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Novel Antibacterial Proteins from the Microbial Communities Associated with the Sponge *Cymbastela concentrica* and the Green Alga *Ulva australis*▽†

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The functional metagenomic screening of the microbial communities associated with a temperate marine sponge and a green alga identified three novel hydrolytic enzymes with antibacterial activities. The results suggest that uncultured alpha- and gammaproteobacteria contain new classes of proteins that may be a source of antibacterial agents.

As a result of the rising number of multidrug-resistant bacteria, recent years have witnessed an increased demand for novel antibiotic compounds. Indeed, examples of multiple resistances have been reported for strains of *Streptococcus pneumoniae* and *Staphylococcus aureus* across Asia, South America, Australia, and Europe (10, 11, 25, 30, 45, 53).

In order to address multidrug resistance in bacteria, sessile marine invertebrates have been explored for the presence of antibiotics and have proven to be a rich source of such novel compounds (13, 17, 21, 37, 41). For example, more than 200 new bioactive metabolites have been reported from sponges per year in the last decade (51). Unfortunately, compounds from marine sources are often available only in low quantities, thus hampering further development into commercial products (18, 21, 23, 24). Due to the fact that numerous natural products isolated from marine invertebrates show structural similarities to known metabolites of microbial origin (41, 44, 47), bioactive screening has also focused on microorganisms associated with such host surfaces. For example, the antibacterial peptide-polyketide andrimid was found in the extract of a sponge as well as in a *Vibrio* sp. isolated from this host (39). Several bacterial strains from the surface of the alga *Ulva australis* are also known to produce an array of compounds effective against bacteria, fungi, diatoms, and other biofouling organisms (15, 16, 19, 42). These observations suggest that surface-associated microbial communities carry a large potential for new antibiotics and bioactive compounds.

Isolation of bioactives from environmental bacteria, however, faces the limitation that many strains are recalcitrant to culturing (1, 33, 48, 51), and this might be particularly true for obligate or facultative symbionts. To access the uncultured majority of the microbial world (43), functional metagenomic approaches that allow for the expression of environmental DNA from uncultured organisms in surrogate hosts have been developed (6, 22, 32, 35, 36, 46). Functional screening of metagenomic libraries has led to the discovery of several novel bioactives and metabolic pathways (5, 36, 52), but the search for new antibiotics has focused mainly on soil-derived samples.

In this study, we explored functional metagenomic libraries from the microbial communities associated with the living surfaces of two marine organisms, the temperate marine sponge *Cymbastela concentrica* and the green alga *Ulva australis*, for the presence of antibacterial activities. We screened these libraries for the inhibition of a range of target strains, identified novel antibacterial genes, characterized their activities, and determined their phylogenetic origins (for further details on the materials and methods, see the supplemental material).

Functional screening of fosmid libraries identified three clones (two from *C. concentrica* and one from *U. australis*) that showed antibacterial activity against the marine *Bacillus* strain Cc6 (where "Cc" indicates "*C. concentrica*"). All clones lacked zones of inhibition in the absence of expression inducers (i.e., arabinose or IPTG [isopropyl-β-D-thiogalactopyranoside]), indicating that genes from the fosmid insert were responsible for the antibacterial activity. The clearance zones had radii of 0.5, 0.8, and 0.3 cm for fosmid clones CcAb1, CcAb2, and UaAb1 (where "Ua" indicates "*U. australis*"), respectively, while the positive control, CBAA11 (8), had a radius of 0.2 cm (see Fig. S1 in the supplemental material). The clone CcAb1 showed further activity against *Staphylococcus aureus* and *Alteromonas* sp. strain CCSH174 (inhibition zones of 0.5 and 0.6 cm, respectively), and UaAb1 was active toward *S. aureus* and *Klebsiella pneumoniae* (both exhibited a 0.2-cm zone of inhibition). CcAb2 did not exhibit antibacterial activity against additional target strains. These results show that the microbial community associated with the two marine eukaryotes contains genes which encode antibacterial activities against bacteria from both environmental and clinical settings.

Through random transposon mutagenesis, six, eight, and

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three mutants were identified to have lost their antibacterial activities for CcAb1, CcAb2, and UaAb1, respectively. Details of the open reading frames (ORFs) identified, namely, *abg1*, *abg2*, and *abg3*, are shown in Table 1. All three genes had clearly recognizable −10 and −35 boxes of bacterial promoters, suggesting that they are under the control of their own promoters. In addition, Abg1 and Abg2 contained predicted signal peptides (of 31 amino acids [aa], MSASTCLRREYFH CFRVLLIASVLLSGNILA, and 26 aa, MNILNKKLLSILLT VATLFLVTVASA, respectively), which indicates that they are secreted. Complementation of the *abg1* and *abg2* genes into *Escherichia coli* containing either their respective transposon mutant fosmids or the empty pCC1FOS vector (the host fosmid of the libraries) restored their antibacterial properties, showing that the genes were solely responsible for the activities (data not shown). Subcloning of *abg3* from UaAb1 was not successful, despite several attempts, and hence subsequent functional characterizations were performed on the original fosmid and its transposon mutants.

