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Microbial Metabolism and Disease Virulence Changes Across Day and Night in Coral Black Band Disease Lesions

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ABSTRACT

Coral black band disease (BBD) is characterised as a cyanobacteria-dominated microbial mat that rapidly kills underlying coral tissue. Solar radiation promotes lesion progression by fuelling the cyanobacterial photosynthesis, while sulphate-reducing bacteria and sulphide-oxidising bacteria are implicated in sulphide dynamics within the mat. How the metabolism of the key microbial communities in the mat varies under light and dark conditions and impacts lesion virulence is poorly characterised, however. To compare microbial gene expression under different light regimes, we recovered 28 near-complete BBD-derived metagenome-assembled genomes (MAGs) using Oxford Nanopore Technologies long-read sequencing and profiled Illumina metatranscriptomic reads from BBD lesions collected at day and night by mapping to these MAGs. Genes from the cyanobacterium *Roseofilum reptotaenium* dominated the differentially expressed genes, with photosynthesis highly represented during the day-time. Relative expression of sulphur and nitrogen metabolism, cofactor biosynthesis, chemotaxis and motility increased among the non-cyanobacterial members at night. Enhanced sulphur reduction by Campylobacteriales and *Desulfovibrionaceae* at night likely supports a sulphide-rich and low oxygen micro-environment in the lesion, while increased chemotaxis and motility by Campylobacteriales and other heterotrophic bacteria drive lesion progression towards healthy coral tissue. This study provides insights into how diurnal light dynamics drive microbial metabolic pathways changes, thereby promoting BBD virulence.

1 | Introduction

Black band disease (BBD) is characterised as a dark-pigmented polymicrobial mat that consists of a complex consortium of bacteria dominated by the cyanobacterium *Roseofilum reptotaenium*, sulphide-oxidising bacteria, and sulphate-reducing bacteria, as well as diverse groups of other bacteria, viruses and archaea (Frias-Lopez et al. 2004; Sato et al. 2013). These dominant microbial groups maintain a sulphide-rich and anoxic microenvironment at the base of the mat, which is toxic to coral tissues (Carlton and Richardson 1995; Glas et al. 2012; Richardson et al. 2009). Due to its polymicrobial nature, BBD

presents a challenge to Koch's postulates and traditional etiological approaches to understanding infectious diseases, as the complex consortium makes it difficult to identify the specific pathogen as the causative agent(s).

Previous studies have recovered metagenome-assembled genomes (MAGs) from BBD lesions to characterise the putative microbial metabolic functions that contribute to disease virulence. For example, Sato et al. (2017) and Meyer et al. (2017) recovered numerous BBD-specific MAGs from dominant taxa including the cyanobacterium *R. reptotaenium* plus Proteobacteria- and Bacteroidota-affiliated taxa. Gene content analysis suggested

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that photosynthetic *R. reptotaenium* has a pivotal role in the production of organic matter and is central to carbon and nitrogen fixation, while Deltaproteobacteria degrade organic compounds while reducing sulphate, thereby producing H_2S , facilitated through sulphate reduction pathways in the microbial mat (Meyer et al. 2017; Sato et al. 2017). Previous studies recovered MAGs of *Arcobacteraceae* and *Rhodobacteraceae* (Meyer et al. 2017; Sato et al. 2017), families that contain potential sulphide-oxidation bacteria, but *sox* gene pathways involved in sulphide oxidation were not identified, likely a result of either low genome completeness of the MAGs. Interestingly, Wada et al. (2023) demonstrated that the relative abundance of *Arcobacteraceae* positively correlated with the BBD mat migration rate, indicating their potential involvement in BBD virulence, with migration rate negatively correlated with the relative abundance of other potential sulphide-oxidising bacteria associated with *Rhodobacteraceae*. While both *Arcobacteraceae* and *Rhodobacteraceae* are likely involved in sulphide oxidation in the BBD mat, their contrasting correlations with mat migration rates suggest that one or both taxa may also perform additional ecological roles that differentially influence lesion progression. Overall, the comparative metagenomic and metatranscriptomic approaches suggested that cyanobacterial photosynthesis supports the metabolic pathways of associated microorganisms in the lesion, contributing directly to BBD lesion progression and disease virulence (Meyer et al. 2017; Sato et al. 2017).

Dynamic changes in oxygen and sulphide driven by light intensity promote lesion progression and alter the physicochemical conditions within the BBD mat, with dark periods causing anoxic conditions and the production of toxic sulphide compounds (Boyett et al. 2007; Sato et al. 2011; Kuehl et al. 2011; Glas et al. 2012). The filamentous cyanobacteria *R. reptotaenium* exhibit positive phototaxis behaviour and chemotaxis towards oxygen and away from sulphide (Myers and Richardson 2009; Richardson et al. 2014), leading the motile cyanobacteria into the upper layer of the BBD mat to facilitate photosynthetic activity during the day. Cell clumping at the surface facilitates oxygen production in *R. reptotaenium*, but also generates steep oxygen gradients underneath, due to depletion of oxygen through microbial respiration and metabolic activity, resulting in an increasing anoxic environment. The anoxic conditions promote the activity of the sulphur-reducing bacteria, resulting in elevated sulphide levels that enhance lesion virulence (Carlton and Richardson 1995; Richardson et al. 2009, 2014). The sulphide-oxidising bacteria also migrate towards the upper layer in darkness to the oxic-anoxic interface to satisfy their metabolic requirements (Jorgensen and Revsbech 1983; Sato et al. 2016), resulting in an increase in sulphide concentrations at the bottom of the mat (Glas et al. 2012). The increasing sulphide at night also contributes to the cyanobacteria exhibiting negative horizontal chemotaxis away from the lesion and migrating towards healthy coral tissue, which promotes the lesion progression (Sato et al. 2016).

