzing activity when the pre-incubation temperature exceeded 25°C, and when the pre-incubation temperature exceeded 40°C, no activity was observed. Under our assay conditions, its activity was highest at 40°C, which is relatively low as compared to the enzymes Tm_Cel5A and FnCel5A, identified in the thermophile *Thermotogales* (around 80°C for both). On the other hand, our enzyme retained

11.8% of its maximum activity at 0°C and 28.7% at 10°C. According to Asperborg et al. (Aspeborg *et al.*, 2012), most of the endoglucanases of this subfamily have been derived from thermophiles, but interestingly, the here-identified cold-active cellulase Cell5.1_3 would appear to come from a mesophilic (or even psychrophilic) bacterium. A cold-active cellulase (CelX) identified in *Pseudoalteromonas sp.* DY3 shows very similar optimal temperature and thermostability (Zeng *et al.*, 2006). However, Cell5.1_3 is active over a broad pH range, from 5 to 8, and is stable at pH values from 4 to 10 (it is still active after a 24-hour pre-incubation in this pH range), which is not the case of the cold-active CelX (Zeng *et al.*, 2006). What's more, other metagenome-derived GH5 cellulases show stability only at acidic (4-6.6, (J. Liu *et al.*, 2011)) or alkaline (6-10, (Pang *et al.*, 2009)) pH. Another advantage of Cell5.1_3 is its halotolerance, as it retains 93% of its activity after a 24-hour pre-incubation in 3 M KCl, and 97% of its activity after a pre-incubation in 4 M NaCl. These remaining activities are much higher than those reported for other halotolerant GH5 enzymes. For example, Cel5A, isolated from a soil metagenome, retains approximately 87% activity after a 20-h pre-incubation in 3M NaCl or 4M KCl (Voget *et al.*, 2006), and a GH5 endoglucanase of the thermophilic eubacterium *Thermoanaerobacter tengcongensis* MB4 retains less than 15% of its activity after a 12-hour pre-incubation in 4 M NaCl (Liang *et al.*, 2011). As GH5 endoglucanases hydrolyze a wide range of cellulose substrates (Aspeborg *et al.*, 2012), we have tested the substrate specificity of Cell5.1_3. The enzyme appears to degrade CMC and mixed glucans (lichenan (ß1,3-ß1,4) and laminarin (ß1,3-ß1,6)), but not ß-1,4 linked xyloglucan. Other metagenome-derived cellulases either fail to degrade laminarin (Voget *et al.*, 2006; Pang *et al.*, 2009; Bao *et al.*, 2011; Nacke *et al.*, 2012) or, like the GH5 enzyme described by Juan et al., show very low specific activity (approximately 0.002 U/mg) (J. Liu *et al.*, 2011). In agreement with its classification as an endoglucanase, Cell5.1_3 failed, in our assay, to degrade the insoluble substrates Avicel and mannan. Triton X-100 enhances significantly the activity of Cell5.1_3. Zheng et al (Zheng *et al.*, 2008) reported three main hypothesis explaining the enhancement of enzymatic cellulose hydrolysis in the presence of non-ionic surfactants: 1) they stabilize the enzyme by reducing thermal and/or mechanical shear forces; 2) they change the substrate structure, enhancing the substrate accessibility; and 3) they affect enzyme-substrate interaction, preventing enzymes inactivation due to non-productive adsorption when hydrolyzing for example lignocellulosic substrates. This last hypothesis doesn't concern the substrate we used (CMC) and as, generally, no significant (positive or negative) effect of Triton X-100 has been observed on other bacterial cellulase activities characterized with CMC (Voget *et al.*, 2006; Feng *et al.*, 2007; Duan *et al.*, 2009; Wang *et al.*, 2010), the second hyptothesis must not be the reason why Cell5.1_3 activity increases to 350% in the presence of Triton

X-100. However, Cell5.1_3 is probably more stable, and therefore more active on CMC, in the presence of Triton X-100.

Competing interests

The authors declare no competing interests.

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Chapter V. Cultivable surface microbiota

Outline

Several bacterial species and strains isolated from seaweeds are known to hydrolyze algal polysaccharides (see chapter II). However, hydrolytic bacteria associated with *Ascophyllum nodosum* have never been explored by culturing. Thus, as a complement to the functional metagenomic approach (chapter IV), which mainly focused on the non-cultivable ones being major in environmental samples, we investigated the enzymatic potential of the cultivable surface microbiota associated with *A. nodosum*. In this fifth chapter, we isolated and identified numerous cultivable bacteria from this alga and tested them for polysaccharolytic activities. This provided us information about the identity of these algal-polysaccharide active bacteria and their proportion in the cutlivable surface microbiota of *A. nodosum*.

Related publication

The cultivable surface microbiota of the brown alga *Ascophyllum nodosum* is enriched in macroalgal-polysaccharide-degrading bacteria

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The cultivable surface microbiota of the brown alga *Ascophyllum nodosum* is enriched in macroalgal-polysaccharide-degrading bacteria

Abstract

Bacteria degrading algal polysaccharides are key players in the global carbon cycle and in algal biomass recycling. Yet the water column, which has been studied largely by metagenomic approaches, is poor in such bacteria and their algal-polysaccharide-degrading enzymes. Even more surprisingly, the few published studies on seaweed-associated microbiomes have revealed low abundances of such bacteria and their specific enzymes. However, as macroalgal cell-wall polysaccharides do not accumulate in nature, these bacteria and their unique polysaccharidases must not be that uncommon. We, therefore, looked at the polysaccharide-degrading activity of the cultivable bacterial subpopulation associated with *Ascophyllum nodosum*. From *A. nodosum* triplicates, 324 bacteria were isolated and taxonomically identified. Out of these isolates, 78 (~25%) were found to act on at least one tested algal polysaccharide (agar, ι- or κ-carrageenan, or alginate). The isolates "active" on algal-polysaccharides belong to 11 genera: *Cellulophaga*, *Maribacter*, *Algibacter,* and *Zobellia* in the class *Flavobacteriia* (41) and *Pseudoalteromonas*, *Vibrio*, *Cobetia*, *Shewanella*, *Colwellia*, *Marinomonas,* and *Paraglaceciola* in the class *Gammaproteobacteria* (37). A major part represents likely novel species. Different proportions of bacterial phyla and classes were observed between the isolated cultivable subpopulation and the total microbial community previously identified on other brown algae. Here, *Bacteroidetes* and *Gammaproteobacteria* were found to be the most abundant and some phyla (as *Planctomycetes* and *Cyanobacteria*) frequently encountered on brown algae weren't identified. At a lower taxonomic level, twelve genera, well-known to be associated with algae (with the exception for *Colwellia*), were consistently found on all three *A. nosodum* samples. Even more interesting, 9 of the 11 above mentioned genera containing polysaccharolytic isolates were predominant in this common core. The cultivable fraction of the bacterial community associated with *Ascophyllum nodosum* is, thus, significantly enriched in macroalgal-polysaccharide-degrading bacteria and these bacteria seem important for the seaweed holobiont even though they are under-represented in alga-associated microbiome studies.

Keywords

Flavobacteriia, *Gammaproteobacteria*, macroalgae, agarase, carrageenase, alginate lyase, *Ascophyllum nodosum*, algal polysaccharidase

1. INTRODUCTION

Polysaccharide-degrading bacteria are key players in the global carbon cycle and have an increasing importance in biotechnology and biomass utilization. In terrestrial environments, plants host a remarkable diversity of microbes, representing a continuum of symbioses ranging from mutualism to commensalism to pathogenic behavior (Bulgarelli *et al.*, 2013). Among these microbes, numerous bacteria possess polysaccharidases involved in the utilization of plant cell wall polymers (Gibson *et al.*, 2011). Soils also constitute an important reservoir of hemicelluloytic microorganisms, which carry on the mineralization of plant polysaccharides (DeAngelis *et al.*, 2010; Leung *et al.*, 2015). In coastal ecosystems, macroalgae are crucial primary producers, constituting a huge biomass and playing an ecological role analogous to that of plants in terrestrial environments. Brown, green, and red macroalgae constitute three independent eukaryotic lineages, and their cell walls contain highly diverse polysaccharides, notably sulfated polysaccharides having no equivalent in land plants (Popper *et al.*, 2011). By comparison with what is known about biomass recycling in terrestrial environments, knowledge of bacterial recycling of macroalgal biomass trails far behind. Nonetheless, the discovery of glycoside hydrolases (GH) and polysaccharide lyases (PL) targeting macroalgal polysaccharides has accelerated in the last fifteen years (Michel and Czjzek, 2013). These particular enzymes were found to constitute either new subfamilies within known Carbohydrate-Active enZYmes (CAZy) families (e.g. κ-carrageenases and β-porphyranases in the GH16 family (Michel, Chantalat, Duee, *et al.*, 2001; Hehemann *et al.*, 2010)) or new CAZy families, such as ι-carrageenases (GH82)(Barbeyron *et al.*, 2000), α-agarases (GH96) (Flament *et al.*, 2007), and fucoidanases (GH107)(Colin *et al.*, 2006).

The majority of these enzymes have been isolated from seaweed-associated bacteria (Martin, Portetelle, *et al.*, 2014). MAPD bacteria have been found essentially in two classes: (i) *Gammaproteobacteria*, in the phylum *Proteobacteria* (e.g. *Agarivorans albus*, *Alteromonas agarilytica*, *Pseudoalteromonas carrageenovora*, *Vibrio* sp. PO-303) and (ii) *Flavobacteriia*, in the phylum *Bacteroidetes* (e.g. *Cellulophaga lytica*, *Flammeovirga yaeyamensis*, *Mariniflexile fucanivorans*, *Zobellia galactanivorans*) (for reviews see Martin et al., 2014b; Michel and Czjzek, 2013). Despite the identification of these bacteria, most MAPD enzymes currently constitute very small protein families: e.g. GH16 κ-carrageenases (6 proteins); GH16 β-agarases (41), GH16 β-porphyranases (7), GH82 ι-carrageenases (19), GH96 α-agarases (4), GH107 fucoidanases (4), and GH118 β-agarases (8) (http://www.cazy.org; (Lombard *et al.*, 2014)). However, as macroalgal cell-wall

polysaccharides do not accumulate in nature, MAPD bacteria and their unique polysaccharidases must not be that uncommon.

The emerging metagenomic exploration of marine environments (Venter *et al.*, 2004; Yooseph *et al.*, 2007; Gómez-Pereira *et al.*, 2012) offers the promise of increasing the discovery of novel enzymes, either through data mining or through activity screening of metagenomic libraries (Lee *et al.*, 2010; Ferrer *et al.*, 2016). Marine metagenomics has yielded disappointing results, however, as regards MAPD enzymes. For instance, in the entire Global Ocean Survey (GOS) dataset (Yooseph *et al.*, 2007), there is not a single κ-carrageenase gene, only one ι-carrageenase gene, thirty coding DNA sequences (CDSs) for α-1,3-(3,6-anhydro)-L-galactosidase (the terminal enzyme of agar degradation, AhgA, GH117) and five times fewer CDSs for β-agarase and β-porphyranase, and seventeen PL17 alginate lyases (Hehemann *et al.*, 2010; Rebuffet *et al.*, 2011; Neumann *et al.*, 2015). Clearly, this relative lack of success in finding MAPD enzymes in the water column suggests that bacterioplanktons rarely possess such enzymes. However, as most MAPD enzymes have been isolated from seaweed-associated bacteria, one should think that seaweed-associated microbiome datasets contain plenty of these specific enzymes. In fact, in the only two existing studies sequencing the microbiota associated with macroalgae (with *Delisea pulchra* and *Ulva australis*), no polysaccharidase gene is discussed (Fernandes *et al.*, 2012) or only genes related to alginate metabolism are briefly mentioned (Burke, Thomas, *et al.*, 2011). Unfortunately, we couldn't find out if MAPD enzymes were present in the these macroalgae associated microbiota, as the metagenomic datasets of these two studies were deposited on the CAMERA database (http://camera.calit2.net/) that has shut down and its successor, iMicrobe (http://data.imicrobe.us/), does not contain these datasets. Also, a transcriptomic analysis of the microbiota of the red macroalga *Laurencia dendroidea* has been performed by RNA-Seq, but again, algal-polysaccharidase genes were either overlooked or absent from the dataset (de Oliveira *et al.*, 2012). The metatranscriptomic datasets associated to *L. dendroidea* is publicly available on the MG-Rast database (de Oliveira et al., 2012). We searched in the protein files corresponding to these datasets sequence identities with the characterized MAPD enzymes (Michel and Czjzek, 2013; Martin, Portetelle, *et al.*, 2014) using BlastP (NCBI), but we did not find any homologous protein (data not shown). Concerning functional metagenomic analyses of seaweed-associated microbiota, only two studies have previously been carried out. On the one hand, the microbial community associated with *Ulva australis* was searched for antibacterial proteins (Yung *et al.*, 2011). On the other hand, our group has recently screened the microbiota of the brown alga *Ascophyllum nodosum* for hydrolytic enzymes, especially glycoside hydrolases. As proof of feasibility, we used established activity tests and identified 13 esterases from

Alphaprotobacteria and *Gammaproteobacteria* members, a GH3 β-glucosidase from an *alphaprotobacterium*, and a GH5 cellulase from a *gammaproteobacterium* (Martin, Biver, *et al.*, 2014). Taking advantage of the ability of κ- and ι-carrageenans, agar and alginate to form gels, we subsequently developed activity tests for screening a second *A. nodusum* metagenomic library for the corresponding polysaccharidases. The quality of this metagenomic library was validated and several esterases and β-glucosidases were again isolated. Surprisingly, however, we did not find any MAPD enzymes (unpublished results).

This disappointing result and the almost absence of MAPD enzymes from other metagenomic studies on seaweed-associated microbiota raised several questions. Are MAPD bacteria sufficiently abundant at the surface of macroalgae? Are metagenomic analyses of entire seaweed-associated microbiota the most adequate approach to finding MAPD enzymes? As a first step towards answering such questions, we have chosen to focus on the cultivable subpopulation of bacteria associated with *A. nodosum* and to establish a direct link with the catabolic capacities of these microorganisms. For this we have isolated, on a marine medium, 324 cultivable bacteria from triplicate *A. nodosum* samples. We have taxonomically identified these bacterial isolates and have screened them for agarases, κ-carrageenases, ι-carrageenases, and alginate lyases. Bacterial isolates found to display at least one such activity were further investigated.

2. MATERIALS AND METHODS

Phylogenetic and statistical analyses of the bacterial communities isolated from the three *Ascophyllum nodosum* samples

Healthy *Ascophyllum nodosum* plants were collected in triplicate from the foreshore in Roscoff (Brittany, France) at the end of March, 2014. The samples were rinsed three times with sterile seawater to remove loosely attached microorganisms and used immediately for microbial isolation. Cultivable microorganisms were isolated by swabbing algal surfaces with sterile cotton tips as in our previous functional metagenomics study (Martin, Biver, *et al.*, 2014) and then inoculating plates of marine medium (Marine Agar, Difco). One plate per sample was inoculated and for each sample we used 20 cm of thallus. Plates were left at room temperature. After 4 days, 108 isolated colonies, representative of our total isolated colonies, were recovered at random from each plate (324 colonies in all) in 96-well plates with marine medium (Marine Broth, Difco). Glycerol (final concentration 20%) was added to the liquid bacterial cultures in the 96-well plates and conserved at -80°C.

To assign taxa to the 324 bacteria isolated from the three alga samples, the genus-specific V3-V4 region of the 16S rRNA (approximately 400 bp) was amplified and sequenced. First, 5

µl stock of bacteria in glycerol was mixed with 10 µl PCR water and heated at 95°C for 10 minutes to lyse the bacteria. Then 5 µl bacterial lysate was added to the PCR mix (200 µM dNTP, 0.4 µM primer F (AllBactF 5'-TCCTACGGGAGGCAGCAGT-3'), 0.4µM primer R (AllBactR 5'-GGACTACCAGGGTATCTAATCCTGTT-3') (Nadkarni et al., 2002; Stroobants et al., 2014), PCR buffer 1x, Taq DNA polymerase, final volume 50 µl) for the PCR (94°C- 7', 35x (95°C-30''), 60°C-30'', 65°C -1'), 68°C-7'). Primers were produced at Eurogentec (BE) and PCR reagents were provided by Roche. PCR were realized in 96-well plates and amplicons were sequenced by Sanger Sequencing at GATC using the AllBactF forward primer (GATC Biotech, Germany). Sequences were aligned with sequences from the GenBank_Bacteria and SILVA SSU databases (E-value threshold 0.01) and each bacterium was taxonomically identified at the genus, family, order, class and phylum level. Averages and percentage ranges of the resulting query coverages and identity percentages have been summarized for each sample and for the total isolated microbiota in the supplementary material (Table V-S1). A Principal Coordinates Analyses (PCoA) was realized with the relative abundance of each genus in each sample to represent the differences in composition of the three samples (Figure V-1). Relative abundances of the genera were log transformed and Bray Curtis matrix distance was used. For each genus, a weight average was calculated based on the abundance of this genus in each sample and the axes-coordinates of the sample, in order to reveal affinities between a genus and one, two or the three samples. The PCoA plot was performed using the VEGAN Package (Dixon, 2003; Oksanen *et al.*, 2008) implemented in the R statistical software on the bacterial genera identified on the three algae samples.

Isolation of bacteria with polysaccharidase activities

The 324 isolated colonies were grown at 22°C for 24 hours in 96-microwell plates containing marine broth and then plated on marine broth containing 1.5 % agar, 1% κ-carrageenan, 2% iota-carrageenan or on modified Zobell medium (24.7g/l NaCl, 6.3g/l $MgSO_4$, 0.7g/l KCl, 5g/l tryptone, 1g/l yeast extract) with 5% alginate salts and 40 mM $MgCl_2$. All chemicals were purchased from Sigma-Aldrich. After 48 hours to one week, bacteria showing hydrolytic activity (a hole in the jellified medium for agarase or κ-carrageenase activity or complete liquefaction of the medium for ι-carrageenase or alginate lyase activity) were recovered and plated in order to obtain isolated colonies. Activity was confirmed by testing again the isolated colonies for the observed activity/activities.

16S rRNA sequence of the bacterial candidates showing polysaccharidase activity

The 16S rRNA genes were amplified by PCR (Taq DNA polymerase, Roche) directly from the isolated colonies. Universal primers were used (8F, 5'-AGAGTTTGATCCTGGCTCAG-3' and

1492R, 3'-TACGGCTACCTTGTTACGACTT-5'). The amplified genes were sequenced by Sanger sequencing from both ends (forward and reverse) (GATC Biotech, Germany). A consensus sequence was produced from the overlapping reads, for the 16S rRNA gene of each MAPD isolate. For each sequence, low quality 5' and 3' extremities were manually trimmed, in order to keep only high quality sequences (DNA base peaks with quality scores above 30). Finally, the BLAST program (NCBI) was used to compare the obtained sequences against the GenBank database and closest neighbors were found by alignment with type strains with verified species names using EzTaxon (O. S. Kim *et al.*, 2012). Sequences were manually aligned and curated with a representative set of 120 sequences of identified members of the family *Flavobacteriaceae* (Figure V-2) and 355 of the class *Gammaproteobacteria* (Figure V-3, Figure V-S2). Phylogenetic trees were constructed with these alignments using the Neighbor Joining Method (Saitou and Nei, 1987). Evolutionary distances were calculated according to Kimura's two-parameter model using the gamma distribution (Kimura, 1980).