Annotations of *abg1*, *abg2*, and *abg3* showed that they encode novel enzymes, with Abg1 having no significant homology to experimentally characterized proteins, while Abg2 and Abg3 have moderate sequence homology to an esterase from *Burkholderia gladioli* (Swiss-Prot accession no. Q9KX40) and a putative hydrolase from *Acanthamoeba polyphaga*, respectively (Table 1). Abg1, -2, and -3 contain the conserved Pfam domains of GDSL-like lipase, beta-lactamases, and abhydrolase_3, respectively (Table 1). Comparison of the three proteins to the noncurated Swiss-Prot database showed homology to proteins putatively annotated as lipolytic enzymes or beta-lactamases (see Table S2 in the supplemental material). Together, these results indicate that the three proteins may have hydrolytic activities.

To further define the postulated hydrolytic activities, we tested the degradation of the lipid analogue tributyrin. Abg1 and Abg2 were capable of degrading tributyrin, with clearance zones of 0.7 and 0.4 cm, respectively. Fosmid clone UaAb1 also degraded tributyrin, with a clearance zone of 0.5 cm. The transposon mutant of UaAb1 with the disrupted *abg3* gene failed to degrade the substrate, indicating that Abg3 mediates hydrolytic activity (see Fig. S2 in the supplemental material).

Abg2 has similarity to beta-lactamase domains (Table 1) and proteins (see Table S2 in the supplemental material), but when we exposed an *E. coli*/pBAD:Chlor-*abg2* clone to five different beta-lactam antibiotics, no resistance was observed. This shows that Abg2 is unlikely to have true beta-lactamase activity.

The three proteins had less than 20% pairwise sequence identity to each other, yet surprisingly, they all produced hydrolytic/lipolytic activities and conferred antibacterial properties to *E. coli*. We therefore propose that these proteins represent three new classes of antibacterial proteins.

The fosmids containing the antibacterial genes were completely sequenced to gain insight into their genomic context and phylogenetic origin. The antibacterial genes *abg1*, *abg2*, and *abg3* were positioned in ORFs 17, 11, and 20 for CcAb1, CcAb2, and UaAb1, respectively (further details appear in Table S3 and Fig. S3 in the supplemental material). Phylogenetic prediction with the Phylopythia algorithm (38) indicated that clone CcAb1 belongs to the class *Deltaproteobacteria*, while taxonomic prediction with MEGAN (26) assigns more

TABLE 1. Antibacterial genes in metagenomic clones[a]

Clone, ORF name	No. of bp in ORF (no. of aa)/sig. pep. (position)	Transcrip. regions/positions	Cons. domain/position/E value/Pfam ID	% identity/% coverage	Amino acid length	Protein (Swiss-Prot accession no.)	Organism
CcAb1, *abg1*	1,245 (414)/yes (aa 31–32)	−10 box (GGTAATGAT)/bp 144; −35 box (TCTCCA)/bp 165	GDSL-like lipase/aa 209-408/2.1 × 10^{-25}/PF00657	26/13	806	Minor extracellular protease *vpr* precursor (P29141)	*Bacillus subtilis*
				35/27	261	Triosephosphate isomerase (A6WC54)	*Kineococcus radiotolerans*
				31/31	382	Subtilisin BPN precursor (P00782)	*Bacillus amyloliquefaciens*
CcAb2, *abg2*	1,308 (435)/yes (1–26)	−10 box (CCATACAAT)/bp 146; −35 box (TTGCTT)/bp 174	Beta-lactamase_related/aa 48-429/ 2.7 × 10^{-67}/PF00144	30/90	377	Uncharacterized protein Rv1367c (Q11037)	*Mycobacterium tuberculosis*
				30/97	392	Esterase *estB* (Q9KX40)	*Burkholderia gladioli*
				24/92	434	UPF0214 protein *yfeW* precursor (P77619)	*Escherichia coli*
UaAb1, *abg3*	975 (325)/no (NA)	−10 box (AGCTATGCT)/bp 36; −35 box (TAGATA)/bp 56	Abhydrolase_3/aa 88-291/4.6 × 10^{-59}/PF07859	29/58	346	Putative alpha/beta hydrolase R526 (Q5UQ83)	*Acanthamoeba polyphaga mimivirus*
				27/52	433	Lipase 2 (P22484)	*Moraxella* sp. (strain TA144)
				24/62	341	AB hydrolase superfamily protein (Q9US38)	*Schizosaccharomyces pombe*

[a] Sig. pep. (position), the presence (yes) or absence (no) of the signal peptide and its respective position in the amino acid sequence; NA, not applicable; Transcrip. regions/positions, the presence of any transcriptional regions and their relative positions upstream of the antibacterial ORF; Cons. domain/position/E value/Pfam ID, the conserved domain identified from the Pfam database and its position in the amino acid sequence and the E value of a matched conserved domain and its respective Pfam identification number. Top hits from a protein similarity search of the antibacterial protein sequence against the curated part of the Swiss-Prot database, along with accession numbers, percentages of identity, percentages of coverage, lengths of the respective proteins, and the organism associated with the protein, are shown.