Despite a strong understanding of the metabolic potential of each microbial member within the BBD mat, corresponding shifts in microbial metabolic pathways across light regimes that cause lesion virulence remain to be elucidated. Current BBD-associated MAGs from Meyer et al. (2017) and Sato et al. (2017) reconstructed using Illumina short-read sequencing primarily

represent dominant microbial taxa but also include a number of low-abundance taxa with poor genome quality. In particular, MAGs corresponding to metabolically important but less abundant groups, such as sulphide-oxidising and sulphate-reducing bacteria, exhibited low completeness and high contamination rates. For example, the sulphide-oxidising *Arcobacteraceae* MAG reconstructed in Sato et al. (2017) only has a ~27% completeness, which undermines the accuracy of inferred metabolic capabilities and compromises the understanding of their functional roles within the BBD mat community. Short-read assemblies often lead to fragmented genomes in less abundant taxa, an issue exacerbated in complex metagenomes like BBD lesions, but one that can be addressed through long-read sequencing methods such as Oxford Nanopore Technologies (ONTs) and Pacific Biosciences (PacBio) (Meslier et al. 2022; Orellana et al. 2023; Sevim et al. 2019). These technologies enable the retrieval of highly complete and accurate MAGs by reducing fragmentation, thus enhancing the quality of MAG recovery from diverse microbiomes (Cuscó et al. 2021; Liu, Yang, et al. 2022; Meslier et al. 2022; Singleton et al. 2021). Additionally, *Arcobacteraceae* has low genomic guanine-cytosine (GC) content (Sato et al. 2017), which presents challenges for GC-biased platforms like Illumina, in contrast to ONT and PacBio platforms (Browne et al. 2020; Sevim et al. 2019). Thus, using long-read sequencing offers a promising solution for achieving more complete microbial genomic representations and associated metabolic pathways within the BBD lesion.

Here, we used long-read Nanopore sequencing derived MAGs to map metatranscriptomes and report differentially expressed genes across light regimes associated with key microbial groups in BBD lesions, including the dominating cyanobacterium *R. reptotaenium*, sulphide-oxidising bacteria, sulphate-reducing bacteria and other heterotrophic bacteria, reflecting the metabolic activities that may contribute to the nighttime virulence of BBD. We constructed complete or near-complete MAGs of BBD-derived microbial communities from diseased *Acropora cf. hyacinthus* using the Nanopore long-read sequencing approach, providing improved genomic resolution of low-abundance but functionally important taxa within the BBD microbial community. Further, meta-transcriptomic sequencing was performed for BBD mat communities subjected to day (light) and night (dark) time conditions from diseased *Pavona duerdeni* and mapped to derived MAGs to compare gene expression differences within the bacterial community under the different light regimes. This study combined long-read based metagenome assemblies with the first diel transcriptomic profiling of the BBD microbial communities, identifying differential gene regulation patterns in key microbial metabolisms between day and night conditions, and provided new insights into processes that contribute to BBD virulence.

2 | Experimental Procedures

2.1 | BBD-Derived Near-Complete Metagenome-Assembled Genome Recovery

Two BBD mats from diseased *A. cf. hyacinthus* colonies were collected from the reef on the northeast side of Orpheus Island (18°34.2' S, 146°29.3' E; NE Orpheus), Palm Island group, Great

Barrier Reef, Australia on 24 February 2023 (GBRMPA permit G22/46534.1). The BBD mat samples were preserved in chilled DMSO-EDTA salt-saturated buffer (DESS; 20% DMSO, 0.25 M disodium EDTA pH 8.0, saturated with NaCl) immediately after collection. High molecular weight (HMW) DNA was extracted using a phenol:chloroform:Isoamyl alcohol (25:24:1) phase separation extraction method modified from O'Brien et al. (2023). In brief, the BBD mat was lysed with the STET lysis buffer (8% sucrose, 5% Triton X-100, 50 mM EDTA, 50 mM Tris-HCl, pH 8.0) and 5% (w/v) lysozyme, without any freeze-thaw cycles before adding sodium dodecyl sulphate (SDS; 10% solution) and lysis at 55°C until fully lysed before proceeding to phase separation and DNA precipitation with alcohol overnight. HMW DNA from each sample was visualised by 1% TAE agarose gel imaging, quantified with Qubit DNA BR Assay (ThermoFisher) and minimal contamination was ensured by Nanodrop spectrophotometer (ThermoFisher).

HMW DNA was subjected to both Oxford Nanopore long-read sequencing and Illumina short-read sequencing to leverage both long-read scaffolding and to provide an additional contig depth profile from short-read sequencing. DNA library preparation was carried out using the ligation sequencing kit SQK-LSK114 (ONT) for sequencing on the MinION R.10.4.1 flowcell (ONT). Long-read sequencing data were generated on the Mk1B device (ONT) with the MinKNOW v22.12.7 software for 72 h, with a 200 bp minimum read length. Super-accurate basecalling was performed at 260 bases per second (bps) and a minimum *q*-score greater than 10 with Guppy v6.4.6. A total of 21.27 M reads and 34.6 Gbp yield were obtained from two MinION sequencing runs. Additionally, paired-end short-read (150 bp) data were generated by the Australian Genome Research Facility (AGRF) using Illumina NovaSeq 300 technology with DNA Prep M library preparation, with a total of 78.23 M paired-end reads and a total of 23.62 Gbp yield from two samples.

