

Metagenomic analysis of the microbial community associated with the coral *Porites astreoides*

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Summary

The coral holobiont is a dynamic assemblage of the coral animal, zooxanthellae, endolithic algae and fungi, *Bacteria*, *Archaea* and viruses. Zooxanthellae and some *Bacteria* form relatively stable and species-specific associations with corals. Other associations are less specific; coral-associated *Archaea* differ from those in the water column, but the same archaeal species may be found on different coral species. It has been hypothesized that the coral animal can adapt to differing ecological niches by 'switching' its microbial associates. In the case of corals and zooxanthellae, this has been termed adaptive bleaching and it has important implications for carbon cycling within the coral holobiont and ultimately the survival of coral reefs. However, the roles of other components of the coral holobiont are essentially unknown. To better understand these other coral associates, a fractionation procedure was used to separate the microbes, mitochondria and viruses from the coral animal cells and zooxanthellae. The resulting metagenomic DNA was sequenced using pyrosequencing. Fungi, *Bacteria* and phage were the most commonly identified organisms in the metagenome. Three of the four fungal phyla were represented, including a wide diversity of fungal genes involved in carbon and nitrogen metabolism, suggesting that the endolithic community is more important than previously appreciated. In particular, the data suggested that endolithic fungi could be converting nitrate and nitrite to ammonia, which would

enable fixed nitrogen to cycle within the coral holobiont. The most prominent bacterial groups were *Proteobacteria* (68%), *Firmicutes* (10%), *Cyanobacteria* (7%) and *Actinobacteria* (6%). Functionally, the bacterial community was primarily heterotrophic and included a number of pathways for the degradation of aromatic compounds, the most abundant being the homogentisate pathway. The most abundant phage family was the ssDNA Microphage and most of the eukaryotic viruses were most closely related to those known to infect aquatic organisms. This study provides a metabolic and taxonomic snapshot of microbes associated with the reef-building coral *Porites astreoides* and presents a basis for understanding how coral-microbial interactions structure the holobiont and coral reefs.

Introduction

The coral holobiont (Rohwer *et al.*, 2002) is a complex assemblage of the coral animal, microbial eukaryotes such as algae, Fungi and protozoa, *Bacteria*, *Archaea* and viruses. The keystone symbiosis for coral reefs is zooxanthellae and coral. By fixing carbon the zooxanthellae provide the animal with energy reserves that are used for constructing the skeleton and producing the mucus sheets (Falkowski *et al.*, 1994). Coral bleaching, one of the major threats to coral reefs, occurs when elevated water temperatures (Jokiel and Brown, 2004), pathogens (Kushmaro *et al.*, 1996) and other factors (Lyons *et al.*, 1998) cause the zooxanthellae-coral association to breakdown. Global warming is predicted to increase the amount of coral bleaching and poses a direct threat to coral reefs (Brown, 1997). Buddemeier and Smith (1999) have raised the possibility that corals will switch their zooxanthellae for communities more thermally tolerant as environmental conditions change (Buddemeier and Smith, 1999; Fautin and Buddemeier, 2004). This adaptive bleaching hypothesis is supported by studies that show ecologically and temporally driven changes in the zooxanthellae associated with corals (Rowan *et al.*, 1997; Baker, 2001).

Endolithic fungi and algae are microbial *Eukarya* ubiquitously associated with corals. Presently, it is believed that the coral-associated fungi are parasitic and attack both the coral polyps and endolithic algae (Kendrick *et al.*,

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1982; Priess *et al.*, 2000; Golubic *et al.*, 2005). However, little is known about the diversity of both endolithic fungi and endolithic algae (Lukas, 1973; Highsmith, 1981; Shashar *et al.*, 1997) or their contributions to metabolic functions in the coral ecosystem. Protists, including the potential pathogen apicomplexa, are also abundant members of the coral holobiont (Upton and Peters, 1986; Toller *et al.*, 2002).

The coral holobiont hosts a highly diverse bacterial community (Rohwer *et al.*, 2001; 2002; Cooney *et al.*, 2002; Frias-Lopez *et al.*, 2002; Bourne and Munn, 2005), which in some cases form species-specific associations with corals (Ritchie and Smith, 1997; Rohwer *et al.*, 2001; 2002). For example, a *Gammaproteobacteria* designated PA1 was always present in 16S rDNA libraries constructed from *Porites astreoides* (Rohwer *et al.*, 2002) and *Porites compressa* (L. Wegley; unpubl. data). Some of this apparent specificity is due to limitations in observing rare species in the environment and some *Bacteria* have been found on multiple species of corals (Casas *et al.*, 2004). Diverse archaeal communities (Kellogg, 2004; Wegley *et al.*, 2004; Beman *et al.*, 2007) are associated with corals. These *Archaea* are coral-specific in the sense that they do not overlap with water-column *Archaea*. However, it appears that this association is fairly cosmopolitan with the same *Archaea* being found on many corals species.

Viruses associated with corals have been shown to be present in coral tissues (Wilson *et al.*, 2005) and have been implicated in coral bleaching and disease (Wilson *et al.*, 2001; 2005; Barash *et al.*, 2005; Davy *et al.*, 2006). Viruses have also been found to be in high abundance on coral surfaces with estimates greater than 10^{10} per cm^2 (K.L. Marhaver, N. Knowlton and F. Rohwer, submitted). Most of these coral-associated viruses are phage, which infect the *Bacteria*.

While cultured and uncultured methods have provided details on the diversity and biogeography of coral-associated microbes, the metabolic contributions of these microbes to the function of the coral holobiont are essentially unknown. Rohwer and colleagues (2002) proposed that the coral holobiont harbours specialized microbiota that may protect the coral animal from pathogens through filling entry niches and/or producing antibiotics and that disrupting this association could lead to coral disease. Corals may also have the ability to switch their prokaryotic communities in order to provide resistance to specific pathogens (Reshef *et al.*, 2006). Genomic characterization of the coral animal and all its symbiotic microorganisms collectively has been recommended (Rosenberg *et al.*, 2007) for eliciting a better understanding of these complex symbiotic interactions. Metagenomics, or community genomics, provides an opportunity to describe the taxonomic components (Tyson *et al.*, 2004), relative abun-

dances (Breitbart *et al.*, 2002; Angly *et al.*, 2005) and metabolic potential (Tringe *et al.*, 2005; Rodriguez-Brito *et al.*, 2006) of all microbes within the coral holobiont. Here we use a metagenomic approach to describe the microbial metagenome of the Caribbean coral *P. astreoides*.

