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Microbial transcriptome profiling of black band disease in a Faviid coral during a seasonal disease peak

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ABSTRACT: The etiology of black band disease (BBD), a persistent, globally distributed coral disease characterized by a dark microbial mat, is still unclear. A metatranscriptomics approach was used to unravel the roles of the major mat constituents in the disease process. By comparing the transcriptomes of the mat constituents with those of the surface microbiota of diseased and healthy corals, we showed a shift in bacterial composition and function in BBD-affected corals. mRNA reads of *Cyanobacteria*, *Bacteroidetes* and *Firmicutes* phyla were prominent in the BBD mat. Cyanobacterial adenosylhomocysteinase, involved in cyanotoxin production, was the most transcribed gene in the band consortium. Pathogenic and non-pathogenic forms of *Vibrio* spp., mainly transcribing the thiamine ABC transporter, were abundant and highly active in both the band and surface tissues. *Desulfovibrio desulfuricans* was the primary producer of sulfide in the band. Members of the *Bacilli* class expressed high levels of rhodanese, an enzyme responsible for cyanide and sulfide detoxification. These results offer a first look at the varied functions of the microbiota in the disease mat and surrounding coral surface and enabled us to develop an improved functional model for this disease.

KEY WORDS: Black band disease · Coral · *Cyanobacteria* · Consortium · Transcriptomics

INTRODUCTION

Over the last 4 decades, coral diseases have caused significant losses in coral populations globally (Rosenberg & Loya 2004, Wilkinson 2008). One such disease, first documented on a reef in Belize in the early 1970s, is the ubiquitous black band disease (BBD) (Antonius 1973, 1985, Garrett & Ducklow 1975), an infectious disease known to be plaguing corals worldwide (Willis et al. 2004). Among the factors that influence the appearance and progression of BBD are depth and water clarity, water temperature, acidity and light intensity (Kuta & Richardson 2002, Zvuloni et al. 2009, Sato et al. 2011).

BBD is mostly active during the summer when water temperatures and irradiation are at their highest levels (Rützler et al. 1983, Kuta & Richardson 1996, Miller & Richardson 2015), and typically appears as a black microbial band or mat (microbial consortium) that migrates across a coral colony, destroying coral tissue and leaving behind a bare skeleton (Carlton & Richardson 1995, Richardson 2004). Microbial compositions of the BBD consortia in the symptomatic mats of the disease have been characterized in samples collected from a variety of geographic locations (Cooney et al. 2002, Frias-Lopez et al. 2002, 2003, Sekar et al. 2006, 2008, Barneah et al. 2007, Voss et al. 2007, Sato et al. 2010, Miller &

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Richardson 2011). These studies showed that although the members of the mats differ slightly across geographic regions in terms of species, the functional groups in the consortium appear to be similar.

Among the major microbial constituents of the disease band, several remain constant throughout the year while others appear to be diverse and dynamic (Arotsker et al. 2015). The most consistent and prominent members of BBD mats are the cyanobacteria that provide its distinctive morphology (Cooney et al. 2002, Frias-Lopez et al. 2003, Barneah et al. 2007, Myers et al. 2007, Sato et al. 2010). Indeed, a meta-analysis of published 16S rRNA gene sequences derived from clone libraries of the BBD microbial consortium by Miller & Richardson (2011) showed that 71% of all BBD samples contained 1 specific type of filamentous cyanobacteria. Moreover, cyanobacteria have been successfully retrieved, identified and analyzed from the black bands of diseased corals from different geographic locations (Gantar et al. 2009, Rasoulouniriana et al. 2009, Sato et al. 2010, Casamatta et al. 2012). In corals from the Red Sea, Israel, cyanobacterial constituents of BBD have been identified as persistent and dominant (Barneah et al. 2007, Kramarsky-Winter et al. 2014, Arotsker et al. 2015), sometimes comprising a sole filamentous cyanobacterium strain BgP10_4S^T (FJ210722), for which the name *Pseudoscillatoria coralii* was proposed (Rasoulouniriana et al. 2009). A meta-analysis of clone libraries of BBD from 10 other geographic locations indicated the presence of a dominant cyanobacterial species at 9 of those sites that was highly homologous (98–99%) to the BgP10_4S^T cyanobacterium strain found in the Red Sea (Miller & Richardson 2011).

BBD-associated cyanobacteria produce cyanotoxins (Richardson et al. 2007, Gantar et al. 2009), and are able to withstand high levels of toxic sulfide (Richardson & Ragoonath 2008), both of which contribute to establishing and maintaining their infection of the coral host. In addition, the cyanobacteria were found to be responsible not only for mat photosynthetic activity, but also for the mat's black color, which is conferred by the bacterium's phycoerythrin pigment (Richardson 1996, 1997). The penetration of cyanobacterial filaments into healthy tissues and even into the coral's calcareous skeleton causes the tissue damage that is characteristic of this disease (Sato et al. 2009, Miller et al. 2011, Kramarsky-Winter et al. 2014). Within established mats, the filamentous cyanobacteria create a stratified oxic/anoxic gradient (Carlton & Richardson 1995) and produce a number of secondary metabolites, including cyanotoxins such as microcystin (Richardson et al. 2007, Gantar et al. 2009) and a number of antimicrobial substances (Gantar et al. 2011).

Additional constituents of the band are also likely to play important roles in disease progression. For example, sulfate-reducing bacteria (SRB), known to produce toxic sulfide (H_2S), are characteristic residents in the anaerobic conditions at the mat/coral interface of BBD (Carlton & Richardson 1995, Richardson 2004). *Gammaproteobacteria* such as *Vibrio* spp., which are considered commensal and opportunistic coral pathogens, were found both in the surface mucus of healthy corals (Ainsworth et al. 2015) and in BBD mats (Barneah et al. 2007, Sekar et al. 2008). As many *Vibrio* spp. have been shown to possess high levels of proteolytic activity (Arotsker et al. 2009), it is likely that pathogenic species of this group found in the mat also play an important role in host tissue degradation. Despite advances in the elucidation of the bacterial composition of the BBD mat consortium, the roles and functions of these microbial constituents still remain largely unknown. Thus, in an attempt to explore the functions of these groups in the BBD mat, we enriched BBD-associated bacterial mRNA and investigated the genes transcribed by the constituent bacteria during the active phases of the disease. Using such a transcriptomic approach allowed us to ascertain the transcribed functional genes of the different members of the consortium during BBD infectious outbreaks.