Nucleotide sequence accession numbers

Sequence data of the V3-V4 region of the 16S rRNA gene of the non-MAPD isolates and the partial 16S rRNA of the 78 MAPD isolates were submitted to the EMBL database under accession numbers LN881131 to LN881422

3. RESULTS AND DISCUSSION

The cultivable microbiota of *Ascophyllum nodosum*: comparison with the total bacterial community of brown algae and highlight of a host-specific common core

Ascophyllum nodosum is a large brown alga (up to 2 m) of the *Fucaceae* family which is common on both sides of North Atlantic ocean (Olsen *et al.*, 2010). This macroalga is dominant along sheltered intertidal rocky shores and is a very long-lived species (10-15 years). The microbial population of *A. nodosum* was studied long ago by culturing (Chan and McManus, 1969) and electronic microscopy (Cundell *et al.*, 1977). Surprisingly, these two old studies are the only ones concerning microorganisms associated with this brown seaweed. Cundell et al. (1977) describe the microbial epiphytes of *A. nodosum* as "*a complex assemblage of end-attached bacteria, filamentous bacteria, flexibacteria, yeasts, and pennate diatoms*". We here report the first extensive characterization of the cultivable bacterial community of this ecologically relevant macroalga.

Approximately 3.10^3 bacteria were isolated from the surface of three *Ascophyllum nodosum* thallus samples. A total of 324 bacteria were recovered (108/sample) for further analysis.

We succeeded in amplifying and sequencing the V3-V4 16S rRNA region from a total of 297 bacteria (98 from S1, 97 from S2, 102 from S3). These sequences were aligned to those from the GenBank and SILVA SSU databases (data not shown), with 291 out of 297 isolates being assigned to a genera. As a proof of quality sequence alignments, we had high average query coverage of 97.2% and identity percentage of 98.9% for the V3-V4 region sequences of these isolates (Table V-S1). For each *A. nodosum* sample, tables were made with the relative abundance and the frequency of each bacterial genus, family, order, class, and phylum identified (Table V-S2).

In the total isolated bacterial population, 36 different genera were identified. The most abundantly represented were *Marinomonas* (13.1%), *Cellulophaga* (10.1%), and *Pseudoalteromonas* (9.1%). Eighteen bacterial families were found, the major ones being *Flavobacteriaceae* (42.8%), *Oceanospirillaceae* (15.5%), and *Rhodobacteraceae* (11.1%). The most abundant phylum was *Proteobacteria* (56%, with class *Alpha*: 11.4%, *Gamma:* 44.1%, *Beta*: 0.7%), closely followed by *Bacteroidetes* (class *Flavobacteriia* 42.8%). *Firmicutes* (1.0%) members were identified only on sample S3 and constituted a very minor fraction of the cultivable community.

Previous molecular studies on the kelp *Laminaria hyperborean* (Bengtsson and Øvreås, 2010; Bengtsson *et al.*, 2012) and on *Fucus vesiculosis* (Lachnit *et al.*, 2011; Stratil *et al.*, 2013, 2014), closely related to *A. nodosum*, reveal the phyla- and class-level structures of their associated microbial communities to be markedly different from the here identified population (see for review Martin et al., 2014b). *Alphaproteobacteria* emerged as a dominant phylum of the total bacterial community of these molecular studies (25-60%), whereas it represents only a minor fraction of the isolated cultivable fraction (11%). In contrast, the abundance of *Bacteroidetes* and *Gammaproteobacteria* members is two- to three-fold higher in our cultivable fraction. Surprisingly, no *Planctomycetes* and *Cyanobacteria* were isolated in our cultivable population even if they represent dominant phyla in the above mentioned reports of microbiota associated with other brown algae. Concerning the absence of *Cyanobacteria*, to our knowledge no representative of this autotrophic class has yet been identified by culturing methods dealing with algal microbiotas (Hollants *et al.*, 2013). Furthermore, as our samples were taken in March 2014, a seasonal shift is likely to explain both the absence of *Cyanobacteria* and the high percentage of *Gammaproteobacteria* members among our isolates, as previously observed for *F. vesiculosus* (Lachnit *et al.*, 2011). The absence of isolated *Planctomycetes* is more surprising, as these heterotrophic bacteria are expected to grow readily on complex culture media.

At lower taxonomic rank, molecular studies have provided evidence that total bacterial communities can differ markedly from one individual alga to another and that the core community specific to the host is limited (~15%) (Burke, Thomas, *et al.*, 2011; Bengtsson *et al.*, 2012). Here, looking solely at the cultivable community of *A. nodosum*, we reach partly similar conclusions as there is some variability between the communities isolated on the three samples, as regards both the genera observed and their relative abundances (Figure V-1, Table V-S2). On the PCoA plot (Figure IV-1), one can see that the samples are equally distant (dissimilar in their bacterial composition) from each other (Bray-Curtis distances between S1-S2=0.45; S1-S3=0.412; S2-S3=0.453). Moreover, some genera are specific to one sample (e. g., *Hydrogenophaga, Ruegeria,* and *Tenacibaculum* were found only on S1) or common to just two samples (e. g., *Leucothrix*, *Psychroserpens*, and *Psychrobacter* were identified only on S2 and S3). However, despite this genus diversity, twelve genera were consistently found on all three *A. nosodum* samples: (i) six *Flavobacteriia* genera, all of the *Flavobacteriaceae* family (*Algibacter*, *Cellulophaga*, *Dokdonia*, *Formosa*, *Maribacter,* and *Zobellia*); (ii) five *Gammaproteobacteria* genera; four genera of the order *Alteromonadales* but of different families (*Colwellia*, *Glaciecola*, *Pseudoalteromonas,* and *Shewanella*) and one genus of the order *Oceanospirillales* (*Marinomonas*, *Oceanospirillaceae* family); and (iii) one *Alphaproteobacteria* genus (*Sulfitobacter*, *Rhodobacteraceae* family) (Figure V-1). These genera represent a core community of 33% of the cultivable subpopulation (12 out of 36 genera in total), which is twice the proportion observed in whole seaweed-associated microbiotas (Burke, Thomas, *et al.*, 2011; Bengtsson *et al.*, 2012). This common core includes several host-specific genera which are well known to associate with macroalgae. *Cellulophaga*, *Pseudolateromonas*, *Shewanella*, *Sulfitobacter,* and *Zobellia,* for example, are among the 33 genera isolated from all three macroalgal groups (brown, green, and red seaweeds), as determined on the basis of a review of 161 studies (mainly culture-based) dealing with bacteria associated with 159 macroalgal species (Hollants *et al.*, 2013). *Formosa*, *Dokdonia*, *Marinomonas,* and *Glaciecola* have been also identified on other brown algae like *Fucus vesiculosus* (Lachnit et al., 2011; Goecke et al., 2013a) , *F. evanescens* (Ivanova, Alexeeva, *et al.*, 2004) or *Laminaria sp.* (Wiese *et al.*, 2009b; Dong *et al.*, 2012). To our knowledge, *Colwellia* is the only genus retrieved here from our three alga samples (representing 3.4% of our total bacterial isolates) that has not previously been isolated from brown algae. The *Colwelliaceae* family (comprising the genera *Colwellia* and *Thalassomonas*) has already been identified on the red alga *Delisea pulchra* (Fernandes *et al.*, 2012). Fernandes et al. found this family to be present on diseased thalli and absent from healthy thalli of this red alga.

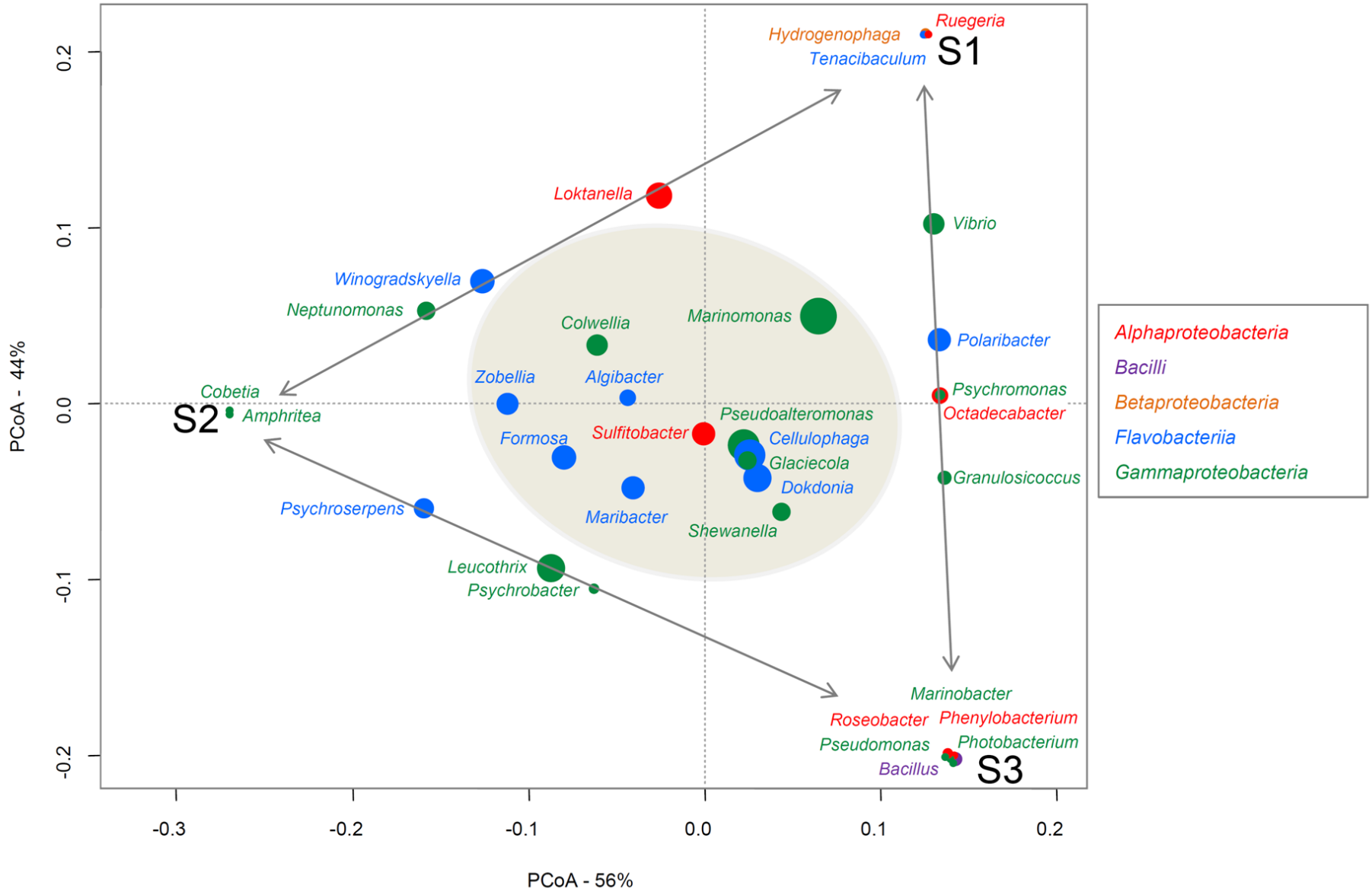

Figure V-1 PCoA of the genera found on the three algae samples. Bacterial classes are indicated in different colors. The size of each spot is proportional to the relative abundance of the genus indicated above or next to it. The closer a spot is to a sample name, the more abundant this genus is in that sample. The genera common to just to sample are aligned along the gray arrows. The genera identified on the three samples (i.e. the common core) are in the gray shadowed circle.

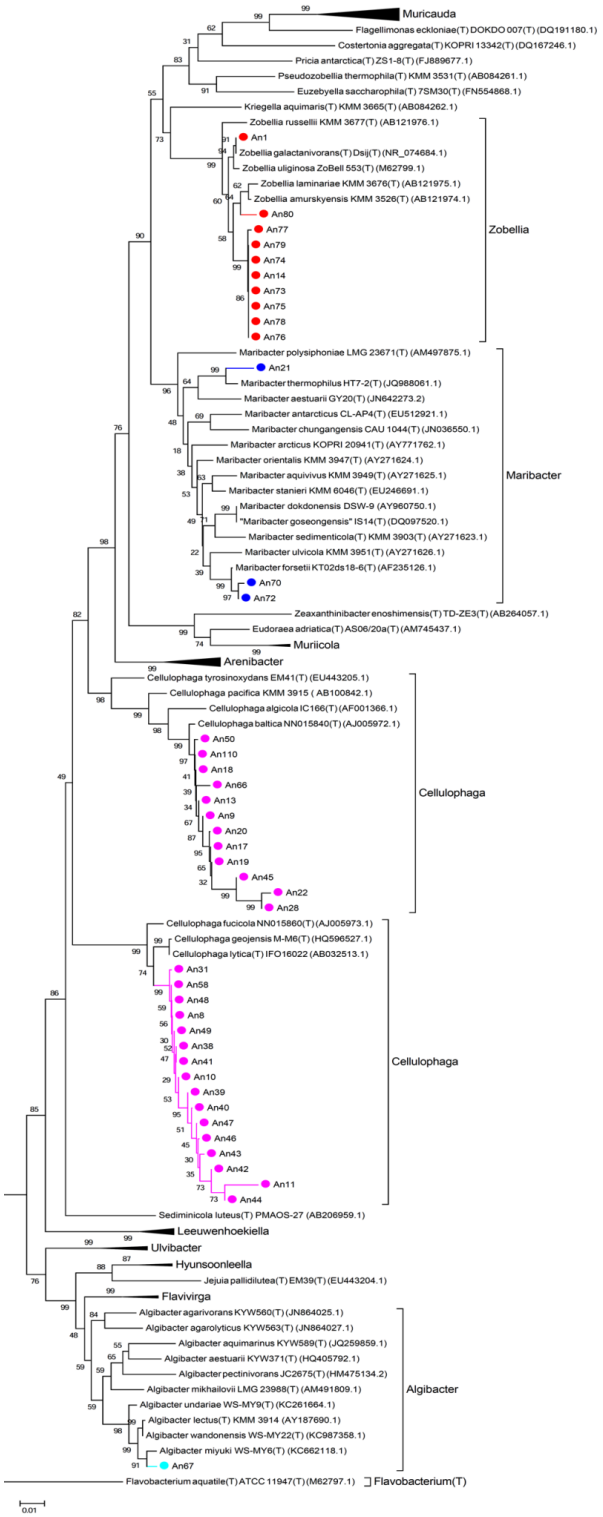

Figure V-2 Phylogenetic tree with the Flavobacteriaceae. 16S rRNA sequences of 120 representatives of the family Flavobacteriaceae were aligned with those of our 41 MAPD flavobacterial isolates. Sequences were manually curated and phylogenetic trees were constructed with these alignments using the Neighbor Joining Method. Evolutionary distances were calculated according to Kimura's two-parameter model using the gamma distribution (Kimura, 1980).

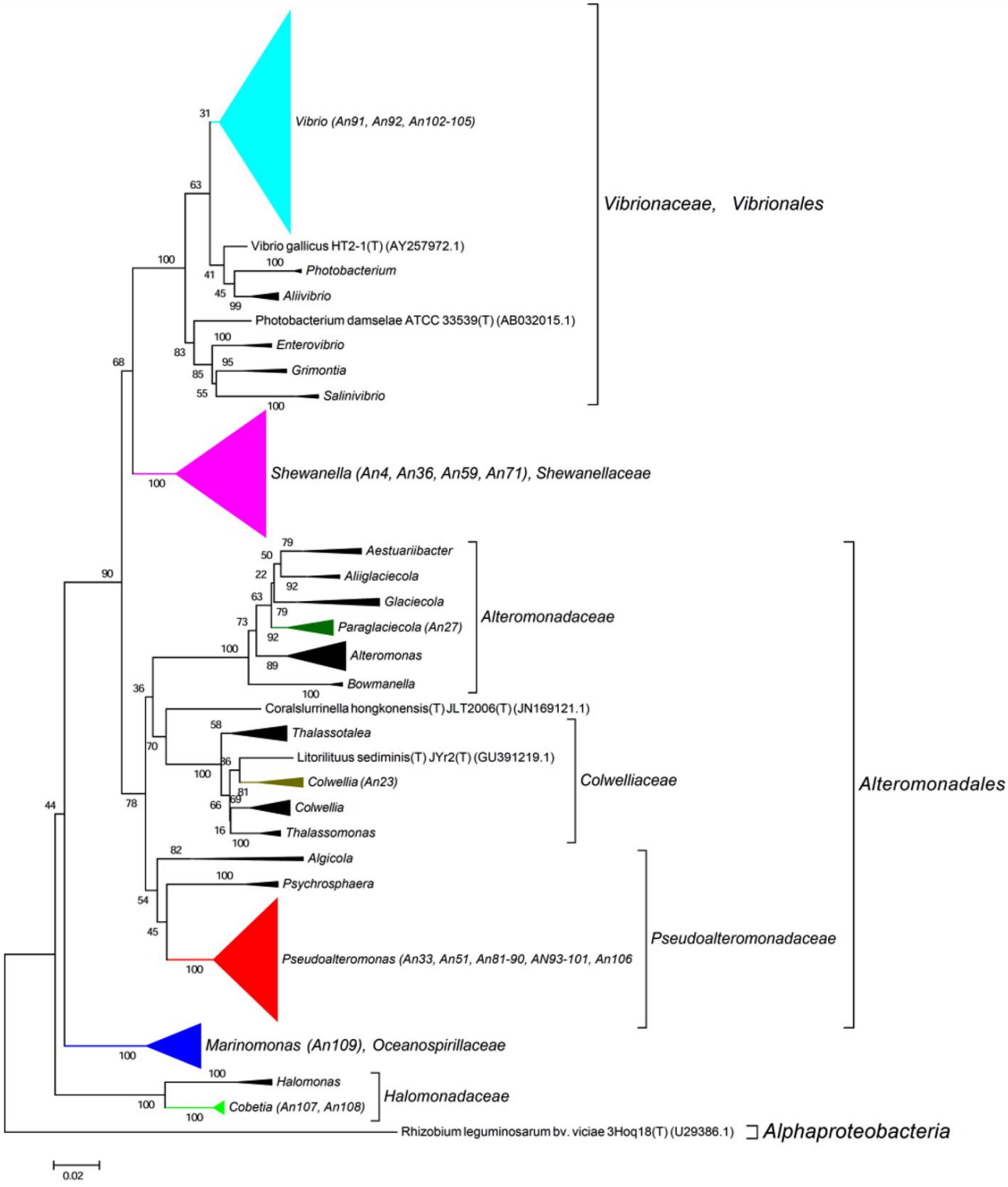

Figure V-3 Phylogenetic tree with the Gammaproteobacteria. 16S rRNA sequences of 355 representatives of the class Gammaproteobacteria were aligned with those of our 37 MAPD gammaproteobacterial isolates. Sequences were manually curated and phylogenetic trees were constructed with these alignments using the Neighbor Joining Method. Evolutionary distances were calculated according to Kimura's two-parameter model using the gamma distribution (Kimura, 1980).

