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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

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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.

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 (1, 2) and explains why metagenomics, a culture-independent approach using total microbial genomes from environmental samples, has met with great success over the past decade (3, 4). Sequence-based metagenomics has already provided information about the composition, organization, function, and hierarchy of microbial communities in particular habitats (5). 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 (6–9) and gut (10–13) samples.

Marine microorganisms represent promising 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 (14). New enzymes have already been identified in marine metagenomic libraries from seawater samples (15, 16) and from microorganisms in symbiosis with marine organisms such as sponges and corals (17). 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 (18). 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 (19–21). 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 (22, 23). Only a few such enzymes have been found 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* (24), *Paenibacillus* sp. strain BME14 (25), *Martelella mediterranea* (26), and *Marinobacter* sp. strain MS1032 (27). 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 being exploited in sustainable applications and bioprocesses (28). Although several cellulases are already in use, there is an increasing demand for cellulases with better or special industrial properties (29). 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 yielded loci 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 newly identified cellulase. To our knowledge, no functional screening of a metagenomic library from alga-associated microbial biofilms has been realized before, and no cellulase has previously been identified by functional metagenomics in a marine environment.

MATERIALS AND METHODS

Environmental sample collection. Several plants (~100 g) 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.

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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 (30). 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% cetyltrimethylammonium bromide [CTAB]) and incubated with 50 μl proteinase K (10 mg/ml) at 37°C. After 1 h, 750 μl of 20% SDS was added, and the cotton tips were incubated for 2 h at 65°C, with manual inversion every 20 min. After centrifugation, 1 volume of chloroform-isoamyl alcohol (24:1 [vol/vol]) was added to the supernatant, and the mixture was centrifuged. The aqueous phase was recovered, and DNA was precipitated with 0.6 volume isopropanol for 1 h at room temperature. DNA was recovered by centrifugation (30 min at 10,000 × *g*) at 4°C and 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 fluorometer (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 England Biolabs). A polyethylene glycol (PEG)–NaCl size-selective precipitation was done as described by Biver and Vandenbol (6) in order to remove small DNA inserts. An equal volume of 6.5% PEG–0.4 M NaCl was added to the restriction fragments (final DNA concentration, 20 ng/μl), and the mixture was incubated for 2 h at room temperature. 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). Electrocompetent Electromax *Escherichia 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% tributyrin 2× yeast-tryptone (YT) agar plates containing 100 μg/ml ampicillin. Beta-glucosidases were sought on 2× YT agar containing 0.5% esculin (Sigma-Aldrich) and 0.1% ammonium iron (III) citrate (Sigma-Aldrich) (12). Cellulolytic activity was detected by plating the library on 2× YT agar plates containing 1 g/liter insoluble synthetic AZCL-HE-cellulose (azurine-cross-linked hydroxypropyl cellulose; Megazyme) and 100 μg/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 (31). All putative proteins corresponding to the identified open reading frames (ORFs) in the inserts were analyzed with BLASTP against the GenBank nonredundant database and Swissprot (curated database). The putative esterases were also analyzed with Pfam (32).

Overexpression and purification of the CellMM5.1 cellulase. The coding sequence of the *Cell5.1_3* locus was amplified, without its putative peptide signal, with Platinum *Pfx* DNA polymerase, 0.5× PCRx enhancer solution (Invitrogen), 5′-gggaattcCATATGCAAACCTCTGACAGCTATGTTCCGC-3′ as the forward primer, and 5′-cgcGGATCCTCATCA**GTGGTGGTGGTGGTGGTG**GCGCCCAAGCAAGGCGT-3′ as the reverse primer (the NdeI and BamHI restriction sites used for subcloning are underlined, and the histidine tag sequence is 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 2× YT medium, 30 μg/ml chloramphenicol and 30 μg/ml kanamycin) onto solid 2× YT medium supplemented with 10 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Fermentas), 1 g/liter 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 an optical density at 600 nm (OD_{600}) of 0.005 in a 1-liter shake flask containing 200 ml 2× YT medium and the corresponding antibiotics. The cultures were grown at 37°C with shaking at 160 rpm until the OD_{600} 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/liter Triton X-100, and 5 mg/ml lysozyme) as described by Biver et al. (33). After sonication on ice (4 cycles of 5 s at 100 W; Sonifier 250; Branson), the lysate was centrifuged at 10,000 × *g* 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/liter 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 nickel-nitrilotriacetic acid (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/liter, 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/liter 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/liter 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/liter 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 to yield E2, which was 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) (34). The enzymatic assay was performed with 1% (wt/vol) carboxymethylcellulose (CMC) (ultralow viscosity; Fluka), 50 mM phosphate buffer (pH 7) for 10 min 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 was heated for 10 min 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 μmol 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 h 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% (wt/vol) 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 a 1 mM final concentration. Effects of the chemicals EDTA (10 mM), SDS (1%), ethanol (10%), Triton (5%), glycerol (15%), and dimethyl sulfoxide (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 to 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 (Table 1).