MATERIALS AND METHODS

Sample collection

Samples (10 ml) of mucus, necrotic and healthy tissue mixtures from healthy and BBD-affected *Favia* sp. coral were collected by scraping over the coral surface with a syringe and were immediately frozen and stored at −80°C until processing. The samples were collected in August 2012 (the most active BBD period) from the coral reef near the Inter-University Institute (IUI) for Marine Science in Eilat (Northern Red Sea, Israel; 29° 51′ N, 34° 94′ E). In total, 3 samples were collected from 1 diseased and 1 healthy *Favia* colony: Aug12-BB, the mat of a highly active black band (BB); Aug12-H, apparently healthy (H) tissue (healthy area) of the same diseased coral colony; and Aug12-Fav, tissue and mucus from a completely healthy looking *Favia* sp. coral (Fav) found in the vicinity of the diseased colony.

Sample processing and prokaryotic mRNA enrichment

Total sample RNA was extracted from coral mucus and tissue using the ZR Soil/Fecal RNA MicroPrep™ (Zymo Research) according to the protocol supplied by the manufacturer, and quality and concentration were assessed using a NanoDrop ND 1000 spectrophotometer (NanoDrop Technologies). Prokaryotic mRNA was enriched using 2 steps and 2 commercial kits. The MICROB*Enrich*™ Kit (Life Technologies) enriches total bacterial RNA by removing eukaryotic 18S rRNA, 28S rRNA and polyadenylated mRNA. In the second step, the MICROB*Express*™ bacterial mRNA Enrichment Kit (Life Technologies) removes the 16S and 23S rRNA from total bacterial RNA resulting in an enrichment of the bacterial mRNA. After processing, 2 samples each from BB and H and 1 Fav sample were of sufficiently high quality for sequencing. These samples underwent whole linear amplification and sequencing using the Ion Torrent PGM system at the MR-DNA Laboratories (Shallowater, TX).

Table 1. Summary of sequencing and annotation of transcriptomes

	Black band mat	Apparently healthy tissue	Completely healthy tissue
Processing of raw sequences			
Number of raw sequences	1 138 044	921 046	709 570
Mean (±SD) length of raw sequences	144 ± 73	186 ± 63	140 ± 65
MG-RAST pipeline results			
Artificial duplicate sequences	0	0	0
Passed quality control	1 057 216	880 423	654 487
Mean (±SD) sequence length after quality control	154 ± 67	189 ± 58	150 ± 57
Predicted protein features	195 402	227 368	241 779
Predicted rRNA features	107 016	42 642	289 070
Identified protein features (annotated)	74 298	82 888	90 591
Unidentified protein features (ORFans)	121 104	144 480	151 188
Assigned to functional categories	34 923	56 998	42 413
Classification of functionally annotated transcripts			
Total	856 177	426 034	299 275
Bacteria (%)	52.92	92.03	10.07
Eukaryota (%)	46.80	7.33	87.06
Archaea (%)	0.13	0.27	0.08
Viruses (%)	0.01	0.30	0.41
Other (%)	0.14	0.07	2.38

Data analysis

The sequenced data were uploaded (MG-RAST IDs 4527293–7) and analyzed using the Metagenomics RAST analysis server (MG-RAST, http://metagenomics.anl.gov/). The analysis algorithms excluded the dereplication function, dynamic trimming, length filtering and ambiguous filtering (noDRISEE, noQualTrim). Only 3 samples (BB, H and Fav) passed quality control and are presented in this paper.

RESULTS

Transcriptome data and annotation

A mean (±SD) of 922 887 (±214 243) reads per sample from the 3 *Favia* sp. coral-associated prokaryote transcriptomes were submitted to the MG-RAST pipeline and the quality control step reduced that number to 864 042 (±201 863) reads (Table 1). A mean of 221 516 (±23 736) reads predicted transcriptional protein features and a mean of 146 243 (±127 811) reads predicted rRNA features. This process resulted in a mean of 82 592 (±8150) annotated features and 138 924 (±15 793) orphan open reading frames (ORFans) per sample. All annotated reads were assigned to 44 778 (±11 226) functional categories using the MG-RAST database M5NR, which integrates 10 sequence databases, including NCBI, KEGG, SEED, and Patric.

In order to obtain high total RNA extraction and bacterial mRNA enrichment yields, it was necessary to collect high quality prokaryotic mRNA from a mixed coral sample (consisting of coral tissues, viruses, and microorganisms, including protists, fungi, algae, bacteria, and archaea). The efficiency of the mRNA enrichment process, which differed between the samples (Table 1), depended on sample properties. Black band (BB) mat prokaryotic mRNA was successfully enriched from less than 5% to approximately 53%, and the prokaryotic mRNA associated with apparently healthy (H) tissue was enriched to 92% (Table 1). On the other hand, mRNA enrichment of the microorganisms associated with completely healthy (Fav) coral yielded only 10%. A rarefaction curve of the total RNA following enrichment of the 3 samples (Fig. 1) shows that the black band and the apparently healthy samples almost plateaued while the completely healthy coral sample continued to rise. Alpha-diversity parameters (Shannon diversity)

