Identification and Characterization of Carboxyl Esterases of Gill Chamber-Associated Microbiota in the Deep-Sea Shrimp *Rimicaris exoculata* by Using Functional Metagenomics

María Alcaide,[a] Anatoli Tchigvintsev,[b] Mónica Martínez-Martínez,[a] Ana Popovic,[b] Oleg N. Reva,[c] Álvaro Lafraya,[a*] Rafael Bargiela,[a] Taras Y. Nechitaylo,[d] Ruth Matesanz,[e] Marie-Anne Cambon-Bonavita,[f] Mohamed Jebbar,[g] Michail M. Yakimov,[h] Alexei Savchenko,[b] Olga V. Golyshina,[i] Alexander F. Yakunin,[b] Peter N. Golyshin,[i] Manuel Ferrer,[a] for The MAMBA Consortium

Consejo Superior de Investigaciones Científicas (CSIC), Institute of Catalysis, Madrid, Spain[a]; Department of Chemical Engineering and Applied Chemistry, University of Toronto, Toronto, Ontario, Canada[b]; Department of Biochemistry, University of Pretoria, Pretoria, South Africa[c]; Insect Symbiosis Research Group, Max Planck Institute for Chemical Ecology, Jena, Germany[d]; Centro de Investigaciones Biológicas, CSIC, Madrid, Spain[e]; Ifremer, Centre de Brest, Laboratoire de Microbiologie des Environnements Extrêmes, REM/DEEP/LM2E, UMR 6197 (Ifremer-CNRS-UBO), ZI de la Pointe du Diable, Plouzané, France[f]; Université de Bretagne Occidentale, Laboratoire de Microbiologie des Environnements Extrêmes-UMR 6197 (CNRS-Ifremer-UBO), Plouzané, France[g]; Institute for Coastal Marine Environment, CNR, Messina, Italy[h]; School of Biological Sciences, Bangor University, Gwynedd, United Kingdom[i]

The shrimp *Rimicaris exoculata* dominates the fauna in deep-sea hydrothermal vent sites along the Mid-Atlantic Ridge (depth, 2,320 m). Here, we identified and biochemically characterized three carboxyl esterases from microbial communities inhabiting the *R. exoculata* gill that were isolated by naive screens of a gill chamber metagenomic library. These proteins exhibit low to moderate identity to known esterase sequences ($\leq$52%) and to each other (11.9 to 63.7%) and appear to have originated from unknown species or from genera of *Proteobacteria* related to *Thiothrix*/*Leucothrix* (MGS-RG1/RG2) and to the *Rhodobacteraceae* group (MGS-RG3). A library of 131 esters and 31 additional esterase/lipase preparations was used to evaluate the activity profiles of these enzymes. All 3 of these enzymes had greater esterase than lipase activity and exhibited specific activities with ester substrates ($\leq$356 U mg^{-1}) in the range of similar enzymes. MGS-RG3 was inhibited by salts and pressure and had a low optimal temperature (30°C), and its substrate profile clustered within a group of low-activity and substrate-restricted marine enzymes. In contrast, MGS-RG1 and MGS-RG2 were most active at 45 to 50°C and were salt activated and barotolerant. They also exhibited wider substrate profiles that were close to those of highly active promiscuous enzymes from a marine hydrothermal vent (MGS-RG2) and from a cold brackish lake (MGS-RG1). The data presented are discussed in the context of promoting the examination of enzyme activities of taxa found in habitats that have been neglected for enzyme prospecting; the enzymes found in these taxa may reflect distinct habitat-specific adaptations and may constitute new sources of rare reaction specificities.

Metagenomics provides a means for the discovery of entirely new enzymes in microorganisms and their communities without the need to culture these microorganisms as individual species, which is technically very difficult (1–5). Although the discovery of new enzyme activities has progressed considerably (6), it has not managed to go beyond the effective identification of enzymatic activities at a rather limited number of environmental sites. While an extensive search in the specialized literature and public databases indicated that microbial communities from approximately 1,800 different sites worldwide have been examined for their genomic content, only in approximately 200 of those locations (11% of the total) have new active clones or enzymes been identified and/or partially characterized. Thus, only a tiny fraction of the Earth's biosphere has been explored for the purpose of enzyme discovery, despite the fact that natural microbial diversity has been recognized to be the major source of new microbes (7). Such diversity also contains novel genes and functions (8) whose activities remain mostly unknown but whose potential to contribute to an innovation-based economy is recognized (9, 10).

One of the habitats less explored to date in terms of examining enzyme repertoires is the deep-sea realm, where microbial communities are shaped by salinity, pressure, and temperature (11). The examination of a set of enzymes recently collected from free-living bacteria from a number of deep-sea, salt-saturated marine habitats (3) reveals that deep-sea marine habitats harbor broad biochemical diversity reflective of the unique environmental conditions under which they evolved. An investigation of these enzymes also demonstrates that enzymes may constitute good model

FIG 1 Global map displaying the locations of the deep-sea sites at which specimens of *R. exoculata* were sampled. Hydrochemistry data for the Mid-Atlantic Ridge (19, 20) are the following: density, 1.000 g liter^{-1}; depth, 2,320 m; total salinity, 23.03 g kg^{-1} (Na$^+$, 12.71 g kg^{-1}; K$^+$, 0.79 g kg^{-1}; Ca^{2+}, 2.67 g kg^{-1}; Cl$^-$, 12.75 g kg^{-1}; Mg^{2+}, 0 g kg^{-1}; SO$_4$$^{2-}$, 0 g kg^{-1}); temperature (average), 8.7°C; pH, 7.0 to 8.0. (Map reproduced with permission from Ifremer; photograph reproduced with permission from Ifremer-Victor/Exomar 2005.)

systems for understanding protein functionality and adaptation under multiple extremes. Although the deep sea is also home to macroorganisms and their associated microbial communities (12, 13), to our knowledge, no previous investigation of the characteristics and habitat-specific adaptations of enzymes from microbial communities associated with deep-sea macroorganisms has been reported. Moreover, only a small number of enzymes from marine sponge- and marine mollusc-associated bacterial communities (14–18) have been characterized, none of which were isolated from marine habitats below a depth of 200 m. Most of these enzymes, for which limited biochemical data are available, show significant homology (54 to 100%) to known proteins, are halo-tolerant, and exhibit an activity-stability trade-off observed in cold-adapted enzymes (16); however, limited biochemical information is known with respect to their substrate profiles. Thus, our understanding of the metabolic significance and protein functionality of bacterial enzymes associated with macroorganisms living within extreme deep-sea habitats is limited.

In this study, we analyzed enzymes from the shrimp *Rimicaris exoculata* (Williams and Rona, 1986), which dominates the fauna at many hydrothermal vent sites along the Mid-Atlantic Ridge (<2,320 m depth). *R. exoculata* thrives along deep chimney walls in the gradient between hydrothermal fluids and cold, oxygenated, and slightly saline ambient waters (19). Based on functional gene surveys, it was found that shrimp specimens, including adult *R. exoculata*, host a dense bacterial community within their gill chambers (12). Such bacteria are known to provide energy and nutrients for the eukaryotic host by cometabolizing inorganic and organic compounds (e.g., H$_2$ and CH$_4$) directly discharged by the hydrothermal system (20–24) or obtained from the water column (25, 26). It remains to be determined, however, how the multiple collective pressures characterizing the habitat in which *R. exoculata* thrives impact the properties and functional characteristics of microbial enzymes such as those from the gill chamber environ-

ment. Following on from this, we aimed to understand the influence of marine conditions, particularly the combined effects of salt, pressure, and temperature, on the properties and functional characteristics of enzymes, as well as to increase the number of characterized enzymes from deep-sea environments. We sequenced and analyzed the biochemical characteristics of three bacterial carboxyl esterases with an α/β hydrolase fold that were identified by naive screens of deep-sea microbiota associated with the gill chambers of *R. exoculata* shrimp inhabiting hydrothermal fluids along the Mid-Atlantic Ridge (Fig. 1). These enzymes first were selected as model enzymes for study because they have been reported to be suitable enzymes to investigate protein functionality and adaptation to polyextremes (3), particularly as a function of temperature, salt, and pressure in deep-sea habitats. In this context, our recent investigation examining the characteristics of a set of esterases has provided the first clear evidence suggesting that, in salt-saturated deep-sea habitats, the adaptation to high pressure is linked to high thermal resistance by unknown structural adaptation mechanisms (3). Therefore, understanding the characteristics of other deep-sea enzymes, particularly esterases for which multiple biochemical data are available compared to other deep-sea enzymes (3), might help in understanding the phenomenon of protein versatility and adaptability to different polyextremes. Second, esterases were selected because they are ubiquitous enzymes that are widespread in nature (at least one per genome), multiple commercial preparations that are used at industrial scale are available, and biochemical data of such enzymes from a number of marine habitats (3, 27, 28) have been reported recently, which collectively may assist to perform an extensive comparative analysis. Finally, they are of great interest as biocatalysts for chemical synthesis (29). A comparative analysis using a set of 31 different enzymes with esterase and lipase activity, including enzymes from marine (deep-sea, hydrothermal vent, and superficial seawater) and brackish water habitats and commercial

preparations, allowed us to infer habitat-specific features of the gill-associated bacterial enzymes as well as to evaluate their specificities and reactivities. Note that two of the enzymes reported in this study originate from an unknown species or possibly from genera related to *Thiothrix* and/or *Leucothrix*, a group of organisms largely neglected with respect to enzyme discovery; only a small number of isolates from these genera are available, and no enzymes from these genera have been characterized.

MATERIALS AND METHODS

Metagenomic library, metagenome screening, and sequence analysis. Gill chamber samples were collected from *R. exoculata* specimens found at locations in the western flank of the Rainbow Ridge in the Atlantic Ocean (36°14′N, 33°54′W; 2,320 m depth; MAR site), where the average seawater temperature is 8.7°C (ranging from 3 to 25°C) (Fig. 1) (19). Total DNA was extracted from the gill chambers of the collected specimens as previously described (19); from this DNA, a large-insert pCCFOS1 fosmid library was generated using the *Escherichia coli* EPI300-T1^R strain (Epicentre Biotechnologies; Madison, WI, USA), and the library was scored for the ability to hydrolyze α-naphthyl acetate and tributyrin (27, 30). Positive clones were selected, and their DNA inserts were sequenced using a Roche 454 GS FLX Ti sequencer (454 Life Sciences, Branford, CT, USA) at Life Sequencing SL (Valencia, Spain) or were completely Sanger sequenced using universal primers and subsequent primer walking. Upon completion of sequencing, the reads were assembled to generate nonredundant metasequences using Newbler GS De Novo Assembler v.2.3 software (Roche, Branford, CT, USA). GeneMark software (31) was employed to predict potential protein-coding regions (open reading frames [ORFs] with ≥20 amino acids) from the sequences of each assembled contig, and deduced proteins were screened using BLASTP and PSI-BLAST searches (32). Multiple protein alignments were performed using the ClustalW program built into BioEdit software, version 7.0.9.0 (33). Domains with a significant propensity to form transmembrane helices were identified with TMpred software (ExPASy, Swiss Institute of Bioinformatics) (34).

