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

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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.

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 (Pace, 1997; 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

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dynamic interactions with their hosts (Holmström et al., 2002; Martin et al., 2014b). 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 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* (Barbeyron et al., 1989; Ivanova et al., 2004; Johansen et al., 1999). 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

2.1. 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. Lipolytic 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 Mattéotti 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.

2.2. 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 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.

2.3. 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 Bactotryptone (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 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.

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

[a] Published in Martin et al., 2015; ++ activity detected within 24 h; + activity detected after more than 24 h; – no activity observed.

2.4. 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™. 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 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.

2.5. 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) (Lombard et al., 2014; Park et al., 2010). 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 1) was restricted and inserted into the *pHT01* cloning vector. The DNA extraction protocol of Cheng and Jiang (2006) enabled us to extract 3–8 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 2, Table S1).

3.1. Flavobacteriia *library*

3.1.1. *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 S1). Six ORFs were identified on the Fvi2 contig (Table 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 S2). The subclone with the *Fvi2_5* gene was able to hydrolyze iota-carrageenans (Table 2, Table S1). The iota-carrageenase Fvi2.5 belongs to the GH82 family.

3.2. Gammaproteobacteria *library*

3.2.1. *Nineteen* Gm *xylanase candidates*

Nineteen clones were found to degrade xylan (Table 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 2, Fig. 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

Table 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	Accessionnumber	ORFsize(aa)	Identity percentage with best hit against GenBank (Source organisms), accession number
Fvi2				
Iota-carrageenase 6.2kb	*Fvi2_1*	LN913026	159[a]	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[a]	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% Crp/Fnr 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[a]	98% endo-1,4-beta-xylanase (*Pseudoalteromonas* sp. Bsw20308) WP_007375178.1
Gmi1				
Iota-carrageenase13.5kb	*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[a]	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[a]	93% Multispecies hypothetical protein (*Pseudoalteromonas*) WP_024603971.1
GmEst				
Carboxy-esterase 9.3 kb	*GmEst_1*	LN913063	126[a]	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% D-beta-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[a]	100% Multispecies H+ gluconate transporter (*alteromonodales*) WP_006992901

[a] Incomplete ORF.

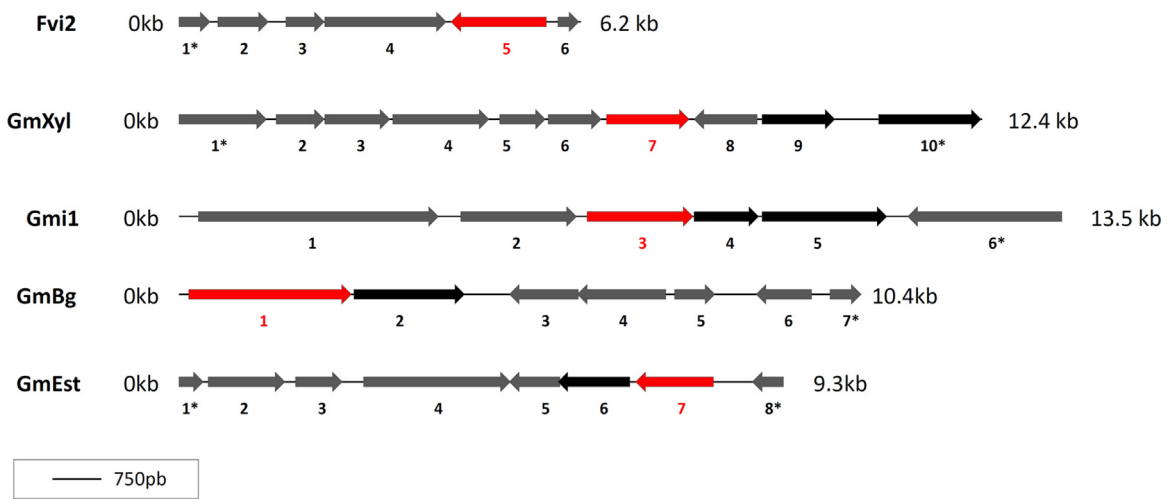

Fig. 1. Plan of the five contigs identified in the two plurigenomic libraries. *: incomplete ORF; →: ORF found by subcloning to be responsible for the observed activity; →: subcloned ORFs that did not confer the observed activity.

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 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.

3.2.2. *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 2, Table S1, Fig. 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 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.

3.2.3. *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 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 2, Table S1). The protein GmBg1 was classified in the GH3CAZyme family.

3.2.4. *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 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 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

4.1. *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 (Ferrer et al., 2016; Liebl et al., 2014; Uchiyama and Miyazaki, 2009). 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 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 (Ferrer et al., 2016; Uchiyama and Miyazaki, 2009). 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 et al., 2014a). 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 et al., 2014a). 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.

4.2. 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 (Deniaud-Bouët et al., 2014; Popper et al., 2011). 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 (Martin et al., 2014b; Michel and Czjzek, 2013). 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 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 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.