RESULTS

DNA extraction, library construction, and functional screening. Bacterial DNA was first extracted as described by Burke et al. (35) with a tri-enzymatic cleaner (Anios PlaII; Anios, France). As this procedure resulted in coextraction of polyphenols, present in brown-algal cell walls (36), the DNA could not be purified (data not shown). Therefore, a method described by Lachnit et al. (30), 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 was 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 2× YT 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 candidates. 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 locus (*CellMM5.1*) or a beta-glucosidase locus (*BglucMM5.1*) accordingly conferred the corresponding activity.

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 identified. Each ORF gene product was analyzed with BLASTP against the GenBank nonredundant database and Swissprot (a curated database). The putative esterases were also analyzed with Pfam (32) (see Data Set S1 in the supplemental material).

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 1). Putative esterase loci were assigned to four carboxylesterase families by amino acid sequence alignment (data not shown). Lip5.8_1 shows sequence similarity to members of bacterial esterase family II (37, 38). 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 products of the *Lip5.7_2* and *Lip5.12_2* loci could not be assigned to any esterase family described by Arpigny and Jaeger (37) or to either of the novel families EstA (39) and LipG (16). However, these locus 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 (32).

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 locus (*Bgluc5.1_1*) was probably responsible for the beta-glucosidase activity and was assigned to family GH3 of the CAZy database (40). The esterase locus (*Bgluc5.1_2*) encodes a putative GDSL lipolytic protein assigned to family II by amino acid sequence alignment (37, 38).

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 no. 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. (41) (Fig. 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 locus (*Cell5.1_3*) product.

It is difficult to attribute a specific biological function to the different locus clusters on the basis of locus annotations, especially for the clusters containing esterases. In contrast, curated locus annotations give insights into the taxonomic affiliation of the *Ascophyllum*-associated bacteria, at least at the class or order level. In most of the clusters, the different loci 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 (42).

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 pu-

TABLE 1 ORFs on the esterase-, cellulase-, and beta-glucosidase-encoding plasmids and sequences showing identities to those for known esterases and glycoside hydrolases

Activity	Candidate name	Locus	ORF size (amino acids)	Best hit against GenBank (source organism); accession no.	Sequence identity (%)	Family assignment[a]	Accession no.
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	GDSL family lipase (*Hyphomonas oceanitis* SCH89); KDA01394.1	46	Est II	KF726992
		Lip5.8_2	221	ABC transporter (*Erythrobacter litoralis* HTCC2594); YP_458689.1	61	/	
		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_012777983.1	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]	Beta-glucosidase (*Hellea balneolensis*); WP_026940963.1	74	GH3	KJ499810
		Bgluc5.1_2	244	GDSL 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.