Protein expression and purification. The cloning, expression, and purification of selected proteins using the p15TV-Lic vector and *E. coli* BL21(DE3) Codon Plus-RIL (for MGS-RG3) and Ek/LIC 46 and *E. coli* BL22 (for MGS-RG1 and MGS-RG2) were performed as described previously (27) using the following primer pairs: MGS-RG1Fwd (5′-GAC GAC GAC AAG ATG ACT GAT TTG TTA CC-3′), MGS-RG1Rev (5′-GAG GAG AAG CCC GGT CAA ATC AAA AC-3′); MGS-RG2Fwd (5′-ATG ATG AAA AAT ATG TCA GAA TTA CC-3′), MGS-RG2Rev (5′-TTA TTT TAA TAT TTT TTG TAG CCA TGC C-3′); and MGS-RG3Fwd (5′-TTG TAT TTC CAG GGC ATG CAG GAA CTT CCC GAT GC-3′), MGS-RG3Rev (5′-CAA GCT TCG TCA TCA GGT CCG GAA CCG CGC CTT G-3′). Purity greater than 98% was obtained after a single His$_6$ tag purification step (see Fig. S1 in the supplemental material). After purification, all enzymes were maintained at a concentration of 2 mg ml^{-1} in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, pH 7.0; the protein stock solution was stored at −20°C until it was used in assays. All chemicals used for enzymatic tests were of the purest grade available and were purchased from either Fluka-Aldrich-Sigma Chemical Co. (St. Louis, MO, USA) or Apin Chemicals (Oxon, United Kingdom).

Hydrolase assays. Carboxyl esterase activity was assayed using *p*-nitrophenyl (*p*NP) esters (read at 410 nm) and structurally diverse esters other than *p*NP esters (read at 540 nm) in 96-well plates as previously described (27). Unless stated otherwise, standard assay reactions were conducted by adding 2 μl of the 2 mg ml^{-1} protein stock solution to an assay mixture containing 2 μl of ester stock solution (100 mM in acetone [for *p*NP esters] or acetonitrile [for other esters]) in 196 μl of 50 mM Tris-HCl buffer, pH 8.0 (for *p*NP esters), or 5 mM *N*-(2-hydroxyethyl) piperazine-*N*′-(3-propanesulfonic acid) (EPPS) buffer, pH 8.0 (for other esters). The final volume of the assay was 200 μl, and the final protein and

substrate concentrations were 10 μg ml^{-1} and 1 mM, respectively. All assays were conducted at pH 8.0 at the optimal temperature for each enzyme (MGS-RG1, 45°C; MGS-RG2, 50°C; MGS-RG3, 30°C). In all cases, absorbance was determined using a microplate reader every 1 min for a total time of 15 min (Synergy HT multi-mode microplate reader; BioTek). All reactions were performed in triplicate. One unit of enzyme activity was defined as the amount of enzyme required to transform 1 μmol of substrate in 1 min under the assay conditions. All values were corrected for nonenzymatic hydrolysis (background rate). If not otherwise stated, 1 mM *p*NP-propionate was used as the standard assay substrate for the determination of the conditions under which each enzyme displayed activity; pH values between 4.5 and 9.0 (at the optimal temperature), temperatures between 4 and 80°C (using 50 mM Tris-HCl buffer, pH 8.0), and NaCl, KCl, and MgCl$_2$ concentrations of up to 4 M (using 50 mM Tris-HCl buffer, pH 8.0, and optimal temperatures) were tested. The buffers used to determine the optimal pH for each enzyme have been described previously (3, 27).

Pressure perturbation studies. The effect of hydrostatic pressure on enzymatic activity was analyzed by placing the reaction mixtures in a high-pressure incubating system consisting of a 2-ml high-pressure cell connected to a pressure generator (High Pressure Equipment, Erie, PA, USA) capable of generating pressures of up to 10,000 lb/in^2. Two pressure values were examined: 23,000 and 35,000 kPa. A total of 20 μg of pure protein was added to 20 ml of freshly prepared 50 mM Tris-HCl buffer, pH 8.0, containing 1.0 mM *p*NP-propionate. A total of 2 ml of the reaction mixture was immediately transferred to the high-pressure reactor, and a separate 2-ml aliquot of the mixture was kept at atmospheric pressure. The experiments were performed at 25°C. In all cases, reactions without protein were used as negative controls. After incubation for 5 min, the samples were immediately depressurized, and the extent of the reaction was monitored by spectrophotometry at 410 nm (as described above). All experiments were performed in triplicate. Note that reactions were performed in (i) the absence of salt, (ii) the presence of 23.03 g kg^{-1} NaCl, which corresponds to the habitat salinity (19), and (iii) the presence of NaCl concentrations required for optimal enzyme activity. In all three cases, similar effects by pressure on enzyme activity were found, and only data in the absence of salt are presented.

CD. Circular dichroism (CD) spectra were acquired between 190 and 255 nm using a Jasco J-720 spectropolarimeter equipped with a Peltier temperature controller (4 to 95°C); the spectra were obtained using a 0.1-cm-path cell and were conducted at 25°C. The protein concentration was determined spectrophotometrically at 280 nm in accordance with the amino acid sequence of the protein (www.expasy.org/tools/protparam .html). The spectra were analyzed, and denaturation temperatures were determined in 50 mM HEPES buffer, pH 7.0, at 220 nm as previously reported (35, 36).

Taxonomic binning. Protein-coding genes in fosmid sequences were predicted using GeneMark software (31) and the antiSMASH server (37) and then were checked manually using the Artemis browser (38). The predicted protein sequences were aligned against the National Center for Biotechnology Information nonredundant (NCBI nr) database using a BLASTP search. Taxonomic binning of the orphan sequences in fosmids was performed by summarizing the top significant BLASTP hits with E values of ≤0.00001. Additionally, genes encoding esterases and their closest homologs identified by BLASTP were compared by patterns of codon usage biases predicted using a Web-based codon usage calculator (39). Composition-based binning of fosmids was performed using the GOH-TAM web server (40). The dissimilarity in synonymous codon usage between two genes was calculated as the Pearson correlation between vectors of fraction values for 59 codons (41). Compositional similarity between fosmid insert sequences was estimated using the SeqWord Genome Browser (42).

Sequence and substrate profile distributions. Multiple protein alignment was conducted using the MUSCLE application in BioEdit software (33) with default settings (43). The resulting alignment then was used for

TABLE 1 General features of the enzymes and amino acid residues potentially involved in catalysis

Enzyme	Molecular mass (Da)	pI	No. of amino acids	Catalytic triad	GXSXG-lipase motif[a]	Oxyanion hole motif[a]	Classified family
MGS-RG1	24,983.6	5.07	222	S118, D/E, H?	G116-F117-S118-Q119-G120-G121	H24-G25-L26-G27-A28	Possibly VI
MGS-RG2	25,363.2	5.31	225	S111, D175, H206	G109-T110-S111-V112-E113-K114	H27-G28-L29-G30-A31	Possibly VI
MGS-RG3	30,611.2	5.51	277	S125, E218, H248	G123-D124-S125-A126-G127-G128	H55-G56-G57-G58-Y59	Possibly IV

[a] Consensus GXSXG-lipase motifs are given for families (46–48). Family IV, GDSAGG; family VI, NGGPG.

phylogenetic reconstruction. A neighbor-joining tree was constructed in MEGA v.6.06 (44) using the settings for the Poisson model and homogenous patterning between lineages. Bootstrap analysis was done with 1,000 sample trees. The scale bar in the figure reflects the number of substitutions per position. Clustering of the substrate spectral profile based on a binomial distribution of the presence or absence of activity for particular ester substrates was performed, and Pearson's correlation coefficient was used to calculate the distances.

Nucleotide sequence accession numbers. The DNA sequences of the carboxyl esterase-positive metagenomic DNA fragments and the polypeptide sequences of the enzymes were deposited in GenBank under the accession numbers KF831416 (for MGS-RG1), KF831417 (for MGS-RG2), and KC986402 (for MGS-RG3).

RESULTS

Metagenome library construction and screening for carboxyl esterase activity. A subset of the 27,200 clones from the *R. exoculata* gill chamber microbiome library generated in this study, which included nearly 816 Mbp of community genomes, was scored for the ability of individual clones to hydrolyze α-naphthyl acetate, as reported previously (30). The subset of clones also was screened for carboxyl esterase activity using tributyrin plates (30). Hydrolysis of both substrates was indicative of esterase/lipase activity. A total of 10 unique positive clones, corresponding to a hit rate of 1:2,720, were identified as active; 3 of these were selected based on activity phenotype (they were among the most active, as judged by their halos/color formation). The inserts were sequenced, analyzed, and compared to the sequences available in the public NCBI nr database (32). Three predicted metagenome sequence (MGS)-encoding carboxyl esterases from *Rimicaris* gill chambers and with an α/β hydrolase fold were identified and successfully expressed as soluble proteins in *E. coli* (see Fig. S1 in the supplemental material), and their properties were investigated. These three proteins were named MGS-RG1, MGS-RG2, and MGS-RG3 (Table 1).

Sequence analysis of carboxyl esterases from the *Rimicaris* gill chamber microbiome. Based on BLAST searches of the NCBI nr database, the 3 studied protein sequences belong to the α/β hydrolase superfamily and had up to 58% sequence identity with homologous proteins in this database. The MGS-RG1 and MGS-RG2 proteins were most similar to presumptive carboxyl esterases from bacteria of the *Thiotrichales* order (41 to 58% similarity; WP_022950445 and Protein Data Bank [PDB] code 4F21 [45]), whereas MGS-RG3 was most similar to α/β hydrolases from *Rhodobacteraceae* (53% similarity; WP_005862880) and esterases/lipases from uncultured bacteria (41% similarity; PDB code 3V46). The deduced molecular masses and estimated pI values of these proteins ranged from 24.9 to 30.69 kDa and from 5.07 to 5.51, respectively (Table 1). The pairwise amino acid sequence

identity ranged from 11.94% to 63.7%; MGS-RG1 and MGS-RG2 were the most similar to each other (63.7% sequence identity), whereas MGS-RG1 and MGS-RG3 (17.3%) and MGS-RG2 and MGS-RG3 (11.9%) were the most divergent at the sequence level. The selected α/β hydrolases contain a classical Ser-Asp-His catalytic triad, but the catalytic elbow and oxyanion hole [i.e., the GXSXGG and H/N-GGG(A)/P-X motifs] often diverged from the consensus, as revealed by sequence analysis (Table 1). Nonetheless, the degree of sequence conservation among these catalytic motifs and in the overall enzyme sequence was sufficient to categorize the enzymes into the following two esterase/lipase subfamilies (46–48): family VI (MGS-RG1 and MGS-RG2) and family IV (MGS-RG3) (see Fig. S2 in the supplemental material). No significant rigorously confirmed transmembrane regions were identified in any of the sequences with TMpred (34); thus, the proteins most likely are intracellular proteins.

