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SHORT COMMUNICATION

Evidence for phosphonate usage in the coral holobiont

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Phosphonates are characterized by a stable carbon–phosphorus bond and commonly occur as lipid conjugates in invertebrate cell membranes. Phosphonoacetate hydrolase encoded by the *phnA* gene, catalyses the cleavage of phosphonoacetate to acetate and phosphate. In this study, we demonstrate the unusually high *phnA* diversity in coral-associated bacteria. The holobiont of eight coral species tested positive when screened for *phnA* using degenerate primers. In two soft coral species, *Sinularia* and *Discosoma*, sequencing of the *phnA* gene showed 13 distinct groups on the basis of 90% sequence identity across 100% of the sequence. A total of 16 bacterial taxa capable of using phosphonoacetate as the sole carbon and phosphorus source were isolated; 8 of which had a *phnA*+ genotype. This study enhances our understanding of the wide taxonomic and environmental distribution of *phnA*, and highlights the importance of phosphonates in marine ecosystems.

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Introduction

Highly efficient nutrient recycling is essential in coral reef environments, and studies have shown that dissolved organic phosphorus may be more effectively used than dissolved organic nitrogen in reef environments (Padayao and San Diego-McGlone, 2000). Phosphonates, which contain a carbon–phosphorus bond, can account for 25% of the dissolved organic phosphorus pool in marine environments (Dyhrman *et al.*, 2006) and for 10% of cellular particulate phosphate in Trichodesmium (Dyhrman *et al.*, 2009), and are important in marine microbial populations (Benitez-Nelson *et al.*, 2004; Karl *et al.*, 2008; Gilbert *et al.*, 2009; Ilikchyan *et al.*, 2009; Martinez *et al.*, 2009). Phosphonates are, by virtue of the carbon–phosphorus bond, both thermally and hydrolytically stable, and form lipid and protein conjugates widely distributed in the Cnidaria (Hilderbrand, 1983). The cleavage of the carbon–phosphorus bond and subsequent regeneration of phosphate is catalysed by a series of substrate-specific phosphonohydrolases (Kulakova *et al.*, 1997; Quinn *et al.*, 2007), the genetic transcription of which is not *pho* regulated, but is rather, substrate dependent. Given the prevalence of the phosphonate moiety in coral membrane conjugates, we postulated the existence of microbial phosphonohydrolase-catalysed phosphonate turnover. Previous studies have shown the presence of *phnA* in open ocean samples (Gilbert *et al.*, 2009). This study further describes the increased diversity of *phnA* in coral-associated bacteria.

Coral-associated bacteria contain *phnA* gene homologues

To search for the presence of *phnA* gene homologues, DNA was extracted from the holobiont of 13 tropical and temperate corals obtained from the National Marine Aquarium, and then PCR amplified using the degenerate nucleotide primers and protocol described by Gilbert *et al.* (2009). *phnA* homologues were detected in eight of the holobionts tested (Table 1). The presence of phosphonolipds in these eight corals was confirmed using the lipid extraction and thin layer chromatography method described by Stillway and Harmon (1980), resulting in characteristic blue spots on the thin layer chromatography plate that did not fade over time.

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Table 1 Presence (+) and absence (−) of the *phnA* gene in bacteria associated with various tropical and temperate coral holobionts

Cnidarian	*phnA gene*
Anthelia sp.[a]	−
Capnella sp.[a]	−
Cladiella sp.[a]	−
Discosoma sp.[a]	+
Eunicella verrucosa[b]	+
Lobophyton sp.[a]	−
Nepthea sp.[a]	+
Pachyclavularia sp.[a]	−
Protopalythoa sp.[a]	+
Rodactis sp.[a]	+
Sarcophyton sp.[a]	+
Sinularia sp.[a]	+
Turbinaria sp.[c]	+

[a]Tropical soft coral.
[b]Temperate gorgonian coral.
[c]Tropical stony coral.

Coral-associated bacteria contain distinct and more diverse communities of *phnA* gene homologues than previously described in open ocean environments

Clone libraries were prepared from the *phnA* PCR products obtained from two of these species, *Sinularia* sp. and *Discosoma* sp., using the methods described in the study by Gilbert *et al.* (2009). A total of 256 *phnA* sequences were characterized (Supplementary Figure S1). As can be seen from Table 2, *phnA* gene homologues found in both *Sinularia* and *Discosoma* are more diverse than those previously described in the open ocean, with clones forming 13 groups with 97% sequence homology in this study as compared with 5 groups from the Western English Channel (Gilbert *et al.*, 2009).

PhnA gene homologues cluster in groups that are both coral and species specific

As shown in Figure 1, when unique *phnA* sequences are clustered at 63% sequence identity (the lowest sequence identifies which defines non-overlapping groups), there are three coral-specific and two coastal water-specific groups (the latter obtained from the study by Gilbert *et al.*, 2009), as well as two mixed groups. There is also a *Discosoma*-specific group that suggests that there are *phnA* sequence types, which are found only in specific holobionts.