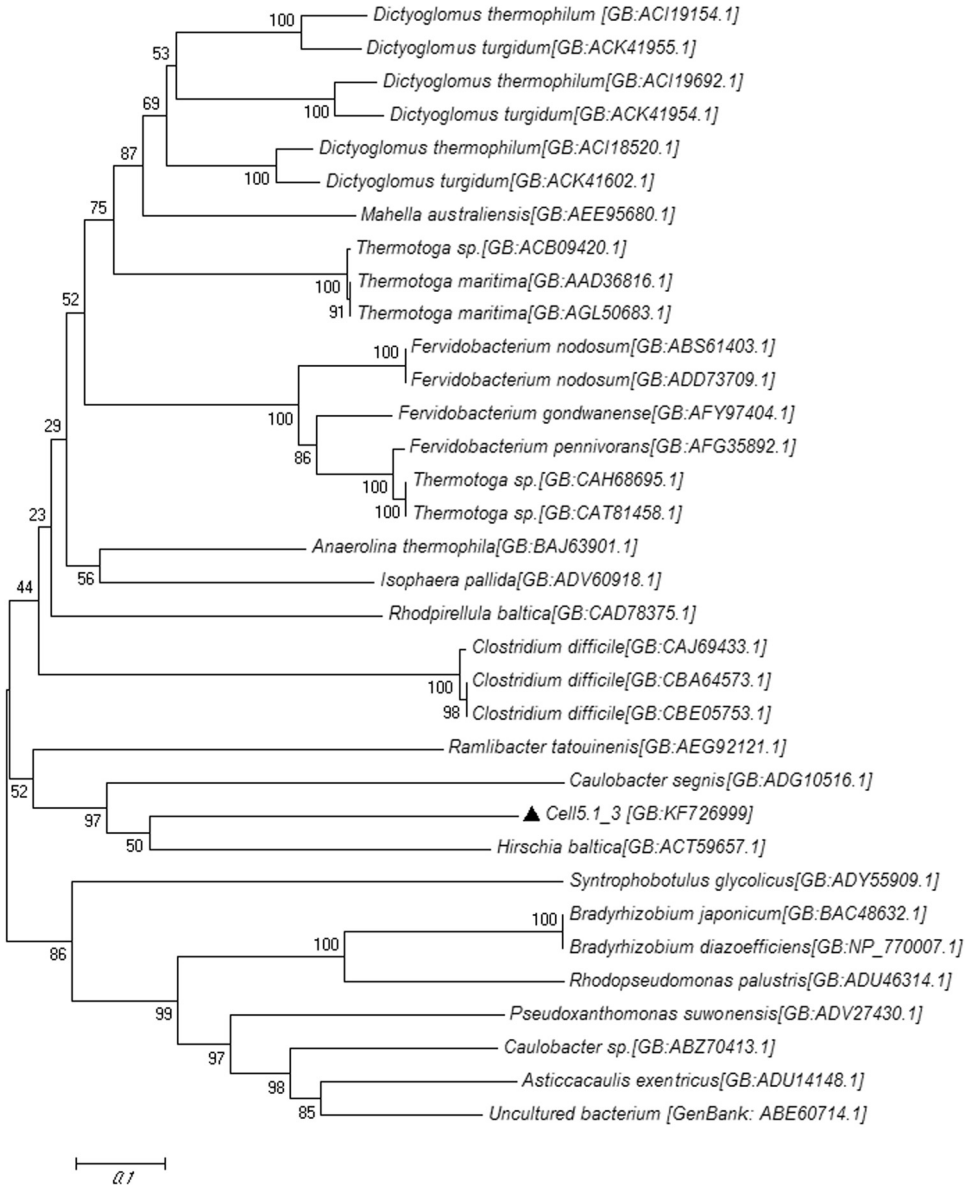

FIG 1 Neighbor-joining phylogenetic tree of subfamily GH5_25 and classification of the cellulase locus *Cell5.1_3*. Bootstrap values higher than 50% are indicated at branch points (1,000 replicates). Reference sequences from the GenBank database are included.

rified by nickel chromatography. SDS-PAGE of the eluted fractions showed a clear band between 30 and 40 kDa (Fig. 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 (Fig. 3A). At 40°C, the optimal pH was pH 7, and the cellulase appeared stable over a wide pH range when preincubated for 24 h at 4°C in different buffers (Fig. 3C and D). The kinetic parameters determined for the reaction with CMC, at optimal pH and temperature, were a K_m of 8.37 μM and a k_{cat} of 10.35 s^{-1}. Halotolerance was also observed: the activity of the enzyme remained unchanged, under standard conditions, after a 24-hour preincubation with 4 M NaCl or 3 M KCl (Fig. 3B). The specificity of the enzyme was tested in the presence of different substrates (Table 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+} (Fig. 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 and glycerol) or totally inhibit (SDS, ethanol, and DMSO) its activity (Fig. 3E).