A search against the GOHTAM database (40) and TBLASTX analysis revealed compositional similarities between the DNA fragment (6,756 bp) containing the gene for MGS-RG3 with genomes of the alphaproteobacterial genus *Rhodobacteraceae* (best hit score, 248), whereas the DNA fragments encoding MGS-RG1 (24,228 bp) and MGS-RG2 (29,267 bp) exhibited similarity to chromosomal sequences of *Gammaproteobacteria*. A BLASTP search demonstrated that the MGS-RG1- and MGS-RG2-coding genes were most similar to those found in the *Thiothrix/Leucothrix* genomes (49–51). DNA fragments bearing MGS-RG1, MGS-RG2, and MGS-RG3 do not include any insertion sequence elements, integrases, or any other phage- or conjugative plasmid-associated genes. The tetranucleotide usage patterns of MGS-RG1 and MGS-RG2 were similar to each other and homogenous for 5,000-bp sliding windows stepping by 500 bp. MGS-RG3 was too short for the above-described analysis of oligonucleotide word usage patterns to be performed using the SeqWord Genome Browser (42). The esterases encoded by MGS-RG1 and MGS-RG2 exhibited very similar synonymous codon usage, with Pearson correlations from 0.81 to 0.84, but they were distinguishable from patterns of synonymous codon usage biases in the homologous genes from *Thiothrix* and *Leucothrix*; for these, the Pearson correlation coefficient was in the range of 0.42 to 0.52. It may be concluded that MGS-RG1 and MGS-RG2 originated from chromosomes of organisms closely related to the *Thiothrix/Leucothrix* lineage, although these two proteins were not members of these genera. This agrees with the close phylogenetic positioning of the sequences encoding such esterases (see Fig. S2 in the supplemental material).

Determination of the requirements for enzyme activity. Using *p*NP-propionate as a model substrate, the purified proteins
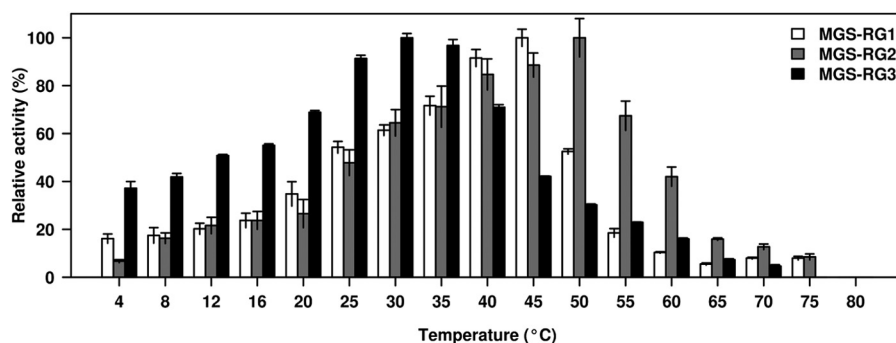
FIG 2 Temperature profiles of the carboxyl esterases from microorganisms inhabiting the gill chamber of *R. exoculata*. The data represent the relative percentages of specific activity (U mg^{-1}) compared with the maximum activity (100%; MGS-RG1, 0.756 U mg^{-1}; MGS-RG2, 1.754 U mg^{-1}; MGS-RG3, 159.2 U mg^{-1}). The specific activities were calculated using 2 μg of protein and 1 mM *p*NP-propionate as the assay substrate at pH 8.0 in 50 mM Tris-HCl, as described in Materials and Methods. Standard deviations (SDs) of the results of assays conducted in triplicate are shown.

exhibited optimal activities at pH values ranging from 8.0 to 8.5 (see Fig. S3 in the supplemental material). At the optimal pH for each enzyme, MGS-RG1 and MGS-RG2 were most active at 45 and 50°C, respectively, and retained ≤16% activity at ≥65°C and 4°C (Fig. 2). In contrast, MGS-RG3 was most active at lower temperatures (its maximal activity occurred at 30°C) and retained approximately 40% activity at 4°C, whereas it was strongly inhibited at ≥45°C. The temperatures found to be optimal for the activity of each enzyme are in agreement with the corresponding protein-denaturing temperatures, as determined by circular dichroism (MGS-RG1, 47.6°C; MGS-RG2, 53.3°C; MGS-RG3, 42°C) (Table 2).

The enzymatic activities of purified MGS-RG1/MGS-RG2 proteins, tested at optimal pH and temperature, were further shown to be stimulated up to 2.4-fold by the addition of NaCl, KCl, and

MgCl$_2$ to the reaction mixture (Fig. 3). Maximal activity for MGS-RG1 and MGS-RG2 was achieved at 3.6 and 3.2 M for NaCl, 3.0 and 2.4 M for KCl, and 0.8 and 0.4 M for MgCl$_2$, respectively. In contrast, MGS-RG3 was strongly inhibited by all three salts (Fig. 3). Note that at 2 M NaCl, commercial esterase/lipase preparations, such as CalA, CalB, Novozym 388 L, and porcine liver esterase (PLE), retained ≤9.1% of the activity they display in the absence of salt (Table 2).

Because pressure is one of the most representative environmental parameters of deep-sea habitats, including hydrothermal vents, the influence of hydrostatic pressure (23,000 and 35,000 kPa) on enzyme performance was further evaluated in high-pressure 2-ml reactors at pH 8.0 and 25°C as described in Materials and Methods. The data presented in Table 2 show that MGS-RG1 and MGS-RG2 are barophilic enzymes; at 23,000 kPa, corre-

TABLE 2 Denaturing temperatures and pressure resistance for activity of the esterases reported here and for other esterases from marine habitats

Enzyme	Origin	$T_d{}^a$ (°C)	Relative activity (%) at:		
			23,000 kPa^b	35,000 kPa^c	2.0 M NaCl^d
MGS-RG1	*R. exoculata* enzymes	47.6	152.0 ± 7.4	97.9 ± 3.5	173 ± 2.7
MGS-RG2		53.3	144.0 ± 7.5	97.7 ± 4.5	136 ± 7.1
MGS-RG3		42.0	77.9 ± 6.7	57.2 ± 2.9	9.3 ± 3.2
CalA^e	Commercial preparations	ND/NA	2.0 ± 0.1	0.2 ± 0.01	7.0 ± 1.5
CalB^e		53.0	8.0 ± 0.4	3.1 ± 0.02	9.1 ± 4.4
Novozym 388 L^e		ND	5.7 ± 0.2	0.4 ± 0.08	6.8 ± 4.0
PLE^e		ND/NA	1.8 ± 0.1	0.2 ± 0.05	1.5 ± 0.7
MGS-M1^f	Deep-sea enzymes	65.2	ND/NA	45.3 ± 3.5	289 ± 5.6
MGS-M2^f		71.4	ND/NA	110 ± 14	1645 ± 32.3
MGS-B1^f		52.4	ND/NA	115 ± 8	84 ± 3.4
MGS-K1^f		40.3	ND/NA	28 ± 7	148 ± 7.1
MGS-MT1^f		55.7	ND/NA	150 ± 15	170 ± 9.4
ABO_1197, ABO_1251, MGS0010, MGS0105, and MGS0109^g	Superficial seawater enzymes	45.7–48.1	ND/NA	ND/NA	~125–40

^{a} Protein denaturation temperature (T_d) as determined by circular dichroism. T_d reported by Qian and Lutz (52). For other commercial preparations, T_d are not shown either because the values, to the best of our knowledge, have been not been reported in the specialized literature or the commercial preparations are not pure enough to perform CD experiments in the present study (they are available as a complex mixture of proteins and additives). ND/NA, data not determined or not available.
^{b} Relative activity at the sampling site (12) refers to that at atmospheric pressure.
^{c} Pressure selected for comparative purposes, as previously reported deep-sea hydrolases have been tested under this pressure (3). The relative activity refers to that shown at atmospheric pressure.
^{d} The relative activity in the presence of 2 M NaCl refers to that shown in the absence of NaCl (data for MGS-RG1 to MGS-RG3 from Fig. 3). Activity measured at 30°C and 50 mM Tris-HCl buffer, pH 8.0, 10 μg ml^{-1} total protein, and 1 mM *p*NP-propionate.
^{e} Commercial esterase/lipase preparations from Novozymes A/S (Bagsværd, Denmark) (CalA, CalB, and Novozyme 388 L) and Sigma Chemical Co. (St. Louis, MO, USA) (PLE).
^{f} Esterases from deep-sea basins of the Mediterranean Sea. Values were reported in reference 3.
^{g} Esterases from superficial seawater at the Mediterranean Sea. Values were reported in reference 28.
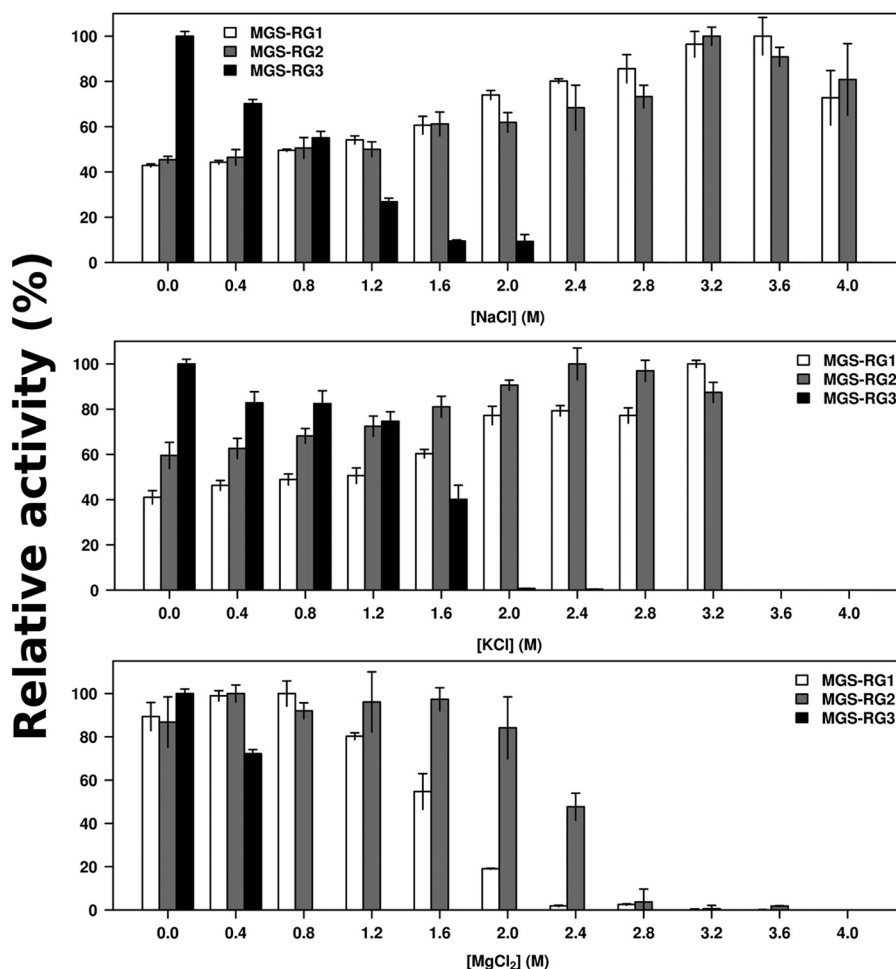
FIG 3 Activity profiles displaying the activities of carboxyl esterases at various concentrations of NaCl, KCl, and MgCl$_2$. The data represent the relative percentages of specific activity (U mg^{-1}) compared with the maximum activity (100%). The specific activities were calculated using 2 µg of protein and 1 mM pNP-propionate as assay substrate at pH 8.0 (50 mM Tris-HCl), and the assays were conducted at the optimal temperature for each enzyme (MGS-RG1, 45°C; MGS-RG2, 50°C; MGS-RG3, 30°C) as described in Materials and Methods. The SDs of the results of assays conducted in triplicate are shown. The activity of each enzyme in the absence of salt is given in the legend to Fig. 2.