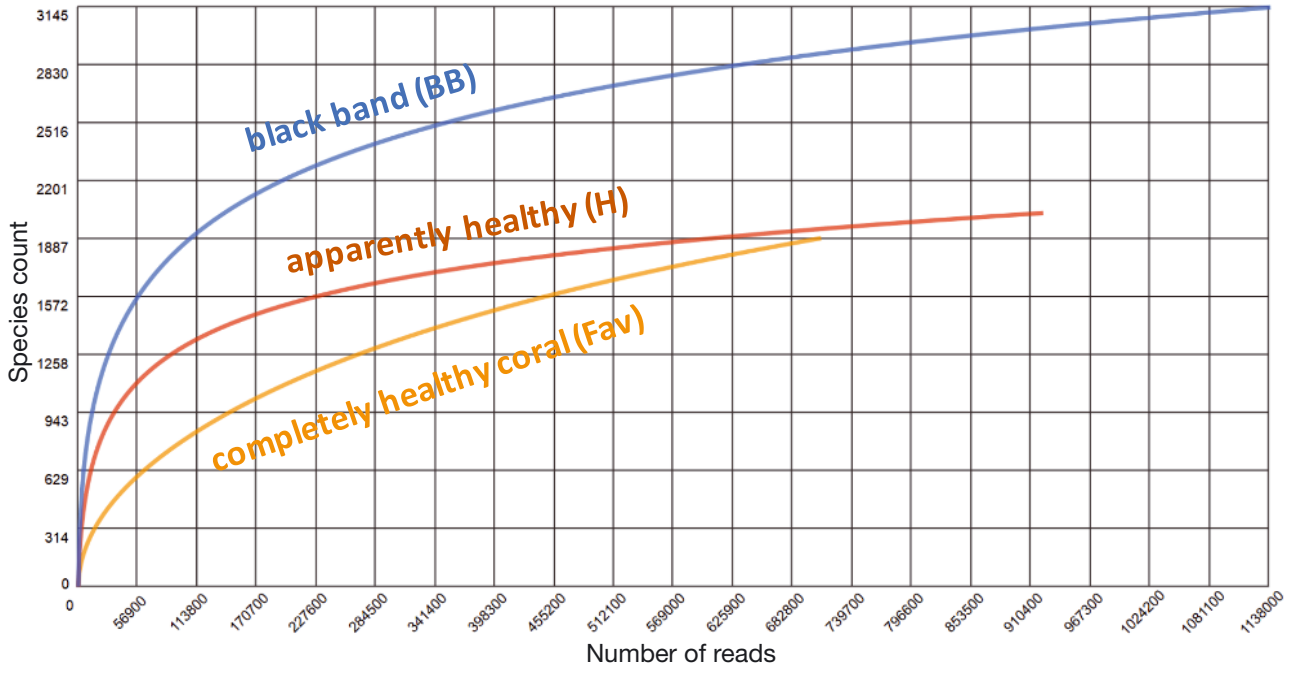

Fig. 1. Rarefaction curve based on rRNA and mRNA reads of the transcriptome samples, produced using the MG-RAST server. (Blue line) BBD consortium, (red line) the apparently healthy tissue, and (yellow line) the completely healthy colony

calculated for the samples were 143.9 for the black band mat, 100.2 for the apparently healthy, and 33.8 for the completely healthy coral tissues.

Diversity and functionality of active bacteria

Using both ribosomal RNA and mRNA with functional annotations (classified by MG-RAST), bacterial sequences were classified into 55 classes that belonged to 28 different phyla. Among these, mRNA reads showed that only 14 classes (Fig. 2) belonging to 5 phyla were highly active in the black band mat, in the apparently healthy tissue and in the tissue of healthy coral. Six functions (Fig. 3) were overexpressed in the black band mat in comparison to the apparently healthy tissue and the completely healthy colony. These functions were the production of amino acids and their derivatives, cell division and the cell cycle, DNA metabolism, protein metabolism, secondary metabolism, and sulfur metabolism.

The *Gammaproteobacteria* class was highly represented in all samples, comprising 26.4% of the black band, 43.9% of the apparently healthy, and 71.2% of the completely healthy coral tissue microbiota (Fig. 2). Of these, *Vibrio* spp. showed the highest ac-

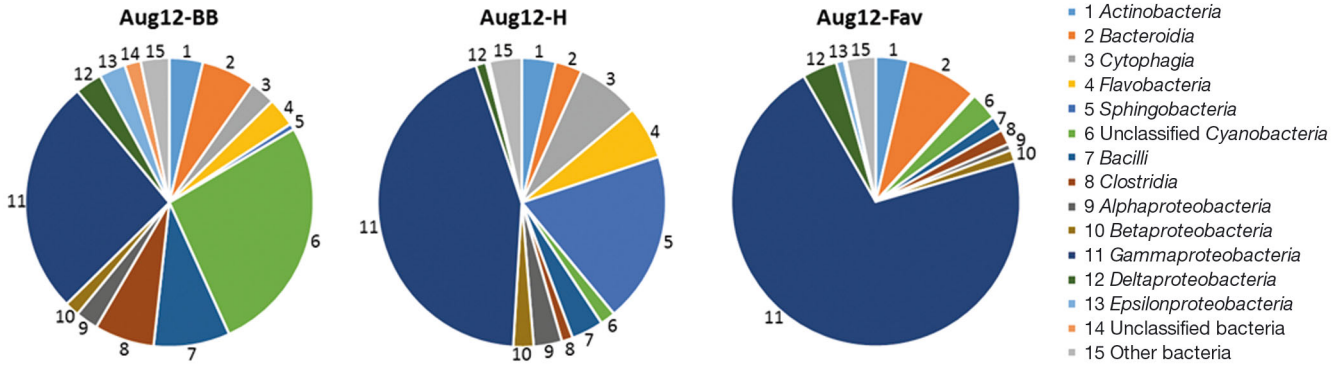

Fig. 2. Bacterial mRNA reads, classified at the class level, of the 14 major active groups. Aug12-BB: the mat of a highly active black band; Aug12-H: apparently healthy tissue (healthy area) of the same diseased *Favia* sp. coral colony; Aug12-Fav: tissue and mucus from a completely healthy looking *Favia* sp. coral. Numbers adjacent to the sections of each graph represent the classes as indicated in the key