Results and discussion

The metagenome of P. astreoides

A mortar and pestle was used to homogenize the *P. astreoides* fragments and the coral nuclei and zooxanthellae were removed using a combination of filtration and Percoll fractionation (modified from Schirmer *et al.*, 2005). DNA was extracted from the top Percoll layer, which contained most of the microbial-sized cells as determined by microscopy (Fig. 1A), amplified using GenomiPhi (Amersham Biosciences, Pittsburgh, PA), and sequenced using 454 Life Sciences pyrosequencing. A total of 316 279 reads, with an average sequence length of 102 base pairs, were obtained from the *P. astreoides* sample and compared with the SEED non-redundant database using BLASTX (Table 1) (Overbeek *et al.*, 2004). The majority of sequences (79%; Fig. 1B) were classified as unknowns (E -value $< 10^{-5}$). The high per cent of unknowns is due to the presence of truly unique sequences in the coral holobiont combined with the shorter sequences obtained with pyrosequencing (i.e. the shorter sequence reads reduces the probability of identifying similarities in the databases). The sequences that were similar to sequences in the database (i.e. the knowns) were grouped as mitochondria (49%), Fungi (38%), *Bacteria* (7%), phage (3%), eukaryotic viruses (2%) and *Archaea* ($< 1\%$; Fig. 1B).

The mitochondrial genome of P. astreoides

The high occurrence of coral mitochondria DNAs (32 102 sequences total) in the metagenome provided enough sequence to reconstruct the coral mitochondrial genome. The large number of mitochondrial sequences was a consequence of our fractionation procedure for isolating the microbes associated with the coral (mitochondria are the same size as microbial cells). The cytochrome-oxidase C subunit 1 (*cox1*) gene, which is commonly used for phylogenetic comparisons (Fukami *et al.*, 2004), was assembled from the *P. astreoides* metagenome and compared with *cox1* from five other coral species. The phylogenetic analysis showed that *P. astreoides* was most closely related to *Porites porites* (data not shown).

Coral-associated fungal communities

Fungi were the most abundant class of organisms represented in the *P. astreoides* microbial metagenome. Members from three of the four fungal phyla (*Ascomy-*

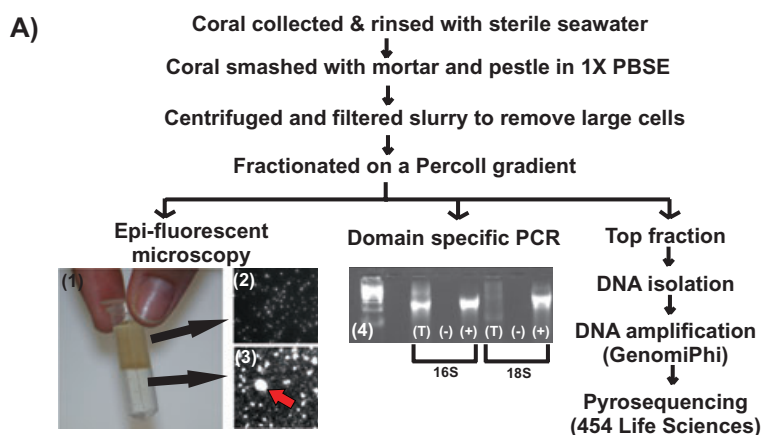
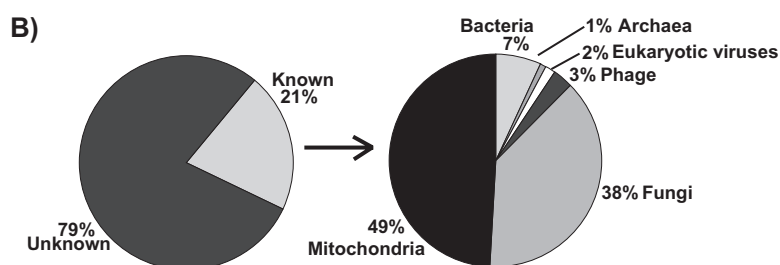


Fig. 1. Flow chart of the methods used to sequence the microbial metagenome associated with *Porites astreoides* (A) and the initial BLASTX results (B). The microbial community was retained in the top Percoll fraction (A1; micrograph A2), while eukaryotic cells (micrograph A3; red arrow) were pelleted. The removal of eukaryotic cells from the top fraction was confirmed using PCR (A4) with 16S rDNA primers (*Bacteria* and *Archaea*) and 18S rDNA primers (*Eukarya*). (T), top fraction; (-), negative control; (+), positive control. BLASTX analyses of the resulting metagenome was used to classify the sequences into taxonomic groups (the cut-off for this analysis was E -value $< 10^{-5}$) (B).



cota, *Basidiomycota* and *Chytridiomycota*) were identified based on comparisons with the Ribosomal Database Project (RDP) (Maidak *et al.*, 1996) and the SEED non-redundant database. The majority of coral-associated fungal sequences were most similar to *Ascomycetes* Fungi (93%) and therefore these were explored in more depth. Within the *Ascomycetes*, most of the Fungi were in the class *Sordariomycetes* (77% for 18S rDNA and 71% for the SEED; Fig. 2). Other fungal classes observed in the library include *Eurotiomycetes*, *Saccharomycetes* and *Schizosaccharomycetes*. Previous microscopic studies have shown that endolithic fungi, classified as

Ascomycetes-like, are ubiquitously found across several genera of healthy corals (Kendrick *et al.*, 1982; Le Campion-Alsumard *et al.*, 1995a,b; Bents *et al.*, 2000). These Fungi associate with the coral early in life and grow with the carbonate skeleton to maintain their position just under the coral tissue (Le Campion-Alsumard *et al.*, 1995b). Corals recognize and defend their tissues against the endolithic fungi by encapsulating the advancing hypha through a dense layer of aragonite (Bents *et al.*, 2000). While endolithic fungi have not proven to be pathogenic, other Fungi, particularly *Aspergillus* spp., are known coral pathogens (Kim *et al.*, 2000; 2006; Alker *et al.*, 2001).