4.3. 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., 2003, 2002) (Table 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 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) (Fig. 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.

(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

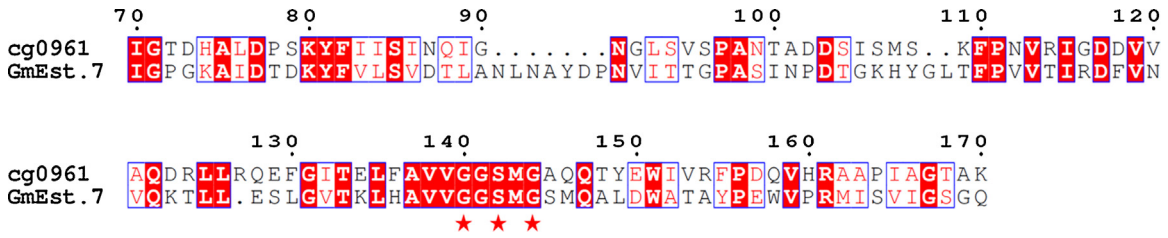

Fig. 2. Multiple sequence alignment of partial amino acid sequences containing the conserved blocks of the HAT-like carboxy-esterases encoded by *GmEst_7* and cg0961. The three stars indicate the GxSxG lipase active site motif.

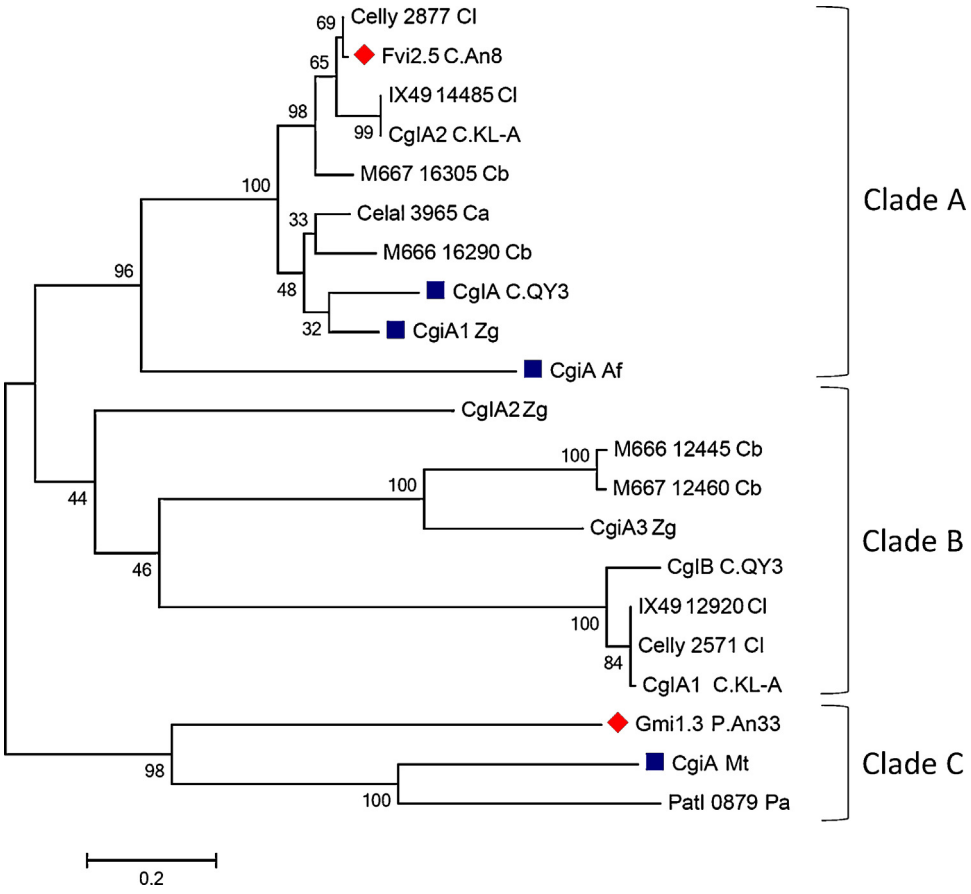

Fig. 3. Phylogenetic tree with the 19 proteins of the GH82 iota-carrageenase family and the two novel iota-carrageenases Fvi2.5 and Gmi1.3 (indicated with red diamonds). The characterized proteins of the GH82 family are indicated with blue squares. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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 and 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 (Fig. 3).

Clade A contains the only iota-carrageenase whose crystal structure has been solved: CgiA_Af of *Alteromonas fortis* (Michel 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) (Fig. 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 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 (Fig. 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, Fig. 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.