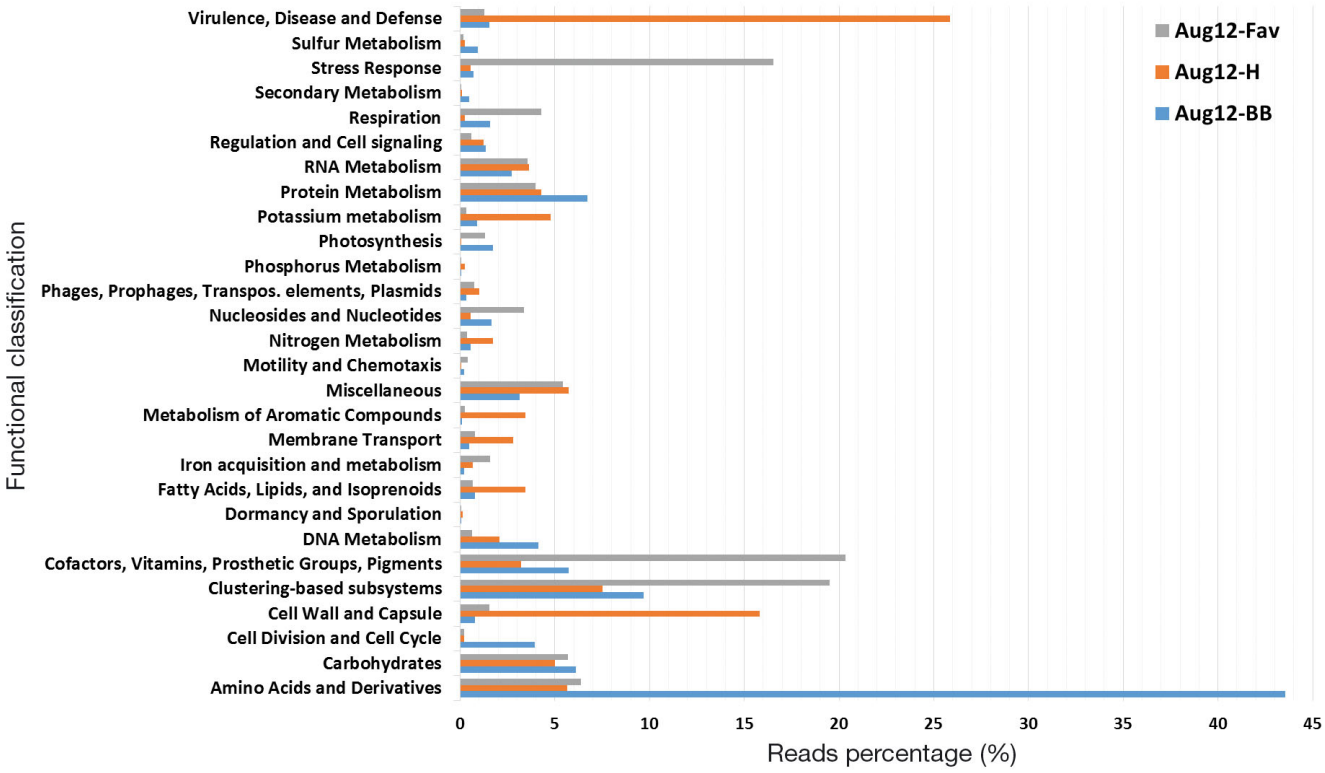

Fig. 3. Percentages of functional classification (mRNA) of (blue) black band (Aug12-BB) sample, (orange) apparently healthy (Aug12-H) tissue, and (grey) a completely healthy (Aug12-Fav) coral colony

tivity (6.2%) in the black band sample compared with the apparently healthy tissue (0.2%) and completely healthy coral tissue (0.2%). Interestingly, at least 40.7% of black band reads affiliated with *Vibrio* were assigned to the thiamine (vitamin B1) ABC transporter in at least 5 different *Vibrio* species. The most active *Vibrio* species in the black band were related to *V. parahaemolyticus*, *V. cholerae*, *V. splendidus*, and *V. shilonii* (Table 2).

Cell division and cell cycle markers were overexpressed (3.9%) in the black band mat sample compared to both the apparently healthy and healthy tissue samples (0.2% each; Fig. 3). Of these, the black band reads were assigned to a total of 112 unique annotations affiliated mainly with *Gammaproteobacteria*. A chromosome segregation ATPase with a high homology to that of *Pseudoalteromonas atlantica* was highly transcribed (42.3%). Additionally, highly expressed (41.1% and 7.7%) transcriptional regulators of the chromosome partitioning protein were found to be affiliated with *Alteromonas macleodii* and *Colwellia psychrerythraea*, respectively. Similar results

Table 2. Ten most abundant functions of *Vibrio* spp. in the black band (BB) sample

Function	Bacteria	Abundance
Thiamine ABC transporter, permease protein	*Vibrio brasiliensis* LMG 20546	3743
Thiamine ABC transporter, transmembrane component	*Vibrio* sp. Ex25	3468
Thiamine ABC transporter, transmembrane component	*Vibrio orientalis* CIP 102891	1708
Thiamine/thiamine-pyrophosphate ABC transporter, permease protein	*Vibrio parahaemolyticus* Peru-466	1300
Thiamine ABC transporter, transmembrane component	*Vibrio sinaloensis* DSM 21326	1273
Hypothetical protein	*Vibrio cholerae* MO10	1199
50S ribosomal protein L3	*Vibrio splendidus* 12B01	642
Queuine tRNA-ribosyltransferase	*Vibrio shilonii* AK1	505
Conserved hypothetical protein	*Vibrio parahaemolyticus* K5030	361
Hypothetical protein	*Vibrio mimicus* VM573	325

Table 3. Eleven most dominant annotated genes of cyanobacteria in the black band (BB) sample. The most dominant function, the enzyme adenosylhomocysteinase, is shown in **bold**

Function	EC	Bacteria	Abundance (reads)
Adenosylhomocysteinase	**EC 3.3.1.1**	*Thermosynechococcus elongatus* BP-1	45 367
Adenosylhomocysteinase	**EC 3.3.1.1**	*Trichodesmium erythraeum* IMS101	44 360
NAD-dependent epimerase/dehydratase		*Trichodesmium erythraeum* IMS101	2145
NAD-dependent epimerase/dehydratase		*Microcoleus chthonoplastes* PCC 7420	1889
Nucleoside-diphosphate-sugar epimerases		*Microcoleus chthonoplastes* PCC 7420	1550
Phycobilisome protein CpeB		*Nostoc punctiforme* PCC 73102	1413
Ribokinase	EC 2.7.1.15	*Synechococcus* sp. RS9917	621
Glutamate dehydrogenase		*Nostoc* sp. PCC 7120	563
Adenosylhomocysteinase	**EC 3.3.1.1**	*Oscillatoria* sp. PCC 9029	556
Adenosylhomocysteinase	**EC 3.3.1.1**	*Arthrospira maxima* CS-328	556
C-phycoerythrin beta chain		*Pseudanabaena* sp. PCC 7409	554

were obtained for DNA metabolism markers, which were found to be associated with the *Alteromonadales* order of the *Gammaproteobacteria*.