Table 1. Databases used in the analysis of the *Porites astreoides*-associated microbial metagenome.

Taxonomical grouping	Database for BLAST comparison	No. of sequence similarities in the library	E -value cut-off
<i>Bacteria</i>	SEED	4 542	10^{-5}
<i>Archaea</i>	SEED	470	10^{-5}
Eukaryotic virus	GenBank	1 111	10^{-4}
Bacteriophage	SCUMS	1 984	10^{-4}
Fungi	SEED and RDP	25 026	10^{-5}
16S rDNA	RDP	387	10^{-6}
16S rDNA (mitochondrial)	RDP	503	10^{-6}
18S rDNA	RDP	47	10^{-6}
Mitochondria (coral)	GenBank	32 051	10^{-5}

The database normalization option in BLAST was used in all analyses; however, we have found that it is still necessary to use different E -value cut-offs based on the size of the database. SEED = SEED non-redundant database; GenBank virus database is at <http://www.ncbi.nlm.nih.gov/genomes/static/vis.html>. The SCUMS phage-prophage list is at http://scums.sdsu.edu/~rob/phage_nt.fasta. RDP, Ribosomal Database Project.

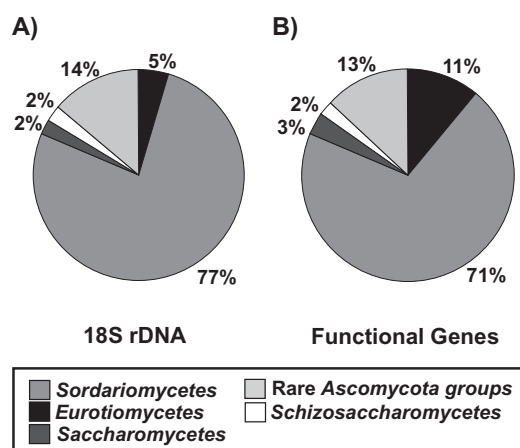


Fig. 2. Composition of the coral-associated *Ascomycetes* Fungi community based on 18S rRNA (A) and the functional genes (B). The 18S rRNA assignments were determined based on comparisons with the Ribosomal Database Project (RDP) and functional genes were determined using the SEED non-redundant database.

The metabolic potential of the fungal sequence similarities were analysed using the SEED non-redundant database. Much of the coral-associated fungal genes encoded proteins involved in cell wall construction [chitin synthase; number of sequence similarities (n) = 51], glutamate/aspartate/asparagine biosynthesis, glutamine metabolism (n = 65) and nitrogen metabolism, including the pathways for nitrate/nitrite ammonification (n = 21) and ammonia assimilation (n = 14; Table 2). These genes belong to multiple species, suggesting that the corals are harbouring a diverse community of endolithic fungi.

Taxonomy of coral-associated Bacteria

The most prominent *Bacteria* phyla in the metagenome were *Proteobacteria* (68%), *Firmicutes* (10%), *Cyanobacteria* (7%) and *Actinobacteria* (6%). These results are similar to a previous 16S rDNA analysis of *Porites*-associated *Bacteria*, where the same three phyla were the most dominant groups (Rohwer *et al.*, 2002). *Gammaproteobacteria* were the most abundant (31%) of all the bacterial sequences in the metagenomic library, once again showing striking similarity to the previous 16S rDNA analyses where 33% of coral-associated *Bacteria* belonged to this group (Rohwer *et al.*, 2002). The most prominent genus was *Acinetobacter* sp. (n = 376). *Bdellovibrio* spp. (n = 206) were also frequently observed on the coral, raising the possibility that this group of *Bacteria* may be important predators within the coral holobiont. The *Cyanobacteria* included representatives from four orders, *Chroococcales*, which includes members of *Synechococcus* sp. and *Gloeobacter* sp., *Nostocales*, *Oscillatoriales* and *Prochlorales*.

Metabolism of coral-associated microbes

It is now clear that metagenomes can be used to predict what microbes are doing in a particular ecosystem (Tringe *et al.*, 2005; Rodriguez-Brito *et al.*, 2006), including the identification of novel metabolisms (Treusch *et al.*, 2005). To characterize the metabolic potential of the *Bacteria* within the metagenome, these sequences were separated (E -value < 10^{-5}) and compared with the functional genes within the SEED non-redundant database. Figure 3 shows 48 metabolic subsystems and their relative abundances within the coral-associated bacterial community. Overall, the community was heterotrophic and acquires carbon, sulfur and nitrogen from the coral animal.

Carbon metabolism of coral-associated microbes

The coral-associated *Bacteria* possessed a large number of genes for the uptake and processing of sugars and proteins (Fig. 3). Utilization pathways for sugars and proteins, as well as the associated transporters, were common in the metagenomic library (n > 250 sequences). In contrast, no transporters for the import of amino acids or fatty acids were observed (Fig. 4A). Peptide transporters (n = 48) were in much higher abundance than sugar transporters (n = 12), but carbohydrate utilization pathways (n = 111) were more common than those for protein utilization (n = 30). Several pathways for the processing of glucose such as glycosyl hydrolases and transferases were observed. Genes for the utilization of monosaccharides (n = 44) were higher than disaccharides (n = 30) and utilization of polysaccharides such as *N*-acetyl-D-glucosamine (n = 15) and sugar alcohols (n = 17) were also observed. Unlike the sugar and protein transporters, amino acid transporters were not observed, therefore the coral-associated microbes probably import the peptides and then recycle the amino acids inside the cell. In contrast, the common marine bacterium, *Pelagibacter ubique*, predominately takes up amino acids from the environment (Giovannoni *et al.*, 2005). The bacterial community has three times the number of genes for degradation of branched amino acids, such as valine and isoleucine, than for other amino acids. In addition, pathways for the breakdown of amino acids with aromatic ring groups were more abundant. Specifically, the homogentisate pathway for degradation of aromatic compounds carries out the breakdown of L-phenylalanine and L-tyrosine (Figs 3 and 4A).