sponding to the site pressure at 2,320 m (19), both enzymes were activated ($\geq$1.4-fold), and at 35,000 kPa, they retain $\geq$97.7% of the activity they display at atmospheric pressure. In contrast, under our assay conditions, MGS-RG3 was inhibited by high pressure (77.9% and 57.2% activity at 23,000 and 35,000 kPa, respectively). Under similar conditions of pH and temperature for assays, commercial esterase/lipase preparations, such as CalA, CalB, Novozym 388 L, and PLE, retained at pressure above 23,000 kPa $\leq$8% of the activity they display at atmospheric pressure.

Taken together, we found that the two barotolerant and moderately halophilic proteins (MGS-RG1 and MGS-RG2) exhibited the highest optimal temperatures (45 to 50°C), whereas the nonbarophilic and nonhalophilic MGS-RG3 protein displayed the lowest optimal temperature (30°C).

Substrate fingerprints: activity against rare, chemically distinct esters. A total of 131 ester-like chemicals were used to evaluate the substrate ranges and specific activities (U mg^{-1}) of the three enzymes at pH 8.0 and at optimal temperature. These chemicals included 11 model esters (7 pNP esters and 4 triacylglycerols) (Fig. 4), as well as a battery of 120 structurally different esters (3, 6,

27, 28, 37, 53) (Fig. 5). Substrate fingerprints revealed that MGS-RG3 (17 positive substrates) exhibited a relatively narrow substrate range compared to those of MGS-RG2 and MGS-RG1, which were active against a larger number of substrates (32 and 37 positive substrates, respectively).

All three ester hydrolases from the α/β-hydrolase family preferred short-to-medium-chain-length pNP-esters and triacylglycerols (Fig. 4) and alkyl, alkenyl, and/or aryl esters but with different orders of preference (Fig. 5). Based on determinations of the specific activity for each enzyme, methyl benzoate (for MGS-RG1; 25.23 $\pm$ 0.95 U mg^{-1}), methyl 2-bromopropionate (for MGS-RG2; 40.7 $\pm$ 0.3 U mg^{-1}), and pNP-butyrate (for MGS-RG3; 356.3 $\pm$ 1.2 U mg^{-1}) were the preferred substrates for these enzymes. The activity ratio between the most and least active enzymes was approximately 1:14.

As shown in Fig. 5, among esters other than common pNP-esters and triacylglycerols, only phenyl acetate was hydrolyzed by all three enzymes, suggesting that these enzymes exhibited a high substrate profile divergence. MGS-RG1 and MGS-RG2 were the most similar, in that they were able to hydrolyze 14 common sub-
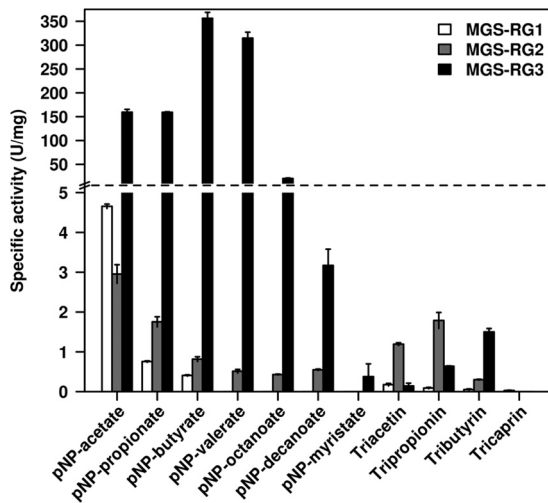
FIG 4 Substrate profiles of the activities of carboxyl esterases from microorganisms inhabiting the gill chamber of *R. exoculata* against model *p*NP-esters and triacylglycerols. The specific activity of each enzyme (in U mg^{-1}) against a set of structurally diverse substrates was measured using 2 μg protein at pH 8.0 in 50 mM Tris-HCl buffer for *p*NP-esters and 5 mM EPPS buffer for triacylglycerols, and the assays were conducted at the optimal temperature for each enzyme (MGS-RG1, 45°C; MGS-RG2, 50°C; MGS-RG3, 30°C) as described in Materials and Methods. The SDs of the results of assays conducted in triplicate are shown.

strates, albeit to different extents; this finding is consistent with their high sequence homology (63.7%) and similar taxonomic origins. However, 13 of the tested esters, including 8 halogenated esters (ethyl chloroacetate, ethyl fluoroacetate, ethyl-2-bromo-propionate, methyl-2-chloropropionate, methyl-2-chloro-3-hydroxypropionate, ethyl-4-bromobutyrate, ethyl-3-bromopropionate, and ethyl-α-isobromobutyrate), methyl glycolate, methyl butyrate, ethyl-*trans*-cinnamate, butyl acetate, and methyl benzoate, were hydrolyzed only by MGS-RG1. Nine esters were hydrolyzed only by MGS-RG2; they included three halogenated esters [methyl 2-bromopropionate, methyl bromoacetate, and methyl

($\pm$)-α-bromophenylacetate], caproic acid methyl ester, methyl-(*R*)-lactate, methyl-(*S*)-lactate, γ-butyrolactone, α-D-glucose pentaacetate, and tri-*O*-acetyl-(D)-glucal. Thus, despite the high sequence homology of these enzymes, distinct substrate preferences were observed; these preferences may be related to distinct architectures and/or to the accessibility of various substrates to the active sites of the enzymes. Finally, MGS-RG3, which shared only 5 common substrates with MGS-RG1 and 3 common substrates with MGS-RG2, was the only enzyme of the 3 able to hydrolyze 4 esters, including vinyl benzoate, (*R*)-menthyl acetate, ethyl 4-bromobutyrate, and methyl chloroacetate.

The ability to hydrolyze halogenated alkyl and aryl esters (including those containing bromide, chloride, fluoride, and iodide) was demonstrated for MGS-RG1 (16 esters), MGS-RG2 (9 esters), and MGS-RG3 (4 esters). Based on specific activity determinations (U mg^{-1}) and using optimal substrates for each enzyme (Fig. 5), MGS-RG2 was found to be the most active carboxyl esterase for haloesters (40.7 ± 0.3 U mg^{-1} for methyl 2-bromopropionate), followed by MGS-RG1 (7.55 ± 0.06 U mg^{-1} for ethyl-α-isobromobutyrate) and, to a far lesser extent, MGS-RG3 (1.66 ± 0.01 U mg^{-1} for ethyl iodoacetate). The activity ratio between the most active and least active enzymes for the hydrolysis of haloesters was approximately 1:25. Notably, whereas MGS-RG2 only degraded bromide-containing esters, MGS-RG1 and MGS-RG3 acted on iodine, chloride, and bromide esters (Fig. 5). Finally, it is notable that methyl-2-bromo-2-butenoate, which was used as a model alkenyl haloester, was the only such substrate for MGS-RG2 (9.76 ± 1.08 U mg^{-1}), followed to a far lesser extent by MGS-RG1 (0.162 ± 0.03 U mg^{-1}).

MGS-RG2 utilized tri-*O*-acetyl-glucal (0.286 ± 0.04 U mg^{-1}) and the carbohydrate ester α-D-glucose pentaacetate (0.373 ± 0.011 U mg^{-1}) as substrates, while the hydroxycinnamic-like ester ethyl-*trans*-cinnamate was a substrate for MGS-RG1 (4.57 ± 0.06 U mg^{-1}) (Fig. 5). The hydrolysis of such compounds is commonly associated with polysaccharide (including mucus) degradation (54–56).