A large percentage of mRNA reads (26.7%) from black band tissues showed cyanobacterial activity, which was found at much lower levels (1.7% and 3.1%) in both apparently healthy and completely healthy tissues, respectively (Fig. 2). Reads showing cyanobacterial activity were assigned to 2107 functional annotations.

Markers for amino acids and their metabolic derivatives were highly overexpressed (43.6%) in the black band sample (Fig. 3) in comparison to both the apparently healthy tissue (5.7%) and the completely healthy coral (6.4%). These reads were assigned to a total of 596 unique annotations, the most dominant of which (90.6%) were assigned to the enzyme adenosylhomocysteinase (EC 3.3.1.1) expressed by several types of cyanobacteria (Table 3).

Epsilonproteobacteria, which comprised 3.0% of the reads in the black band mat, reached only 0.3% in apparently healthy tissue and 0.9% in the completely healthy sample. *Alphaproteobacteria* were present at higher percentages in the diseased colony, where they constituted 2.5% of the bacteria consortium in the BBD mat and 3.2% in the apparently healthy tissue, compared to completely healthy coral (0.7%). Interestingly, *Beta-* and *Deltaproteobacteria* showed similar distributions in all samples (1.7% and 3.0% in the black band, 2.3% and 1.2% in the apparently healthy tissue, and 1.3% and 3.9% in the completely healthy coral, respectively).

Members of the *Bacteroidetes* phylum, the *Cytophaga*, *Flavobacteria*, and *Sphingobacteria* classes, were present in the black band (6.8%) and in apparently healthy (32.2%) tissues of the diseased coral, but were present only in very low percentages in the completely healthy coral (0.3%). *Sphingobacteria* were highly active (19.1%) in apparently healthy tissue, less so (0.7%) in the black band and not active in the healthy colony. Both the *Bacilli* and *Clostridia* classes of the *Firmicutes* phylum were more highly active in the black band tissue (8.6% and 6.7%, respectively) than in apparently healthy tissue (3.6% and 1.2%) or in the completely healthy colony (1.7% and 1.7%). Members of the *Actinobacteria* class were present at the same level (3.8%) in all 3 samples. Other classified bacteria were present in very low numbers. In addition, the black band mat had a higher portion of unclassified bacteria (1.8%) than the apparently healthy tissue (0.08%) or the completely healthy colony (0.3%).

The SRB *Desulfovibrio* spp. were found to be the most dominant genus of the *Deltaproteobacteria* class (2.0%) in the black band mat, while their activity was low (0.07%) in the apparently healthy tissue and absent from completely healthy coral. At least 42.5% of *Desulfovibrio* spp. reads, related mostly to *D. desulfuricans*, were transcripts of genes that are responsible for sulfide production (Table 4). Although *Deltaproteobacteria* class bacteria were present in all tissues (Fig. 2), no transcripts for sulfide production were found in healthy tissues. Sulfur metabolism was associated with 0.9% of the reads from the black band mat sample, 5-fold more than in the apparently healthy tissue or the completely healthy colony (each about 0.2%) (Fig. 3). Black band sulfur metabolism reads were assigned to a total of 84 unique annotations, among which the most dominant function (50.5%) was assigned to genes associated with sulfite reduction from several *Desulfovibrio* spp. (Table 4). In addition, sulfate adenylyltransferase, responsible

Table 4. Sulfur metabolism functional classification, bacteria responsible for each function and read abundance in the black band (BB) sample

Function	EC	Bacteria	Abundance
Sulfite reduction-associated complex DsrMKJOP protein DsrM		*Desulfovibrio desulfuricans* G20	557
Sulfite reduction-associated complex DsrMKJOP protein DsrM		*Desulfovibrio vulgaris*	335
Thioredoxin reductase	EC 1.8.1.9	*Clostridium botulinum*	251
Sulfite reduction-associated complex		*Desulfovibrio vulgaris*	176
Thioredoxin reductase	EC 1.8.1.9	*Alkaliphilus metalliredigens*	152
Antioxidant, AhpC/Tsa family		*Vibrio harveyi*	94
Peroxiredoxin	EC 1.11.1.15	*Streptosporangium roseum*	60
Sulfate adenylyltransferase	EC 2.7.7.4	*Desulfovibrio desulfuricans*	54
Molybdate/tungstate binding		*Burkholderia xenovorans*	42
Sulfate adenylyltransferase	EC 2.7.7.4	*Desulfovibrio desulfuricans* G20	36
Peroxiredoxin, AhpC-type		*Aliivibrio salmonicida*	33
Sulfate adenylyltransferase	EC 2.7.7.4	*Desulfovibrio vulgaris*	33
Sulfate adenylyltransferase	EC 2.7.7.4	*Desulfotalea psychrophila*	31
Sulfate adenylyltransferase	EC 2.7.7.4	*Desulfomicrobium baculatum*	25
Sulfite reductase alpha subunit	EC 1.8.99.1	*Desulfovibrio vulgaris*	4
Sulfite reductase alpha subunit	EC 1.8.99.1	*Desulfovibrio desulfuricans* G20	1
Sulfite reduction-associated complex DsrMKJOP protein DsrM		*Desulfovibrio desulfuricans*	1
Sulfite reductase alpha subunit	EC 1.8.99.1	*Desulfovibrio vulgaris*	1

for adenosine-5′-phosphosulfate (APS) production, was transcribed by 10 different bacterial species, 5 of which were from the black band, where they are also responsible for sulfide production. On the other hand, healthy tissue transcripts showed no expression of genes associated with sulfide production.