The microbial communities metabolic characteristics reflect the compounds found in coral mucus, where concentrations of proteins and polysaccharides are high, lipid content is lower and amino acids are variable (Ducklow and Mitchell, 1979a; Meikle *et al.*, 1988; Wild *et al.*, 2004). A study of the coral mucus content of six different species

Table 2. Functional genes in metagenome with significant similarities to Ascomycetes Fungi (based on an *E*-value < 10⁻⁵ to the SEED non-redundant database).

Functional gene	No. of hits	Subsystem	Similarities by family, > 90%	Includes species	No. of hits
Chitin synthase	51	Cell wall biosynthesis	Chaetothyriomycetes	<i>Exophiala dermatitidis</i>	3
				<i>Phaeosphaeria nodorum</i>	1
			Eurotiomycetes	<i>Emericella nidulans</i>	
				<i>Aspergillus oryzae</i>	
				<i>Aspergillus fumigatus</i>	
				<i>Penicillium chrysogenum</i>	9
				<i>Coccidioides immitis</i>	
				<i>Coccidioides posadasii</i>	
				<i>Paracoccidioides brasiliensis</i>	
				<i>Botryotinia fuckeliana</i>	3
<i>Fusarium oxysporum</i>					
Nitrite reductase	21	Nitrate/nitrite ammonification	Sordariomycetes	<i>Gibberella zeae</i>	24
				<i>Magnaporthe grisea</i>	
				<i>Phialophora verrucosa</i>	
				<i>Glomerella graminicola</i>	
				<i>Neurospora crassa</i>	3
Ammonium transporter	7	Ammonia assimilation	Dothideomycetes	<i>Phaeosphaeria nodorum</i>	2
				<i>Gibberella zeae</i>	
				<i>Magnaporthe grisea</i>	
				<i>Tuber borchii</i>	1
				<i>Gibberella zeae</i>	1
				<i>Gibberella zeae</i>	1
				NA	
Ammonium transporter family	3		Sordariomycetes	<i>Tuber borchii</i>	1
				<i>Gibberella zeae</i>	1
				<i>Gibberella zeae</i>	1
				NA	
				NA	
Uridyltransferase	2		Sordariomycetes	<i>Gibberella zeae</i>	1
				NA	
				NA	
				NA	
				NA	
Nitrogen regulation NR(I)	1		Sordariomycetes	<i>Gibberella zeae</i>	1
				NA	
				NA	
				NA	
				NA	
Sigma-54 factor rpoN	5	Glutamate, aspartate and asparagine biosynthesis	Eurotiomycetes	<i>Neurospora crassa</i>	1
				<i>Emericella nidulans</i>	1
				<i>Tuber borchii</i>	1
				<i>Saccharomyces cerevisiae</i>	3
				<i>Schizosaccharomyces pombe</i>	2
Glutamine synthetase	60		Sordariomycetes	<i>Gibberella zeae</i>	2
				<i>Magnaporthe grisea</i>	35
				<i>Neurospora crassa</i>	
				<i>Gibberella zeae</i>	
				<i>Magnaporthe grisea</i>	

Listed in the right-hand column are the proportions of sequences where the similarity was > 90% to specific species.

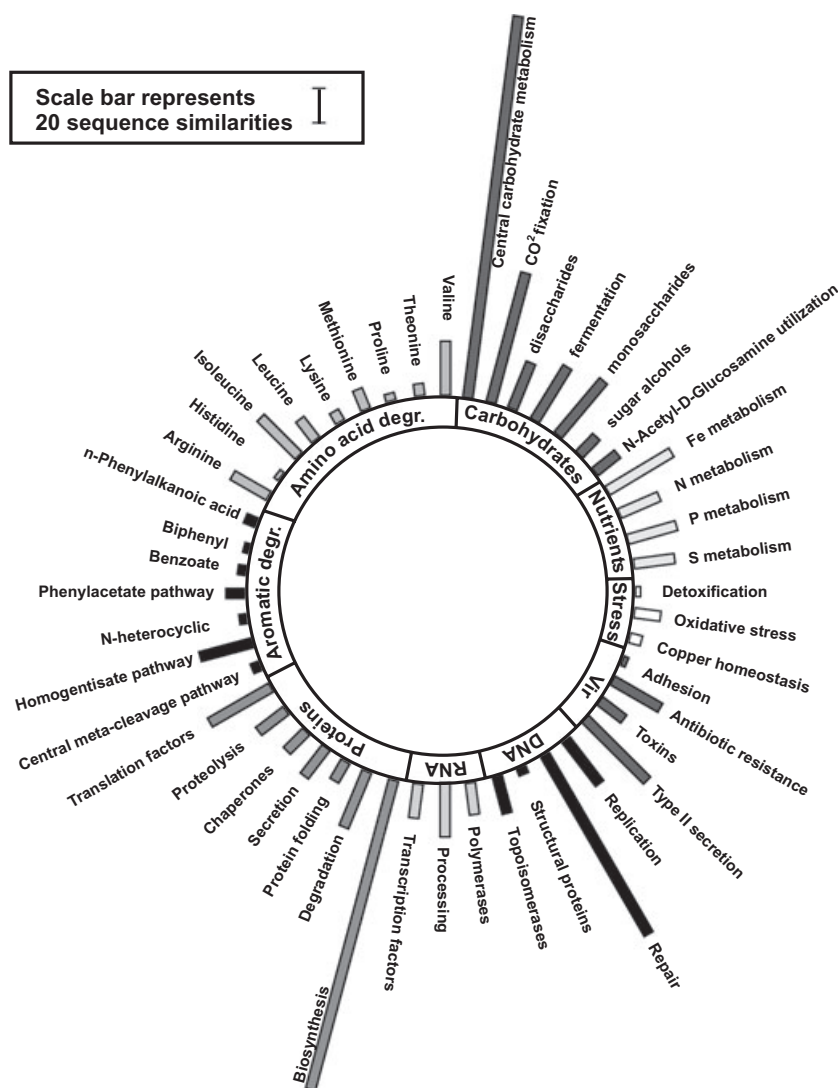


Fig. 3. The metabolic potential of the coral-associated *Bacteria* within the metagenome. Sequences with best similarities to *Bacteria* (E -value $< 10^{-5}$) were classified into functional groups using SEED subsystems. Vir, virulence. Aromatic degr., aromatic compound degradation.