The cultivable microbiota from *A. nodosum* is enriched in MAPD bacteria: novel activities and putative novel species

The 324 isolated bacteria were plated and tested on culture media gelified with agar, κ-carrageenan, ι-carrageenan, or alginate salts. We have found 78 isolates (24%) with polysaccharidase activity against at least one of the tested substrates (Table V-1). Similar proportions of such bacteria were isolated from the three specimens collected (S1: 20%, S2: 23%, S3: 34%). Thus our study clearly indicates that the cultivable microbiota of *A. nodosum* is significantly enriched in MAPD bacteria as compared to the total bacterial community (based on molecular studies and on to the difficulty to identify MAPD enzymes by functional genomics). Nonetheless, MAPD bacteria remain a minority even within the cultivable microbiota (24%).

The entire 16S rRNA genes of the various candidates were amplified and sequenced. Sequences were aligned with those of type strains with valid species names (EzTaxon, Kim et al., 2012). The percentage of identity to the closest type strain and the polysaccharidase activities of each isolate are listed in Table V-1. The first remarkable result is that all the MAPD isolates belong to only two bacterial classes, despite the taxonomic diversity of the cultivable community: 41 isolates were identified as *Flavobacteriia* members and 37 as *Gammaproteobacteria* members (Table V-1).

These isolates were assigned to 11 genera (out of the 36 genera isolated) and 8 families. Among the ten most abundant genera in our total isolated population (Figure V-4a), only four (*Marinomonas*, *Cellulophaga*, *Pseudoalteromnas*, and *Maribacter*) are represented by isolates with at least one polysaccharolytic activity. The genera *Cellulophaga* and *Pseudoalteromonas* are exceptional, being both abundant in the cultivable community (the second and third most abundant genera, respectively) and representing together more than 60% of the MAPD isolates (27/78 and 22/78, respectively) (Figure V-4a,b). In contrast, the most abundant genus (*Marinomonas*) is represented here by only one MAPD isolate (with a single, alginolytic activity). Similarly, *Formosa* is the 6th most abundant genus, but none of the *Formosa* isolates displays any MAPD activity. This is particularly unexpected, since the characterized species *Formosa agariphila* is agarolytic (Nedashkovskaya, 2006) and its genome analysis has revealed a broad potential for degrading algal polysaccharides (Mann *et al.*, 2013). This striking example is a reminder that extrapolating the biological or ecological function of a particular species to a larger bacterial group can lead to incorrect interpretations. Among the less abundant genera, *Zobellia* (13th, 10/297), *Vibrio* (15th, 7/297), and *Shewanella* (17th, 6/297) are also remarkable by their overrepresentation

among the MAPD isolates (Figure V-4b). Notably, 100% of the *Zobellia* isolates display MAPD activity.

Table V-1 Identified MAPD isolates, each with the closest type strain, the corresponding percentage of 16S rRNA gene similarity and the observed polysaccharidase activities. 16S rRNA perecentage lower than 97% were indicated in red.

Isolated bacterial strain	Closest bacterial strain (EZTaxon)	% identity	Polysaccharidase activities[1]			
			Ag	ι-C	κ-C	AL
	Flavobacteriia					
FLAVOBACTERIACEAE						
***ALGIBACTER* sp.**						
An67	*Algibacter miyuki* WS-MY6	97.75	+	+	-	-
***CELLULOPHAGA* sp.**						
An8	*Cellulophaga geojensis* M-M6	97.88	+	+	+	+
An10	*Cellulophaga geojensis* M-M6	97.88	+	+	+	+
An42	*Cellulophaga geojensis* M-M6	97.66	+	+	+	-
An44	*Cellulophaga geojensis* M-M6	96.28	+	+	+	+
An47	*Cellulophaga geojensis* M-M6	96.6	+	+	+	-
An48	*Cellulophaga geojensis* M-M6	97.65	+	+	+	-
An11	*Cellulophaga fucicola* NN015860	95.56	+	+	+	+
An31	*Cellulophaga fucicola* NN015860	97.22	+	+	+	+
An38	*Cellulophaga fucicola* NN015860	97.51	+	+	+	+
An39	*Cellulophaga fucicola* NN015860	98	+	+	+	+
An40	*Cellulophaga fucicola* NN015860	96.75	+	+	+	+
An41	*Cellulophaga fucicola* NN015860	97.58	+	+	+	+
An43	*Cellulophaga fucicola* NN015860	96.83	+	+	+	+
An46	*Cellulophaga fucicola* NN015860	96.82	+	+	+	-
An49	*Cellulophaga fucicola* NN015860	97.65	+	+	+	+
An9	*Cellulophaga baltica* NN015840	98.27	+	+	+	+
An13	*Cellulophaga baltica* NN015840	98.64	+	+	+	+
An17	*Cellulophaga baltica* NN015840	97.75	+	+	+	+
An18	*Cellulophaga baltica* NN015840	98.49	+	+	+	+
An19	*Cellulophaga baltica* NN015840	98.20	+	+	+	+
An20	*Cellulophaga baltica* NN015840	97.45	+	+	+	+
An22	*Cellulophaga baltica* NN015840	95.87	+	+	+	-
An28	*Cellulophaga baltica* NN015840	96.32	+	+	+	+
An45	*Cellulophaga baltica* NN015840	98.63	+	+	+	+
An50	*Cellulophaga baltica* NN015840	97.68	+	+	+	+
An110	*Cellulophaga baltica* NN015840	98.53	+	+	+	+
An66	*Cellulophaga pacifica* KMM3664	96.21	+	+	+	+

***MARIBACTER* sp.**						
An21	*Maribacter aestuarii* GY20	95.33	+	+	+	+
An70	*Maribacter forsetii* KT02ds 18-6	99.28	-	+	-	+
An72	*Maribacter forsetii* KT02ds 18-6	99.06	-	+	-	+
***ZOBELLIA* sp.**						
An1	*Zobellia galactanivorans* DsiJ	99.4	+	+	+	+
An14	*Zobellia galactanivorans* DsiJ	97.63	+	+	-	-
An73	*Zobellia galactanivorans* DsiJ	97.7	+	+	-	+
An75	*Zobellia galactanivorans* DsiJ	98	+	+	-	-
An76	*Zobellia galactanivorans* DsiJ	97.8	+	+	-	+
An78	*Zobellia galactanivorans* DsiJ	97.5	+	+	-	-
An79	*Zobellia galactanivorans* DsiJ	97.8	+	+	-	-
An74	*Zobellia laminariae* KMM3676	97.6	+	+	-	-
An77	*Zobellia laminariae* KMM3676	96.8	+	+	-	-
An80	*Zobellia laminariae* KMM3676	98.9	+	+	+	-
	Gammaproteobacteria					
ALTEROMONADACEAE						
***PARAGLACIECOLA* sp.**						
An27	Paraglaciecola mesophila KMM241	96.49	+	+	+	-
COLWELLIACEAE						
***COLWELLIA* sp.**						
An23	Colwellia meonggei MA1-3	96.17	+	+	+	-
HALOMONODACEAE						
***COBETIA* sp.**						
An107	Cobetia litoralis KMM 3880	99.31	-	-	-	+
An108	Cobetia litoralis KMM 3880	98.96	-	-	-	+
OCEANOSPIRILLACEAE						
***MARINOMONAS* sp.**						
An109	Marinomonas ushuaiensis U1	97.14	-	-	-	+
PSEUDOALTEROMONODACEAE						
***PSEUDOALTEROMONAS* sp.**						
An88	Pseudoalteromonas aliena KMM 3562	99.57	-	-	-	+
An89	Pseudoalteromonas aliena KMM 3562	99.2	-	-	-	+
An97	Pseudoalteromonas aliena KMM 3562	98.3	-	-	-	+
An33	Pseudoalteromonas espejiana NCIMB 2127	99.4	-	+	+	+
An51	Pseudoalteromonas espejiana NCIMB 2127	98.1	-	-	-	+
An81	Pseudoalteromonas espejiana NCIMB 2127	99.34	-	-	-	+
An82	Pseudoalteromonas espejiana NCIMB 2127	99.42	-	-	-	+
An83	Pseudoalteromonas espejiana NCIMB 2127	99.13	-	-	-	+
An84	Pseudoalteromonas espejiana NCIMB 2127	99.71	-	+	+	+
An85	Pseudoalteromonas espejiana NCIMB 2127	98.84	-	-	-	+

An86	Pseudoalteromonas espejiana NCIMB 2127	99.05	-	-	-	+
An87	Pseudoalteromonas espejiana NCIMB 2127	98.11	-	-	-	+
An90	Pseudoalteromonas espejiana NCIMB 2127	98.98	-	+	-	+
An93	Pseudoalteromonas espejiana NCIMB 2127	99.6	-	-	-	+
An94	Pseudoalteromonas espejiana NCIMB 2127	98.8	-	-	-	+
An95	Pseudoalteromonas espejiana NCIMB 2127	99.7	-	-	-	+
An99	Pseudoalteromonas espejiana NCIMB 2127	99.7	-	+	+	+
An100	Pseudoalteromonas espejiana NCIMB 2127	99.6	-	+	+	+
An101	Pseudoalteromonas espejiana NCIMB 2127	98.1	-	-	-	+
An106	Pseudoalteromonas espejiana NCIMB 2127	98.76	-	-	-	+
An96	Pseudolateromonas nigrifaciens NCIMB 8614	99.2	-	-	-	+
An98	Pseudolateromonas nigrifaciens NCIMB 8615	99.2	-	-	-	+
SHEWANELLACEAE						
***SHEWANELLA* sp.**						
An36	Shewanella pacifica KMM3597	98.3	-	+	+	+
An59	Shewanella pacifica KMM3597	97.6	-	+	+	+
An71	Shewanella pacifica KMM3597	97.6	-	+	+	+
An4	Shewanella japonica KMM 3299	98.3	-	+	-	+
VIBRIONACEAE						
***VIBRIO* sp.**						
An91	Vibrio hemicentroti AlyHp32	99.17	-	-	-	+
An92	Vibrio splendidus ATCC 33125	97.2	-	-	-	+
An102	Vibrio splendidus ATCC 33125	98.99	-	-	-	+
An103	Vibrio splendidus ATCC 33125	99.14	-	-	-	+
An104	Vibrio splendidus ATCC 33125	99.21	-	-	-	+
An105	Vibrio splendidus ATCC 33125	99.28	-	-	-	+

[1] Ag = agarase ;i-C= ι-carrageenase ; κ-C = κ-carrageenase ;AL = alginate lyase

Interestingly, the polysaccharidase activities identified here do not necessarily reflect the cell wall composition of *A. nodosum*. Brown alga cell walls are largely composed of alginates and sulfated fucoidans, whereas red macroalgae mainly contain sulfated galactans (agars or carrageenans) (Popper *et al.*, 2011). Unexpectedly, we have found similar proportions of bacterial isolates degrading polysaccharides of red (64/78) and brown (61/78) macroalgae (Table V-1, Figure V-4b), but these activities are not equally distributed between *Gammaproteobacteria* and *Flavobacteriia.* Most gammaproteobacterial isolates (26/37) exclusively degrade alginate (including all *Cobetia*, *Marinomonas*, and *Vibrio* isolates). All *Pseudoalteromonas* isolates are alginolytic, but a minority is also able to hydrolyze carrageenans (22%), and none of them is agarolytic. MAPD activities are more evenly

distributed in *Shewanella*, all isolates being both alginolytic and carrageenolytic. More surprisingly, the *Paraglaciecola* and *Colwellia* isolates are not alginolytic, but can hydrolyze both agars and carrageenans. In contrast, the *Flavobacteriia* isolates are much more generalistic degraders. All the MAPD *Flavobacteriia* isolates hydrolyze red algal sulfated galactans (agars and/or carrageenans) and most of them (26/41) also alginate. This suggests that the MAPD *Gammaproteobacteria* isolates are more specific to brown algae, while the MAPD *Flavobacteriia* strains or species isolated from *A. nodosum* are likely to be found also on the surfaces of agarophytic and carrageenophytic red seaweeds.

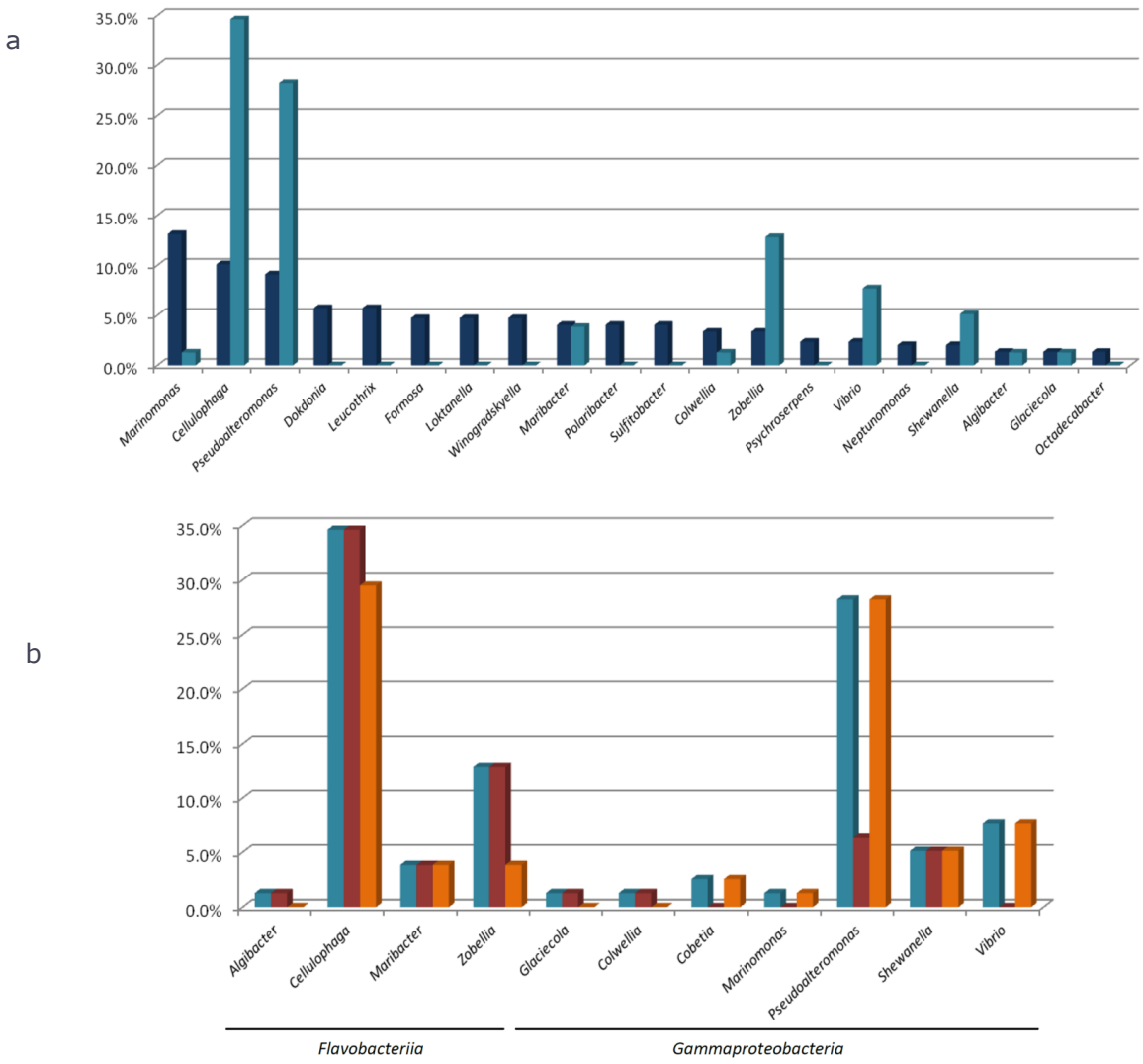

Figure V-4 a) Percentage proportions of the most represented genera in the total isolated bacterial population ■ and of the MAPD isolates belonging to these genera in the whole set of 78 MAPD isolates ■ ; **b)** Percentage proportions of MAPD isolates belonging to each MAPD-isolate-containing genus in the whole set of 78 MAPD isolates ■, with their activities on red ■ or brown ■ seaweed galactans.

It is also noteworthy that we have discovered MAPD activities in genera that were not previously known to include MAPD bacteria (*Marinomonas* and *Colwellia* strains) or to display the activities observed here (ι- and κ-carrageeenases in *Algibacter* and *Maribacter* isolates, respectively) (Table V-2).

Table V-2 Activities identified in our study and previously described for MAPD species or strains from the genera to which our 78 MAPD isolates were assigned.

Genera to which the 78 MAPD-isolates were assigned	Activities identified in our study[1]				Previously described activities[1]				References
	Ag	ί-C	κ-C	AL	Ag	ί-C	κ-C	AL	
Flavobacteriia									
Algibacter	•	■			•			•	(Park *et al.*, 2013; Tanaka *et al.*, 2015)
Cellulophaga	•	•	•	•	•	•	•	•	(Johansen *et al.*, 1999; Park *et al.*, 2012; Yao *et al.*, 2013)
Maribacter	•	•	■	•	•	•		•	(Barbeyron, Carpentier, *et al.*, 2008)
Zobellia	•	•	•	•	•	•	•	•	(T. Barbeyron *et al.*, 2001; Nedashkovskaya *et al.*, 2004)
Gammaproteobacteria									
Paraglaciecola	•	•	•		•	•	•		(Romanenko *et al.*, 2003; Yong *et al.*, 2007)
Colwellia	■	■	■						(Browman, 2013; Liu *et al.*, 2014; Wang *et al.*, 2015)
Cobetia				•				•	(Lelchat *et al.*, 2015)
Marinomonas				■					(Macián *et al.*, 2005; Lucas-Elió *et al.*, 2011)
Pseudolateromonas		•	•	•	•	•	•	•	(Akagawa-Matsushita *et al.*, 1992; Chi *et al.*, 2014)
Shewanella		•	•	•	•	•	•	•	(Ivanova *et al.*, 2001, 2003; Ivanova, Gorshkova, *et al.*, 2004; Wang *et al.*, 2014)
Vibrio				•	•			•	(Sugano *et al.*, 1993; Kim *et al.*, 2013)

• Activities found in our study and found previously for species/strains of this genus; ■ Novel activities, that weren't identified for any species/strains of this genus previously.

These novel activities may be explained by the likely isolation of novel MAPD species. Indeed, 63% of the MAPD isolates identified here have less than 98.65% sequence identity at 16S rRNA level to a known species (Figure V-5). Therefore they could represent putative novel species even if there is still some discussion regarding the threshold percentage of 16S rRNA gene identity at which two species can be distinguished. A commonly accepted value is 97%. Recently, Kim et al. (2014), having compared the average nucleotide identities of almost 7000 prokaryotic genomes and their 16S rRNA gene identities, propose a threshold of 98.65%, while Tindall et al. (2010) stress that the 16S rRNA alone does not describe a species but only provides a putative indication of a novel species. Nevertheless, further taxonomic analyses and DNA-DNA hybridization experiments should be performed or average nucleotide identities determined to confirm this (Tindall *et al.*, 2010; Stackebrandt, 2011). However, to strengthen the taxonomic identification of these MAPD isolates and the assumption that most of these isolates represent new species, phylogenetic trees of entire 16S rRNA genes were constructed

for the *Flavobactericeae* (Figure V-2) and *Gammaproteobacteria* members (Figure V-3, Figure V-S2).The *Flavobacteriaceae* phylogenetic tree strongly suggests that we have identified three novel *Zobellia* species (represented by An80, An77, and the seven strains of the An14 clade) and a novel *Maribacter* species (An21) (Figure V-2). Furthermore, in this phylogenetic tree, the *Cellulophaga* genus clearly appears non-monophyletic as the MAPD *Cellulophaga* isolates are separated into two clades, 12 of them having a common ancestor with *C. baltica* and the other 16 a common ancestor with *C. lytica*. This result appears to confirm the doubts raised in Bergey's manual of Systematic Bacteriology concerning the monophyletic character of the *Cellulopha* genus (Krieg *et al.*, 2011). In the detailed *Gammaproteobacteria* tree (Figure V-S2), the *Colwellia sp.* An23, the *Paraglaciecola sp.* An27, the four *Shewanella* isolated and the *Marinomonas sp.* An 109 seem very likely to represent novel species.