Raw long-reads were de novo assembled using the Flye v. 2.9.1 assembler (Kolmogorov et al. 2020) with the '-meta' setting enabled and the '-nano-hq' option (Figure S1). Assemblies were then polished with three rounds of Racon v1.5.0 (<https://github.com/isovic/racon>) and one round of Medaka v1.4.4 (<https://github.com/nanoporetech/medaka>) to improve accuracy by correcting residual base-level errors in reads. Automated binning was carried out using MetaBAT2 and MaxBin2 binning tools (Kang et al. 2019; Wu et al. 2016). To aid with the binning process, contig coverage profiles from both Nanopore long-reads and Illumina short-reads were provided as input to the two binning tools. Contig coverage was profiled using Minimap2, BWA and samtools. The binning output of MetaBAT2 and MaxBin2 was then integrated and refined using DAS Tool (Sieber et al. 2018). CoverM v. 0.6.1 (<https://github.com/wwood/CoverM>) was applied to de-replicate MAGs with >95% ANI (using the 'coverm genome --dereplication-ani' setting) and to calculate the bin coverage (using the 'coverm contig -m mean' setting) and the relative abundance ('coverm contig -m relative_abundance'). The completeness and contamination of the genome bins were estimated using CheckM v1.2.2 (Parks et al. 2015) and the quality of each MAG was evaluated based on Bowers et al. (2017) with its completeness and contamination. The low quality (< 50% completeness and/or > 10% contamination) MAGs were removed, keeping only high and medium quality MAGs from

downstream analyses. The bins were taxonomically classified using GTDB-Tk v 2.3.0 (Chaumeil et al. 2020) and functionally annotated using Prokka v 1.14.6 (Seemann 2014), GhostKOALA v 2.0 (Kanehisa et al. 2016) and egg-nog-mapper v 2.1.12 (Cantalapiedra et al. 2021).

2.2 | BBD Mat Gene Expression Comparison Between Day and Night Conditions

To study metatranscriptomic profiles of BBD-associated microbial communities under day and night conditions, we collected BBD mat specimens once at daytime and again under nighttime conditions from identical lesions. Five colonies of BBD-infected *P. duerdeni* of approximately 8 cm in diameter were collected from NE Orpheus and transported back to outdoor flowthrough raceways at the Orpheus Island Research Station on 5 March 2013 (GBRMPA permit G11/34003.1). After the initial 24 h acclimation period in filtered (5 µm) seawater under outdoor natural daylight and temperature conditions, the BBD mats from each colony were collected at 1 PM (daytime, 7 h after sunrise) and again at 10 pm (nighttime, 4 h after sunset) on 6 March 2013. In total, 10 BBD lesion samples comprising five 'Day' samples and five 'Night' samples were collected. However, temperature, light intensity, water DO level were not measured. The BBD mat samples were preserved in RNA-later solution and stored at -20°C until RNA extraction.

Total RNA was extracted from the preserved BBD mat, using the RNeasy PowerMicrobiome kit (Qiagen) with a modified phenol-based lysis protocol by adding Phenol:Chloroform:Isoamyl alcohol (25:24:1) to the sample prior to following the manufacturer's instructions. The Invitrogen DNA-free DNA Removal kit (ThermoFisher) was used to remove residual DNA contamination, and the total RNA was further purified with a RNeasy MinElute Cleanup kit (Qiagen). The quality and quantity of RNA recovered were estimated using a TapeStation high-sensitivity RNA system (Agilent), before cDNA synthesis using the Ovation system cDNA synthesis kit (NuGEN Technologies Incorporated), following the manufacturer's instructions. The quality and quantity of the resulting cDNA was determined using a Nanodrop spectrophotometer (ThermoFisher) and Qubit DNA BR Assay (ThermoFisher), ensuring that cDNA samples met the quality requirements for Illumina NextSeq500, 75 bp paired-end read sequencing, at the Ramaciotti Sequencing Centre (UNSW, Sydney). A total of 30 Gb raw paired-end sequence data derived from the 10 samples was obtained, and the sequence reads were subjected to quality control checking and adaptor removal using the MultiQC and Trimmomatic pipelines.

To profile the gene expression patterns of BBD-associated microbial at the community level, focusing on the dominant but diverse bacterial taxa captured in our new set of BBD-derived MAGs, the clean RNA reads were mapped against the 28 MAGs generated above using Bowtie2. The counts of the mapped reads were used for assessing gene expression levels using RSEM (Li and Dewey 2011). The between-sample normalisation, differential expression analysis and hypothesis testing were performed using the standard workflow implemented in the DESeq2 package (Love et al. 2014) in the R environment (R Core Team 2023). An additional non-zero and low-counts pre-filtering step was

applied to exclude genes with less than 10 read counts in more than 3 samples. Although taxon-specific normalisation has gained traction in recent metatranscriptomic studies (Imminger et al. 2024; Klingenberg and Meinicke 2017; Shakya et al. 2019), we adopted a gene-centric normalisation across all MAGs, which we found to be more suitable for our dataset due to sparsity in many taxa (see Supplementary A for details and justification). The differential expressed (DE) genes between the day and night samples were detected with paired analysis, and the Benjamini–Hochberg false discovery rate (FDR) was applied to limit the number of false-positive results to less than 5%. For the interpretation of functional aspects, only functionally-annotated DE genes were studied and all the uncharacterised and hypothetical proteins were excluded. The TopGO package (Alexa and Rahnenführer 2009) was used to perform Gene Ontology (GO) enrichment analysis for the biological processes of the DE genes, using the ‘weight01’ algorithm. The clusterProfiler package (Yu et al. 2012) was used to perform KEGG (Kyoto Encyclopaedia of Genes and Genomes) orthologue enrichment analysis of the DE genes. The results of the GO and KO enrichment analysis were visualised in the R environment.