of corals showed that the mucus compounds were variable between all species (Meikle *et al.*, 1988). It has been suggested that mucus of different coral species enriches for different bacterial communities (Ducklow and Mitchell, 1979b; Ritchie and Smith, 1995; 1997), and the community on the *P. astreoides* coral has likely evolved to efficiently utilize the coral's mucus. Addition of poly- or monosaccharides can disrupt this relationship and allow the coral-associated microbes to overgrow and kill the coral (Kuntz *et al.*, 2005; Kline *et al.*, 2006).

Sulfur metabolism of coral-associated microbes

All living organisms require sulfur for the synthesis of proteins and essential cofactors. Both inorganic sulfur sources, such as sulfate, and organic sulfur sources are assimilated by microbes for the biosynthesis of amino

acids, such as cystine and methionine. *Escherichia coli*, for example, uses glutathione in sulfur metabolism, redox regulation and adaptation to stresses (Smirnova and Oktyabrsky, 2005). Genes for both the transport and degradation of glutathione were observed in the coral metagenome ($n = 23$). The abundance of genes for using glutathione as a sulfur source was three times higher than that of genes for assimilation of inorganic sulfur sources, such as sulfate (Fig. 4B). Therefore, the coral-associated microbes are probably using glutathione as a sulfur source, which is abundant in eukaryotic cells (Hopkins, 1945).

Nitrogen metabolism of coral-associated microbes

Coral reefs often reside in oligotrophic waters and it has been suggested that the coral animal may acquire nutri-

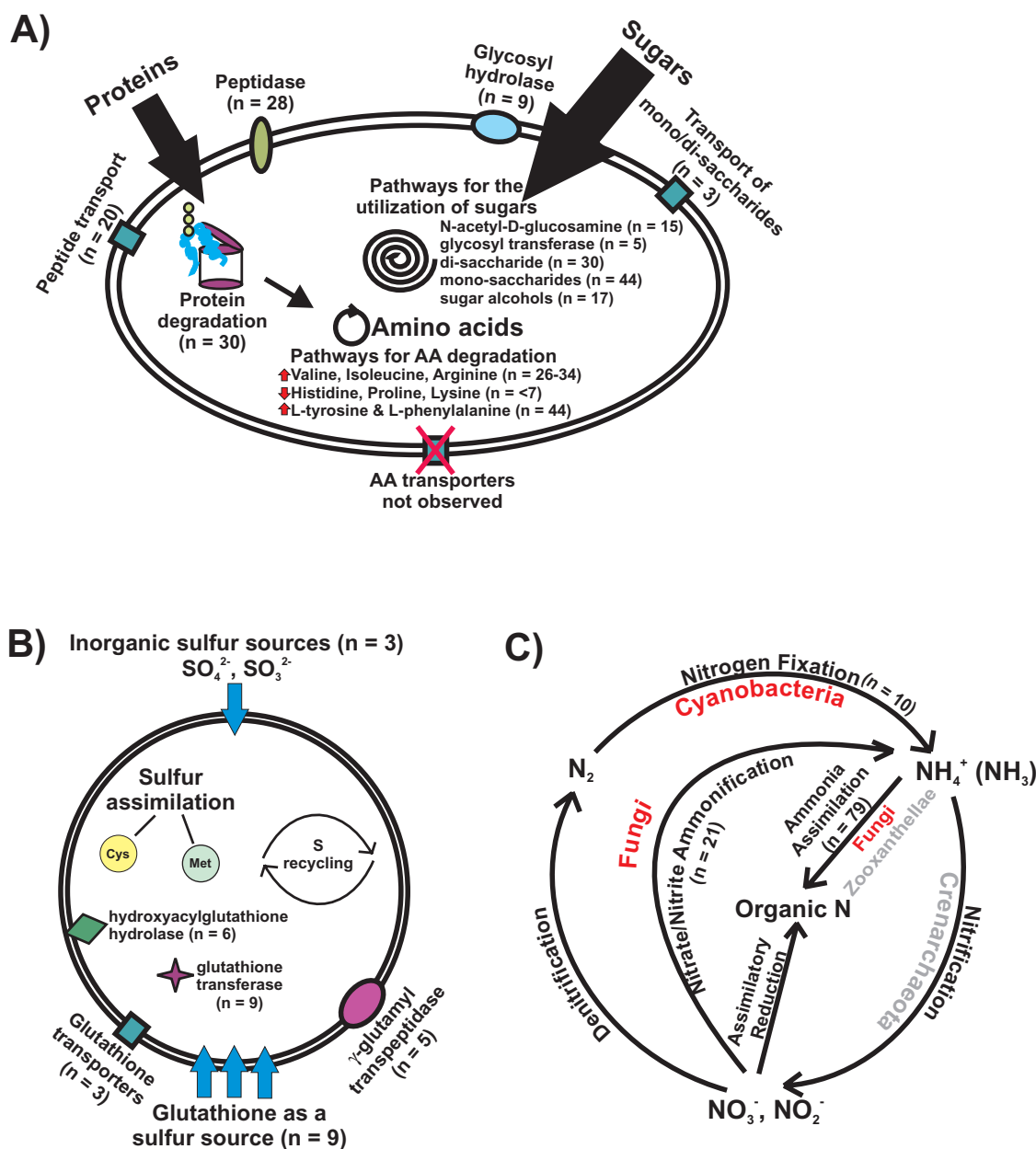


Fig. 4. Proposed models for carbohydrate protein (A), sulfur (B) and nitrogen (C) cycling in the *Porites astreoides*-associated microbial community. n = number of times a particular class of enzymes was found in the metagenome.