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 environ-

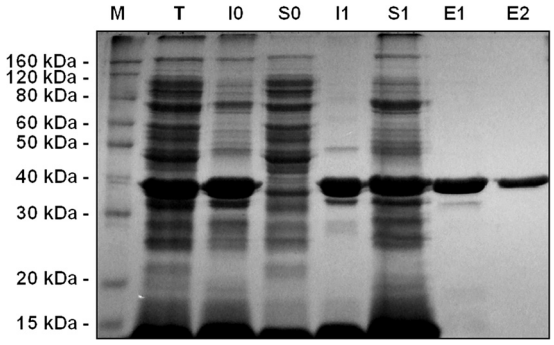

FIG 2 SDS-PAGE analysis of the recombinant cellulase. M, molecular mass; T, total extract. I0 and S0 are the insoluble and soluble fractions obtained by sonication, followed by centrifugation, of *E. coli* cells overexpressing the cellulase locus *Cell5.1_3*. As the GH was mostly present in the insoluble 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). This fraction was then purified twice by chromatography (E1 and E2).

TABLE 2 Substrate specificity of Cell5.1_3

Substrate	Main linkage type	Sp act[a] (U/mg)
Soluble substrates		
Carboxymethylcellulose	β-1,4-Glucan	10.22 ± 0.002
Lichenan	β-1,3–1,4-Glucan	12.61 ± 0.008
Laminarin	β-1,3/1,6-Glucan	5.08 ± 0.004
Xyloglucan	β-1,4-Xyloglucan	UD
Insoluble substrates[b]		
Mannan	β-1,4-Mannan	UD
Avicel	β-1,4-Glucan	UD

[a] One unit of enzyme activity corresponds to 1 μmol of glucose released in 1 min. Measurements were performed in triplicate, and values are means ± standard deviations. UD, undetectable.
[b] For these substrates, the reaction time was extended to 5 h at 20°C, with automatic inversion.

ment in which these microorganisms live leads to the production of specific enzymes with original biochemistry (18). Here we have constructed a metagenomic library from the genomic DNA of the bacterial communities associated in February 2012 with the