Finally, under our assay conditions, all three carboxyl esterases
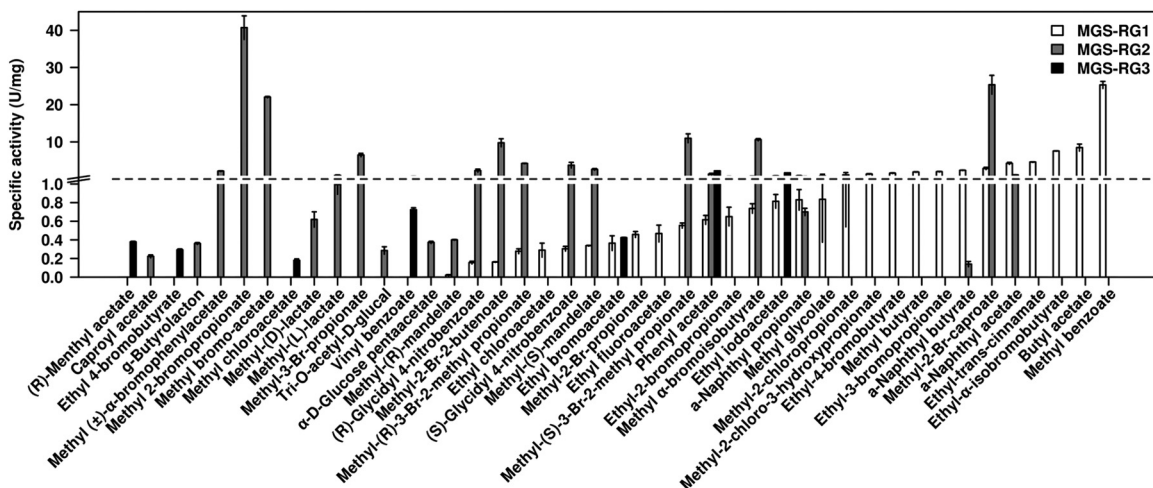
FIG 5 Substrate profiles of the activities of carboxyl esterases from microorganisms inhabiting the gill chamber of *R. exoculata* compared with those of a set of structurally distinct esters. The specific activities (U mg^{-1}) of each enzyme were measured against a set of structurally diverse substrates using 2 μg of protein at pH 8.0 in 5 mM EPPS buffer, and the assays were conducted at the optimal temperature for each enzyme (MGS-RG1, 45°C; MGS-RG2, 50°C; MGS-RG3, 30°C) as described in Materials and Methods. The SDs of the results of assays conducted in triplicate are shown.
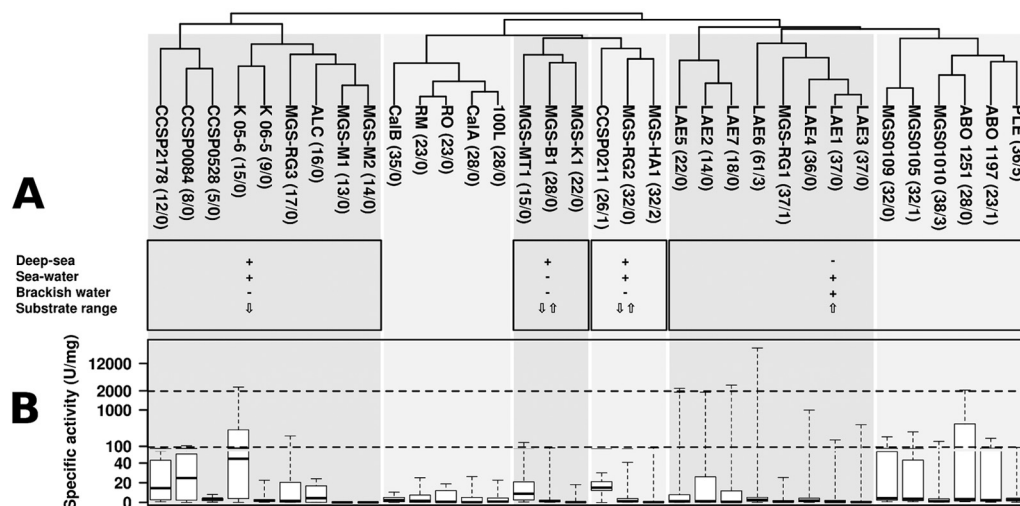
FIG 6 Clustering and specific activities (U mg^{-1}) of a set of 131 ester substrates of carboxyl esterases isolated from the gill chamber-associated microbiota from the deep-sea shrimp *R. exoculata* and from commercial and previously reported preparations. The source of chemicals are as reported previously (3, 6, 27, 28, 37, 53). Pearson's correlation was used to calculate the distances. (A) Hierarchical clustering was based on a binomial distribution of the presence or absence of activity for a portfolio of 131 substrates. The type of habitat (deep sea, superficial seawater, or brackish water) and the estimation of enzyme versatility, as estimated by the number of substrates (substrate range, low [⇓], medium [⇓ ⇑], or high [⇑]) upon which a given enzyme acts are shown for the enzymes in each cluster. (B) Box plots of the specific activity (U mg^{-1}) for the set of enzymes and esters shown in panel A. (A complete list of the esters tested is given in the materials and methods section of the supplemental material.) The activity values for enzymes other than those investigated in this study are reported elsewhere (3, 6, 27, 28, 37, 53). The values for MGS-RG1, MGS-RG2, and MGS-RG3 are shown in Fig. 4 and 5.

also were found to be enantioselective to different degrees for 5 chiral esters, which were found to serve as substrates for these enzymes (Fig. 5). Whereas methyl-(±)-mandelate, (±)-glycidyl-4-nitrobenzoate and (±)-methyl-3-bromo-2-methyl propionate were hydrolyzed by MGS-RG1 and MGS-RG2, methyl-(±)-lactate was a substrate for MGS-RG2 only, and menthyl-(±)-acetate was a substrate for MGS-RG3 only. The specific activities of the three enzymes for chiral esters ranged from 0.021 ± 0.006 to 10.97 ± 1.18 U mg^{-1}, and methyl-(*S*)-3-bromo-2-methyl propionate was the preferred chiral substrate. Based on a calculation of the apparent enantiomeric ratios for separate enantiomers (6), the enantiomeric ratios and substrate preferences are tentatively the following: (i) 158.4 ± 11.0 [preference for methyl-(*S*)-mandelate], 19.6 ± 0.8 [for (*S*)-glycidyl-4-nitrobenzoate], and 20.0 ± 0.7 [for (*S*)-methyl-3-bromo-2-methyl propionate] for MGS-RG1; (ii) 16.1 ± 1.3 [for methyl-(*S*)-lactate], 16.3 ± 1.1 [for (*S*)-glycidyl-4-nitrobenzoate], 26.1 ± 2.2 [for (*S*)-methyl-3-bromo-2-methyl propionate], and 65.4 ± 7.9 [for methyl-(*S*)-mandelate] for MGS-RG2; and (iii) 300 ± 9.3 for MGS-RG3 and menthyl-(*R*)-acetate.

Substrate fingerprints: comparison with known enzymes. The substrate profiles of the enzymes reported in this study against a set of 131 ester substrates (see the section on materials and methods in the supplemental material) were compared to a set of well-characterized commercial preparations with high substrate versatility (including CalA, CalB, Novozym 388 L, and PLE) and a set of carboxyl esterases obtained via genomic mining of marine bacteria (ABO_1197, ABO_1251, CCSP0084, CCSP0211, CCSP0528, and CCSP2178) or via metagenomic approaches of communities from marine (27, 28) and brackish sediment samples (6).

A cluster analysis (Fig. 6A), which was generated from a binomial distribution based on the presence or absence of activity at 30°C and at pH 8.0 against the set of 131 different esters used in

this study and reported previously (3, 6, 27, 28, 37, 53), revealed that the three characterized carboxyl esterases clustered separately, consistent with the finding that they possess different substrate spectra (Fig. 4 and 5). Notably, the positioning of the enzymes based on their substrate profiles did not correlate with the scattered positioning based on sequence analysis (see Fig. S2 in the supplemental material), suggesting that the phylogenetic positions of these enzymes are independent of their biochemical profiles.

As shown in Fig. 6A, a number of observations can be made from the data. First, MGS-RG1 clustered more closely with a group of enzymes from marine (28) and brackish lake (6) samples that have been reported to be cold adapted and that show a broad substrate range. Interestingly, MGS-RG2 was functionally closer and possesses a substrate spectrum similar to that of a group of enzymes with a substrate portfolio of medium size; this group includes a recently reported carboxyl esterase isolated from a 100-m-depth seawater sample obtained from a hydrothermal vent at Saint Paul Island (3). Finally, MGS-RG3 is located within a group of enzymes with a highly restricted substrate range; this group includes two enzymes from deep-sea saline lakes in the Mediterranean Sea (3) and enzymes from oil-contaminated seawater samples (27). Of the enzymes tested, MGS-RG1 was the only enzyme able to hydrolyze methyl-2-Br-propionate. In addition, MGS-RG1 and MGS-RG3 showed various capacities to hydrolyze (*R,S*)-glycidyl 4-nitrobenzoate and methyl-2-bromo-2-butenoate, esters that otherwise were hydrolyzed only by MGS-HA1, an enzyme isolated from a hydrothermal vent (3).

DISCUSSION

Hydrothermal vents in the Mid-Atlantic Ridge hosting the deep-sea hydrothermal vent shrimp *R. exoculata* (19) lie 2,320 m below sea level, exhibit a salinity slightly lower than that of seawater

($\sim$23.03 versus $\sim$35.5 g kg^{-1}), and are characterized by high pressure ($\sim$23,000 kPa) and by a moderately low-to-warm temperature gradient that consistently ranges from 3 to 25°C (19). Using a metagenomic approach, the DNA of microbial communities inhabiting the gill chamber of *R. exoculata* was harvested and cloned to establish the fosmid library, which was used to screen for enzymatic activities of interest. This screening led to the discovery of three new carboxyl esterases, which were biochemically characterized after expression in a surrogate microbial host (*E. coli*). The results of this study demonstrate that microbes associated with the gill chamber of *R. exoculata* contain biochemically heterogeneous carboxyl esterases belonging to the α/β-hydrolase family; the properties of the newly discovered esterases correlate with their differing bacterial origins and most likely with different adaptations of their hosts to the prevailing environmental conditions.

The taxonomic distribution of top protein hits, as well as genome linguistics analysis, suggested that the metagenomic fragments containing these enzymes belong to *Alphaproteobacteria* (*Rhodobacterales*) and *Gammaproteobacteria* (unknown species or a genus related to *Thiothrix* and/or *Leucothrix*) from the *R. exoculata* gill chamber. This finding is consistent with the fact that *Alphaproteobacteria* and *Thiothrix/Leucothrix* (the closest cultured relative of which is the sulfur-oxidizing bacterium *L. mucor* [57]) accounted for approximately 4% and 7.5%, respectively, of the total *R. exoculata* gill chamber microbial population, as reported earlier, and that they were absent from the habitat seawater (19). Notably, *Thiothrix/Leucothrix*-related bacteria are of interest not only because of their location in a presently genomically uncharacterized region of the tree of life (51) but also because no functional data with respect to their metabolic capacities or their enzyme arsenal have been reported in the specialized literature.

The activity levels of the characterized enzymes (maximum for best substrates of 25.2 to 356 U mg^{-1}, depending on the carboxyl esterase) are in the range of other reported enzymes with esterase and lipase activity from macroorganism-associated microbiota. The only such enzymes previously reported are associated with marine sponges and have maximum activities of 2.8 to 2,700 U mg^{-1} (15, 16). The activity levels of the esterases reported here against ester substrates under conditions similar to those used in this study were similar to or somewhat lower than those of commercial preparations, as well as to those of previously reported enzymes isolated from marine seawater and from brackish water (average of up to 1,227 U mg^{-1}) (Fig. 6B). Their activities also were comparable to the activities of previously characterized carboxyl esterases from Mediterranean deep-sea locations (average, 54.5 U mg^{-1} [3]) and other marine deep-sea sediment ($\sim$1.70 to 560 U mg^{-1} [58–60]). The specific activities of these enzymes also were in the range of that observed for most similar proteins (34 to 39% sequence identity) that have been characterized (150 to 2,500 U mg^{-1} [61, 62]).