3 | Results

3.1 | Near-Complete BBD-Derived MAGs Recovered From Diseased *A. cf hyacinthus* Colonies

The metagenomic de-novo assembly yielded 79,360 contigs with a combined length of approximately 0.69 gigabase pairs (Gbps), obtained from two ONT gDNA ligation sequencing runs utilising MinION R10.1.4 flowcells. The average N50 length of the contigs was 14.5kbp, whereas the largest identified contig had a size of 5.8Mbps. Subsequent genome binning and refinement procedures culminated in the retrieval of 28 high and medium quality MAGs (>90% completeness and <5% contamination, >50% completeness and <10% contamination, respectively). These 28 BBD-derived MAGs collectively represented approximately 81% of relative read abundance within the metagenome from the BBD mat on *A. cf hyacinthus* coral, assessed by the number of mapped reads (Table 1). Specifically, 14 high-quality MAGs, with completeness above 90% and contamination not exceeding 5%, accounted for over 69% of the metagenomic reads from the entire BBD mat bacterial community. Notably, two MAGs within the high-quality subset achieved 100% completeness and less than 1% contamination, that is, Campylobacterales *Halarcobacter* sp. and Desulfovibrionales *Maridesulfovibrio* sp. In addition, a Cytophagales *UBA2561 sp002340355* MAG displayed 98% completeness across one single contig with 4.7Mbps in size. The remaining 14 medium-quality MAGs, with completeness more than 50% and contamination less than 10%, contributed an additional 12% to the sequence composition.

The 28 BBD-derived MAGs represented 21 families in 8 bacterial phyla, with 20 MAGs taxonomically assigned at the genus level and two MAGs at the species level (Table 1). Cyanobacterium *R. reptotaenium* dominated the BBD-derived metagenome, with 28% of reads assembled to this MAG. The MAG assigned Cytophagales *UBA2561 sp002340355* ranked as the second most abundant taxon, accounting for 14% of the

reads. Key BBD bacterial members including sulphur-oxidising bacteria Campylobacterales (three MAGs, previously described as sulphur-oxidising bacteria in BBD mats), accounted for 6.5% of the reads, while sulphur-reducing bacteria Desulfovibrionales and Desulfobacterales (one MAG each, Desulfovibrionales previously characterised as sulphate-reducing bacteria in BBD mats), constituted collectively 6.5% of derived reads. Gammaproteobacteria (Enterobacterales and Pseudomonadales) were represented across seven distinct MAGs, collectively constituting 5% of the reads, while Clostridia (Peptostreptococcales and Lachnospirales) and Bacteroidales constituted 8.6% and <1% of the reads, respectively (Table 1). Other recovered bacterial MAGs affiliated with XYD2-FULL-50-16 from the SAR324 clade (1%), Chitinophagales (1.8%), Flavobacteriales (7.8%), Oligoflexales (0.35%) and Rhodobacterales (0.8%).

3.2 | Mapping BBD Metatranscriptomes to BBD-Derived MAGs

A total of 997 million metatranscriptomic reads were obtained from 10 samples comparing day and night conditions from 5 BBD-infected *P. duerdeni* colonies (between 59 and 153 million reads per sample). Between 10.7% and 32.7% of the total raw reads per sample were mapped to the 28 BBD-derived MAGs, with an average of 25.9% for daytime samples and 21.81% for nighttime samples. Approximately 43% of the total mapped reads (approximately 40 million reads) were mapped to the cyanobacterium *R. reptotaenium*, followed by approximately 28.9% of the reads mapping to Cytophagales *UBA2561 sp002340355* (Figure 1a). The remaining mapped reads were distributed across other taxa, including Clostridia Peptostreptococcales and Lachnospirales (7.2%), Chitinophagales (6.9%), Campylobacterales (2.8%), Desulfovibrionales and Desulfobacterales (2.2%), Gammaproteobacteria Enterobacterales and Pseudomonadales (2.1%), Flavobacteriales (1.5%), Bacteroidales (1.3%), SAR324 (0.7%), Oligoflexales (<0.2%) and Rhodobacterales (<0.01%).

3.3 | Differential Expression in Response to Day and Night Regimes in the BBD Community

BBD lesion gene expression data (i.e., CDS and tRNA) across both day and night samples were dominated by the cyanobacterium *R. reptotaenium* (Figure 1b). Differential expression analysis unveiled 414 DE genes between day and night samples with statistical significance (DESeq2, FDR adjusted $p < 0.05$; Figure 2; Table S1). The overrepresentation analysis (ORA) identified GO and KEGG orthology (KO) terms that were overrepresented in the set of all DEGs including those with higher expression in either day or night conditions. The significantly enriched DE genes are largely associated to photosynthesis, including biological processes in photosynthesis (GO: 0015979) and photosynthetic electron transport chain (GO: 0009076) in GO terms, with photosynthesis (ko00195) and photosynthesis antenna proteins (ko00196) in KO terms (Figure S2).

Among all the DE genes, the 93 genes exhibiting significantly elevated expression during the daytime were exclusively

TABLE 1 | BBD-derived MAGs recovered from the BBD lesion from diseased *Acropora cf. hyacinthus*.