TABLE 2. Predicted phylogenetic origins of the antibacterial fosmid insert DNA using the Phylopythia and MEGAN algorithms[a]

Program	Clone	Domain	Phylum	Class	Order	Genus	Others
Phylopythia	CcAb1	*Bacteria*	*Proteobacteria*	*Deltaproteobacteria*			
	CcAb2	*Bacteria*	*Proteobacteria*	*Deltaproteobacteria*			
	UaAb1	*Bacteria*	*Proteobacteria*	*Alphaproteobacteria*			
MEGAN	CcAb1	*Bacteria* (2)	*Proteobacteria* (4)	*Gammaproteobacteria* (17)			Not assigned/ no hits (9)
	CcAb2	*Bacteria* (4)		*Gammaproteobacteria* (2)		*Solibacter usitatus* (4)	Not assigned/ no hits (11)
	UaAb1				*Sphingomonadales* (16)	*Sphingomonas* sp. (1)	

[a] Numbers in parentheses indicate the number of ORFs assigned to that particular category.

than 50% of its ORFs to the *Gammaproteobacteria* (Table 2). This clone had a high correlation index of tetranucleotide composition (0.7) to a fosmid clone previously described to be derived from a novel gammaproteobacterium in the bacterial community of the sponge *C. concentrica* (54) (GenBank accession number GQ160460). It is therefore likely that the source of the CcAb1 fosmid is a novel gammaproteobacterium. Clone CcAb2 also had a high index of correlation (0.68) to this gammaproteobacterial sequence, while the Phylopythia algorithm and MEGAN analysis gave inconclusive results. We therefore postulate that fosmids CcAb1 and CcAb2 have been derived from the same organism. Both Phylopythia and MEGAN analysis showed that clone UaAb1 is most likely derived from a bacterium in the class *Alphaproteobacteria*, with the majority of ORFs taxonomically assigned to the *Sphingomonadales* order (Table 2).

We have here identified three novel hydrolytic enzymes from sponge- and alga-associated microbial communities that are responsible for antibacterial activities. These enzymes were identified to possibly originate from alpha- and gammaproteobacteria, which highlights the utility of screening functional metagenomic libraries for discovery of novel antibacterial activities. Most of the antibacterial agents that have been identified by metagenomic screening are small molecules, for example, palmitoylputrescine (7), violacein (6), turbomycin A and B (20), and indirubin and indigo (34). The results presented in this study suggest the possibility of hydrolases as alternative sources of antibacterial activity from host-associated microorganisms.

Microbial hydrolytic enzymes (e.g., lipases and esterases) play a major role in biotechnological applications as detergents, in food processing, and in stereospecific organic synthesis, catalyzing both the hydrolysis and synthesis of long-chain acyl glycerols (2). Previous functional screening of microbial metagenomic libraries associated with the sponges *Aplysina aerophoba* and *Hyrtios erecta* also found novel lipolytic enzymes (28, 40); however, no antibiotic activity was reported. Lipases act on lipids to release fatty acids of different chain lengths, which are known to have a broad spectrum of antibacterial activity (12, 27). The mode of action is thought to be related to the detergent properties of these acids, which allow them to create pores or, at high concentrations, to cause cell lysis through cell wall degradation (12). Free fatty acids released through the actions of lipases have been shown to protect human skin against infection from opportunistic pathogens such as *S. aureus* (14) and to protect the gastrointestinal tract against pathogens such as *Helicobacter pylori*, *Enterococcus faecalis*, and *Klebsiella pneumoniae* (49, 50). Lipases have also been associated with antibacterial activity in sand flies (4), suggesting a broad biological role for lipases in the protection against bacterial infection. Further biochemical characterization is necessary to define the substrate and product range of the hydrolytic enzymes identified here. This will provide insight into the modes of action of these novel enzyme classes.

Free fatty acids with antimicrobial properties have also been identified from algae and sponges (3, 9, 29, 31), and it is possible that the role of the hydrolases detected from the sponge- and alga-associated microbial communities is the conversion of lipids excreted by the eukaryotic host to free fatty acids with antibacterial properties. This in turn may prevent the colonization or growth of certain bacteria and hence may have an impact on the community composition of the host's microbiota.

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