Further, the biochemical properties of the novel carboxyl esterases reported in this study revealed that although all three enzymes showed an activity-stability trade-off characteristic of cold-adapted enzymes, MGS-RG1 and MGS-RG2 exhibit higher temperature windows and greater thermostability than MGS-RG3. This finding is in agreement with the stability trade-off found via determination of the aggregation/denaturation temperatures (T_d) of these enzymes. Thus, the T_ds for MGS-RG1 (47.6°C) and MGS-RG2 (53.3°C) are higher than the T_d for MGS-RG3 (42.0°C). The T_d for the first subgroup of enzymes is comparable to that of the OLEI01171 carboxyl esterase from the cold-adapted marine bacterium *Oleispira antarctica* RB-8 (45.7°C), as well as to those of previously described esterases from superficial seawater (45.7 to 48.1°C [28]) and deep-sea (40.3 to 71.4°C [3]) (Table 2) and mesophilic bacteria (51 to 60°C [63]), whereas that for MGS-RG3 is slightly lower. The higher (for MGS-RG2) and lower (for MGS-RG3) T_ds are consistent with the fact that MGS-RG2 was the most active of the three enzymes at 55°C (70% of maximal activity), and that MGS-RG3 was the most active at 4°C (40% of maximal activity). The optimal temperatures for the activities of MGS-RG1 (45°C) and MGS-RG2 (50°C) are within the range of optimal temperatures of most similar characterized enzymes, including the top hit, EstB, from *Pseudomonas fluorescens* (34 to 37% amino acid sequence identity; UniProtKB/Swiss-Prot code Q51758.1), which has an optimal temperature for activity of 45°C (61). A similar observation was made for MGS-RG3; in that case, the most similar previously characterized hydrolase, MLH (39% amino acid sequence identity), showed an optimal temperature of 30°C and similar residual activity at low temperature (50% of the maximum activity at 10°C; UniProtKB/Swiss-Prot code Q9EX73.1 [62]). Differences in optimal temperature of at least 10 to 20°C between the two groups of enzymes investigated in this study also have been reported for several carboxyl esterases from the marine bacteria *Alcanivorax borkumensis* SK2 (28) and *Oleispira antarctica* RB-8 (64), as well as from uncultured bacteria from seawater samples (28), suggesting that such heterogeneous profiles are common in cold or moderate-temperature marine habitats.

Similarly, while the MGS-RG1 and MGS-RG2 enzymes exhibited similar hyperactivation at high salt concentrations and similar resistance to high pressure, MGS-RG3 exhibited low remaining activity in the presence of salt and pressure. Because salt and temperature dependence have not been reported for most similar enzymes, a direct comparison could not be done. However, examination of the effect of salt concentration on the activities of metagenomic esterases (3, 28) revealed that heterogeneous effects also are common among enzymes found in marine habitats, including deep-sea habitats (Table 2). Notably, the level of activation of both MGS-RG1 and MGS-RG2, which was as high as 2.4-fold at 3.2 to 3.6 M salt, and the significant pressure tolerance of these enzymes (>97% at 35,000 kPa) are similar to the salt and pressure dependence reported for other deep-sea enzymes (3), with similar optimal temperatures for activity. This is an important factor, because it was recently found that the development of enzymes (i.e., MGS-M2 [Table 2]) with higher optimal temperatures for activity by organisms in deep-sea environments can be linked to pressure and salt resistance, implicating yet-unknown mechanisms in this type of adaptation (3). In agreement with this, MGS-RG3, which exhibits low resistance to salt and pressure, was cold adapted, whereas the halophilic and barotolerant MGS-RG1 and MGS-RG2 enzymes were more active at moderate temperatures.

The different sensitivities of the three enzymes to environmental constraints, namely, temperature, pressure, and salinity, also were reflected at the level of reaction specificities and reactivities, with MGS-RG1 and MGS-RG2 being at least 14-fold less active than MGS-RG3 yet capable of accepting a broader range of ester substrates. Based on these findings, we speculate that the existence of two distinct subgroups of these enzymes based on their biochemical properties is a direct consequence of the different bacterial origins of the enzymes, as well as of the different adaptation

capacities of the corresponding bacterial hosts to the prevailing environmental conditions (pressure, salinity, and temperature). In addition, we infer that such differences also become marked at the highest level of functional hierarchy, that is, in relation to the substrate spectra of the enzymes, differences that are likely to support the different abilities of the corresponding bacterial hosts to metabolize nutrients. In agreement with this, we found that, in contrast to MGS-RG3, MGS-RG1 and MGS-RG2 are able to hydrolyze triacylglycerols; (non)halogenated alkyl, alkenyl, and aryl esters; lactones; chiral epoxides; and/or cinnamoyl and carbohydrate esters to similar extents. Such broad promiscuity is rare among enzymes with esterase and lipase activity (6, 28). In addition, both MGS-RG1 and MGS-RG2 can support polysaccharide degradation, an enzymatic activity that is critical for the ecological success of microorganisms in deep-sea environments, as well as for the utilization by these microorganisms of nutrients produced or liberated by the host in the gill chamber (50, 56). The polysaccharide degradation capability of these enzymes was demonstrated by their ability to degrade tri-O-acetyl-glucal, α-D-glucose pentaacetate, and ethyl-*trans*-cinnamate.

Finally, it should be emphasized that *R. exoculata* has been reported to move from one vent to another; thus, the microbes inhabiting the gill chamber of this species might contain versatile enzymes capable of adaptation to environmental and nutrient-availability gradients. This hypothesis is supported by the results presented here, which demonstrate the heterogeneous salt, pressure, and temperature dependence and stability tradeoff of the three enzymes investigated. As shown in Fig. 2 and 3 and Table 2, all three enzymes, albeit to different extents, showed enzymatic activity under the relevant conditions in the hydrothermal vent (3 to 25°C; 23.03 g kg^{-1} salt [or 0.42 M NaCl]; pressure, 230 atm at depth of 2,320 m). In addition, the heterogeneous substrate profiles of the three enzymes also agree with previous considerations. Further experimental evidence on this issue with a larger set of enzymes will be required to determine whether heterogeneity is common in other marine habitats. Related to this point, Fig. 6A of our work clearly shows that 3 of 4 enzymes from the polyaromatic-degrading marine bacterium *Cycloclasticus* sp. strain ME7 (CCSP codes), 2 from seawater at Kolguev Island (K codes), 2 from deep-sea Medee Lake (MGS-M codes), 3 from other deep-sea habitats (MGS-B/K/MT codes), 7 from a karst lake (LAE codes), and a set of 5 from oil-contaminated marine seawater samples and bacteria isolated from those samples (ABO codes and MGS0105, MGS0109, and MGS1010) formed separated homogeneous clusters in their reactivity profiles. Accordingly, the fact that other enzymes from different locations were ranked functionally closer to each other compared to enzymes from other locations while those from *R. exoculata* were scattered within the reactivity-based tree may support the existence of a wide functional heterogeneity in the gill chamber of *R. exoculata*. Note that the substrate profiles of the enzymes reported in this study also differ significantly from those of the most similar (34 to 39% sequence identity) characterized enzymes (61, 62); thus, structural factors may account for such differences.

Using a library of structurally diverse esters, we further demonstrated that the enzymes described in this study are characterized by high hydrolytic rates and broad substrate spectra together with enantioselectivity, all of which are desired features for biocatalysis. As an example, an enantiomeric ratio greater than 20 typically is associated with hydrolases with high enantioselectivity

(6). Notably, the high and broad activity of these enzymes toward halogenated esters also may be of use in the optical enrichment of starting haloesters (65). We also found that while MGS-RG1 accepts methyl benzoate as the substrate, MGS-RG3 accepts only vinyl benzoate; therefore, we speculate that MGS-RG1 and MGS-RG3 can be used for selective transesterification procedures that rely upon irreversible benzoylation with methyl or vinyl esters (66).

The enzyme characteristics and versatile reactivity of the enzymes reported in this study, from a biochemically unexploited marine habitat and from a neglected taxonomic group of bacteria associated with macroorganisms inhabiting deep-sea hydrothermal vents, must be further evaluated and exploited on a larger scale.

Finally, one question that might rise after examining the phylogenetic positioning of sequences (see Fig. S2 in the supplemental material) and the biochemical clustering (Fig. 6) of all deep-sea enzymes, including the ones reported here, is whether the evolution of these model enzymes suggests any particular constraint in the deep-sea environment. The limited number of esterases (about 8) from deep-sea environments identified and characterized to date (3) does not allow us to reach a clear-cut conclusion. However, the distinct placement of some of the deep-sea enzymes at the level of substrate profiles (i.e., MGS-MT1, B1, K1, M1, and M2) (Fig. 6) and at the level of sequence (see Fig. S2) may point at the drivers of the evolution of enzymes in the deep sea. However, before drawing any conclusions, a further experimental analysis with a larger set of deep-sea enzymes is needed.

REFERENCES

1. **Singh BK.** 2010. Exploring microbial diversity for biotechnology: the way forward. Trends Biotechnol **28**:111–116. http://dx.doi.org/10.1016/j.tibtech.2009.11.006.
2. **Chistoserdova L.** 2014. Is metagenomics resolving identification of functions in microbial communities? Microb Biotechnol **7**:1–4. http://dx.doi.org/10.1111/1751-7915.12077.
3. **Alcaide M, Stogios PJ, Lafraya A, Tchigvintsev A, Flick R, Bargiela R, Chernikova TN, Reva ON, Hai T, Leggewie CC, Katzke N, La Cono V, Matesanz R, Jebbar M, Jaeger KE, Yakimov MM, Yakunin AF, Golyshin PN, Golyshina OV, Savchenko A, Ferrer M.** 20 October 2014. Pressure adaptation is linked to thermal adaptation in salt-saturated marine habitats. Environ Microbiol http://dx.doi.org/10.1111/1462-2920.12660.
4. **Guazzaroni ME, Silva-Rocha R, Ward RJ.** 13 August 2014. Synthetic biology approaches to improve biocatalyst identification in metagenomic library screening. Microb Biotechnol http://dx.doi.org/10.1111/1751-7915.12146.
5. **Puspita ID, Kamagata Y, Tanaka M, Asano K, Nakatsu CH.** 2012. Are uncultivated bacteria really uncultivable? Microbes Environ **27**:356–366. http://dx.doi.org/10.1264/jsme2.ME12092.