MAG ID	Classification	Completeness (%)	Contamination (%)	Heterogeneity (%)	Length (bp)	Mean GC (%)	# contigs	Mapped reads (%)
BBD_MAG01	p_Bacteroidota;c_Bacteroidia;o_Bacteroidales;f_UBA6680;g_UBA6680;s_	84.32	3.28	20	5,366,523	34.51	561	0.25
BBD_MAG02	p_Bacteroidota;c_Bacteroidia;o_Bacteroidales;f_Mariniifilaceae;g_Mariniifilum;s_	54.46	1.37	18.18	2,259,833	32.58	423	0.28
BBD_MAG03	p_Campylobacterota;c_Campylobacteriia;o_Campylobacteriales;f_Arcobacteraceae;g_Halarcobacter;s_	100	0.2	0	3,614,370	27.96	9	4.06
BBD_MAG04	p_Campylobacterota;c_Campylobacteriia;o_Campylobacteriales;f_Arcobacteraceae;g_Poseidonibacter;s_	98.92	2.71	50	3,315,793	27.644	34	0.73
BBD_MAG05	p_Campylobacterota;c_Campylobacteriia;o_Campylobacteriales;f_Sulfurospirillaceae;g_Sulfurospirillum_A;s_	99.49	2.44	0	2,872,862	32.45	22	1.68
BBD_MAG06	p_Bacteroidota;c_Bacteroidia;o_Chitinophagales;f_Saprospiraceae;g_Saprospira;s_	99.26	1.98	16.67	4,222,953	43.48	22	1.85
BBD_MAG07	p_Cyanobacteriia;c_Cyanobacteriia;o_Cyanobacteriales;f_Desertifilaceae;g_Roseofilum;s_Roseofilum reptotaenium	98.22	0.67	0	5,832,788	44.73	5	27.93
BBD_MAG08	p_Bacteroidota;c_Bacteroidia;o_Cytophagales;f_Bernardetiaceae;g_UBA2561;s_UBA2561 sp002340355	97.92	1.34	16.67	4,706,775	34.31	1	14.73
BBD_MAG09	p_Bacteroidota;c_Bacteroidia;o_Cytophagales;f_Bernardetiaceae;g_s_	92.94	5.37	35.71	4,233,716	44.65	432	0.76

(Continues)

TABLE 1 | (Continued)

MAG ID	Classification	Completeness (%)	Contamination (%)	Heterogeneity (%)	Length (bp)	Mean GC (%)	# contigs	Mapped reads (%)
BBD_MAG10	p_Desulfobacterota;c_Desulfobacteria;o_Desulfobacteriales;f_Desulforegulaceae;g_s_Maridesulfovibrio;s	98.39	2.15	14.29	4,123,670	35.33	5	2.99
BBD_MAG11	p_Desulfobacterota;c_Desulfovibrionia;o_Desulfovibrionales;f_Desulfovibrionaceae;g_Maridesulfovibrio;s	100	0.07	0	3,973,970	47.90	6	3.54
BBD_MAG12	p_Proteobacteria;c_Gammaproteobacteria;o_Enterobacterales;f_Alteromonadaceae;g_Psychrosphaera;s	94.94	9.58	65.28	4,416,166	40.18	200	1.34
BBD_MAG13	p_Proteobacteria;c_Gammaproteobacteria;o_Enterobacterales;f_Alteromonadaceae;g_Alteromonas;s	95.4	2.09	80	4,343,435	44.42	185	0.59
BBD_MAG14	p_Proteobacteria;c_Gammaproteobacteria;o_Enterobacterales;f_Alteromonadaceae;g_Thalassotalea;s	83.05	6.34	38.89	3,901,495	42.17	378	0.43
BBD_MAG15	p_Proteobacteria;c_Gammaproteobacteria;o_Enterobacterales;f_Vibrionaceae;g_s	93.87	0.72	50	3,125,021	44.24	52	0.58
BBD_MAG16	p_Bacteroidota;c_Bacteroidia;o_Flavobacteriales;f_Flavobacteriaceae;g_DT-34;s	98.18	0.77	50	3,208,200	32.82	12	2.34
BBD_MAG17	p_Bacteroidota;c_Bacteroidia;o_Flavobacteriales;f_Flavobacteriaceae;g_Winogradskyella;s	55.28	0.66	0	2,090,678	33.81	21	3.01
BBD_MAG18	p_Bacteroidota;c_Bacteroidia;o_Flavobacteriales;f_Flavobacteriaceae;g_Winogradskyella;s	67.28	2.9	33.33	2,517,146	50.25	556	0.81

(Continues)

TABLE 1 | (Continued)

MAG ID	Classification	Completeness (%)	Contamination (%)	Heterogeneity (%)	Length (bp)	Mean GC (%)	# contigs	Mapped reads (%)
BBD_MAG19	p_Bacteroidota;c_Bacteroidia;o_Flavobacteriales;f_Salibacteraceae;g_UBA6049;s_	94.98	1.08	100	2,950,036	36.29	183	0.32
BBD_MAG20	p_Firmicutes;c_Clostridia;o_Lachnospirales;f_Vallitaleaceae;g_s_	98.43	2.5	0	3,424,281	32.51	5	3.13
BBD_MAG21	p_Bdellovibrionota;c_Oligoflexia;o_Oligoflexales;f_Oligoflexaceae;g_s_	81.34	7.38	16.67	4,568,950	36.28	420	0.36
BBD_MAG22	p_Firmicutes;c_Clostridia;o_Peptostreptococcales;f_Acidaminobacteraceae;g_JAFGTX01;s_	94.33	7.09	15	5,253,171	37.20	320	3.36
BBD_MAG23	p_Firmicutes;c_Clostridia;o_Peptostreptococcales;f_Acidaminobacteraceae;g_JABXX01;s_	96.81	8.63	0	5,530,963	33.83	64	2.15
BBD_MAG24	p_Proteobacteria;c_Gammaproteobacteria;o_Pseudomonadales;f_DSM-6294;g_s_	93.33	5.53	62.16	3,686,387	48.22	142	1.12
BBD_MAG25	p_Proteobacteria;c_Gammaproteobacteria;o_Pseudomonadales;f_Endozoicomonadales;g_Endozoicomonadales;s_	76.38	7.93	33.33	4,640,658	48.63	813	0.47
BBD_MAG26	p_Proteobacteria;c_Gammaproteobacteria;o_Pseudomonadales;f_Oleiphilaceae;g_PZPK01;s_	97.54	1.6	11.11	3,586,715	48.03	111	0.57
BBD_MAG27	p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Cognatishimia;s_	76.83	5.2	45.71	2,572,824	55.46	306	0.80
BBD_MAG28	p_SAR324;c_SAR324;o_XYD2-FULL-50-16;f_XYD2-FULL-50-16;g_s_	89.44	2.52	0	4,707,090	39.40	42	1.04

Note: Taxonomy classification of the MAGs based on GTDB-Tk v 2.3.0. The mapped reads show the percentage of the total reads that mapped to each MAG, representing the relative proportion of metagenomic data.