ents from their microbial counterparts. Although the coral animal is able to assimilate organic nitrogen either directly from the water column (Muscatine and Delia, 1978) or from their prey (Piniak *et al.*, 2003), efficiently recycling nutrients are still important and nitrogen-fixing microbes may subsidize the holobiont nitrogen budget. Several microbes potentially involved in nitrogen cycling have been identified within the holobiont and the current metagenome confirmed their presence, as well as identifying new taxa (Fig. 4C). Previously identified groups include *Cyanobacteria* (Williams *et al.*, 1987; Shashar

et al., 1994; Lesser *et al.*, 2004; Kayanne *et al.*, 2005), non-photosynthetic *Bacteria* (Williams *et al.*, 1987) and ammonia-oxidizing *Crenarchaeotes* (Treich *et al.*, 2005; Beman *et al.*, 2007) (Fig. 4C). It has been estimated that nitrogen-fixing *Bacteria* could provide up to 6% of the total nitrogen requirements of the coral community (Williams *et al.*, 1987). High numbers of *Cyanobacteria* cells (10^7 per cm^2) have also been observed on the surface of *Montastrea cavernosa* and the presence of nitrogenase proteins suggested these *Cyanobacteria* were capable of fixing nitrogen (Lesser *et al.*, 2004). In the metagenomic

Table 3. Number of sequences in the metagenome with significant similarities to eukaryotic viruses and phage.

Virus family	Host	No. sequence similarities in library	% similarities to viral or phage database	Examples of observed taxa
dsDNA viruses				
<i>Baculoviridae</i>	Crustacea, insects	23	2	
<i>Caulimoviridae</i>	Plants	9	1	
<i>Herpesviridae</i>	Molluscs, fish, mammals, turtles	354	32	
<i>Iridoviridae</i>	Molluscs, fish, frogs	412	37	<i>Lymphocystivirus</i> (LCDV), <i>Ranavirus</i>
<i>Mimiviridae</i>	Protozoa	105	9	<i>Acanthameoba</i> mimivirus
<i>Nimaviridae</i>	Crustacea	5	1	<i>Whispovirus</i> (white spot syndrome virus)
<i>Phycodnaviridae</i>	Algae, protozoa	71	6	<i>Coccolithovirus</i> (EhV-86), <i>Ecotocarpus</i> virus (EsV1)
<i>Polydnaviridae</i>	Insects	19	2	
<i>Poxviridae</i>	Birds, crustacea, insects	26	2	<i>Avipoxvirus</i> (canarypox and fowlpox virus)
<i>Myoviridae</i>	Bacteria	588	30	<i>Cyanophage</i> , <i>Aeromaonas</i> and <i>Vibrio</i> phage
<i>Podoviridae</i>	Bacteria	52	3	<i>Cyanophage</i> (P-SSP7)
<i>Siphoviridae</i>	Bacteria	226	11	<i>Mycobacterium</i> phage
Unclassified	Bacteria, Archaea	47	2	<i>Psuedomonas</i> and <i>Actinoplanes</i> phage
ssDNA viruses				
<i>Circoviridae</i>	Birds	17	2	<i>Circovirus</i> (beak and feather disease virus)
<i>Nanoviridae</i>	Plants	30	3	Coconut foliar decay virus
<i>Inoviridae</i>	Bacteria	45	2	<i>Enterobacteria</i> phage
<i>Microviridae</i>	Bacteria	1026	52	<i>Enterobacteria</i> and <i>Chlamydia</i> phage

library from *P. astreoides* the *Cyanobacteria* accounted for 7% of the bacterial groups and included nitrogen fixers, such as *Chroococcales* and *Nostocales*. Nitrogenase ($n=6$) and *nif*-specific regulatory protein ($n=4$) were also observed in the metagenome. Because the growth of zooxanthellae is nitrogen-limited (Cook and Delia, 1987; McAuley, 1987), it has been suggested that the symbiosis is dependent on the coral providing this nutrient (Rees, 1986), and these microbial symbionts could be important for providing fixed nitrogen. The endolithic fungi, described by the metagenomic library, were potentially involved in at least two processes of the nitrogen cycle within the coral (Fig. 4C), including reduction of nitrate/nitrite to ammonia and assimilating ammonia for use in biosynthesis (Table 2), suggesting that the endolithic fungi play a previously undescribed role in the nitrogen cycle within the coral holobiont. Denitrification is the only nitrogen-cycling process which has not been described in corals.

Stress and virulence genes in the metagenome

Oxidative stress response genes were present in the metagenome ($n=16$), suggesting that the community is experiencing reactive oxygen conditions (Fig. 3). This hypothesis is strengthened by the abundance of genes involved in DNA repair found in the library ($n=73$). Coral-associated microbes have been shown to be exposed to high levels of ultraviolet radiation (Lyons *et al.*, 1998) and the metagenome suggests that coral microbes have repair mechanisms to deal with this stress.

The microbes associated with *P. astreoides* coral have virulence genes (Fig. 3). Several genes for the resis-

tance of antibiotics and toxic compounds were observed ($n=34$), specifically resistance to fluoroquinolones ($n=13$) and toxic compounds cobalt, zinc and cadmium ($n=19$). Genes for the synthesis and secretion of toxins were also present in the library ($n=73$). Taken together, these pathways suggest that the microbial community benefits from carrying genes for waging chemical warfare.

Coral-associated phage

Five phage (viruses that infect prokaryotes) families were observed in the microbial metagenome (Table 3). Microphage, the most abundant phage family, accounted for 51% of the phage similarities. These ssDNA phages were also abundant in other marine viromes (Angly *et al.*, 2006). It was hypothesized that the abundances of these ssDNA viruses could be biased by the GenomiPhi DNA amplification procedure; however, this has not been proven. Cyanophages made up 17% of all the phage sequences, suggesting that coral-associated phages may help regulate the microbial populations on the coral.