6. Martínez-Martínez M, Alcaide M, Tchigvintsev A, Reva O, Polaina J, Bargiela R, Guazzaroni ME, Chicote A, Canet A, Valero F, Rico Eguizabal E, Guerrero Mdel C, Yakunin AF, Ferrer M. 2013. Biochemical diversity of carboxyl esterases and lipases from Lake Arreo (Spain): a metagenomic approach. Appl Environ Microbiol 79:3553–3562. http://dx.doi.org/10.1128/AEM.00240-13.

7. Yarza P, Yilmaz P, Pruesse E, Glöckner FO, Ludwig W, Schleifer KH, Whitman WB, Euzéby J, Amann R, Rosselló-Móra R. 2014. Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences. Nat Rev Microbiol 12:635–645. http://dx.doi.org/10.1038/nrmicro3330.

8. Kyrpides NC, Hugenholtz P, Eisen JA, Woyke T, Göker M, Parker CT, Amann R, Beck BJ, Chain PS, Chun J, Colwell RR, Danchin A, Dawyndt P, Dedeurwaerdere T, DeLong EF, Detter JC, De Vos P, Donohue TJ, Dong XZ, Ehrlich DS, Fraser C, Gibbs R, Gilbert J, Gilna P, Glöckner FO, Jansson JK, Keasling JD, Knight R, Labeda D, Lapidus A, Lee JS, Li WJ, Ma J, Markowitz V, Moore ER, Morrison M, Meyer F, Nelson KE, Ohkuma M, Ouzounis CA, Pace N, Parkhill J, Qin N, Rossello-Mora R, Sikorski J, Smith D, Sogin M, Stevens R, Stingl U, Suzuki K, et al. 2014. Genomic encyclopedia of bacteria and archaea: sequencing a myriad of type strains. PLoS Biol 12:e1001920. http://dx.doi.org/10.1371/journal.pbio.1001920.

9. Jemli S, Ayadi-Zouari D, Hlima HB, Bejar S. 2014. Biocatalysts: application and engineering for industrial purposes. Crit Rev Biotechnol 6:1–13.

10. Timmis K, de Lorenzo V, Verstraete W, Garcia JL, Ramos JL, Santos H, Economidis I, Nogales B, Timmis JK, Fonseca C, Pruzzo C, Karagouni A, Panopoulos N, Dixon B. 2014. Pipelines for new chemicals: a strategy to create new value chains and stimulate innovation-based economic revival in southern European countries. Environ Microbiol 16:9–18. http://dx.doi.org/10.1111/1462-2920.12337.

11. Harrison JP, Gheeraert N, Tsigelnitskiy D, Cockell CS. 2013. The limits for life under multiple extremes. Trends Microbiol 21:204–212. http://dx.doi.org/10.1016/j.tim.2013.01.006.

12. Schmidt PS, Serrão EA, Pearson GA, Riginos C, Rawson PD, Hilbish TJ, Brawley SH, Trussell GC, Carrington E, Wethey DS, Grahame JW, Bonhomme F, Rand DM. 2008. Ecological genetics in the North Atlantic: environmental gradients and adaptation at specific loci. Ecology 89:S91–S107. http://dx.doi.org/10.1890/07-1162.1.

13. Petersen JM, Zielinski FU, Pape T, Seifert R, Moraru C, Amann R, Hourdez S, Girguis PR, Wankel SD, Barbe V, Pelletier E, Fink D, Borowski C, Bach W, Dubilier N. 2011. Hydrogen is an energy source for hydrothermal vent symbioses. Nature 476:176–180. http://dx.doi.org/10.1038/nature10325.

14. Karpushova A, Brümmer F, Barth S, Lange S, Schmid RD. 2005. Cloning, recombinant expression and biochemical characterisation of novel esterases from *Bacillus* sp. associated with the marine sponge *Aplysina aerophoba*. Appl Microbiol Biotechnol 67:59–69. http://dx.doi.org/10.1007/s00253-004-1780-6.

15. Okamura Y, Kimura T, Yokouchi H, Meneses-Osorio M, Katoh M, Matsunaga T, Takeyama H. 2010. Isolation and characterization of a GDSL esterase from the metagenome of a marine sponge-associated bacteria. Mar Biotechnol 12:395–402. http://dx.doi.org/10.1007/s10126-009-9226-x.

16. Selvin J, Kennedy J, Lejon DP, Kiran GS, Dobson AD. 2012. Isolation identification and biochemical characterization of a novel halo-tolerant lipase from the metagenome of the marine sponge *Haliclona simulans*. Microb Cell Fact 11:72. http://dx.doi.org/10.1186/1475-2859-11-72.

17. Kim D, Kim SN, Baik KS, Park SC, Lim CH, Kim JO, Shin TS, Oh MJ, Seong CN. 2011. Screening and characterization of a cellulase gene from the gut microflora of abalone using metagenomic library. J Microbiol 49:141–145. http://dx.doi.org/10.1007/s12275-011-0205-3.

18. Sim SJ, Baik KS, Park SC, Choe HN, Seong CN, Shin TS, Woo HC, Cho JY, Kim D. 2012. Characterization of alginate lyase gene using a metagenomic library constructed from the gut microflora of abalone. J Ind Microbiol Biotechnol 39:585–593. http://dx.doi.org/10.1007/s10295-011-1054-0.

19. Jan C, Petersen JM, Werner J, Teeling H, Huang S, Glöckner FO, Golyshina OV, Dubilier N, Golyshin PN, Jebbar M, Cambon-Bonavita MA. 2014. The gill chamber epibiosis of deep-sea shrimp *Rimicaris exoculata*: an in-depth metagenomic investigation and discovery of Zetaproteobacteria. Environ Microbiol 16:2723–2738. http://dx.doi.org/10.1111/1462-2920.12406.

20. Charlaou JL, Donwal JP, Fouquet Y, Jean-Baptiste P, Jean-Luc C, Jean-Pierre D, Yves F, Jean-Baptiste P, Holm N. 2002. Geochemistry of high H_2 and CH_4 vent fluids issuing from ultramafic rocks at the Rainbow hydrothermal field (36°14′N, MAR). Chem Geol 191:345–359. http://dx.doi.org/10.1016/S0009-2541(02)00134-1.

21. Zbinden M, Shillito B, Le Bris N, de Villardi de Montlaur C, Roussel E, Guyot F, Gaill F, Cambon-Bonavita M-A. 2008. New insights on the metabolic diversity among the epibiotic microbial community of the hydrothermal shrimp *Rimicaris exoculata*. J Exp Mar Biol Ecol 359:131–140. http://dx.doi.org/10.1016/j.jembe.2008.03.009.

22. Hügler M, Petersen JM, Dubilier N, Imhoff JF, Sievert SM. 2011. Pathways of carbon and energy metabolism of the epibiotic community associated with the deep-sea hydrothermal vent shrimp *Rimicaris exoculata*. PLoS One 6:e16018. http://dx.doi.org/10.1371/journal.pone.0016018.

23. Guri M, Durand L, Cueff-Gauchard V, Zbinden M, Crassous P, Shillito B, Cambon-Bonavita MA. 2012. Acquisition of epibiotic bacteria along the life cycle of the hydrothermal shrimp *Rimicaris exoculata*. ISME J 6:597–609. http://dx.doi.org/10.1038/ismej.2011.133.

24. Ponsard J, Cambon-Bonavita MA, Zbinden M, Lepoint G, Joassin A, Corbari L, Shillito B, Durand L, Cueff-Gauchard V, Compere P. 2013. Inorganic carbon fixation by chemosynthetic ectosymbionts and nutritional transfers to the hydrothermal vent host-shrimp *Rimicaris exoculata*. ISME J 7:96–109. http://dx.doi.org/10.1038/ismej.2012.87.

25. Konn C, Testemale D, Querellou J, Holm NG, Charlou JL. 2011. New insight into the contributions of thermogenic processes and biogenic sources to the generation of organic compounds in hydrothermal fluids. Geobiology 9:79–93. http://dx.doi.org/10.1111/j.1472-4669.2010.00260.x.

26. Wahl M, Goecke F, Labes A, Dobretsov S, Weinberger F. 2012. The second skin: ecological role of epibiotic biofilms on marine organisms. Front Microbiol 3:292. http://dx.doi.org/10.3389/fmicb.2012.00292.

27. Alcaide M, Tornés J, Stogios PJ, Xu X, Gertler C, Di Leo R, Bargiela R, Lafraya A, Guazzaroni ME, López-Cortés N, Chernikova TN, Golyshina OV, Nechitaylo TY, Plumeier I, Pieper DH, Yakimov MM, Savchenko A, Golyshin PN, Ferrer M. 2013. Single residues dictate the co-evolution of dual esterases: MCP hydrolases from the α/β hydrolase family. Biochem J 454:157–166. http://dx.doi.org/10.1042/BJ20130552.

28. Tchigvintsev A, Tran H, Popovic A, Kovacic F, Brown G, Flick R, Hajighasemi M, Egorova O, Somody JC, Tchigvintsev D, Khusnutdinova A, Chernikova TN, Golyshina OV, Yakimov MM, Savchenko A, Golyshin PN, Jaeger KE, Yakunin AF. 7 September 2014. The environment shapes microbial enzymes: five cold-active and salt-resistant carboxylesterases from marine metagenomes. Appl Microbiol Biotechnol http://dx.doi.org/10.1007/s00253-014-6038-3.

29. Turner NJ, Truppo MD. 2013. Biocatalysis enters a new era. Curr Opin Chem Biol 17:212–214. http://dx.doi.org/10.1016/j.cbpa.2013.02.026.

30. Reyes-Duarte D, Ferrer M, García-Arellano H. 2012. Functional-based screening methods for lipases esterases and phospholipases in metagenomic libraries. Methods Mol Biol 861:101–113. http://dx.doi.org/10.1007/978-1-61779-600-5_6.

31. Lukashin AV, Borodovsky M. 1998. GeneMark.hmm: new solutions for gene finding. Nucleic Acids Res 26:1107–1115. http://dx.doi.org/10.1093/nar/26.4.1107.

32. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402. http://dx.doi.org/10.1093/nar/25.17.3389.

33. Hall TA. 1999. Bioedit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98.

34. Hofmann K, Stoffel W. 1993. TMbase—a database of membrane spanning proteins segments. Biol Chem Hoppe-Seyler 374:166.

35. Pace N, Scholtz JM. 1997. Measuring the conformational stability of a protein, p 299–322. *In* Creighton TE (ed), Protein structure: a practical approach. IRL Press at Oxford University Press, Oxford, United Kingdom.

36. Schmid FZ. 1997. Optical spectroscopy to characterize protein conformation, p 261–298. *In* Creighton TE (ed), Protein structure: a practical approach. IRL Press at Oxford University Press, Oxford, United Kingdom.