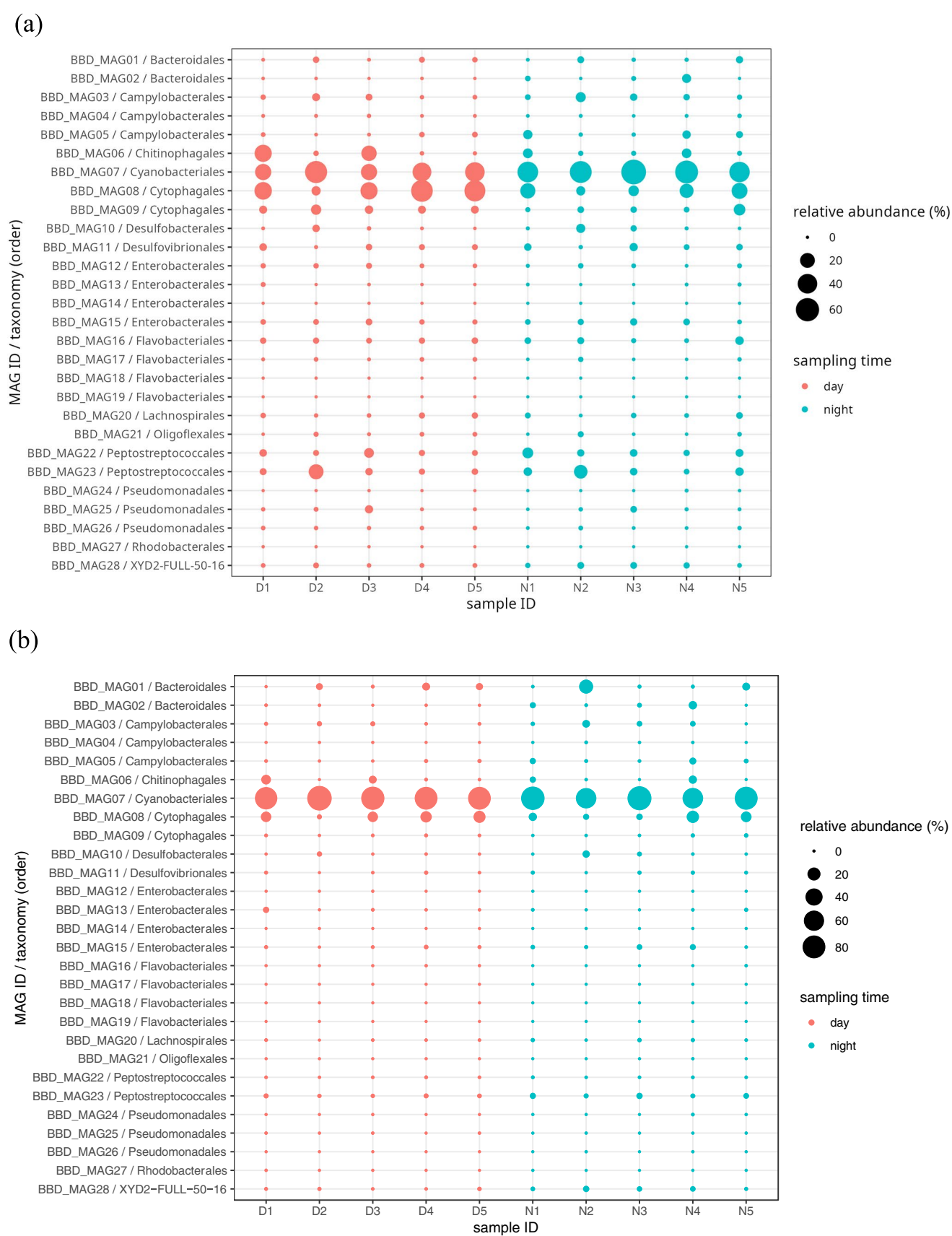


FIGURE 1 | (a) Relative abundance of total metatranscriptomic reads mapped to the 28 BBD-derived MAGs compared against the (b) relative gene expression (i.e., CDS and tRNA, excluding non-coding RNA from the total transcripts) reads. The y-axis displays each of the MAG IDs and bacteria taxonomy at the order level from the 28 BBD-specific MAGs, while the x-axis shows each BBD mat sample collected between day (D1–D5) and night (N1–N5).

derived from *R. reptotaenium* and Cytophagales, encompassing 83 and 10 DE genes, respectively (Figure 2; Table S1). The *R. reptotaenium* expressed genes were predominantly photosynthesis-related and included Photosystem II (PSII)