Last but not least, one can observe that the proportion of MAPD bacteria increases dramatically while looking only at the core group of cultivable bacteria. Indeed, MAPD activity was detected in 75% of the core genera (*Algibacter, Cellulophaga, Colwellia, Glaciecola Maribacter, Marinomonas, Pseudoalteromonas, Shewanella* and *Zobellia*) (Table V-1, Figure V-1). Thus, even though MAPD bacteria constitute a minor fraction of both the total and cultivable bacterial communities, they apparently belong to the core group of bacteria living at the surface of *A. nodosum* and likely exert functions that are important for their macroalgal host and/or within the microbiota as a whole. How harboring MAPD bacteria might be beneficial to the host is not obvious. Such bacteria have mostly been described as detrimental to macroalgae, being responsible for diseases, providing an entry for opportunistic bacteria, or accelerating algal degradation (Goecke *et al.*, 2010; Egan *et al.*, 2013; Hollants *et al.*, 2013). Recently, Marzinelli et al. (2015) compared microbial communities on healthy and bleached thalli of the brown kelp *Ecklonia radiata*. They found *Flavobacteriaceae* and *Oceanospirillaceae* representatives to be more present on diseased tissues. Within these families, however, some genera were found in much higher proportion on healthy samples than on bleached ones, suggesting a role favorable to the macroalgal host. Interestingly, these genera include several of those represented by MAPD isolates obtained from *A. nodosum*: *Zobellia*, *Maribacter*, *Pseudoalteromonas*, *Vibrio, Marinomonas*, and *Cobetia*. Beyond their MAPD activities, species of these genera may have additional metabolic capacities advantageous for their hosts. This hypothesis is plausible at least for *Zobellia* species, which are known to synthesize an algal morphogenesis inducer (Matsuo *et al.*, 2003). The role of MAPD bacteria within the total seaweed-associated microbiota is more obvious. These bacteria are essential for degrading intact cell-wall polysaccharides, and thus for releasing hydrolysis products assimilable by the much more abundant bacteria (e.g. *Alphaproteobacteria*) lacking these unique MAPD enzymes.

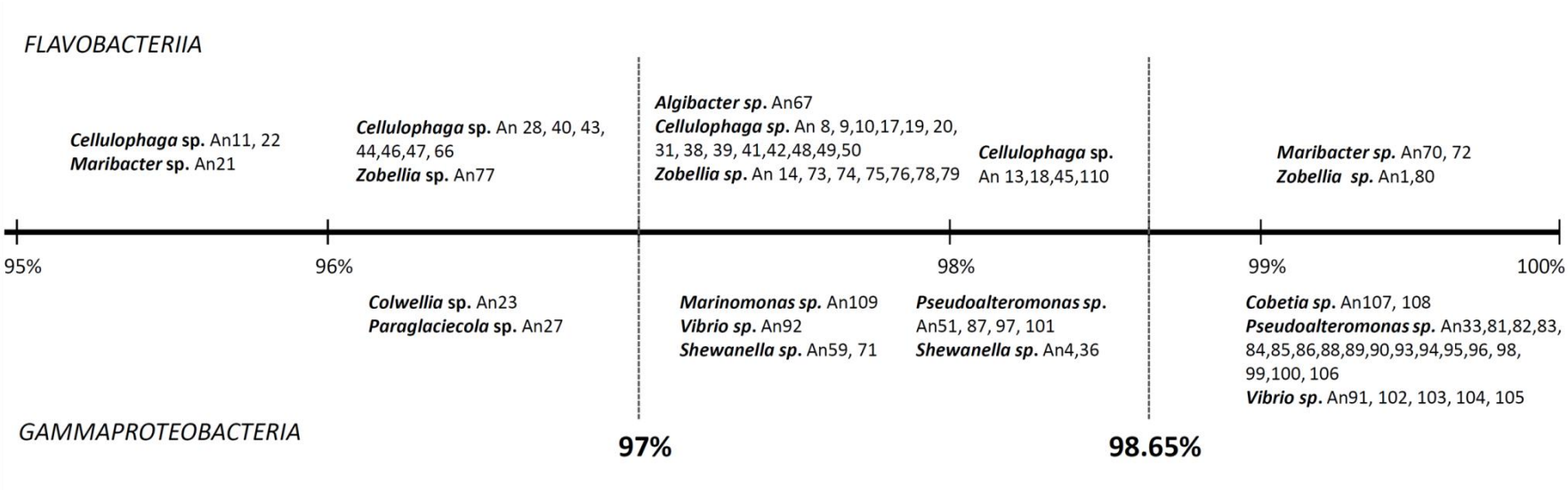

Figure V-5 Ranges of 16S rRNA identity percentages for the identified MAPD isolates versus known species. Two third of the MAPD isolates (< 98.65% 16S rRNA identities) likely represent novel species. Indeed, 97% is the commonly accepted threshold percentage at which two species can be distinguished and 98,65% is the threshold proposed by Kim et al. (2014) which have compared the average nucleotide identities of almost 7000 prokaryotic genomes and their 16S rRNA gene identities.

4. CONCLUSION

In terrestrial environments, the bacteria involved in recycling plant polysaccharides are essentially found both on living plants and in the soils that immediately surround them. The situation is more complex for marine macroalgae. They live attached to rocks, and when algal fragments are released, they are quickly dispersed by the waves and tides. The available marine metagenomic data show that the water column is a habitat poor in MAPD bacteria and, for a macroalga, not equivalent to a surrounding soil. Tidal sediments could be crucial reservoirs of MAPD bacteria, but this remains an open question. A third environment likely to be a habitat for MAPD bacteria is the surface of the macroalgae themselves. We have shown here that this is indeed the case and that the cultivable microbiota of healthy *A. nodosum* specimens is enriched in MAPD bacteria. These bacteria, however, are not the most abundant ones associated with brown seaweeds; they constitute a minority fraction even within the cultivable subpopulation. An attractive hypothesis is that this low abundance of MAPD bacteria is due to active and/or passive defense systems of the macroalga, preventing proliferation of these potentially harmful bacteria. Evidence of such defense systems in macroalgae has been accumulating over the last decade (Potin *et al.*, 2002; Egan *et al.*, 2014). If this hypothesis is correct, one can expect MAPD bacteria to bloom on weakened or dead macroalgae, thus contributing significantly to recycling of macroalgal biomass. As regards bioprospecting, our work demonstrates that culturing (combined, for instance, with subsequent genome sequencing of cultivable isolates) is an efficient strategy for finding new MAPD bacteria and their corresponding polysaccharidases.

Non-standard abbreviations

MAPD : Macroalgal-polysaccharide-degrading bacteria

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Chapter VI. Functional screening of plurigenomic libraries

Outline

In the previous chapter, we demonstrated that the cultivable surface microbiota of *A.nodosum* is considerably enriched in macroalgal-polysaccharide-degrading (MAPD) bacteria and 78 such bacteria were isolated. Most of these isolates, belonging to the *Flavobacteriia* or the *Gammaproteobacteria* classes, are distantly related to described strains or species from the literature. Furthermore, some MAPD bacterial isolates belonged to genera that weren't previously known to hydrolyze algal-polysaccharides. The originality of our findings, led us to explore the genomic potential of these uncommon isolates for hydrolytic enzymes. In this last research chapter, we describe the functional screening of plurigenomic libraries constructed with some of these original MAPD bacteria genomic DNA. This resulted in the identification of several functional genes.

Related publication

Discovering novel enzymes by functional screening of plurigenomic libraries from alga-associated *Flavobacteriia* and *Gammaproteobacteria*.

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Discovering novel enzymes by functional screening of plurigenomic libraries from alga-associated *Flavobacteriia* and *Gammaproteobacteria*

ABSTRACT

Alga-associated microorganisms, in the context of their numerous interactions with the host and the complexity of the marine environment, are known to produce diverse hydrolytic enzymes with original biochemistry. We recently isolated several macroalgal-polysaccharide-degrading bacteria from the surface of the brown alga *Ascophyllum nodosum*. These active isolates belong to two classes: the *Flavobacteriia* and the *Gammaproteobacteria*. In the present study, we constructed two "plurigenomic" (with multiple bacterial genomes) libraries with the 5 most interesting isolates (regarding their phylogeny and their enzymatic activities) of each class (Fv and Gm libraries). Both libraries were screened for diverse hydrolytic activities. Five activities, out of the 48 previously identified in the natural polysaccharolytic isolates, were recovered by functional screening: a xylanase (GmXyl7), a beta-glucosidase (GmBg1), an esterase (GmEst7) and two iota-carrageenases (Fvi2.5 and Gmi1.3). We discuss here the potential role of the used host-cell, the average DNA insert-sizes and the used restriction enzymes on the divergent screening yields obtained for both libraries and get deeper inside the "great screen anomaly". Interestingly, the discovered esterase probably stands for a novel family of homoserine o-acetyltransferase-like-esterases, while the two iota-carrageenases represent new members of the poorly known GH82 family (containing only 19 proteins since its description in 2000). These original results demonstrate the efficiency of our uncommon "plurigenomic" library approach and the underexplored potential of alga-associated cultivable microbiota for the identification of novel and algal-specific enzymes.

KEYWORDS

alga-associated microflora, marine bacteria, agarase, iota-carrageenase, kappa-carrageenase, esterase, beta-glucosidase, xylanase, marine enzymes.

1. INTRODUCTION

For a long time, researchers have investigated environmental samples to identify novel microbial biocatalysts. Early efforts focused on discovering novel enzymes of known or newly described cultivable microorganisms. More recently, (sequence- or function-based) metagenomic screening has been included among the tools used to mine for novel microbial enzymes (Handelsman, 2004). Sequence-based metagenomic approaches provide access to non-cultivable microorganisms, but as enzymes are sought by sequence comparisons with known biocatalysts, only new variants of existing enzymes are discovered (Simon and Daniel, 2011). In contrast, function-based screening of metagenomic libraries can lead to discovering completely novel enzymes, while looking only at the function of the gene and not its similarities with known sequences. Yet searching for a particular enzyme type in a metagenome, is somewhat like looking for a needle in a haystack. It is fastidious, and yields are poor (Ekkers *et al.*, 2012; Ferrer *et al.*, 2016).Therefore the old-fashioned culturing approach, followed by selection of isolates showing activities of interest, still has a place in the hunt for novel enzymes. Preselection of "active" strains limits the number of genomes to be prospected and allows more focused work. Furthermore, even though culturing methods restrict the diversity of the findings, as only a very low percentage of existing microbes are cultivable (estimated at less than 1% in most environmental samples (Torsvik and Øvreås, 2002)), novel enzymes and enzyme families far different from known ones can still be discovered in underexplored bacterial taxa and environments.

Bacteria associated with algae have been shown to produce many polysaccharide hydrolases, because of their complex and dynamic interactions with their hosts (Holmström *et al.*, 2002; Martin, Portetelle, *et al.*, 2014). We have recently demonstrated that the cultivable microbiota associated with the brown alga *Ascophyllum nodosum* is rich in macroalgal-polysaccharide-degrading bacteria (Martin *et al.*, 2015). Furthermore, many novel species and genera have been identified in alga-associated microbiotas, suggesting that they constitute an interesting biotic environment for the discovery of new bacterial taxa and hence, original biocatalysts (Goecke, Thiel, *et al.*, 2013). In the class *Flavobacteriia* (*Bacteroidetes*) several polysaccharolytic genera were first identified on a macroalga: for example, *Cellulophaga* on the brown alga *Fucus serratus*, *Formosa* on the brown alga *Fucus evanescens*, and *Zobellia* on the red alga *Delesseria sanguine* (Johansen *et al.*, 1999; T. Barbeyron *et al.*, 2001; Ivanova, Alexeeva, *et al.*, 2004). Novel glycoside hydrolase families have also been discovered in these genera (Michel and Czjzek, 2013), such as the iota-carrageenase (GH82) and β-porphyranase (GH86) families in *Zobellia galactanivorans* (Barbeyron *et al.*, 2000; Hehemann *et al.*, 2010). On the other hand, few studies have

focused on identifying more classical hydrolytic enzymes in alga-associated bacterial species, such as cellulases, xylanases, beta-glucosidases, or esterases, even though marine hydrolases differ markedly from their terrestrial homologs (Dalmaso *et al.*, 2015). It thus seems that cultivable alga-associated bacteria constitute an interesting source of a large range of novel hydrolytic enzymes.

In a previous study, we isolated and identified several algal-polysaccharide-degrading bacteria from the microflora associated with the brown alga *A. nodosum* (Martin *et al.*, 2015). All the isolates, some of which very probably represent new species, were taxonomically assigned to the classes *Flavobacteriia* and *Gammaproteobacteria*. They display diverse hydrolytic activities. In the present work, to identify novel enzyme-encoding genes from these original alga-associated bacteria, we have used an uncommon approach: constructing and screening "plurigenomic" (multiple bacterial genomes) libraries. One library was constructed with the genomes of five *Flavobacteriia* isolates and one with the genomes of five *Gammaproteobacteria* isolates. Screening was done for agarase, iota-carrageenase and kappa-carrageenase, beta-glucosidase, endo-cellulase, xylanase, and esterase activity.

2. MATERIALS AND METHODS

Isolation and functional screening of alga-associated *Flavobacteriia* and *Gammaproteobacteria*

Diverse algal-polysaccharide-degrading bacteria were isolated from the brown alga *A. nodosum*, as described in our previous study (Martin et al., 2015). These bacteria were assigned to the classes *Flavobacteriia* and *Gammaproteobacteria*. The isolates were tested for diverse hydrolytic activities at room temperature. Agarase, iota-carrageenase and kappa-carrageenase activities were detected on Marine Broth (Difco) containing, respectively, 1.5% agar, 2% iota-carrageenan, or 1% kappa-carrageenan (Sigma). Isolates showing hydrolytic activity were detected by a hole in the surrounding jellified medium for agarase and κ-carrageenase activities or the complete liquefaction of the medium for ι-carrageenase activity. Marine Agar (Difco) and AZCL-HE-cellulose, -amylose or -xylan (birchwood) (Megazyme) was used to detect endo-cellulase, alpha-amylase, or endo-1,4-β-D-xylanase activity, respectively. A blue halo around a colony was indicative of hydrolase activity. Esterase activity was detected on Marine Agar and 3% Difco Lipase Reagent (Difco). Lipolytic isolates were detected by a clear halo around the colony. Beta-glucosidase activity was detected as described by Matteotti et al. (2011) on Marine Agar containing 0.5% esculin and 0.1% ammonium iron (III) citrate (Sigma-Aldrich). A bacterium was identified as positive when a brown precipitate appeared around its colony.

Construction of plurigenomic libraries with *Flavobacteriia* and *Gammaproteobacteria* isolates

Two plurigenomic libraries were constructed, one for each class. Five isolates per class were used. The *Flavobacteriia* used were the *Cellulophaga* isolates An8, An9, and An20 (16s rRNA sequence accession numbers: LN881186, LN881202, LN881252), the *Zobellia* isolate An14 (LN881227), and the *Maribacter* isolate An21 (LN881276). The *Gammaproteobacteria* used were the *Shewanella* isolates An4 and An36 (LN881152, LN881379), the *Pseudoalteromonas* isolate An33 (LN881360), the *Colwellia* isolate An23 (LN881284), and the *Paraglaciecola* isolate An27 (L881305). These ten isolates were selected on the basis of their original phylogeny and the intensity and/or diversity of their activities (Table VI-1). They were grown for 24-48 h at 20°C in 3 ml Zobell medium (Difco Marine Broth). Genomic DNA from each isolate was extracted as described by Cheng and Jiang (2006). DNA quantity and quality were checked, respectively, with the Qubit fluorometer (Invitrogen) and by gel electrophoresis through a 0.8% agarose gel. The genomic DNA of each strain was tested for restriction by *Sau3AI* or its isoschizomer *DpnII*. We then pooled, on the one hand, 3 µg genomic DNA of each *Flavobacteriia* isolate, and on the other hand, 3 µg genomic DNA of each *Gammaproteobacteria* isolate. The pool of genomic DNA from the *Flavobacteriia* isolates was partially restricted for 1-3 min with 0.2 U/µg DpnII (NEB), and the *Gammaproteobacteria* pool for 1-2 min with 0.3 U/µg *Sau3AI* (Roche). After elimination of small DNA inserts by size-selective polyethylene glycol-NaCl precipitation as described by Biver and Vandenbol (2013), the DNA was purified by migration through a 1% low-melting-point agarose gel (Promega). DNA inserts exceeding 5 kb were recovered by beta-agarase digestion (NEB). The cloning vector *pHT01* (MoBiTec, Germany) was linearized with *BamHI* (Roche) and dephosphorylated (Dephos and Ligation kit, Roche). A vector:insert ratio of 1:3 was used to ligate the DNA inserts of each library into the *pHT01* cloning vector at 16°C overnight (T4 DNA ligase, Roche). Electrocompetent Electromax™ *Escherichia coli* DH10B cells (Life Technologies) were transformed with 1 µl ligation products. The average DNA insert size in each plurigenomic library was estimated by isolation and purification of 20 randomly chosen plasmids.

Functional screening of the plurigenomic libraries

Almost 12600 clones of the *Gammaproteobacteria* (*Gm*) library and 15000 clones of the *Flavobacteriia* (*Fv*) library were isolated in 96-well plates and grown overnight in 2xYT liquid culture medium at 37°C. The liquid cultures were then replicated onto the various screening media and incubated at room temperature. Similar screening tests were used as described above, but Marine Broth was replace by minimal medium (1 g/l Yeast Extract, 5 g/l Bacto-

tryptone (MP, Biomedicals), 5 g/l NaCl (Merk)) and Marine Agar by minimum medium with 1.5% agar. The *Fv* library was screened for agarase, iota- and kappa-carrageenase, esterase, cellulase and xylanase activities and the *Gm* library was additionally screened for beta-glucosidase activity, as regards the hydrolytic activities observed for the natural strains of both libraries (Table VI-1). Positive clones were isolated and their plasmids purified. Activity was confirmed by transforming other *E. coli* strains (DH5α) with the purified plasmid and testing recombinant clones on the screening medium corresponding to the observed activity.