Coral-associated viruses have hosts that are mostly aquatic organisms

The eukaryotic viruses associated with *P. astreoides* were identified by comparison with the GenBank viral database (<http://www.ncbi.nlm.nih.gov/genomes/static/vis.html>; all sequences included in this section had an E -value $< 10^{-4}$). The viral species were then categorized at the family level. Most of the eukaryotic viruses found on corals were similar to those known to infect aquatic organisms (59%),

such as fish, seabirds, crustaceans, algae and protists (Table 3). The *Iridoviridae* are mostly known pathogens of aquatic organisms and they represented 37% of the viral similarities in the metagenome. Lymphocystis virus, which accounted for 17% of the coral-associated eukaryotic virus similarities, has been shown to infect many species of coral reef fish (Panek, 2005). Lymphocystis virus causes a contagious, chronic infection leading to skin lesions in fish. While the disease is not lethal, it has been shown to reduce fitness of the host by affecting growth and increasing susceptibility to predation (Munn, 2006). The identification of a high percentage of potentially pathogenic viruses on *P. astreoides* suggests that the coral surface may act as a reservoir for pathogens. Corals have been previously implicated in spreading disease; for example, corals act as vectors for transmitting the digenean fluke, *Podocotyloides stenometra*, to butterfly fish (Aeby, 1998). Other marine animals have also been suggested to aid in the transfer of viruses to aquatic organisms; these include waterborne insect larvae (Roekring *et al.*, 2002), polychaete worms (Vijayan *et al.*, 2005) and seabirds (Vanpatten *et al.*, 2004).

Water collected directly above four coral reefs in the Northern Line islands (E.A. Dinsdale, O. Pantos, S. Smrigra, R.A. Edwards, F.E. Angly, D. Hall, E. Brown, M. Haynes, L. Krause, E. Sala, S.A. Sandin, R.V. Thurber, B.L. Willis, F. Azam, N. Knowlton, and F. Rohwer, submitted) was used to compare viruses on or within the coral to viruses found in the water column adjacent to the coral. The reef water was collected no more than 5 cm from the reef. Most of the viruses associated with coral (76%) were most similar to viral families that infect metazoans. In contrast, 61% of the viruses found in the reef water were most similar to families that infect algae and protozoa (Fig. 5).

How well did this approach work?

While initially unanticipated, the co-purification of coral mitochondria with the other microbes was a useful way of obtaining taxonomic information on the animal host at the same time as the microbial constituents. Currently, coral taxonomy is in disarray (Fukami *et al.*, 2004) and this method would provide much needed data for better classification of corals (i.e. whole mitochondrial genomes).

PA1 is a *Gammaproteobacteria* that has been shown to be ubiquitously associated with *P. astreoides* (Rohwer *et al.*, 2002) and *P. compressa* (L. Wegley, unpubl. data). The current approach did not provide specific information on PA1. This may be rectified with more sequencing, but it would be nearly impossible to reconstitute the PA1 genomes from this mixture. Currently, it appears that the BAC/cosmid approaches would be best for

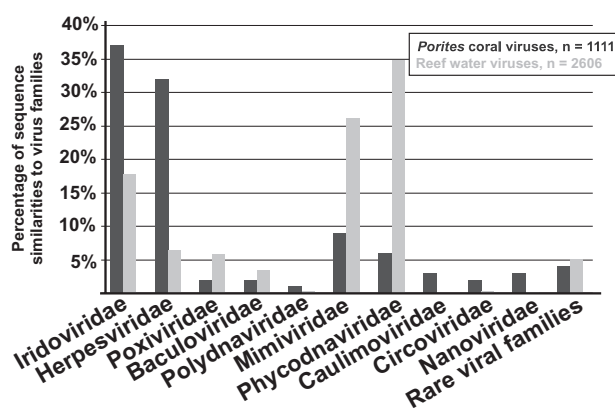


Fig. 5. Comparison of viruses that infect *Eukarya* found on *Porites astreoides* and in coral reef water. *Porites astreoides* associated coral viruses are depicted by black bars and reef water viruses are depicted by grey bars. Similarities to eukaryotic viruses were determined by comparing the metagenomes to the viral genome list in GenBank (<http://www.ncbi.nlm.nih.gov/genomes/static/vis.html>; E-value cut-off used for this analysis was $< 10^{-4}$) and grouped into viral families.

constructing environmental microbial genomes (Suzuki *et al.*, 2004).

The approach was very useful for generating hypotheses about potential roles of coral-associated microbes. In the future, we expect this approach will be used to compare multiple metagenomes from different environmental and anthropogenic regimes using bioinformatics tools like XIPE (Rodriguez-Brito *et al.*, 2006) and cross-contig analyses (Angly *et al.*, 2006).

Conclusions

The interactions of corals and microbes are one of the fundamental dynamics on reefs. Corals are increasingly faced with changing conditions on both regional and global scales. To understand how these changes will affect the coral holobiont, it is necessary to understand the roles of microbes and their responses to these changes. All of the current evidence suggests that corals and their microbial consortiums exist in a delicate balance (Kuntz *et al.*, 2005; Kline *et al.*, 2006; Smith *et al.*, 2006), which can be disrupted such that microbes behave as specific and opportunistic pathogens (Harvell *et al.*, 2007). Based on the microbial metagenome presented here, we suggest that the roles summarized in Table 4 are important to the functioning of the holobiont. There are almost certainly other important microbial-coral interactions still waiting to be uncovered in the current and future metagenomes. The analyses and techniques presented here will provide another tool for determining how these interactions change under different stressors with the goal of preventing coral holobiont collapse.

Table 4. Proposed roles of microbes inhabiting *Porites astreoides*.

Microbe type	Predicted functional roles
Endolithic fungi <i>Bacteria</i>	Nitrogen cycling, including ammonification. Probably not living strictly parasitically as previously described. Mostly heterotrophs, utilizing complex carbon compounds such as proteins and polysaccharides. Some carbon fixation and nitrogen fixation. Genes for stress response and virulence, in particular, DNA repair and antibiotic resistance appear to be important
Eukaryotic viruses Phages	The most abundant viruses present were most similar to viruses that infect coral reef fishes Preying on the bacterial community on the coral

Experimental procedures

Coral sampling

Porites astreoides samples were collected from Bocas del Toro, Panama (9°19'50"N, 82°14'58"W) using a punch and hammer. Coral fragments were placed in Ziploc bags, rinsed with sterile seawater at the surface and placed on ice until processed. Ten coral fragments (~2 cm²) from different *P. astreoides* colonies were collected and subjected to the fractionation procedure. The resulting fractions were all assayed for the highest-quality sample (described below).