PhnA gene homologues from isolates cultured from the coral tissue largely cluster in one group, despite the distant relationship of the host bacteria

To identify the bacterial species that contained these *phnA* homologues, samples of *Sinularia* spp. coral tissue were plated onto a marine agar containing phosphonoacetate as the sole carbon and phosphorus source (Gilbert *et al.*, 2009). Isolate taxonomy was verified by 16S rDNA sequencing as described in the study by Gilbert *et al.* (2009), resulting in 16 distinct bacterial taxa (<97% nucleotide identity). The *phnA* status of representatives of each taxon was determined using the protocol described in the study by Gilbert *et al.* (2009). Only eight of these taxa had a *phnA* homologue, five Gammaproteobacteria (*Vibrio*, *Pseudoalteromonas*, *Alteromonas*, *Psychrobacter*), one Alphaproteobacteria (*Thalassospira*), one Actinobacteria (*Mycobacterium*) and one Bacteroidetes (*Flavobacterium*). However, the homology of *phnA* genes was not reflected in the phylogeny of the host bacteria (Supplementary Figure S1). For instance, seven of the eight sequences cluster as a near identical group (group 1); only the *Alteromonas*

Table 2 Summary of diversity indices derived from *phnA* gene homologues obtained from the corals *Sinularia sp*, *Discosoma sp* and the previous study by Gilbert *et al.* (2009)

	Sinularia	*Discosoma*	*WEC* (*Gilbert* et al., *2009*)
H	2.47	2.38	1.34
J	0.73	0.75	0.51
1-D	0.85	0.88	0.54
MVDISP	1.051	1.302	0.531

Abbreviations: D, Simpson's diversity index; H, Shannon's diversity indices; J, Pielouse eveness index; MVDISP, multivariate dispersion; WEC, Western English Channel.

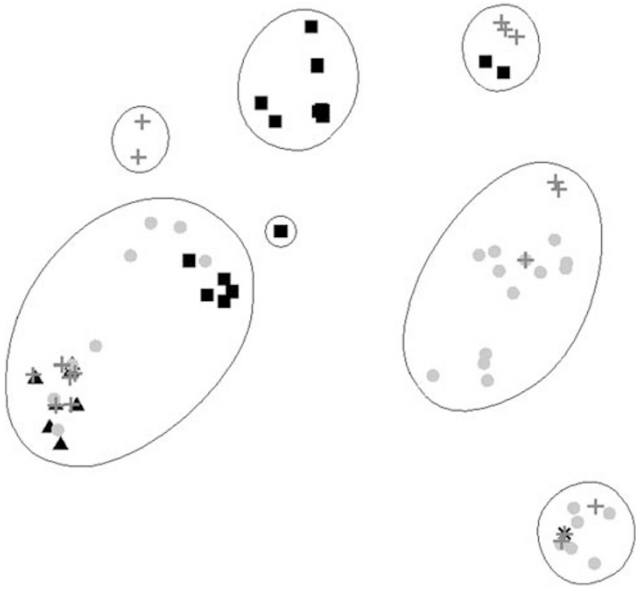

Figure 1 Nonmetric multidimensional scaling plot of the unique *phnA* sequences from coral species, coral-associated bacterial isolates and coastal pelagic samples (Gilbert *et al.*, 2009). All sequences-identity comparisons (Clustal W) were used to create a sequence identity matrix. ■—coastal water-derived sequences, ●—Sinularia-derived sequences, +—Discosoma-derived sequences, X—group 2-cultured isolates, ▲—group 1-cultured isolates. 2D Stress: 0.09.

phnA sequence does not (group 2; Figure 1). This may reflect the highly conserved nature of the active site of the *phnA* gene or possibly infer lateral gene transfer between taxa. The eight *phnA* isolates indicate either the presence of another pathway or a different class of phosphonoacetate hydrolase genes not amenable to amplification with these primers.

Potential coral pathogens contain multiple phosphonate degradation pathways

In all, 3 strains (97% 16S rDNA nucleotide identity) of the 50 cultured isolates belonged to the genus *Vibrio* (which includes several proposed coral pathogens (Reshef *et al*., 2006). All sequenced *Vibrio* representatives in the NCBI database contain the phosphonatase pathway encoded by *phnW* (2-AEP transaminase) and *phnX* genes (phosphonoacetaldehyde hydrolase). phnX was identified in all *Vibrio* isolates using degenerate primers designed to amplify a 154-bp fragment of the *phnX* gene (PA154R CA ATSACRTTYTTSAGTGCC; PA154F ATCGGNCTT GYTCTGGTTA). It is believed the phosphonoacetaldehyde from the transaminase reaction is also converted into phosphonoacetate by an unidentified enzyme; hence, *phnA* could have a role in this phosphonatase pathway (Quinn *et al*., 2007).

This study constitutes the first direct identification of *phnA* homologues from a coral holobiont. The *phnA* pathway is prevalent within both commensal bacteria and potential coral pathogens, and has a greater diversity than previously found in coastal pelagic waters. Work to determine whether the presence and expression of this functional gene in Cnidarian-associated bacteria is important in both the recycling of phosphorus within reef systems, and the potential pathogenicity of microorganisms associated with coral disease is ongoing.

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