Sequencing, identification, and subcloning of the gene(s) putatively responsible for the observed activities

The DNA inserts of confirmed positive candidates were sequenced by Sanger sequencing (Germany) at GATC Biotech, and open reading frames (ORFs) were identified with ORF Finder (NCBI) and SnapGene^{TM}. All putative proteins corresponding to ORFs identified in the inserts were analyzed with BlastP (NCBI) against the non-redundant GenBank database and the curated Swissprot database. The genes putatively conferring the observed activities (as judged by sequence similarity) were subcloned. Genes for subcloning were amplified with primers chosen to anneal in the 200-basepair region located upstream (forward primer) or downstream (reverse primer) from the coding sequence. A *BamHI* restriction site was added on each primer. PCR amplifications were done with the Phusion® High-Fidelity DNA Polymerase according to the manufacturer's instructions (NEB, New England Biolabs). After amplification, the PCR products were restricted with *BamHI* (Roche). The vector *pHT01* was also linearized with *BamHI*. Vector dephosphorylation and ligations were done with the Roche Dephos&Ligation kit. *E. coli* DH5α cells were transformed with the ligation products. Subclones were tested on the screening medium corresponding to the expected activity. The DNA insert of each positive subclone was checked at GATC Biotech. In order to know from which alga-associated strain the contig derived, we looked at the Blast results of the different ORFs constituting the contig (Table VI-2) and verified our hypotheses by PCR amplification (Taq polymerase, Roche) of part of each candidate insert in the genomic DNA of the bacterial isolate from which it was assumed to derive.

Table VI-1 Enzymatic activities of the *Flavobacteriia* and *Gammaproteobacteria* strains used to construct the plurigenomic libraries.
Ag : agarase, İ-carr: iota-carrageenase, κ-carr: kappa-carrageenase, Amyl: alpha-amylase, Cell: endo-cellulase, β-gluc : Beta-glucosidase, Est : Esterase, Xyl : xylanase

Strains	16S rRNa accession numbers[1]	Closest bacterial strain (EZTaxon)	Enzymatic activities detected with our screening tests							
			Ag	İ-carr	κ-carr	Amyl	Cell	β-gluc	Est	Xyl
Bacterial isolates of the *Flavobacteriia* plurigenomic library *Fv*										
Cellulophaga sp. An8	LN881186	97.9% *Cellulophaga geojensis* M-M6	++	+	+	-	++	-	-	+
Cellulophaga sp. An9	LN881202	98.3% *Cellulophaga baltica* NN015840	++	+	+	-	++	-	+	+
Cellulophaga sp. An20	LN881252	97.5% *Cellulophaga baltica* NN015840	++	+	++	-	++	-	+	+
Maribacter sp. An21	LN881276	95.3% *Maribacter aestuarii* GY20	+	++	++	-	+	-	+	++
Zobellia sp. An14	LN881227	97.6% *Zobellia laminariae* KMM3676	-	+	-	-	-	-	-	-
Bacterial isolates of the *Gammaproteobacteria* plurigenomic library *Gm*										
Cowellia sp. An23	LN881284	96.2% *Colwellia meonggei* MA1-3	+	++	++	-	+	-	++	+
Paraglaciecola sp. An27	LN881305	96.5% *Paraglaciecola mesophila* KMM241	+	+	+	-	+	+	+	++
Pseudoalteromonas sp. An33	LN881360	99.4% *Pseudolalteromonas espeijana* NCIMB 2127	-	+	+	-	++	+	++	++
Shewanella sp. An4	LN881152	97.6% *Shewanella japonica* KMM 3299	-	+	-	-	-	-	+	-
Shewanella sp. An36	LN881379	99.0% *Shewanella dovemarinesis* MAR441	-	+	+	-	-	-	+	-

[1] Published in Martin et al., 2015; ++ activity detected within 24 hours; + activity detected after more than 24 hours; - no activity observed

Additional bioinformatic analyses

Conserved domains and family motifs were sought in the Conserved Domain Database (NCBI), the Pfam database (Finn *et al.*, 2014), and the CAZyme database using the CAZyme Analysis Toolkit (CAT) (Park *et al.*, 2010; Lombard *et al.*, 2014). Operons were predicted with the Softberry FGENESB software (Solovyev and Salamov, 2011). Protein sequences were aligned with MAFFT (Katoh *et al.*, 2002). The sequence alignement with the iota-carrageenases were manually edited with Bioedit (© Tom Hall), based on the protein sequence of CgiA_Af from *Alteromonas fortis.* Sequence alignment figures were arranged with EsPript3 (http://espript.ibcp.fr, Robert and Gouet, 2014). The maximum likelihood method with bootstrap values was used to construct phylogenetic trees with MEGA6 (Tamura *et al.*, 2013).

3. RESULTS

To identify novel hydrolases from marine bacteria, two plurigenomic libraries were constructed and screened for enzymatic activities. Pooled genomic DNA from the five *Fv* isolates and the five *Gm* isolates (Table VI-1) was restricted and inserted into the *pHT01* cloning vector. The DNA extraction protocol of Cheng and Jiang (2006) enabled us to extract 3 to 8 µg genomic DNA from 3 ml of each individual bacterial culture. The genomic DNA of the five *Fv* isolates was restricted with *DpnII* (since the restriction with *Sau3AI* was unsuccessful) and that of the five *Gm* isolates was restricted with *Sau3AI*. The average insert size was estimated at 6.5 kb for the *Fv* library and 9 kb for the *Gm* library. Screening of the libraries for agarase, iota- and kappa-carrageenase, esterase/lipase, xylanase, endocellulase, and beta-glucosidase activity yielded five contigs containing functional genes from three of the ten bacterial isolates (An8, An33, and An27) (Table VI-2, Table VI-S1).

Flavobacteriia library

One Fv Iota-carrageenase candidate

In the *Fv* library only one activity (iota-carrageenase) was observed, two months after inoculation of the screening medium. The corresponding clone was Fvi2 (Table VI-S1). Six ORFs were identified on the Fvi2 contig (Table VI-2). Only the protein encoded by *Fvi2_5*, showing sequence identity to a hypothetical protein, could be responsible for the observed activity, as the other ORFs display sequence identity to characterized proteins that are not iota-carrageenases. Furthermore, the second-best hit in GenBank for the sequence of the protein encoded by *Fvi2_5* is an iota-carrageenase of a *Cellulophaga sp.* (90% identity) (Table VI-S2). The subclone with the *Fvi2_5* gene was able to hydrolyze iota-carrageenans (Table VI-2, Table VI-S1). The iota-carrageenase Fvi2.5 belongs to the GH82 family.

Table VI-2 Gene name, accession number, ORF size, sequence identities of the diverse coding sequences identified on the constructed contigs from the active clones**.** ORFs that were subcloned are indicated in bold. ORFs indicated in red were found by subcloning to be responsible for the observed activity

Clone name Activity Size	**Gene name**	**Accession number**	**ORF size** (aa)	**Identity percentage with best hit against GenBank (Source organisms), accession number**
Fvi2 Iota-carrageenase 6.2kb	*Fvi2_1*	LN913026	159*	87% 2OG-Fe(II) oxygenase (*Maribacter forsetii*) WP_036154505.1
	Fvi2_2	LN913027	261	74% Short-chain dehydrogenase (*Maribacter forsetii*) WP_034666723.1
	Fvi2_3	LN913028	197	80% NAD(P)H oxidoreductase (*Zobellia uliginosa*) WP_038232878.1
	Fvi2_4	LN913029	626	91% Potassium transporter (*Cellulophaga geojensis*) EWH14754
	Fvi2_5	LN913030	491	97% Hypothetical protein (*Cellulophaga lytica*) WP_013622437.1
	Fvi2_6	LN913031	108	63% Competence protein TfoX (*Zobellia galactanivorans*)WP_013995580.1
GmXyl Xylanase 12.4kb	*GmXyl_1*	LN913032	451*	99% Ton B-dependent receptor (*Pseudoalteromonas haloplanktis*) WP_024599008.1
	GmXyl_2	LN913033	247	99% Multidrug transporter (*Pseudoalteromonas haloplanktis*) WP_024599009.1
	GmXyl_3	LN913034	336	97% Cupin (*Pseudoalteromonas haloplanktis*) WP_024599009.1
	GmXyl_4	LN913035	494	98% Tryptophan halogenase (*Pseudalteromonas citrea*) WP_033028792.1
	GmXyl_5	LN913036	236	97% Hypothetical protein (*Pseudoalteromonas haloplanktis*) WP_024599012.1
	GmXyl_6	LN913037	273	98% Transcriptional regulator (*Pseudoalteromonas* sp. BSi20429) WP_007585178.1
	GmXyl_7	LN913038	426	99% endo-1,4-beta-xylanase (*Pseudoalteromonas arctica*) CBY88881.1
	GmXyl_8	LN913039	324	96% Gluconolactonase (*Pseudoalteromonas haloplanktis*) WP_024599014.1
	GmXyl_9	LN913040	377	99% endo-1,4-beta-xylanase (*Pseudoalteromonas haloplanktis*) WP_024599015.1
	GmXyl_10	LN913041	528*	98% endo-1,4-beta-xylanase (*Pseudoalteromonas* sp. Bsw20308) WP_007375178.1
Gmi1 Iota-carrageenase 13.5kb	*Gmi1_1*	LN913042	1236	99% TonB-dependent receptor (*Pseudoalteromonas sp. BSW* 20308) WP_007375140.1
	Gmi1_2	LN913043	595	91% Hypothetical protein *(Pseudoalteromonas sp.* BSW 20308) WP_007375139.1
	Gmi1_3	LN913044	545	100% Hypothetical protein (*Pseudoalteromonas sp.* P1-11) WP_055255568.1
	Gmi1_4	LN913045	333	90% Glycoside Hydrolase (*Pseudalteromonas sp.* S3431) ; WP_033028792.1
	Gmi1_5	LN913046	642	94% Hypothetical protein (*Pseudoalteromonas haloplanktis*) ; WP_024594190.1
	Gmi1_6	LN913047	793*	98% Multispecies TonB-dependant receptor (*Pseudoalteromonas).* ; WP_007378531.1
GmBg Beta-glucosidase 10.4kb	*GmBg_1*	LN913056	838	86% Beta-glucosidase (*Pseudoalteromonas sp.* ECSMB14103) WP_039037212.1
	GmBg_2	LN913057	571	73% Glycoside hydrolase family 9 *(Pseudoalteromonas marina*) WP_024599009.1
	GmBg_3	LN913058	354	70% Hypothetical protein *(Pseudoalteromonas haloplanktis*) WP_024594157.1
	GmBg_4	LN913059	456	93% MATE family efflux transporter (*Pseudoalteromonas haloplanktis)* WP_024603968.
	GmBg_5	LN913060	208	90% Riboflavin synthase subunit α (*Pseudoalteromonas haloplanktis*) WP_024599030.1
	GmBg_6	LN913061	287	88% Hypothetical protein (*Pseudoalteromonas agarivorans*) WP_004586762.1
	GmBg_7	LN913062	205*	93% Multispecies hypothetical protein (*Pseudoalteromonas*) WP_024603971.1
GmEst Carboxy-esterase 9.3 kb	*GmEst_1*	LN913063	126*	95% Prepilin cleavage protein (*Pseudoalteromonas Atlantica*) ABG40490.1
	GmEst_2	LN913064	395	94% Serine/thréonine protein kinase *(Paraglaciecola mesophila*) GAC24755.1
	GmEst_3	LN913065	241	43% 2OG-Fe(II) oxygenase (*Brevundimonas sp*. KM4) KJV41957.1
	GmEst_4	LN913066	754	99% Peroxidase *(Pseudoalteromonas sp. PLSV*) WP_033185651.1
	GmEst_5	LN913067	261	99% hydroxubutyrate dehydrogenase (*Paraglaciecola mesophila*) WP_006992904.1
	GmEst_6	LN913068	368	98% Polyhydroxybutyrate depolymerase (*Pseudoalteromonas sp.*) WP_033185653.1
	GmEst_7	LN913069	399	99% Homoserine o-acetyltransferase (*Paraglaciecola mesophila*) GAC24751
	GmEst_8	LN913070	161*	100% Multispecies H+ gluconate transporter (*alteromonodales*) WP_006992901

* incomplete ORF

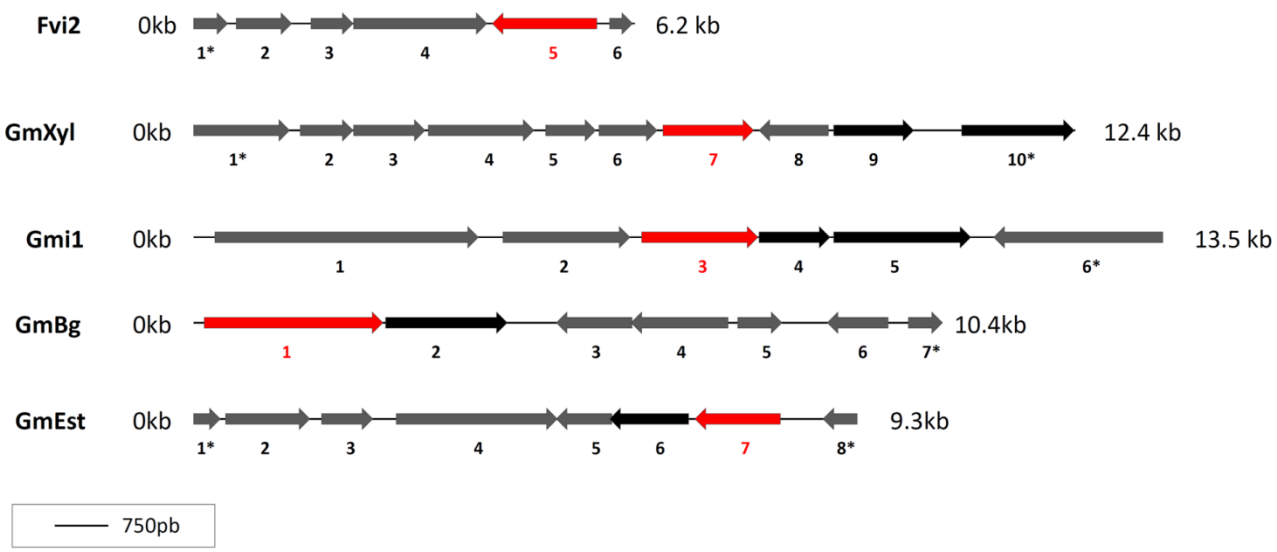

Figure VI-1 Plan of the five contigs identified in the two plurigenomic libraries.
* : incomplete ORF; →: ORF found by subcloning to be responsible for the observed activity; → : subcloned ORFs that did not confer the observed activity.

Gammaproteobacteria library

Nineteen Gm xylanase candidates

Nineteen clones were found to degrade xylan (Table VI-S1). Their DNA inserts showed similar sequences. With the DNA insert sequences of the 19 clones, we were able to reconstitute a 12.4-kb GmXyl contig containing 10 ORFs, originating from the *Pseudoalteromonas sp*. An33 (Table VI-2, Figure VI-1). Two complete ORFs (GmXyl_7 and GmXyl_9) coding for proteins close to known endo-β1,4-xylanases were identified on the 19 clone inserts. The last gene locus (*GmXyl_10*), closely related to other xylanases, was found only on some plasmids from positive clones and was always incomplete. We were able to retrieve the complete *GmXyl_10* gene from the genomic DNA of the *Pseudoalteromonas* sp. An33 by PCR amplification (a reverse primer was designed on the basis of a sequence alignment with the ten closest proteins). Each putative xylanase-encoding gene (*GmXyl_7*, *GmXyl_9*, *GmXyl_10*) was subcloned separately (Table VI-S1). Only the subclone containing the *GmXyl_7* gene could hydrolyze xylan. The GmXyl7 protein was found to be a GH8-family xylanase. GmXyl9 and GmXyl10 belong to the GH10 family, composed essentially of endoxylanases.

Ten Gm Iota-carrageenase candidates

Ten clones of the *Gm* library were able to hydrolyze iota-carrageenans. Their DNA insert sequences were found to contain identical ORFs and were used to construct a 13.5-kb contig (Table VI-2, Table VI-S1, Figure VI-1). This contig, called Gmi1, also originates from the An33 *Pseudoalteromonas sp*. isolate. No gene on this contig was found to be closely related to a known iota-carrageenase, but three ORFs (*Gmi1_2, Gmi1_3*, *Gmi1_5*) appeared to code for proteins closely related to uncharacterized hypothetical proteins and one (*Gmi1_4*) for a protein closely related to an uncharacterized GH16-family protein (Table VI-2). As *Gmi1_2* was not found on the DNA inserts of all ten positive clones, we discounted it as potentially responsible for the iota-carrageenase activity. The three other ORFs were subcloned, and the subclone containing the *Gmi1_3* gene was the only one found to hydrolyze iota-carrageenans. This iota-carrageenase (Gmi1.3) was assigned to the only known GH family containing iota-carrageenases: GH82.

One Gm beta-glucosidase candidate

The GmBg contig was identified on the basis of beta-glucosidase activity. The protein encoded by its first gene showed low sequence identity to beta-glucosidases (Table VI-2). As the protein encoded by its second gene showed low sequence identity to endo-1,4- beta-glucanases (endocellulases), the corresponding clone was also tested on AZCL-cellulose, but no activity was observed under our screening conditions. The beta-glucosidase activity of *GmBg_1* was confirmed by subcloning (Table VI-2, VI-S1). The protein GmBg1 was classified in the GH3 CAZyme family.

One Gm lipolytic candidate

Lastly, one clone was found to hydrolyze tributyrin. The sequence of its DNA insert revealed 8 ORFs with no sequence identity to any known lipolytic enzyme (Table VI-2). Nevertheless, the sequences of the proteins encoded by *GmEst_6* and *GmEst_7* were found to contain an α/β-hydrolase domain (found in lipolytic enzymes). Subcloning of these two ORFs showed that only *GmEst_7* was responsible for the esterase activity (Table VI-S1). The subclones were also tested for lipase activity on minimal medium containing olive oil and trioctanoate, but proved unable to degrade these substrates. The GmEst contig was found, by PCR amplification, to originate from the *Paraglaciecola sp.* isolate An27.