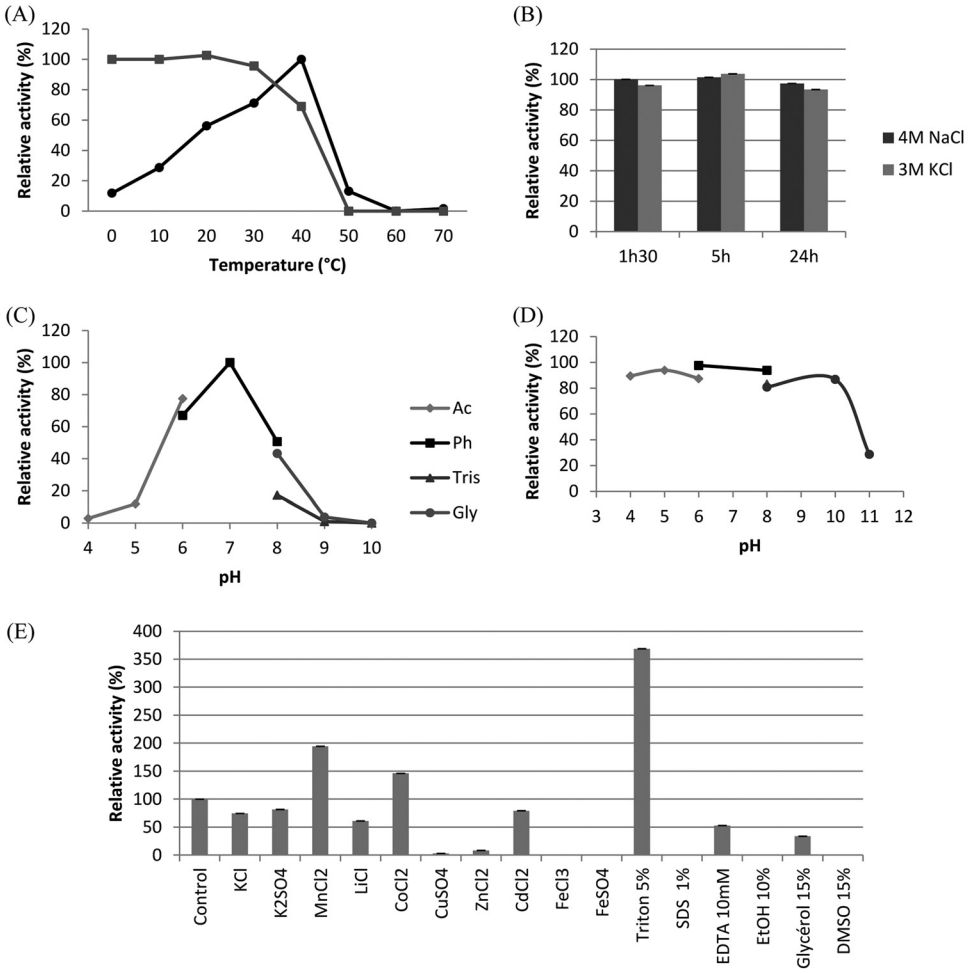

FIG 3 Characterization of the activity of Cell5.1_3. (A) Influence of temperature on Cell5.1_3 activity (●) and thermostability after 1 h incubation at indicated temperature (■). (B) Halotolerance of Cell5.1_3. (C) Influence of pH on Cell5.1_3 activity. (D) Residual activity of Cell5.1_3 after a 24-hour 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 the Cell5.1_3.

brown alga *Ascophyllum nodosum* (order *Fucales*) and have screened it for diverse hydrolytic enzymes. From this library we have identified a range of new esterase loci and two new glycoside hydrolase loci, thus demonstrating the pertinence of seeking new enzymes in such microbial communities by functional metagenomics. All these hydrolase loci can most likely be assigned to the phylum *Proteobacteria* and largely to the class *Alphaproteobacteria*, with a few in 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 to be dominated by *Alphaproteobacteria* and *Gammaproteobacteria* species (20). Nonetheless, *Bacteroidetes* and *Planctomycetes* species are also relatively abundant on brown algal surfaces (20, 43), and one might be surprised that our functional metagenomic screen yielded no loci 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 loci by screening a relatively low number of megabases, approximately 180 Mb, which makes the average 1 esterase per 14 Mb screened. This is much higher than in other metagenomic studies (see the review by Steele et al. [44]). The 13 esterase loci 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*, and *Lip5.19_3*) have been assigned to family IV. This esterase family appears to be 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 (39, 45–51). We have assigned one of the esterase loci 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 (37, 38). Only two esterases of marine origin (FJ483459 and FJ483468), identified in a metagenomic library from marine sediments, have been assigned to this family previously (48). 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 mass, around 42 kDa (38). The predicted Lip5.11_3 esterase shows sequence identity to β-lactamases and has an estimated molecular mass of 41.4 kDa. The predicted Lip5.15 esterase has an estimated molecular mass 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 locus (*Bgluc5.1_2*) found on the DNA insert of the beta-glucosidase candidate *BglucMM5.1* and in the esterase locus (*Lip5.8_1*) of *LipMM5.8*. Only one marine-metagenome-derived esterase (AB432912), identified in a sponge-associated bacterial metagenome (50), 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 (52). 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 loci identified on *LipMM5.11* (*Lip5.11_4* and *_5*) seem to be in an operon. The product of the first locus (*Lip5.11_4*) has sequence identity to a thioesterase of *Caulobacter* sp. strain K31 (Caul_4896; YP_001686513.1), whose gene is located with that for another thioesterase in a 3′-5′-oriented operon (Caul_4895; YP_001686512.1). The other thioesterase locus (*Lip5.11_5*) has sequence identity to a thioesterase locus of *Parvibaculum lavamentivorans* DS-1 (Plav_0440; YP_001411720.1), which is also in a 3′-5′-oriented operon with another thioesterase locus (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 13 loci encoding esterases, (ii) we have assigned these loci 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.