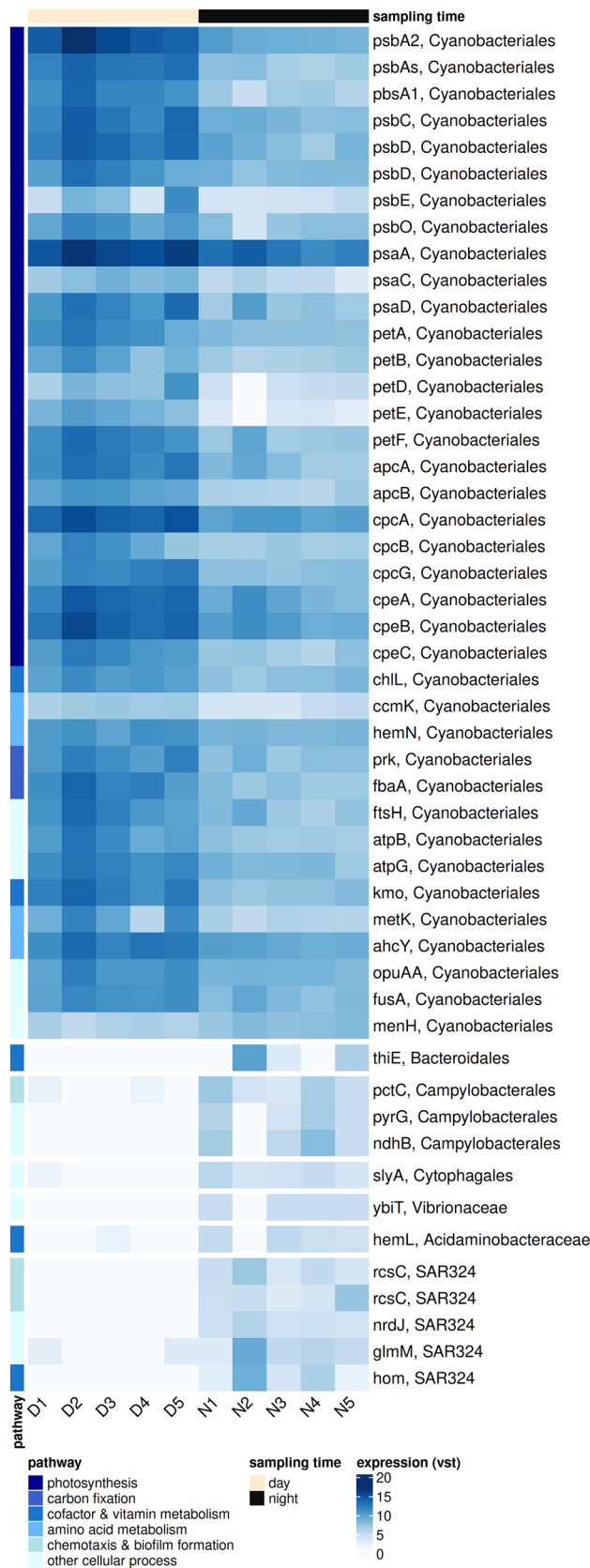


FIGURE 2 | Expression levels of the Top 50 significantly DE genes between day and night BBD lesion samples across five colonies. Each row represents a single DE gene and its affiliated bacteria and each column indicates one of the samples. Columns are ordered with all daytime samples on the left and nighttime on the right. The blue colour scale shows the variance stabilising transformation (VST) counts of each gene in each sample.

protein D *psbA*, Plastocyanin *petE* and phycobilisome *cpcA* (Figure 2; Table S1). The gene involved in pantothenate biosynthesis (*panB*) from Cytophagales also showed higher daytime expression. Conversely, the 306 DE genes that were more highly expressed at night were from diverse bacterial groups. During nighttime, significantly expressed DE genes included those encoding chemotaxis proteins (*CheA*, *CheW*, *PctB*, *PctC*, *PomA*) in Campylobacteriales, SAR324, Vibrionaceae, Acidaminobacteraceae and Cytophagales (Figure 2; Table S1). The biofilm formation regulators (*rssB*, *rcsC*) from Campylobacteriales and SAR324 were highly expressed at night (Figure 2; Table S1).

3.4 | Shifts in the Carbon Fixation, Nitrogen and Sulphur Metabolism Gene Expression Patterns of the BBD Community Across Day and Night

Carbon fixation metabolic pathways dominated gene expression patterns with components of photosystems PSI and PSII (*psaABCDIK*, *psbACDEHMO*) from *R. reptotaenium* abundant and significantly highly expressed (DESeq2, FDR adjusted $p < 0.05$) during the day (Figure 2, Table S1). Other *R. reptotaenium* genes involved in light-harvesting, including components of the cytochrome b6/f complex and electron transport (*petAB-CDEFH*), ATP synthase (*atpABCDEFHG*) and light-harvesting pigments (*cpcABG*, *cpeABC*) were also highly expressed during the day. Genes encoding components of the Calvin-Benson cycle carbon fixation pathway, including *prk*, *fba* and *glpX* were significantly upregulated in *R. reptotaenium* in the daytime samples (Figure S3). In contrast, genes involved in the reductive citrate cycle (rTCA), such as fumarate reductase (*frdA*), succinyl-CoA synthetase (*sucD*), isocitrate dehydrogenase [NADP] (*icd*) and homoisocitrate dehydrogenase (*hicDH*) were upregulated in Bacteroidales, Campylobacteriales and SAR324 taxa at night.

Nitrogen metabolism encoding genes, including nitrogen fixation (*nifDKH*) and assimilatory nitrate reduction (*narB*, *nirA*), showed consistent expression patterns in both day and night in *R. reptotaenium*. Similarly, the denitrification related gene in Chitinophagales also showed consistent expression patterns in both day and night (Figure 3). In contrast, dissimilatory nitrate reduction genes (*napAB*, *nrfA*) were significantly highly expressed (DESeq2, FDR adjusted $p < 0.05$) in Campylobacteriales, with a similar increased expression pattern in SAR324. Nitrogen fixation genes from Bacteroidales and Acidaminobacteraceae also showed an elevated expression pattern at nighttime.