4. DISCUSSION

Functional screening of plurigenomic libraries: probing the "great screen anomaly"

Functional metagenomic screening has emerged as the trendy approach to discovering novel enzymes. Yet its yield is generally poor, and this has led to intense discussion of its challenges. The studied environment as well as the host cells, expression systems, DNA extraction methods, DNA insert sizes, and screening methods used have all been pinpointed as bias-creating factors (Uchiyama and Miyazaki, 2009; Liebl *et al.*, 2014; Ferrer *et al.*, 2016). Here we have constructed plurigenomic libraries, which can be viewed as "small-scale" metagenomic libraries. Knowing which microorganisms contributed their genomic DNA to our libraries and which enzymatic activities they displayed, we can get a closer look at the so-called "great screen anomaly" (Ekkers *et al.*, 2012). Five *Flavobacteriia* isolates were used to construct one library, and five *Gammaproteobacteria* isolates to construct the other. *E. coli* cells transformed with these libraries were screened for hydrolytic enzyme activities and the inserts of positive clones were analyzed. This has enabled us to attribute functions to five genes, three of which (*Fvi2_5, Gmi1_3, GmEst_7*) were not previously known to confer the observed activity. Yet only five activities were recovered, out of the 48 observed prior to screening (Table VI-1) for these 10 isolates: the iota-carrageenase activity of An8, the iota-carrageenase, xylanase, and beta-glucosidase activities of An33, and the esterase activity of An27. This screening yield seems rather low, especially since the bacterial isolates were preselected as displaying the activities for which we screened. Nevertheless, the yield is definitely higher than those generally obtained in less restricted studies using functional metagenomics (Uchiyama and Miyazaki, 2009; Ferrer *et al.*, 2016). It is noteworthy that the yields of the two screens were not equal: only one active clone (1 pos/97 Mb screened) was detected in the *Fv* library, under our screening conditions, versus 31 (1 pos/3.6 Mb screened) in the *Gammaproteobacteria* library. A first obvious explanation could be the host chosen for cloning and screening the genomic DNA. *E. coli*, the host used here, is a gammaproteobacterium. It is therefore probably best equipped genetically (in terms of promoter recognition, transcription, translation, and post-translational modifications such as protein folding and secretion) to express genes of other *Gammaproteobacteria* (Liebl et al., 2014). This hypothesis is supported by our previous functional metagenomic study of the microbiota associated with *A. nodosum*, where the esterase and glycoside hydrolase genes identified were mostly from *Alpha*- and *Gammaproteobacteria* (Martin, Biver, *et al.*, 2014). Furthermore, even within each library screened here, the genes of different bacterial genera do not seem to have been equally expressed. In the *Gm* library, for instance, no genes from

the two *Shewanella* isolates were identified on the basis of expression in *E. coli*, whilst of the four contigs retrieved from this library, three were from the single *Pseudoalteromonas* isolate. In the *Fv* library, a contig was retrieved only from the *Cellulophaga* isolate An8, even though the other four *Flavobacteriia* used were active against most of the tested substrates. Assuming that the level of heterologous expression increases when the donor of the foreign DNA is closely related to the expression host, it might be possible to solve these expression problems by using a marine host. *Pseudomonas antartica*, for example, appears to be an excellent psychrophilic expression host; it has few interfering natural enzymatic activities and is easily transformable by electroporation (see for review Liebl et al., 2014). *Rhodobacter capsulatus* is another promising host for producing functional membrane-bound enzymes from heterologous genes (Liebl *et al.*, 2014). Yet these bacteria are both *Proteobacteria*, and we have failed to find any *Bacteroidetes* member (liable to better express flavobacterial genomes) that has already been used for library constructions.

Another explanation for our different screening yields might be the different average DNA insert sizes of our two plurigenomic libraries (*Fv*: 6.5 kb, *Gm*: 9 kb). Even though the number of megabases screened was the same for both libraries, the presence of smaller inserts reduces the probability of having an entire gene or operon, complete with upstream promoter and downstream terminator, expressed (Ekkers *et al.*, 2012). Functional screening of metagenomic libraries, constructed in plasmids from similar environmental samples, has been found to have a better yield (expressed in 1 positive/Mb screened) when the insert size is greater (for a review see Uchiyama and Miyazaki, 2009).

A last issue worth mentioning is the choice of the restriction enzyme used to generate the library inserts. In a previous study focusing on the microbiota associated with *A. nodosum*, we found the extracted DNA to be much more easily restricted with *DpnII* than with *Sau3AI* (Martin, Biver, *et al.*, 2014). We therefore constructed and screened a metagenomic library containing only *DpnII* restriction fragments as inserts. Here, however, we see that *DpnII* fails to restrict the genomic DNA from some marine bacteria. This suggests that in our previous study, some genes were probably not inserted into the DNA library and thus not screened. To our knowledge, this particular source of bias has never been mentioned in relation to the poor yields of functional metagenomics.

Cultivable macroalgal-polysaccharide-degrading bacteria are specialized in the hydrolyzation of sugars

Marine macroalgae contain various sulfated and non-sulfated polysaccharides. According to their cell-wall composition and phylogeny, they are divided into three phyla: red, brown, and green seaweeds. Brown algae mainly contain alginates (uronic acids) , fucans (sulfated

polysaccharides), β1,3 - β1,4 mixed linkage glucans, cellulose, and xylan or arabinoxylan (Popper *et al.*, 2011; Deniaud-Bouët *et al.*, 2014). In terrestrial environments, specific cellulolytic and hemicellulolytic bacteria are known to be specialized in the hyrolyzation and mineralization of plant polymers (DeAngelis *et al.*, 2010; Gibson *et al.*, 2011; Leung *et al.*, 2015). Similar observations have been made on seaweed-associated microbiotas, where certain bacterial groups have emerged as being specialized in the use of algal polysaccharides (Michel and Czjzek, 2013; Martin, Portetelle, *et al.*, 2014). Here we have found our macroalgal-polysaccharide-degrading isolates to hydrolyze other sugars as well: alongside their ability to degrade algal polysaccharides, they exhibit xylanase, endocellulase, and beta-glucosidase activities. Furthermore, on the five sequenced genome contigs from such bacteria we have identified nine genes (*Fvi2_5, GmXyl_7, GmXyl_9, GmXyl_10, Gmi1_3, Gmi1_5, GmBg_1, GmBg_2*) coding for proteins having sequence identity to known GH enzymes (Table VI-2), and we have proven by subcloning that four of these genes (*Fvi2_5*, *GmXyl_7*, *Gmi1_3* and *GmBg_1*) confer the predicted activity in the presence of a relevant substrate (Table VI-S1). By using the genomes of only ten preselected cultivable polysaccharolytic isolates, we have discovered several novel GH genes, whereas glycosidases represent less than 15% of the enzymes identified by functional screening of metagenomic libraries (Ferrer *et al.*, 2016). The genomes of cultivable macroalgal-polysaccharide-degrading bacteria thus appear particularly rich in genes involved in sugar hydrolyzation, and preselecting polysaccharolytic bacteria obviously increases the identification of novel GH genes.

Identification of genes encoding original functional enzymes in cultivable alga-associated isolates

The discovery of novel enzyme and protein families from marine organisms is interesting from the standpoint of both basic science and biotechnology. Some enzymes from marine microbes show unusually high stability or display diverse genetic and biochemical characteristics that distinguish them from their counterparts in terrestrial organisms (Zhang and Kim, 2010). Here we have identified from marine isolates five novel functional-enzyme-encoding genes, three of which (*Fvi2_5, Gmi1_3,* and *GmEst_7*) were not previously known to confer the observed activity and were not assigned to that function in protein databases.

(i) The ***GmXyl_7*** gene encodes a putative GH8 xylanase, GmXyl7, having high sequence identity (98%) to an enzyme that has been characterized and crystallized: the endo-1,4-beta xylanase pXyl (Q8RJN8) of *Pseudoalteromonas haloplanktis* (Collins *et al.*, 2002; Van Petegem *et al.*, 2002, 2003) (Table VI-S2). Interestingly, the latter enzyme appears most

active towards xylan from the red alga *Palmaria palmata*. This xylan is a linear β1,3-β1,4 mixed-linkage seaweed xylan (Collins *et al.*, 2002). This suggests that the main source of xylan in the natural environment of *pXyl* could be of algal origin, as it is for *GmXyl7.* Only a few xylanases have been classified in the GH8 family so far. The potential of GH8 xylanases as technological aids in baking has been clearly demonstrated, particularly with the cold-active pXyl from *P. haloplantkis* (Collins *et al.*, 2006). Furthermore, functional analyses have shown that these enzymes have narrow substrate specificity and low affinity for smaller xylan units. This is an advantage in industrial applications, as the enzymes will not hydrolyze the released degradation products (Pollet *et al.*, 2010). The GmXyl contig bears two other genes (*GmXyl_9 and GmXyl_10*) closely related to known xylanases. Several hypotheses might be proposed to explain why *GmXyl_9* and *GmXyl_10* do not confer any endoxylanase activity when subcloned. It could be that our screening conditions (temperature, pH, substrate...) were not appropriate for observing the activity of the encoded proteins. Alternatively, the genes *GmXyl_7, GmXyl_9, and GmXyl_10* might work in an operon regulated by a promoter in front of *GmXyl_7*. We found no such operon, however, in operon databases such as ProOpDB (Taboada *et al.*, 2012) and OperonDB (Pertea *et al.*, 2009), and no operon was predicted in the Softberry FGENESB software (Solovyev and Salamov, 2011). Moreover, all attempts to produce the GmXyl9 protein in *E. coli* under the control of an IPTG-inducible promoter (expression vector pET30b), and under various conditions in a bioreactor, proved unsuccessful (data not shown). On the other hand, *GmXyl_9* and *GmXyl_10* might be pseudogenes. Pseudogenes are sequences sharing homology with active genes but having lost their ability to function as transcriptional units. They are found in high number in bacterial genomes but are still difficult to predict (Lerat, 2005; Rouchka and Cha, 2009).

(ii) The putative beta-glucosidase encoded by ***GmBg_1*** should also show interesting properties, as the few characterized beta-glucosidases isolated from marine bacteria have been found to be alkali-stable and cold-active (Chen *et al.*, 2010; Mao *et al.*, 2010).

(iii) Interestingly, the sequence of the potential esterase encoded by the ***GmEst_7*** gene is practically identical to proteins annotated as homoserine o-acetyltransferases (HAT) (Table VI-S2), but contains an esterase/lipase domain and hydrolyzes tributyrin. Closely related HAT-annotated proteins may thus be wrongly annotated. Another similar protein, *Cg*Hle of *Corynebacterium glutamicum*, also referred as a HAT in protein databases, likewise contains an esterase/lipase domain and displays esterase activity (Tölzer *et al.*, 2009). HATs are required in methionine biosynthesis (Bourhy *et al.*, 1997), but *Cg*Hle was not found to play a role in the main methionine pathway or in any alternative one, and thus appears to have

been (wrongly) assigned as a HAT on the sole basis of its structure (Rückert *et al.*, 2003; Tölzer *et al.*, 2009). Lastly, by aligning the amino acid sequences of the *Cg*Hle and GmEst7 proteins, we were able to retrieve the GxSxG amino acid motif typically found in lipolytic enzymes (Arpigny and Jaeger, 1999) (Figure VI-2), but were unable to assign these two esterases to any known esterase family. Hausmann and Jaeger (2010) note that many esterases in protein databases remain unassigned to already described esterase families. The proteins GmEst7 and *Cg*Hle could thus be members of a novel family of HAT-like carboxy-esterases.

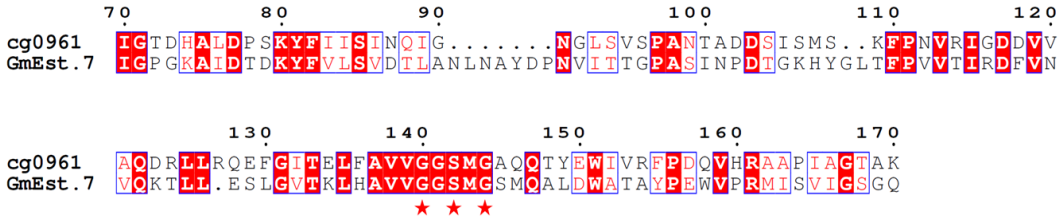

Figure VI-2 Multiple sequence alignment of partial amino acid sequences containing the conserved blocks of the HAT-like carboxy-esterases encoded by *GmEst_7* and cg0961. The three stars indicate the GxSxG lipase active site motif.

(iv) Lastly, we have identified two functional iota-carrageenase genes (*Fvi2_5* and *Gmi1_3*). Both of the encoded proteins belong to the GH82 family. Iotase activity was described for the first time in 1984 (Greer and Yaphe, 1984), but the iota-carrageenase enzymes and family (GH82) were not defined until 2000 (Barbeyron *et al.*, 2000). Since then, only 19 proteins have been assigned to this family (CAZy Database, Lombard et al., 2014). Iota-carrageenases have been divided by Michel & Czjzek (2013) into three clades, according to their phylogeny. In the constructed phylogenetic tree, the iota-carrageenase Fvi2.5 appears to belong to clade A and Gmi2.3 to clade C (Figure VI-3).

Clade A contains the only iota-carrageenase whose crystal structure has been solved: CgiA_Af of *Alteromonas fortis* (Michel, Chantalat, Fanchon, *et al.*, 2001). This enzyme folds into a right-handed β-helix flanked by two additional domains (domains A and B). Domain A has been found to be highly conserved in clade A iota-carrageenases and to be responsible for their processive character (Michel *et al.*, 2003). This domain is indicated in the protein sequence of Fvi2.5, by sequence alignment with other characterized iota-carrageenases of this clade (CgiA_Af (CGIA_ALTFO), CgiA1_Zg, CgiA_C.QY3) (Figure VI-4). Domain A is absent from the two other clades (containing only non-processive enzymes), and domain B is found in some iota-carrageenases of these clades (Rebuffet *et al.*, 2010).

The enzyme Gmi1.3 is related (30% sequence identity) to the characterized clade C iota-carrageenase CgiA_Mt of *Microbulbifer thermotolerans* (Hatada *et al.*, 2011) (Table VI-S2).

Only two enzymes (CgiA_Mt and Patl-0879 of *Pseudoalteromonas atlantica*) belong to this clade so far. Adding this novel iota-carrageenase will reinforce the coherence of this group (Figure VI-3). Gmi1.3 is very distant from the clade-A sequences (only 18% sequence identity to CgiA_Af). This is notably due to the absence of Domain A in clade C sequences. In contrast, Gmi1.3 features several large insertions as compared to CgiA_Af (mainly between the strands β13 and β14, β25 and β26, and β27 and β28, Figure VI-4). The absence of domain A suggests that Gmi1.3 is not a processive enzyme, but the large inserts in this new iota-carrageenase may influence its mode of action.

In Fvi2.5, the essential residues of the catalytic site, E245, D247, Q222, and H281 (Rebuffet *et al.*, 2010), are strictly conserved (Figure VI-4). Residue E310 of domain A, involved in stabilizing the intermediate substrate-bound conformation, is also recovered in the sequence of Fvi2.5 (Michel *et al.*, 2003; Michel and Czjzek, 2013). Gmi1.3 also features most of the essential residues of the CgiA_Af catalytic machinery (E245, Q222 and H281), but the base catalyst D247 is replaced by a glycine. This substitution is also observed in the characterized iota-carrageenase CgiA_Mt. Thus, the identity of the base catalyst in clade C enzymes remains an open question.

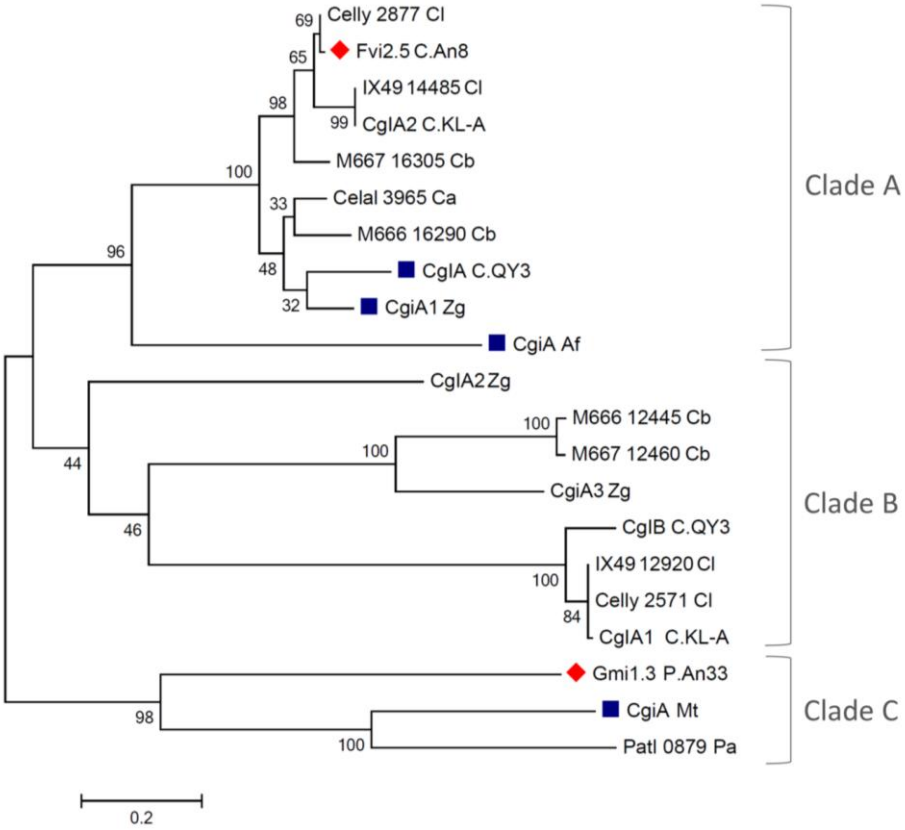

Figure VI-3 Phylogenetic tree with the 19 proteins of the GH82 iota-carrageenase family and the two novel iota-carrageenases Fvi2.5 and Gmi1.3 (indicated with red diamonds). The characterized proteins of the GH82 family are indicated with blue squares.

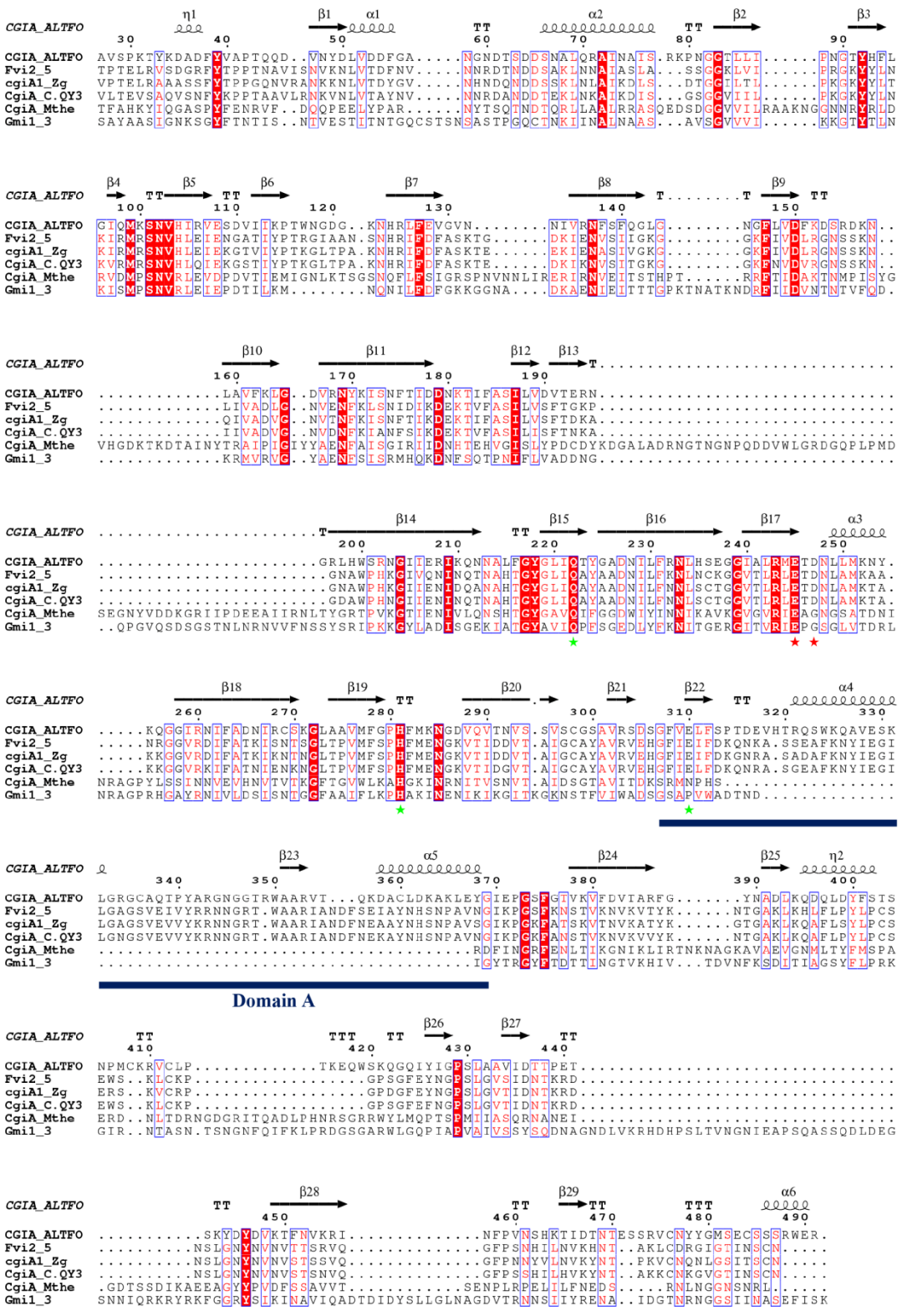

Figure VI-4 Multiple sequence alignments with the characterized iota-carrageenases of the GH82 family and Fvi2.5 and Gmi1.3 The secondary structural elements of the crystallized iota-carrageenase of *Alteromonas fortis* are found above the sequences. Domain A is underlined in blue. The proton donor and the base catalyst (DE245 and D247) are indicated with red stars. The other residues important for catalysis in CgiA_Af (Q222 and H281; E310 from domain A) are indicated with green stars.