Compared to esterase genes, few cellulase genes have been identified by functional metagenomics. To our knowledge, no cellulase gene has been identified by metagenomics in a marine environment before. All of the cellulases identified by analysis of soil (53–56) or rumen (57, 58) 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 to be new and only distantly related to known cellulases. We have assigned the Cell5.1_3 cellulase to endo-β-1,4-glucanase family GH5, subfamily 25. Although most cellulases identified by metagenomics have been classified as GH5-family enzymes (8, 54, 57–59), only one of them (ABE60714.1), identified in a pulp sediment metagenome from a paper mill effluent, belongs to subfamily 25 (60). The other members of this subfamily were identified in cultivable microorganisms such as *Caulobacter*, *Clostridium*, *Dictyoglomus*, *Fervidobacterium*, and *Thermotoga* species isolated from diverse environments (Carbohydrate-Active Enzymes database [www.cazy.org]). Only two subfamily 25 enzymes have been characterized so far, encoded by *Tm_Cel5A* (Q9X273) of *Thermotoga maritima* (61, 62) and *FnCel5A* (UniProt accession no. A7HNC0) of *Fervidobacterium nodosum* (63). Both of these appear to be thermostable. Cell5.1_3, in contrast, shows low thermostability: when preincubated for 1 h at various temperatures, it began to show decreased CMC-hydrolyzing activity when the preincubation temperature exceeded 25°C, and when the preincubation temperature exceeded 40°C, no activity was observed. Under our assay conditions, its activity was highest at 40°C, which is relatively low compared to the activities of the enzymes Tm_Cel5A and Fn_Cel5A, 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 Aspeborg et al. (41), most of the endoglucanases of this subfamily have been derived from thermophiles, but interestingly, the cold-active cellulase identified here, Cell5.1_3, would appear to come from a mesophilic (or even psychrophilic) bacterium. A cold-active cellulase (CelX) identified in *Pseudoalteromonas* sp. strain DY3 shows very similar optimal temperature and thermostability (64). 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 preincubation in this pH range), which is not the case for the cold-active CelX (64). What is more, other metagenome-derived GH5 cellulases show stability only at acidic (4 to 6.6 [59]) or alkaline (6 to 10 [55]) pH. Another advantage of Cell5.1_3 is its halotolerance, as it retains 93% of its activity after a 24-hour preincubation in 3 M KCl and 97% of its activity after a preincubation 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 preincubation in 3 M NaCl or 4 M KCl (54), and a GH5 endoglucanase of the thermophilic eubacterium *Thermoanaerobacter tengcongensis* MB4 retains less than 15% of its activity after a 12-hour preincubation in 4 M NaCl (65). As GH5 endoglucanases hydrolyze a wide range of cellulose substrates (41), we have tested the substrate specificity of Cell5.1_3. The enzyme appears to degrade CMC and mixed glucans (lichenan [β-1,3–β-1,4] and laminarin [β-1,3–β1,6]) but not β-1,4-linked xyloglucan. Other metagenome-derived cellulases either fail to degrade laminarin (53–55, 58) or, like the GH5 enzyme described by Liu et al., show very low specific activity (approximately 0.002 U/mg) (59). 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. (66) reported three main hypotheses explaining the enhancement of enzymatic cellulose hydrolysis in the presence of nonionic surfactants: (i) they stabilize the enzyme by reducing thermal and/or mechanical shear forces; (ii) they change the substrate structure, enhancing substrate accessibility; and (iii) they affect enzyme-substrate interaction, preventing enzyme inactivation due to nonproductive adsorption when, for example, lignocellulosic substrates are being hydrolyzed. This last hypothesis does not 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 (9, 54, 63, 67), the second hypothesis 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.

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We have no competing interests to declare.

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