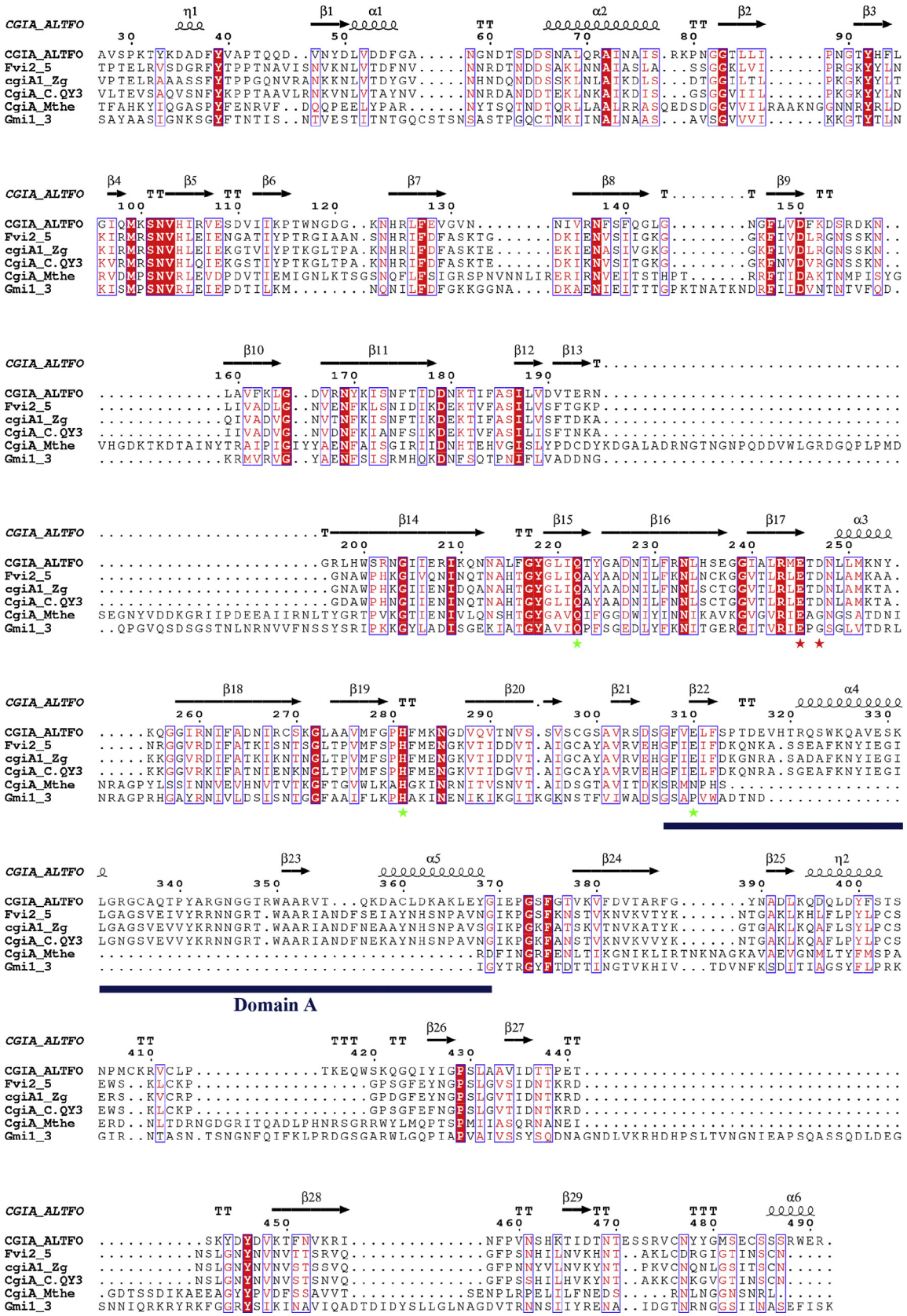

Fig. 4. Multiple sequence alignments with the characterized iota-carrageenases of the GH82 family and Fvi2.5 and Gmi1.3 The secondary structural elements of the crystallized iota-carrageenase of *Alteromonas fortis* are found above the sequences. Domain A is underlined in blue. The proton donor and the 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In Fvi2.5, the essential residues of the catalytic site, E245, D247, Q222, and H281 (Rebuffet et al., 2010), are strictly conserved (Fig. 4). Residue E310 of domain A, involved in stabilizing the intermediate substrate-bound conformation, is also recovered in the sequence of Fvi2.5 (Michel and Czjzek, 2013; Michel et al., 2003). 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.

Increasingly interest is taken on iota-carrageenans, and their derived oligosaccharides, as immo-stimulators in plant, human or animal health (Bhattacharyya et al., 2010; Vera et al., 2011), as prebiotics (O'sullivan et al., 2010) or as thickeners and gelling agents for the food and medical industry (Bixler and Porse, 2010; De Ruiter and Rudolph, 1997). In order to purify this polysaccharide and its derivatives, specific iota-carrageenases are still needed and searched for.

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) contributes to the novelty of our discoveries.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.micres.2016.03.005.

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