5. CONCLUSION

The plurigenomic libraries screened in the present study were constructed with the genomic DNA of bacteria preselected for the presence of specific enzymatic activities. Yet only five activities out of the 48 identified in these natural polysaccharolytic isolates were recovered by functional screening. Expression in a heterologous host, DNA insert size, and/or the restriction enzyme used may at least partly explain this low yield. These limitations are obviously magnified in functional metagenomic analysis, as there is no preselection of specific bacterial isolates that act on the screening substrates. This explains why even lower yields are obtained by this approach. These results highlight, once again, the difficulty of identifying novel enzyme genes by functional analysis. Nevertheless, we also demonstrate that cultivable bacteria should not be left out, as with only ten bacteria we have discovered two novel iota-carraageenase genes (acquiring knowledge about this poorly known enzyme family) and a putative novel HAT-like esterase family. The originality of the cultivable isolates used (low identity of their 16S rRNA genes to those of known species), of the environment from which they were isolated (few functional analyses have focused on alga-associated microbiotas), and of the method used here (construction of plurigenomic libraries from preselected original bacteria) contribute to the novelty of our discoveries.

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Chapter VII. General discussion, Conclusions and Future prospects

1. SUMMARY OF THE RESULTS

The aim of this work was to investigate the bacterial microbiota associated with the surface of the brown alga *Ascophyllum nodosum*. We wanted to examine its hydrolytic potential and particularly its ability to degrade algal cell-wall polysaccharides.

Two approaches were employed.

- We first used functional metagenomics to investigate the whole bacterial population (composed mainly of not-yet-cultivable bacteria) associated with *A. nodosum* (Chapter IV).
- We then looked specifically at the small fraction of cultivable bacteria associated with *A. nodosum* (Chapter V and VI).

The results of these two approaches are summarized below.

Analyzing the whole bacterial population associated with *A. nodosum*

Algal thalli were collected in winter (February 2012) and in summer (July 2012) from the foreshore in Roscoff. Total DNA was extracted from the bacterial population present at the surface of each set of thalli and used to construct a "summer" and a "winter" metagenomic library. Both libraries were functionally screened in *Escherichia coli* for hydrolytic activities.

Who's in there?

Screening of the **winter library** resulted in 13 positive clones, whose DNA inserts were sequenced. The identified gene loci were annotated. To most DNA fragments it was possible to assign, consistently, a phylum (based on the bacterial origin of the closest homologues of the gene considered). This provided information on the bacterial taxa associated with *Ascophyllum nodosum* during the winter (Chapter IV):

- Most of the identified loci were assigned to the phylum *Proteobacteria* and largely to the class *Alphaproteobacteria* (mainly of the order *Rhodobacterales*), a few being assigned to the classes *Beta-* and *Gammaproteobacteria*.
- No gene originating from a member of the phylum *Bacteroidetes* or *Planctomyces* (both known to be relatively abundant on brown algae) was identified.

What are they doing?

Established screening tests were used to screen the **winter library** for common hydrolytic activities (esterase, xylanase, beta-glucosidase, cellulase, protease, arabinanase, amylase) and for MAPD enzymes (agarase, iota- and kappa-carrageenase). We thus identified 13 loci encoding esterases, an incomplete (but active) beta-glucosidase gene, and an endocellulase gene. The product of this last gene was purified and biochemically characterized (Chapter IV). Here are the most interesting results of this chapter:

- Approximately 180 Mb were screened, with a high average hit rate: 1 discovered gene per 12 Mb screened;
- Most of the predicted protein sequences corresponding to the 15 identified genes had low sequence identity to known esterases, beta-glucosidases, or endocellulases present in protein databases;
- The 13 esterases identified in this winter library were assigned to four different esterase families (some of which had hardly ever been identified before in the marine environment);
- Characterization of the discovered cellulase revealed interesting biotechnological properties: activity at low temperature, high halotolerance, stability over a wide pH range, and ability to hydrolyze mixed glucans;
- No MAPD enzymes were identified.

The originality of our findings left no doubt as to the wealth and diversity of this winter library or regarding the utility of investigating *Ascophyllum nodosum*-associated bacteria by functional metagenomics.

These encouraging results prompted us to construct the **summer library** and to screen it for the same activities and in the same manner as described above.
This screen led to identifying 5 esterase genes and a beta-glucosidase gene (unpublished results). No MAPD enzymes were identified. Approximately 150 Mb of DNA was screened. The average hit rate was lower (1 gene per 25 Mb screened) than for the winter library, and the findings were less original. For the culture-based approach, we thus decided to continue working with winter alga samples.

Analyzing the cultivable surface microbiota associated with *A. nodosum*

Cultivable bacteria are known to represent only a tiny part of the total microflora in an environmental sample, but a potentially interesting one. As a complement to functional metagenomics, we thus investigated the cultivable surface microbiota associated with *A. nodosum.*

From winter *A. nodosum* triplicates (March 2014), we isolated 324 bacteria (108/sample). A taxonomic affiliation was assigned to 297 of these isolates, which were tested for MAPD activities (Chapter V). Some of the polysaccharolytic isolates where used to construct plurigenomic libraries, which were then screened for common hydrolytic enzymes and MAPD enzymes (Chapter VI).

Who's in there?

The 297 isolates were assigned to three phyla. In order of abundance in the cultivable surface microbiotas associated with the three *A. nodosum* samples, these phyla were:

- *Proteobacteria* (56.2%, including class *Gamma*: 44.1%, class *Alpha*: 11.4%, and class *Beta*: 0.7%);
- *Bacteroidetes* (42.8%, represented only by the class *Flavobacteriia*);
- *Firmicutes* (1%, isolated from only one sample).

At lower taxonomic rank, 36 genera were isolated from the *Ascophyllum nodosum* triplicates. PCoA performed on the basis of the relative abundances of the genera identified on the three samples led to the following findings (Figure V-1):

- Dissimilar bacterial compositions for the three samples, as regards both the genera identified and their abundances;
- Some genera (and classes) were isolated from just one sample and others were common to just two samples;
- 12 genera were consistently found on all three algal samples :
    - Six *Flavobacteriia* genera, all of the *Flavobacteriaceae* family : *Algibacter*, *Cellulophaga*, *Dokdonia*, *Formosa*, *Maribacter,* and *Zobellia;*
    - Five *Gammaproteobacteria* genera, of different orders and families: *Colwellia*, *Glaciecola*, *Pseudoalteromonas, Shewanella,* and *Marinomonas;*
    - One *Alphaproteobacteria* genus, of the family *Rhodobacteraceae*: *Sulfitobacter*.

What are they doing?

Chapter V presents the results obtained when the 324 isolates were tested for MAPD activity against agar, iota-carrageenase, kappa-carrageenase, or alginate. The most relevant results are listed below (Table V-1, Figure V-4):

- Less than a fourth (78/324) of the isolates composing the cultivable microbiota associated with *A. nodosum* were found to hydrolyze at least one of the tested algal polysaccharides. Thus, MAPD bacteria remain a minority within the cultivable microbiota.
- These polysaccharolytic bacteria were found to belong to two bacterial classes, eight families, and eleven genera:
    - Class *Flavobacteriia* (41), all belonging to the *Flavobacteriaceae* family : *Cellulophaga*, *Maribacter*, *Algibacter,* and *Zobellia* ;
    - Class *Gammaproteobacteria* (37), belonging to different families: *Pseudoalteromonas*, *Vibrio*, *Cobetia*, *Shewanella*, *Colwellia*, *Marinomonas,* and *Paraglaceciola*.
- Nine of these 11 genera were predominant in the common core of the three *A. nodosum* samples (Figure V-1).
- 63 of the 78 isolates were able to degrade alginate. This is not surprising, given the nature of the host: alginate is the main cell wall component of brown algae (Chapter I).
- The other polysaccharides, which are found only in red algae, were degraded in similar proportion (52 iota-carrageenase, 41 agarase, and 39 kappa-carrageenase activities were observed).
- The MAPD *Gammaproteobacteria* isolates appeared more specific to brown algae (most of them exclusively degraded alginate).
- The MAPD *Flavobacteriia* isolates emerged as more generalistic degraders (all of them degrade red-alga polysaccharides and most of them degrade alginate).
- Several MAPD isolates most probably represent novel species (Figure V-5).
- Some MAPD bacteria were assigned to genera not previously known to hydrolyze algal polysaccharides (Table V-2).

Chapter VI focuses on the genomic potential of 10 of these active and phylogenetically original isolates: five *Flavobacteriia* isolates (*Cellulophaga sp*. An8, An9 and An20, *Maribacter sp.* An21, *Zobellia sp*. An14) and five *Gammaproteobacteria* isolates (*Colwellia* sp. An23, *Paraglaciecola* sp. An27, *Pseudoalteromonas* sp. An33, *Shewanella* sp. An4 and An36). These isolates were first tested for a variety of additional hydrolytic activities (esterase, protease, endocellulase, beta-glucosidase, alpha-amylase, xylanase,

arabinanase) (Table VI-1) and then pooled by class in order to construct a Flavobacteriia (Fv) library and a Gammaproteobacteria (Gm) library. Both plurigenomic libraries were then functionally screened for MAPD enzymes and common hydrolytic enzymes. The main results of this sixth chapter are as follows:

- 48 hydrolytic activities were observed for these 10 natural isolates (Table VI-1).
- It proved necessary to use two different restriction enzymes: *DpnII* for the gDNA of the *Flavobacteriia* isolates and *Sau3AI* for that of the *Gammaproteobacteria* isolates).
- Five of the 48 activities were retrieved by functional screening of the plurigenomic libraries constructed with these strains :
  - Four functional genes were identified in the *Gm* library:
    - GmXyl7, encoding a xylanase of *Pseudoalteromonas* sp. An33;
    - GmBg1, encoding a beta-glucosidase of *Pseudoalteromonas* sp. An33;
    - Gmi1.3, encoding an iota-carrageenase of *Pseudoalteromonas* sp. An33;
    - GmEst7, encoding an esterase of *Paraglaciecola* sp. An27;
  - One functional gene was identified in the *Fv* library:
    - Fvi2.5, encoding an iota-carrageenase of *Cellulophaga* sp. An8.
- The encoded esterase probably represents a novel family of HAT-like esterases.
- Gmi1.3 displayed very low sequence identity (<30%) to known iota-carrageenases.

2. ABOUT THE ASSOCIATED BACTERIAL TAXA

At the phylum and class levels

As mentioned in the review in Chapter II, the most represented bacterial phyla on brown algae (Table II-1) are *Proteobacteria (Alpha, Gamma and Beta)*, *Bacteroidetes*, and *Planctomycetes*, and to a lesser extent, *Cyanobacteria* and *Firmicutes*. Bacteria belonging to these phyla should also be associated with *A. nodosum*, as algae of the same group (brown, red, or green) tend to show similar bacterial diversity at phylum level.

- ***Proteobacteria*** is the most abundant phylum in marine environments (particularly the class *Alphaproteobacteria*). Not surprisingly, therefore, both our culture-dependent and our culture-independent approach revealed this phylum as dominant on *A. nodosum*. At the class level, however, the two approaches yielded different results.
  - In the DNA inserts of the positive clones obtained by functional metagenomics, *Alphaproteobacteria* genes were dominant, and *Gamma-* and *Betaproteobacteria* genes were scarce (Chapter IV). The low abundance of *Gammaproteobacteria* genes was surprising, as this class has often been identified on other brown algae

(representing 10-20% of the total identified classes) and as the heterologous expression of *Gammaproteobacteria* genes in *E. coli* should not be that problematic (as suggested by the results of the functional screen of the plurigenomic Gm library (Chapter VI)). Yet the work described in Chapter VI also revealed that *Gammaproteobacteria* genes are poorly restricted by *DpnII* (the restriction enzyme used to construct the metagenomic library - Chapter IV). This might have biased the DNA insert population of the metagenomic library against this class.

- On the other hand, more than half of the isolated cultivable bacteria belonged to the class *Gammaproteobacteria*, representatives of the class *Alphaproteobacteria* being much less abundant. Only one *Betaproteobacteria* representative was identified, on one algal sample (Chapter V). *Gammaproteobacteria* are well known to contain lots of carbohydrate metabolism genes (Edwards et al., 2010) and are thus expected to grow readily on culture media. Marine alphaproteobacteria, in contrast, seem harder to isolate by traditional culturing. In recent studies applying innovative culturing approaches to marine samples, the authors identified many more *Alphaproteobacteria* members (Jung et al., 2014; Yang et al., 2016).

For sure, the phylum *Proteobacteria* is present in high profusion in the epiphytic microflora of *A. nodosum*, but it is hard to compare the abundances of *Alphaproteobacteria* and *Gammaproteobacteria* members. *Betaproteobacteria* are probably scarce, as observed on other brown algae (Chapter II).

- ***Bacteroidetes*** bacteria are well known to live preferentially attach to surfaces, rather than in a plantktonic state. They are able to consume polymers, contain lots of carbohydrate metabolism genes, and are easily grown on marine culture media (Edwards et al., 2010; Fernández-Gómez et al., 2013). The high representation of this phylum among the cultivable bacteria thus came as no surprise (Chapter V). The class *Flavobacteriia* is often the most abundantly represented *Bacteroidetes* group in marine environments (Alonso et al., 2007; Buchan et al., 2014). All the *Bacteroidetes* members isolated in this work belong to this class and to the family *Flavobacteriaceae*. *Bacteroidetes* members are present for sure on *A. nodosum*, but their abundance is difficult to estimate, as no genes corresponding to this phylum were identified by functional metagenomics (Chapter IV). We think that expression problems linked to the choice of the cloning host (*E. coli*) may partially explain this result. The poor yield of the functional screen of the *Fv* plurigenomic library strongly supports this hypothesis (Chapter VI).

- The absence of ***Planctomyces*** and ***Cyanobacteria*** members with both approaches may be due to different factors:
  - The sampling season (winter): other authors have found these phyla to be less abundant in winter brown algal samples than in summer or autumnal samples (Bengtsson et al., 2010; Lachnit et al., 2011). They are sometimes even absent from winter samples (particularly *Cyanobacteria*).
  - Bad heterologous expression: these phyla are not closely related to *E.coli*, and expression problems in this cloning and screening host might explain why we didn't retrieve genes originating from these phyla by functional metagenomics.
  - Unsuitable culture media : *Cyanobacteria* have not been identified previously on algae by culturing approaches (Hollants et al., 2013), and *Planctomyces* members require appropriate complex culture media (Ward et al., 2006).

- In agreement with previous studies on brown alga symbionts, the phylum ***Firmicutes*** appeared poorly represented. Representatives of this phylum are probably less abundant on brown algae, at the chosen sampling period, or on the studied algal species. One should keep in mind, however, that DNA from Gram-positive bacteria might be difficult to extract because of their cell wall thickness and not readily expressed in *E. coli*. This might have biased the results of the functional metagenomic approach against them.

At lower taxonomic rank

Previous studies have shown the bacterial genera and species associated with algae to vary between algal species and even between individual algal specimens (Chapter II). Nevertheless, small core communities (~15%) characteristic of the host species have also been described (Bengtsson et al., 2012; Burke et al., 2011). In our functional metagenomic study, the percentages of identity between the gene loci identified and entries in genomic databases were, on the average, too low (<40%) to allow assigning a genus or species, but by culturing we obtained results in agreement with previous studies: we observed **differences between the three *A. nodosum* samples** (as regards genera and their abundance) and a major **common core** (a third of the identified genera) was also found. Most of the isolated genera had already been identified on algae, and all the genera of the common core (except *Colwellia*) had previously been identified on brown algae. On *Ascophyllum nodosum* samples collected more than 40 years ago, Cundell *et al*. (1977) identified a *Leucothrix* species and Chand and McManus (1969) isolated mainly *Vibrio*, *Flavobacter*, and *Pseudomonas* species. In this work, all these genera except *Flavobacter* were identified in the cultivable surface microbiota of *A. nodosum*, but none of them belonged to the common core of the three samples. The genus *Leucothrix* was the fifth most abundant genus in the isolated bacterial population,

Vibrio was the fifteenth, and *Pseudomonas* species were isolated from only one sample (Figure V-1, V-4A).

Fifteen of the isolated genera might be considered characteristic of algal associations, as they have been found consistently on brown, red, and green seaweeds (Chan and McManus, 1969; Cundell et al., 1977; review of Hollants et al., 2013) and in this work as well (Figure V-1):

- Identified in the common core: *Cellulophaga*, *Pseudoalteromonas*, *Shewanella*, *Sulfitobacter*, and *Zobellia*;
- Identified on two samples: *Loktanella* (S1, S2), *Octadecabacter*, *Vibrio* (S2,S3), *Leucothrix*, *Psychrobacter*, (S2, S3);
- Identified on one sample: *Ruegeria* (S1), *Cobetia* (S2), *Bacillus*, *Pseudomonas*, *and Roseobacter* (S3).

Conclusion, prospects for improvement and further study

Using functional analysis of the underexplored bacterial population associated with *Ascophyllum nodosum*, we have gained an idea of the bacterial taxa that are present on this alga. Our two approaches (functional metagenomics, Chapter IV and culturing, Chapter V) have yielded complementary data regarding the taxa present at the surface of this alga. Our functional screens of plurigenomic libraries (Chapter VI) have further highlighted problems that may arise when one attempts to express foreign DNA in *E. coli*. This work has shown, on the one hand, that several bacterial phyla and genera well known to associate with (brown) algae are present on *Ascophyllum nodosum* as well. On the other hand, it has also revealed the presence of bacterial genera not previously known to associate with brown algae. It has highlighted both inter-individual differences in cultivable bacterial community composition and the existence of a common core.