37. Blin K, Medema MH, Kazempour D, Fischbach MA, Breitling R, Takano E, Weber T. 2013. AntiSMASH 2.0—a versatile platform for genome mining of secondary metabolite producers. Nucleic Acids Res 1:W204–W212.

38. **Rutherford K, Parkhill J, Crook J, Horsnell T, Rice P, Rajandream MA, Barrell B.** 2000. Artemis: sequence visualization and annotation. Bioinformatics **16:**944–945. http://dx.doi.org/10.1093/bioinformatics/16.10.944.

39. **Stothard P.** 2000. The Sequence Manipulation Suite: JavaScript programs for analyzing and formatting protein and DNA sequences. Biotechniques **28:**1102–1104.

40. **Ménigaud S, Mallet L, Picord G, Churlaud C, Borrel A, Deschavanne P.** 2012. GOHTAM: a website for genomic origin of horizontal transfers, alignment and metagenomics. Bioinformatics **28:**1270–1271. http://dx.doi.org/10.1093/bioinformatics/bts118.

41. **Suzuki H, Saito R, Tomita M.** 2009. Measure of synonymous codon usage diversity among genes in bacteria. BMC Bioinformatics **10:**167. http://dx.doi.org/10.1186/1471-2105-10-167.

42. **Ganesan H, Rakitianskaia AS, Davenport CF, Tümmler B, Reva ON.** 2008. The SeqWord Genome Browser: an online tool for the identification and visualization of atypical regions of bacterial genomes through oligonucleotide usage. BMC Bioinformatics **9:**333. http://dx.doi.org/10.1186/1471-2105-9-333.

43. **Edgar RC.** 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics **5:**113. http://dx.doi.org/10.1186/1471-2105-5-113.

44. **Tamura K, Stecher G, Peterson D, Filipski A, Kumar S.** 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol **30:**2725–2729. http://dx.doi.org/10.1093/molbev/mst197.

45. **Filippova EV, Weston LA, Kuhn ML, Geissler B, Gehring AM, Armoush N, Adkins CT, Minasov G, Dubrovska I, Shuvalova L, Winsor JR, Lavis LD, Satchell KJ, Becker DP, Anderson WF, Johnson RJ.** 2013. Large scale structural rearrangement of a serine hydrolase from *Francisella tularensis* facilitates catalysis. J Biol Chem **288:**10522–10535. http://dx.doi.org/10.1074/jbc.M112.446625.

46. **Arpigny JL, Jaeger KE.** 1999. Bacterial lipolytic enzymes: classification and properties. Biochem J **343:**177–183. http://dx.doi.org/10.1042/0264-6021:3430177.

47. **Hausmann S, Jaeger K-E.** 2010. Lipolytic enzymes from bacteria, p 1099–1126. *In* Timmis KN (ed), Handbook of hydrocarbon and lipid microbiology. Springer, Berlin, Germany.

48. **Kourist R, Jochens H, Bartsch S, Kuipers R, Padhi SK, Gall M, Böttcher D, Joosten HJ, Bornscheuer UT.** 2010. The alpha/beta-hydrolase fold 3DM database (ABHDB) as a tool for protein engineering. Chembiochem **11:**1635–1643. http://dx.doi.org/10.1002/cbic.201000213.

49. **Howarth R, Unz RF, Seviour EM, Seviour RJ, Blackall LL, Pickup RW, Jones JG, Yaguchi J, Head IM.** 1999. Phylogenetic relationships of filamentous sulfur bacteria (*Thiothrix* spp. and Eikelboom type 021N bacteria) isolated from wastewater-treatment plants and description of *Thiothrix eikelboomii* sp. nov., *Thiothrix unzii* sp. nov., *Thiothrix fructosivorans* sp. nov. and *Thiothrix defluvii* sp. nov. Int J Syst Bacteriol **4:**1817–1827.

50. **Brock TD.** 2006. The genus *Leucothrix*. Prokaryotes **6:**931–938.

51. **Lapidus A, Nolan M, Lucas S, Glavina Del Rio T, Tice H, Cheng JF, Tapia R, Han C, Goodwin L, Pitluck S, Liolios K, Pagani I, Ivanova N, Huntemann M, Mavromatis K, Mikhailova N, Pati A, Chen A, Palaniappan K, Land M, Brambilla EM, Rohde M, Abt B, Verbarg S, Göker M, Bristow J, Eisen JA, Markowitz V, Hugenholtz P, Kyrpides NC, Klenk HP, Woyke T.** 2011. Genome sequence of the filamentous, gliding *Thiothrix nivea* neotype strain (JP2ᵀ). Stand Genomic Sci **5:**398–406. http://dx.doi.org/10.4056/sigs.2344929.

52. **Qian Z, Lutz S.** 2005. Improving the catalytic activity of *Candida antarctica* lipase B by circular permutation. J Am Chem Soc **127:**13466–13467. http://dx.doi.org/10.1021/ja053932h.

53. **Martínez-Martínez M, Lores I, Peña-García C, Bargiela R, Reyes-Duarte D, Guazzaroni ME, Peláez AI, Sánchez J, Ferrer M.** 2014. Biochemical studies on a versatile esterase that is most catalytically active with polyaromatic esters. Microb Biotechnol **7:**184–191. http://dx.doi.org/10.1111/1751-7915.12107.

54. **Beloqui A, Nechitaylo TY, López-Cortés N, Ghazi A, Guazzaroni ME, Polaina J, Strittmatter AW, Reva O, Waliczek A, Yakimov MM, Goly-
shina OV, Ferrer M, Golyshin PN.** 2010. Diversity of glycosyl hydrolases from cellulose-depleting communities enriched from casts of two earthworm species. Appl Environ Microbiol **76:**5934–5946. http://dx.doi.org/10.1128/AEM.00902-10.

55. **Vieites JM, Ghazi A, Beloqui A, Polaina J, Andreu JM, Golyshina OV, Nechitaylo TY, Waliczek A, Yakimov MM, Golyshin PN, Ferrer M.** 2010. Inter-conversion of catalytic abilities in a bifunctional carboxyl/feruloyl-esterase from earthworm gut metagenome. Microb Biotechnol **3:**48–58. http://dx.doi.org/10.1111/j.1751-7915.2009.00135.x.

56. **Werner J, Ferrer M, Michel G, Mann AJ, Huang S, Juarez S, Ciordia S, Albar JP, Alcaide M, La Cono V, Yakimov MM, Antunes A, Taborda M, da Costa MS, Hai T, Glöckner FO, Golyshina OV, Golyshin PN, Teeling H, Consortium MAMBA.** 2014. *Halorhabdus tiamatea*: proteogenomics and glycosidase activity measurements identify the first cultivated euryarchaeon from a deep-sea anoxic brine lake as potential polysaccharide degrader. Environ Microbiol **16:**2525–2537. http://dx.doi.org/10.1111/1462-2920.12393.

57. **Grabovich MY, Muntyan MS, Lebedeva VY, Ustiyan VS, Dubinina GA.** 1999. Lithoheterotrophic growth and electron transfer chain components of the filamentous gliding bacterium *Leucothrix mucor* DSM 2157 during oxidation of sulfur compounds. FEMS Microbiol Rev **178:**155–161. http://dx.doi.org/10.1111/j.1574-6968.1999.tb13772.x.

58. **Hårdeman F, Sjöling S.** 2007. Metagenomic approach for the isolation of a novel low-temperature-active lipase from uncultured bacteria of marine sediment. FEMS Microbiol Ecol **59:**524–534. http://dx.doi.org/10.1111/j.1574-6941.2006.00206.x.

59. **Jeon JH, Kim JT, Kim YJ, Kim HK, Lee HS, Kang SG, Kim SJ, Lee JH.** 2009. Cloning and characterization of a new cold-active lipase from a deep-sea sediment metagenome. Appl Microbiol Biotechnol **81:**865–874. http://dx.doi.org/10.1007/s00253-008-1656-2.

60. **Jeon JH, Kim JT, Lee HS, Kim SJ, Kang SG, Choi SH, Lee JH.** 2011. Novel lipolytic enzymes identified from metagenomic library of deep-sea sediment. Evid Based Complement Alternat Med **2011:**271419. http://dx.doi.org/10.1155/2011/271419.

61. **Hong KH, Jang WH, Choi KD, Yoo OJ.** 1991. Characterization of *Pseudomonas fluorescens* carboxylesterase: cloning and expression of the esterase gene in *Escherichia coli*. Agric Biol Chem **55:**2839–2845. http://dx.doi.org/10.1271/bbb1961.55.2839.

62. **van der Vlugt-Bergmans CJ, van der Werf MJ.** 2001. Genetic and biochemical characterization of a novel monoterpene epsilon-lactone hydrolase from *Rhodococcus erythropolis* DCL14. Appl Environ Microbiol **67:**733–741. http://dx.doi.org/10.1128/AEM.67.2.733-741.2001.

63. **Lemak S, Tchigvintsev A, Petit P, Flick R, Singer AU, Brown G, Evdokimova E, Egorova O, Gonzalez CF, Chernikova TN, Yakimov MM, Kube M, Reinhardt R, Golyshin PN, Savchenko A, Yakunin AF.** 2012. Structure and activity of the cold-active and anion-activated carboxyl esterase OLEI01171 from the oil-degrading marine bacterium *Oleispira antarctica*. Biochem J **445:**193–203. http://dx.doi.org/10.1042/BJ20112113.

64. **Kube M, Chernikova TN, Al-Ramahi Y, Beloqui A, Lopez-Cortez N, Guazzaroni ME, Heipieper HJ, Klages S, Kotsyurbenko OR, Langer I, Nechitaylo TY, Lünsdorf H, Fernández M, Juárez S, Ciordia S, Singer A, Kagan O, Egorova O, Petit PA, Stogios P, Kim Y, Tchigvintsev A, Flick R, Denaro R, Genovese M, Albar JP, Reva ON, Martínez-Gomariz M, Tran H, Ferrer M, Savchenko A, Yakunin AF, Yakimov MM, Golyshina OV, Reinhardt R, Golyshin PN.** 2013. Genome sequence and functional genomic analysis of the oil-degrading bacterium *Oleispira antarctica*. Nat Commun **4:**2156. http://dx.doi.org/10.1038/ncomms3156.

65. **Azim A, Sharma SK, Olsen CE, Parmar VS.** 2001. Lipase catalysed synthesis of optically enriched α-haloamides. Bioorg Med Chem **9:**1345–1348. http://dx.doi.org/10.1016/S0968-0896(01)00006-2.

66. **Ciffreda P, Alessandrini L, Terraneo G, Santaniello E.** 2003. Lipase-catalyzed selective benzoylation of 1,2-diols with vinyl benzoate in organic solvents. Tetrahedron Asymmetr **14:**3197–3201. http://dx.doi.org/10.1016/j.tetasy.2003.07.003.