Members of the *Bacilli* class (*Firmicutes*) were found to constitute a unique feature of the BBD mat, and were assigned to 1348 distinctive annotations. The most dominant transcript (6.4%) was that of thiosulfate sulfurtransferase (rhodanese), an enzyme involved in 'virulence, disease and defense' processes and that is responsible for cyanide and sulfide detoxification (Billaut-Laden et al. 2006). In black band tissue, 2 bacteria species in particular, *Oceanobacillus iheyensis* and *Bacillus* sp., were responsible for the production of the rhodanese transcripts, which were absent from both the apparently healthy and completely healthy coral tissues.

Additional functions found to be overexpressed in the BBD mat were those of protein and secondary metabolism (Fig. 3). Protein metabolism was slightly higher (6.7%) in the black band sample than in the apparently healthy tissue (4.3%) or in the completely healthy colony (4.0%). Assigned to a total of 1405 unique features, none of these black band reads were dominant. The 7 most abundant reads were assigned to ribosomes, heat shock proteins and translation factors of *Vibrio* spp. and *Desulfovibrio* spp. (Table 5) that were found mainly in the black band mat sample.

Secondary metabolism markers, assigned to a total of 25 unique annotations, represented 0.5% of the black band sample, and were present at levels of only 0.08% in apparently healthy tissue and 0.06% in the completely healthy colony. The most dominant function (25.1%) was assigned to the dihydrokaempferol 4-reductase activity of *Burkholderia* spp. These belong to the *Betaproteobacteria*, a key black band microbial feature. An additional 9.9% of the reads were assigned to the cinnamyl-alcohol dehydrogenase of *Cytophaga* spp. (aerobic gliding bac-

Table 5. Protein metabolism functional classification, bacteria responsible for each function and read abundance in the black band (BB) sample

Function	Bacteria	Abund.
50S ribosomal protein L3	*Vibrio* spp.	642
50S ribosomal protein L10	*Desulfovibrio desulfuricans* G20	475
50S ribosomal protein L10	*Desulfovibrio vulgaris* RCH1	376
Heat shock protein Hsp90	*Desulfovibrio desulfuricans* G20	326
Translation initiation factor 3	*Vibrio harveyi* ATCC BAA-1116	312
Heat shock protein Hsp90	*Desulfovibrio vulgaris* Miyazaki F	306
Translation elongation factor G	*Vibrio vulnificus* CMCP6	306

teria). These enzymes are oxidoreductases involved in flavonoid and phenylpropanoid biosynthesis.

Iron metabolism was one of the functions that were under-transcribed in the black band (0.19%) compared to the apparently healthy tissues (0.65%) or the healthy colony (1.57%) (Fig. 3). The majority of the black band reads were assigned to heme oxygenase, an enzyme used to acquire iron (Frankenberg-Dinkel 2004), and to the ferric uptake regulation protein, a negative regulator of the aerobactin operon (Bagg & Neilands 1987). The apparently healthy and completely healthy tissues, on the other hand, transcribed a different set of enzymes belonging to the ferric siderophore transport system (and its associated binding proteins) and sensor proteins.

DISCUSSION

Metatranscriptomic analysis provides valuable information about the ribosomal and messenger RNA associated with microbial activity in environmental niches, offering us a glance at the structures and functions of active microorganisms. However, the many technical challenges of the technology precluded the publication of more than a few studies that present prokaryotic mRNA expression patterns from field samples (Sorek & Cossart 2010). Indeed, metatranscriptomic research in lower marine invertebrate biology, including that of corals and sponges, is still a new field (Radax et al. 2012, Closek et al. 2014, Gust et al. 2014, Mayfield et al. 2014, Moitinho-Silva et al. 2014, Shinzato et al. 2014). Therefore, the development of this tool to enable more research of the diseases plaguing marine organisms is an important step in understanding the drivers of, and the possible solutions to, many such diseases.

To the best of our knowledge, this is the first study to present a metatranscriptomic comparison of the microbial constituents of healthy and disease-affected corals. The functions of members of the BBD consortium in the disease process were analyzed by comparing the prokaryotic mRNA activity in the black band, the apparently healthy coral tissues of the affected colony, and the healthy tissues of another colony. Following enrichment of the prokaryotic mRNA, a relatively high percentage (~24%) of reads that received annotated functions were obtained. To obtain analyses that were rigorous, samples with less than 10% enrichment that had been obtained from additional corals were disregarded.

Based on mRNA reads, alpha-diversity indices and rarefaction analyses (Fig. 1), the bacterial consortium of the black band mat in August was found to be much more active than those in either the completely healthy coral or the apparently healthy tissues of the affected coral. On the other hand, diversity indices of the BBD mat measured by genomics analyses (amplification of 16S rRNA gene) at disease peak activity (August) were much less diverse than those in apparently healthy tissues and in diseased tissues in non-active and waning seasons (Arotsker et al. 2015). This implies that the bacterial consortium of the BBD mat may be dominated by the specific community that is responsible for the characteristic appearance and progression of the black band. Despite the presence of a wide variety (28 phyla, 55 classes) of different types of bacteria found assembling the band, according to the mRNA data, only 14 classes showed high activity (>96% of total activity). Interestingly, the most prominent of the overexpressed functions in the black band were cell cycle and division (Fig. 3), indicating the presence of higher levels of bacterial cell proliferation in the advancing cyanobacterial mat.

Members of the *Cyanobacteria*, *Bacteroidetes*, and *Firmicutes* phyla were found in high numbers in the black band. These findings correlate strongly with previous studies of black band consortia (Cooney et al. 2002, Frias-Lopez et al. 2002, Richardson 2004, Barneah et al. 2007, Sekar et al. 2008, Arotsker et al. 2009, 2015, Miller & Richardson 2011). Indeed, in an earlier study, we used the 16S rRNA gene as a marker for diversity in BBD-affected colonies and found that mat diversity decreased during the peak disease period, indicating that certain species were dominant (Arotsker et al. 2015). In the present study, we investigated the activity of these bacterial groups in diseased corals and found that the high numbers of RNA reads in the black band mat reflect the high band activity.