Our screens have failed to detect some bacterial taxa that are considered characteristic of algal surfaces. This may be due in part to inappropriate culturing methods or to use of an inadequate expression host or DNA extraction technique.

Investigating the bacterial taxa associated with *A. nodosum* through **functional metagenomics** might be improved:

- by constructing large-insert libraries in fosmids, cosmids, or BACs, so as to obtain more information on the phylogenetic affiliation of the DNA inserts;
- by constructing libraries in broad-host-range vectors for further screening in multiple bacterial hosts (marine hosts such as *Pseudomonas antarctica* and *Rhodobacter capsulatus* have emerged as excellent expression hosts (see the review by Liebl et al., 2014)). This could make it possible to identify bacterial taxa whose genes are not readily expressed in *E. coli*;

- by restricting the eDNA with diverse restriction enzymes: this should avoid biases caused by poor restriction of genomic DNA from certain bacterial taxa, resulting in non-inclusion of corresponding inserts in the library (see Chapter VI).

To isolate a wider range of alga-associated bacteria by **culturing**, one could:

- increase the number of samples: the greater the number of samples, the more information we will get about the bacteria associated with the studied alga, notably as regards their abundance, their systematic association with the alga (their presence in the common core), or their occasional presence on the alga;
- use very low-nutrient media and longer incubation times, an approach known as extinction culturing: this method has been shown to enhance the isolation of marine bacteria other than those isolated on rich media (Choi et al., 2015; Connon and Giovannoni, 2002; Jensen et al., 1996; Yang et al., 2016). The authors using these methods explain that low-nutrient media mimic the real nutritional conditions of the natural environment, thus avoiding "nutrient shocks" (upon transfer from the nutrient-poor natural environment to a rich laboratory medium) and allowing the growth of oligotrophic bacteria;
- use a modern *in situ* culturing technique allowing interactions with other microorganisms and with chemical components from the natural environment. Two such methods have been tested on sponge-associated bacteria: the use of diffusion growth chambers (Steinert et al., 2014) and the I-tip technique (*in situ* cultivation in tips) (Jung et al., 2014). This has led to the successful isolation of previously uncultivable marine bacteria.

As the main objective of the present work was to investigate the hydrolytic potential of the bacterial population associated with *A. nodosum*, we have chosen to use the functional metagenomics and culturing approaches. It would be possible, however, to use **sequence-based metagenomics** to investigate the total bacterial population associated with *A. nodosum*, independently of its functional potential. For instance, the best way to visualize the bacterial population of an environmental sample is to amplify and sequence a taxonomic genetic marker (generally a region of the 16S rRNA genes) in the extracted eDNA and then to search for sequence homologies with known bacterial taxa. Yet one should bear in mind that this method has limitations (e.g. mutations in the 16S rRNA gene or horizontal gene transfer might lead to misrepresentations) (Das et al., 2014). Furthermore, there is still lots of controversy regarding the sequence similarity percentage threshold for assigning a genus or a species to a sequence (Kim et al., 2014).

3. ABOUT THE ENZYMATIC ACTIVITIES

Common hydrolytic enzymes

As compared to other metagenomic studies on the marine environment, our functional metagenomic approach has yielded very interesting results in terms of the hit rate achieved, the diversity of the findings, the novelty of the enzymes identified, and the biotechnological characteristics of the purified endocellulase.

By culturing, we isolated 78 MAPD bacteria. We next chose ten of these isolates (representative of the 78 isolates) to construct **plurigenomic libraries**, which were then screened. Together these 10 isolates displayed 48 hydrolytic activities, and functional screening of the two constructed libraries led to identifying five functional genes, some of which are original and only distantly related to known genes.

Two types of hydrolytic enzymes have emerged from our functional metagenomics study (Chapter IV) and through functional screening of bacterial isolates and plurigenomic libraries (Chapter VI):

- **Esterases.** Esterases have been isolated from a wide variety of environments. They constitute a huge enzyme class, which emphasizes their biological importance. In the bacterial population associated with *A. nodosum*, we have here identified several carboxylesterase activities and genes, using tributyrin as substrate (Chapters IV and VI). All of the identified esterase genes have very low sequence identity to known ones, and some of the corresponding families have almost never been identified before in the marine environment. The abundance and diversity of such enzymes in this microbiota might be due to the various physiological functions that carboxylesterases exert in bacteria (reviewed by Hausmann and Jaeger, 2010). Here are some esterase functions that could be relevant to microbial epibionts associated with algae:

    - Releasing fatty acids for use as carbon sources;
    - Regulating important interspecies communications and interactions, by processing quorum-sensing signal molecules;
    - Releasing fatty acids for use in antibiotic synthesis;
    - Enabling bacteria to defend themselves against antimicrobials produced by other bacteria in the biofilm;
    - Contributing to the virulence of pathogenic bacteria by degrading plant cell walls. A similar "function" could be imagined for the esterases of MAPD bacteria;
    - Playing a role in cell motility and biofilm formation.

- **Glycoside hydrolases.** These enzymes are five times less frequently identified than esterases by functional metagenomics, and even less in the marine environment (Ferrer et al., 2016). In Chapters IV, V, and VI, we describe the identification of several and diverse glycoside hydrolase activities and genes. The most obvious and main function of glycoside hydrolases of alga-associated bacteria is converting organic matter to available carbon sources for the other bacteria on the alga and for the algae and planktonic bacteria in the surrounding seawater. Below, we examine the common GH enzymes identified in this work in relation to the composition of the algal cell wall.

  - Cellulose is only a minor component of the the cell wall of brown algae. This might suggest that cellulase-encoding genes are scarce in the genome of the bacteria associated with the brown alga *A. nodosum*. Yet in this work we have identified by functional metagenomics an endocellulase and a beta-glucosidase gene, although previous functional metagenomics studies have failed to reveal any cellulase genes in a marine environment. Moreover, cellulase activities were detected for seven of the ten MAPD bacteria used to construct the plurigenomic libraries (Table VI-1). In contrast, these bacteria displayed fewer beta-glucosidase activities (2/10). There is thus no correlation between the fraction of cellulose in the algal cell wall and the potential of its associated bacteria to exert cellulase activity.

  - The several xylanase activities identified in this work (Table VI-1) might enable brown-alga-associated MAPD bacteria to partially hydrolyze heterofucans. As stated in Chapter I, brown algal cell walls are composed mainly of sulfated homo- or heterofucans and alginate. Heterofucans can consist of low-sulfate polymers with significant proportions of xylose, galactose, and mannose (Mabeau et al., 1990).

We have suggested a range of potential biological and physiological functions that the esterases and glycoside hydrolases identified here might exert in the alga-associated microbial biofilm, but what about their **biotechnological potential**?

Partial characterization of the endocellulase identified here revealed interesting biotechnological properties, detailed in Chapter IV, including particularly strong halotolerance and interesting activity at low temperature.

Although the proteins encoded by the other functional genes identified in this work have not yet been purified and characterized, we might expect equally interesting properties. On the one hand, all the recovered functional genes originate from halotolerant (or halophilic), cold-adapted natural strains. On the other hand, numerous reviews present diverse characterized marine esterases, xylanases, cellulases, and beta-glucosidases showing exceptional activity at high pressure, low temperature, high salt concentration, or over a broad pH range (see for review Lee *et al.*, 2010; Kennedy *et al.*, 2011; de Lourdes Moreno *et al.*, 2013; Dalmaso *et al.*, 2015).

Most of these characterized interesting enzymes originate from extremophilic genera. Here, from the surface microbiota of *A. nodosum*, we have also isolated bacteria of the following genera (Figure V-1), for example:

- Stenopsychrophiles (growing at temperatures between 10 and 20°C) : *Colwellia, Glaciecola, Halomonas, Marinobacter, Pseudoalteromonas, Pseudomonas, Psychrobacter, Shewanella*;
- pH extremophiles: *Bacillus, Photobacterium, Pseudomonas, Shewanella*;
- Piezophiles: *Colwellia, Photobacterium, Psychromonas, Shewanella.*

These potentially (poly)extremophilic bacteria associated with *A. nodosum* most probably synthesize a huge range of robust enzymes with exceptional biotechnological potential.

Enzymes acting on algal polysaccharides

Glycoside hydrolases acting on algal polysaccharides tend to be structurally far different from common hydrolytic enzymes. Most of them represent separate GH families, and novel families are continuously being discovered (Michel and Czjzek, 2013).

We couldn't investigate the microbiota associated with an alga without taking an interest in MAPD enzymes, as alga-associated bacteria constitute the most obvious source of these particular, underexplored enzymes.

By **functional metagenomics,** however, we failed to identify any agarase or iota- or kappa-carrageenase[1] activity under the conditions used, although several common hydrolytic enzymes were identified. This result raises several questions:

- Are MAPD bacteria scarce in the bacterial population associated with *A. nodosum*?
- Are MAPD-enzyme-encoding genes scarce in the genomes of such bacteria?
- Can the screening host readily express MAPD-enzyme-encoding genes?
- Are the screening tests sensitive enough?
- Do the genomes of brown-alga-associated bacteria contain genes encoding enzymes that degrade polysaccharides of red algae?

In contrast, 78 **cultivable bacteria** isolated from the three *A. nodosum* samples proved active against agar, iota-carrageenan, kappa-carrageenan, and/or alginate (Chapter V). The identified polysaccharolytic bacteria belong to 11 genera and two classes: the *Gammaproteobacteria* and the *Flavobacteriia.* No *Alphaproteobacteria* (likely abundant in the bacterial population), *Betaproteobacteria*, or *Firmicutes* isolates were able to degrade the tested polysaccharides under the conditions used.

By subsequent functional screening of the **plurigenomic libraries** constructed with 10 of these 78 isolates (five representatives of each class), we identified two MAPD (iota-carrageenase) activities. The two corresponding genes code for original and novel members of the GH82 family, in which only 19 proteins have been included since its initial description (Barbeyron et al., 2000)).

It seems certain, therefore, that further exploration of the entire genomes of the 78 polysaccharolytic isolates will lead to identifying other novel MAPD-enzyme-encoding genes.

[1] The jellified alginate medium is hard to pour; this is why we couldn't perform high-throughput screening of DNA libraries for this activity.

These interesting results described in Chapters V and VI provide partial answers to the questions raised above.

Are MAPD bacteria scarce in the bacterial population associated with A. nodosum?

MAPD bacteria do appear to constitute a minority within the bacterial population associated with *A. nodosum* (and probably of those associated with algae in general).
Within the cultivable fraction of this population (known to be quite small), MAPD isolates already appear to be a minor component (<25% of the total isolated population), and previous molecular studies have revealed that the most abundantly represented alga-associated genera are ones not known to act on algal polysaccharides (Barott et al., 2011; Burke et al., 2011; Lachnit et al., 2011; Miranda et al., 2013; Wu et al., 2014). It is noteworthy that the present work has revealed MAPD isolates belonging to genera not previously known to act on algal polysaccharides (Table V-2). Improved culturing should allow isolating other bacterial genera and taxa not previously known to act on algal polysaccharides. It should give a better idea of the proportion of MAPD bacteria in alga-associated microbiota.

Are MAPD-enzyme-encoding genes scarce in the genomes of such bacteria?

Probably not, but it is too early to answer this question.
As shown in Chapter V, several isolates can degrade more than one algal polysaccharide. This suggests that diverse MAPD-enzyme-encoding genes are present in MAPD bacterial genomes. Furthermore, in the genome of the extensively studied polysaccharide-degrading model bacterium *Zobellia galactanivorans*, genes encoding two beta-agarases, two iota-carrageenases, one kappa-carrageenase, two beta-porphyranases, three alginate lyases, and one laminarinase have already been discovered (Table II-2). This suggests that this bacterium is highly specialized in the hydrolyzation of diverse algal polysaccharides.
Is this bacterium exceptional, or are other MAPD bacteria also full of MAPD-enzyme-encoding genes? Further genome sequencing and annotation of the 78 polysaccharolytic isolates described in Chapter V will certainly contribute to answering this question.

How readily does the screening host express MAPD-enzyme-encoding genes?

So-so, it would seem. The results in Chapter VI show that *E. coli* was able to express one MAPD-enzyme-encoding gene from a *Gammaproteobacteria* isolate and one from a *Flavobacteriia* isolate, but given the hydrolytic potential of the MAPD bacteria used, this is not much.

Furthermore, even though the screening yield of the *Gm* library was better than that of the *Fv* library, it was still poor. This means that heterologous expression of other *Gammaproteobacteria* genes in *E. coli* is still difficult, despite the close relatedness of the organism from which the foreign DNA was derived and the screening host. One would expect genes belonging to other bacterial classes or phyla to be expressed even less readily. Yet *E. coli* has proved able, in this work and in previous studies, to express some MAPD-enzyme-encoding genes from distantly related bacteria. The use of (marine) host cells should be tested with a view to improving heterologous expression, as discussed above.

Are the screening tests sensitive enough?

Yes/no. For sure, jelly-degrading activities are not always easily detected. With the natural MAPD isolates, the holes in the jellified medium were very impressive, and activities were easily detected. In contrast, the iota-carrageenase activities expressed in *E. coli* were less considerable (Chapter VI).

One should know however, that for the functional screening of the winter metagenomic library, the *E. coli* recombinant clones were pooled and densely plated on each medium. The colonies thus remained relatively small on the screening medium, making it hard to detect such activity. In subsequent functional screens, we tried to improve the method, by first isolating individual *E. coli* clones in 96-well plates and then inoculating each plate of screening medium with only 96 clones. The plating density was thus lower and the colonies bigger, which enhanced the sensitivity of the test. Nevertheless, picking into 96-well plates is fastidious and time consuming. The use of a picking robot would considerably facilitate this step.

Do the genomes of brown-alga-associated bacteria contain genes coding for enzymes that degrade polysaccharides from red algae?

Yes! Under laboratory conditions, we identified numerous agarase, iota-carrageenase, and kappa-carrageenase activities in the 78 polysaccharolytic isolates. Some bacteria associated with *A. nodosum* are, for sure, able to degrade red-alga polysaccharides, although these activities are probably not expressed in the natural environment of the isolated strains (i.e. on a brown alga). This could be confirmed (or not) by transcriptomic or proteomic approaches.

Conclusion, prospects for improvement and further study

Using a combination of culture-dependent and -independent approaches, we have demonstrated that the microbiota associated with *Ascophyllum nodosum* is a rich and diverse source of hydrolytic bacteria producing original enzymes.
The identified activities give us an idea of the physiological and biological functions these alga-associated bacteria are likely to exert. We have also identified genes coding for enzymes capable of degrading polysaccharides that are not present in the cell wall of the host (e.g. agarase- and carrageenase-encoding genes).

Alga-associated bacteria are thus worthy of future investigation in order to learn more about their particular ecological roles and functions in the seaweed holobiont. Furthermore, the enzymes produced by these robust marine bacteria might be interesting for practical purposes, as they seem to exhibit very particular and searched-for bioindustrial characteristics.

We list below some improvements that might enhance the search for hydrolytic enzymes in alga-associated microbiotas by **functional screening of (meta)genomic libraries**:

- Constructing long-DNA-insert libraries in cosmids, fosmids, or BACs.
  Longer DNA inserts:
  - will provide information about the genes downstream and upstream from the identified functional genes and hence about their biological and physiological functions;
  - will increase the chances of obtaining a complete operon. This might be essential to the expression of some screened-for activities (as in the case of the two alginolytic operons of *Zobellia galactanivorans* (Thomas et al., 2012)).
- Using a different screening host might allow expression of functional genes not readily expressed in *E. coli*;
- Using a picking robot will increase the number of recombinant *E. coli* clones screened (particularly for functional metagenomics) and hence, the hit rate.

To identify more hydrolytic activities through **culturing,** one can culture more bacteria from more samples and use a variety of culture methods as discussed above (see point 2 of this chapter: About the associated bacterial taxa).

We have chosen to work with DNA and with cultivable bacteria as a first step in investigating the hydrolytic potential of the bacteria associated with *A. nodosum.* The results obtained open the door to further investigation of this particular microbiota.
Here are some suggested short- and long-term prospects:

- **Genome sequencing and annotation of the 78 MAPD isolates.** This will allow the identification of MAPD-encoding genes and will provide information about the abundance of these genes in the genomes of MAPD bacteria. It will also allow better understanding of the biological functions of these bacteria;
- **Construction of plurigenomic libraries with the other 68 MAPD bacteria and screening of these libraries**. This could lead to identifying other MAPD or original hydrolytic enzymes that might be only distantly related to known ones and thus unlikely to be detected during genome annotation (i.e. likely to be annotated as "hypothetical proteins");
- **Developing screening tests for other MAPD activities** such as laminarinase, porphyranase, fucoidanase, and ulvan lyase activities or other enzymes or bio-active compounds of interest;
- **(Meta)transcriptomics and (meta)proteomics** applied to this algal microbiota should provide information regarding which genes are really expressed on this alga at the time of sampling or under particular laboratory conditions. These approaches will be complementary to the metagenomic and culturing approaches used in this work, which have provided a first estimate of the potential of the studied alga-associated bacteria to produce hydrolytic enzymes.

4. A FEW FINAL WORDS

Alga-associated bacteria have been studied since the end of the 19th century. It is now clear that they are highly diverse and interact closely with the host alga, in ways that can be beneficial or detrimental to one partner or the other.

In the present work we have identified bacterial taxa that typically associate with the brown alga *Ascophyllum nodosum* and that seem to entertain a privileged relation with seaweeds.
We find that several of these typically alga-associated bacteria have developed a range of hydrolytic activities whose assumed biological and physiological functions should enable them to gain a competitive advantage in colonizing algal surfaces.
We have isolated numerous macroalgal-polysaccharide-degrading bacteria which seem able to associate with different algal species, as a look at their genomes reveals the potential to degrade polysaccharides of both red and brown algae. Yet the genera to which these bacteria belong have seldom been mentioned in molecular studies investigating whole alga-associated bacterial populations. This means that polysaccharolytic bacteria, although characteristic of algae and essential to the seaweed holobiont, might not be the most abundant ones.

At this point, we can only speculate about the abundance and ecological functions of such hydrolytic bacteria in the seaweed holobiont. The difficulty of studying macroalgae and bacteria as a whole maintains a shroud of mystery around their interactions. Only further investigations using different techniques and disciplines will lead to a better understanding of the particular relationships between these two ecologically relevant partners.

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Supplementary material

Supplementary material of Chapter IV, related to the publication "Identification and characterization of a halotolerant, cold-active marine endo-β-1,4-glucanase by using functional metagenomics of seaweed-associated microbiota", Applied and Environmental Microbiology, 2014, 80 (16): 4958-67, is available at:
http://aem.asm.org/content/80/16/4958/suppl/DCSupplemental

Supplementary material of Chapter V, related to the publication " The cultivable surface microbiota of the brown alga *Ascophyllum nodosum*" is enriched in macroalgal-polysaccharide-degrading bacteria, Frontiers in Microbiology, 2015, 6, 1487, is available at:
http://journal.frontiersin.org/article/10.3389/fmicb.2015.01487/abstract

Supplementary material of Chapter VI, related to the publication "Discovering novel enzymes by functional screening of plurigenomic libraries from alga-associated *Flavobacteriia* and *Gammaproteobacteria*", Microbiological Research, 2016, 186: 52-61, is available at:
http://www.sciencedirect.com/science/article/pii/S0944501316300519