Removal of zooxanthellae and coral animal cells

Coral fragments were diluted with PBSE, 1× phosphate-buffered saline plus EDTA (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, 1.4 mM KH₂PO₄ and 10 mM EDTA) and ground into slurry using a mortar and pestle. Coral slurries were transferred to 15 ml centrifuge tubes and slowly spun in a table-top centrifuge at 100 *g* to pellet the skeletal components. The supernatant was poured off into a glass homogenizer and mixed until the slurry appeared homogeneous (about 5 min). The coral slurry was then pressed through an 8.0 μm nucleopore filter (Whatman, UK) and separated into 2 ml polypropylene centrifuge tubes (four to six replicates were made per coral sample). Approximately 500 μl of coral slurry after the filtration step was stored at -20°C for DNA isolation. Tubes were centrifuged at 500 *g* at room temperature for 15 min. After this step, the two fractions contained the following: the pellet consisting of coral nuclei, zooxanthellae, and other large entities, and the supernatant consisting of mostly microbes and viruses. The pellet was rinsed twice with 1× PBSE, re-suspended in 1 ml of 1× PBSE, and fixed with 2% paraformaldehyde for microscopy. The supernatant (containing microbes and viruses; Fig. 1A) was poured off into a fresh 2 ml tube and spun at 8800 *g* for 20 min at room temperature. The supernatant was again poured off and fixed with 2% paraformaldehyde for microscopy (this fraction was essentially free of larger organisms). The pellet was re-suspended in 1 ml of 1× PBSE and 500 μl of this solution was layered onto a cushion made of 15% Percoll (Fluka Chemika, Switzerland) in 1× PBSE. The coral solution was carefully layered using a sterile pipette onto a 1 ml Percoll cushion in a 2 ml centrifuge tube. Tubes were spun at 750 *g* for 20 min at room temperature. The top layer (first 500 μl) contained almost solely microbial cells (Fig. 1A), while the bottom fraction harboured the eukaryotic cells (Fig. 1A). All top layers from the replicate tubes were collected using a plastic pipette with a cut-off tip and combined

(as were the bottom fractions). A small amount from both the top and bottom fraction were fixed with 2% paraformaldehyde for microscopy. The top and bottom fractions of each coral sample were stored at -20°C until DNA isolation. All fractions that had been fixed for microscopy were stored at 4°C.

DNA extraction and amplification

The top and bottom fractions of the gradient, as well as the coral slurry before centrifugation, were subjected to DNA extraction using the Ultraclean Power Soil DNA Extraction Kit (Mo Bio, Solano Beach, CA). In order to acquire sufficient concentrations (> 1 μg per 10 μl) for metagenomic sequencing, the DNA was amplified using φ29 polymerase (Genomi-Phi; Amersham Biosciences, Pittsburgh, PA) (Yokouchi *et al.*, 2006). DNAs from the top fractions were assayed for eukaryotic contamination using PCR. Primer sets used for this test included the *Bacteria*-specific 16S rDNA gene (27F, 5'-AGAGTTTGATCMTGGCTCAG and 1492R, 5'-TACGGYTACCTTGTTACGACTT), the eukaryote-specific 18S rDNA gene (EukA-F, 5'-AACCTGGTTGATCCTGCCAGT and EukB-R, 5'-TGATCCTTCTGCAGGTTCCACCTAC) and zooxanthellae-specific primers (ZOOXss3Z, 5'-AGCACTGCGTCAGTCCGAATAATTCACCGG and ZOOXss5L, 5'-GGTTGATCCTGCCAGTAGTCATATGCTTG). The coral sample for metagenomic sequencing was chosen based on the quality and concentration of the DNA, the presence of a strong 16S band and absence of bands for 18S and zooxanthellae in the PCR reaction, and by visualization of the cells using epifluorescence microscopy. The selected DNA sample, which represented one coral fragment, was sequenced using pyrophosphate sequencing (454 Life Sciences, Branford, CT).

Bioinformatics

The metagenome sequences from each of the libraries were compared with the SEED non-redundant database using BLASTX (Altschul *et al.*, 1990; Overbeek *et al.*, 2004). The SEED includes the GenBank database supplemented with other complete and draft genome sequences. The environmental database consists of the microbial assemblages from the Iron Mountain acid mine drainage (Tyson *et al.*, 2004), Sargasso Sea (Venter *et al.*, 2004), whale fall (Tringe *et al.*, 2005) and Minnesota farm soil (Tringe *et al.*, 2005). The contaminating sequences have been removed from the Sargasso Sea data set (DeLong, 2005; Mahenthiralingam *et al.*, 2006). All large-scale computational analyses were performed on the Terraport and NMPDR cluster at Argonne

National Laboratory and the Life Sciences Gateway to the Teragrid. Individual analyses were performed on a 12-node Orion desktop cluster (Orion, Santa Clara, CA).

These comparisons were supplemented with more extensive TBLASTN and TBLASTX comparisons (Altschul *et al.*, 1990) of either selected portions of the data against the complete non-redundant database or the whole library compared with boutique databases. The same cut-off *E*-value was always used for the same database and search method. In addition, the sequences were compared with the phage and prophage sequences from 510 genomes of the phage genome database and the 2323 genomes of the viral genome database (R. Edwards, unpublished). These sequences are available at <http://phage.sdsu.edu/phage>.

In an approach similar to previous work (Breitbart *et al.*, 2002; Edwards *et al.*, 2006), the best hit for each metagenomic sequence was automatically parsed and assigned as 'known' if there was a significant hit ($E < 10^{-5}$) to a sequence from the non-redundant nucleotide database, else 'unknown' (if there was no significant hit to any database). The number of hits in each group was then counted.

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