Cyanobacteria were not only the dominant component of the black band mat, they were also found to be the most highly active group, accounting for 26.7% of total mat activity (Fig. 2). This finding coincides with those of previous studies showing that these cyanobacteria appear to be present and dominant in black band outbreaks around the world (Sussman et al. 2006, Sato et al. 2010, Casamatta et al. 2012, Arotsker et al. 2015). In the Red Sea (Gulf of Eilat) black band sample, the BgP10_4S^T cyanobacterium strain (FJ210722) named *Psuedoscillatoria coralii* by Rasoulouniriana et al. (2009) was closely related (>97%) to *Roseofilum reptotaenium* (*Oscilla-*

toriales, *Cyanobacteria*) isolated from Caribbean BBD-affected corals (Casamatta et al. 2012) and was also closely related (99%) to the Cyano OCN074 strain (KJ914890) from Hawaiian BBD (Aeby et al. 2015). A major portion of the cyanobacteria activity in the transcriptome correlates with the presence of this cyanobacterium in the band (Arotsker et al. 2015). It is therefore likely that this strain provides much of the cyanobacterial activity, though due to database limitations, we cannot specifically assign the activity we observed to this BBD strain.

One of the most overexpressed mRNA reads in the BBD mat was affiliated with adenosylhomocysteinase hydrolase (from a family of thioether and trialkylsulfonium hydrolases) (Table 3). This enzyme is involved in the biosynthesis of cyanotoxins such as saxitoxins (Taroncher-Oldenburg & Anderson 2000, Moustafa et al. 2009, Wang et al. 2013), the high levels of expression of which are not surprising, since cyanotoxin production has been previously documented in black band research (Richardson et al. 2007, Gantar et al. 2009). It is possible that this function has a role in cyanobacterial penetration into apparently healthy tissue, compromising coral health and initiating consortium establishment (Arotsker et al. 2015, Meyer et al. in press). It is also possible that the *Sphingobacteria* activity noted in the microbial transcriptome of apparently healthy tissue was quenched by the increasing toxic activity of the cyanobacteria in the black band mat (Fig. 2) which might diffuse to nearby tissues and displace members of the resident microbiome (Meyer et al. in press).

The *Bacilli* and *Clostridia* classes of the *Firmicutes* phylum were also found to be associated with the black band (8.56% and 6.71%, respectively), and both have been previously documented in high numbers in earlier black band studies of *Favia* sp. (Barneah et al. 2007, Arotsker et al. 2009, 2015). Of these, 2 bacillus species (*Oceanobacillus iheyensis* and *Bacillus* sp.) express high levels of rhodanese (thiosulfate sulfurtransferase), which detoxifies cyanide and sulfide (Villarejo & Westley 1966, Cipollone et al. 2004, Billaut-Laden et al. 2006, Liu et al. 2014, Shen et al. 2015). Well-established black bands contain considerable concentrations of toxic hydrogen sulfide, and therefore, the presence of rhodanese may reflect a hydrogen sulfide detoxification mechanism. On the other hand, although known bacterial genes for cyanide production (glycine oxidation by HCN synthase; Blumer & Haas 2000) were not transcribed in the black band we sampled, there are metabolic reactions (both eukaryotic and prokaryotic) that can produce cyanide that, in turn, negatively affects the coral and its microbiota (Cervino et al. 2003). It is also possible that some microorganisms found in the band produce cyanide through an allelopathic reaction (Vanelslander et al. 2012) to control assemblies of other microorganisms in their immediate microenvironment. Thus, the activity of rhodanese may promote the survival of bacteria species sensitive to sulfide and cyanide by reducing its local concentration. From this perspective, the production of rhodanese may play an important role in BBD consortium maintenance and its specific activity.

Gammaproteobacteria were found to be part of the natural coral flora and major contributors to cell division and cell cycle transcripts of the black band. This class also contains a group of known coral pathogens, the vibrios, which in this study, were highly active only in the black band sample. Indeed, vibrios are an active and important part of the black band (Barneah et al. 2007, Sekar et al. 2008, Arotsker et al. 2009, 2015). The most transcribed mRNA associated with *Vibrio* spp. in the black band was found to be the thiamine (vitamin B1) ABC transporter (Table 2). A required cofactor, thiamine has a critical metabolic role as an electron carrier and nucleophile for several enzymes. Although the thiamine ABC transporter is involved in the specific translocation of thiamine and its phosphoesters across the inner membrane of the cell, the specific role of thiamine in bacteria metabolism has yet to be fully understood. However, the finding that its transporters are transcribed in the black band is indicative of the high level of metabolic activity of the band's resident *Vibrio* spp., which also contributed significantly to the levels of protein metabolism in the band (Table 5). Among the most dominant vibrios present in the band, we were able to find several species that are closely related to known pathogens, including *V. parahaemolyticus* and *V. cholerae* (human pathogens), and *V. splendidus* and *V. shilonii* (pathogens of marine animals, including corals) (Kushmaro et al. 1996, Jensen et al. 2003). Vibrios typically use extracellular metalloproteases as part of their mode of pathogenicity, activity that occurs in *Vibrio* spp. associated with BBD (Arotsker et al. 2009).

SRB, an integral part of the black band, are responsible for the oxide/sulfide gradient found in the band (Richardson 2004). *Desulfovibrio* spp. are vital members of the black band (Cooney et al. 2002, Kuta & Richardson 2002, Viehman et al. 2006, Barneah et al. 2007, Sato et al. 2009, Bourne et al. 2011, Arotsker et al. 2015), and *D. desulfuricans* has been found to be

the most active species of the *Deltaproteobacteria* class (Barneah et al. 2007, Arotsker et al. 2009). In the current study, almost half of the transcripts associated with *Desulfovibrio* spp. were genes involved in sulfide production (Table 4) that, together with the transcripts for APS production, may play a major role in the metabolism of proteins and sulfur in the black band. Therefore, among all the SRB found in the band, *Desulfovibrio* spp. is likely to be the principal bacteria responsible for sulfide production, thereby contributing greatly to disease initiation and progression. Involved in the reductive arm of the sulfur cycle, *D. desulfuricans* plays a critical role in degrading organic compounds in sulfate- and organic-rich environments (Hansen 1994). Although *Desulfovibrio* spp. representatives were also present in the apparently healthy area of the diseased coral, no transcripts for sulfide production were evident in this sample. This finding indicates that *Desulfovibrio* spp. may be the first colonizers of apparently healthy tissue and that they eventually shift their activity toward sulfide production under favorable conditions, such as during the initiation and establishment of the black band mat.

Together with the necrotic coral tissue, the BBD bacterial consortium creates a micro-environment that is highly rich in organic matter, rendering the BBD mat microbiota on corals that live in highly oligotrophic waters a virtual 'gold mine' for opportunists. The most dominant carbon utilization function in the BBD-affected coral bacteria (0.54 %) was that of transketolase, an enzyme integral to the pentose phosphate pathway during carbohydrate metabolism in all organisms and in the Calvin cycle of photosynthesis. The finding that this enzyme was transcribed, therefore, is not surprising, as the BBD mat and its associated host tissues provide a niche that is very rich in different types of available carbon (Viehman et al. 2006). Additionally, the dissolved organic carbon produced during cyanobacterial photosynthesis stimulates heterotrophs in the microbial mat, including the SRB (Bourne et al. 2011). Upregulation of transketolase in BBD-affected tissue may explain previous observations that elevated temperatures favor bacterial degradation of the organic carbon, promoting the BBD disease processes (Hoppe et al. 2008, Miller & Richardson 2015).

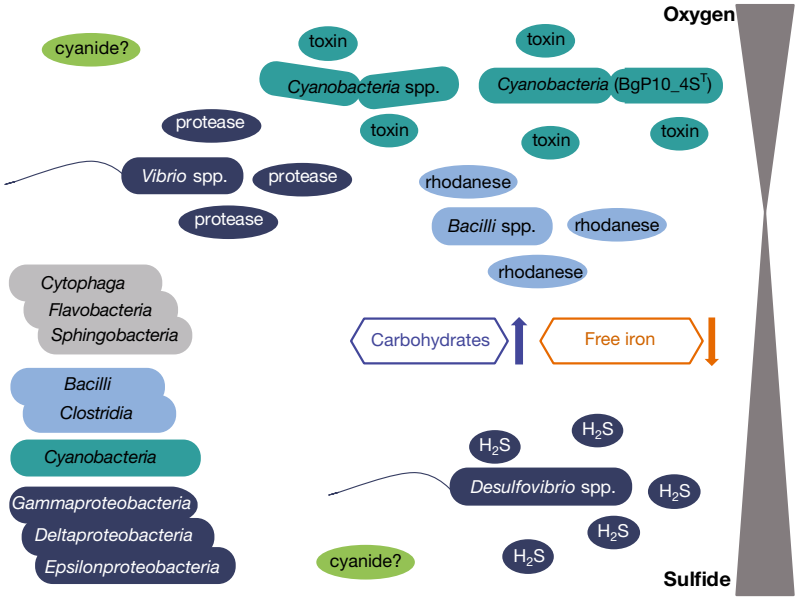

Fig. 4. Black band model. The most active bacterial classes are listed on the left. Levels of oxygen decrease from the band surface, through the soft tissues, to the skeleton/band interphase where elevated restricting levels of anoxia and sulfide are found. Coral tissue breakdown is caused by the amplified growth of the cyanobacteria and accompanying bacteria. Width and depth of the bacterial invasion front and proliferation of the bacteria adjacent to the healthy tissues result in a reduction of the available oxygen at the tissue–mat interface, concurrently creating a sulfide- and carbohydrate-rich environment. This environment is iron limited due to the enhanced iron consumption by the resident cyanobacteria. *Desulfovibrio* spp., the main producers of sulfide in the band, cause additional degradation of underlying coral tissues. The micro-environmental changes confer an opportunity for commensals and opportunistic coral pathogens, such as some *Vibrio* spp., to further degrade the tissues using proteases, while some *Bacilli* spp. express rhodanese, an enzyme responsible for cyanide and sulfide detoxification

Proposed BBD functional model

The unique conditions found in the black band probably account for the complexity of its microbial consortium and support the persistence and advance of the band. Fig. 4 summarizes our new insights into the structure and function of the black band mat and its components. Compared to the consortium of bacteria found on healthy tissues, the black band mat is dominated by *Cyanobacteria*, *Cytophaga*, *Flavobacteria*, *Sphingobacteria*, *Bacilli*, *Clostridia*, *Gamma-*, *Delta-*, and *Epsilonproteobacteria*. In terms of function, cyanobacteria, present at high numbers in the black band mat where they proliferate profusely, express genes that are involved in toxin production. *Desulfovibrio* spp. are

probably the main producers of sulfide in the band, which causes additional degradation of underlying coral tissues. This tissue breakdown may result in the reduction of available oxygen at the tissue–mat interface, thus creating a sulfide-, carbohydrate-, and possibly cyanide-rich environment that seems to be iron limited due to the enhanced iron consumption of the highly active cyanobacteria (Singh & Sherman 2000, Frias Lopez et al. 2004). The sulfur supplier rhodanese, present in various forms in the heterotrophic bacteria of the mat, may be involved in cyanide and sulfide detoxification (Billaut-Laden et al. 2006, Liu et al. 2014, Shen et al. 2015) to maintain a balance in the relative numbers of BBD consortium members. Additionally, due to these microenvironmental changes, the numerous *Vibrio* strains (commensals and opportunistic coral pathogens) found on healthy colonies make a pathogenic contribution to the black band mat, where they produce proteases that further degrade underlying coral tissues. As the consortium continues to develop, its bacteria members continue to degrade the coral tissue below and adjacent to the mat, thereby producing the materials necessary for further growth of the consortium members. This process ensures the slow but persistent advance of the mat over the adjacent coral tissue.

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