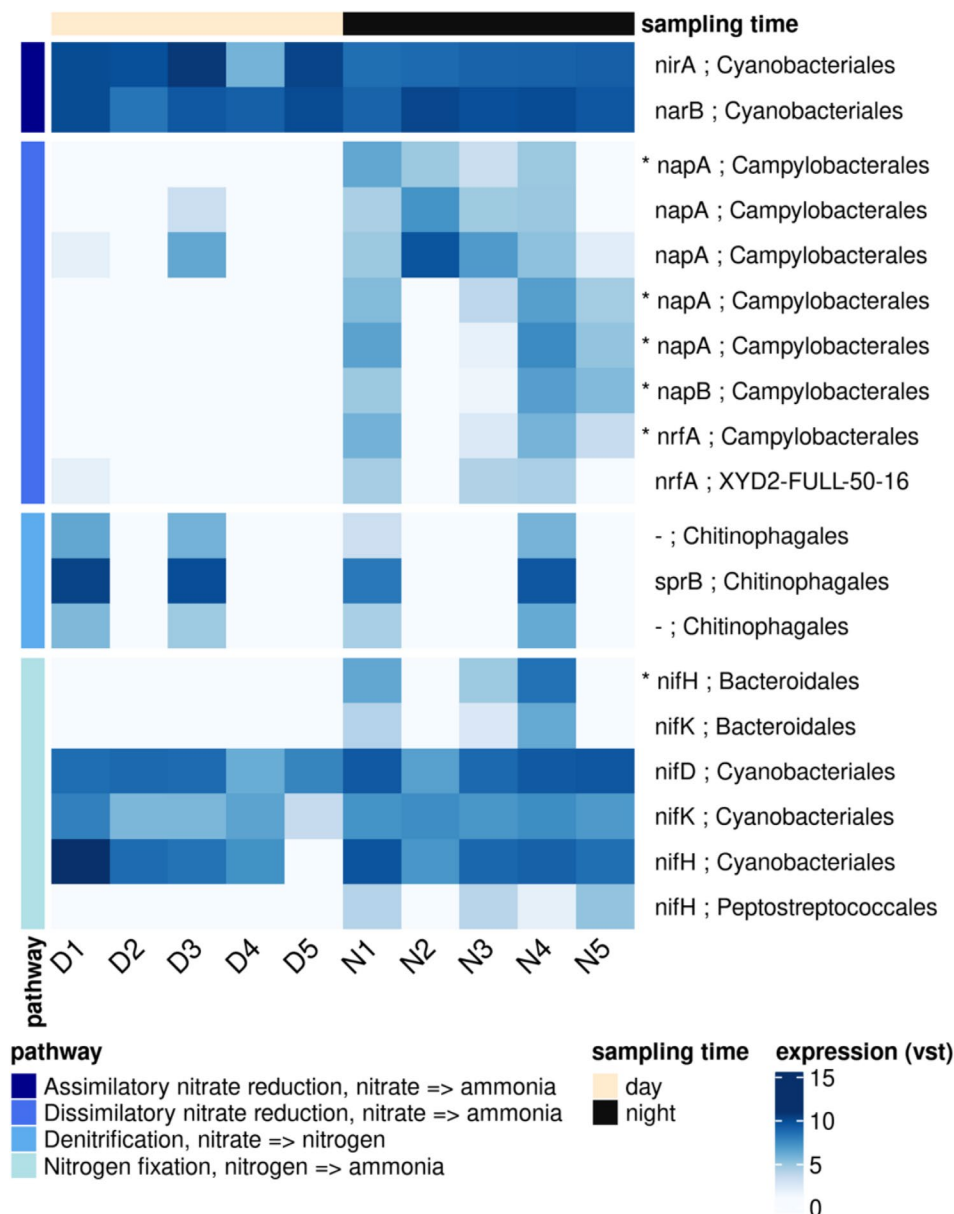


FIGURE 3 | Expression levels of the nitrogen metabolism genes between day and night BBD lesion samples across five colonies. Each row represents a single gene and its affiliated bacteria. Columns indicate samples and are ordered with all daytime samples on the left and nighttime on the right. The blue colour scale shows the VST transformed counts of each gene in each sample. Asterisks (*) indicate genes with statistically significant differential expression between day and night (adjusted $p < 0.05$).

The most abundant expressed sulphur metabolism encoding genes across taxa were derived from the *R. reptotaenium* assimilatory and dissimilatory sulphate reduction pathways (*sat*, *cysCHI*, *sir*, *sat*), though the expression patterns for these genes were consistent between day and night samples (Figure 4). In contrast, other bacterial groups generally exhibited higher expression of sulphur metabolism pathways during the night. For example, Campylobacteriales displayed higher expression of genes involved in both assimilatory and dissimilatory sulphate reduction (*sir*, *pyk*), thiosulphate reduction to sulphide (*phsA*) and thiosulphate oxidation SOX pathway (*soxD*) during the night. Sulphate reduction encoded genes, *pyk* and *cysHI*, in Cytophagales also showed higher expression patterns at night time. Dissimilatory sulphate reduction genes (*aprAB*, *dsuC*, *dsrP*) were only expressed in *Desulfovibrionaceae*, with dissimilatory

sulphate reduction complex *dsrP* significantly highly expressed at night.

4 | Discussion

4.1 | Long-Read MAGs Enable Improved Metabolic Profiling of Diverse Microbial Taxa in the BBD Lesion

This study improves our molecular-based insights into the metabolic activities and gene regulations of the complex microbial environment contributing to BBD virulence. Though the biogeochemical microenvironment and chemotaxis behaviour within the BBD mat have been characterised (Glas et al. 2012;

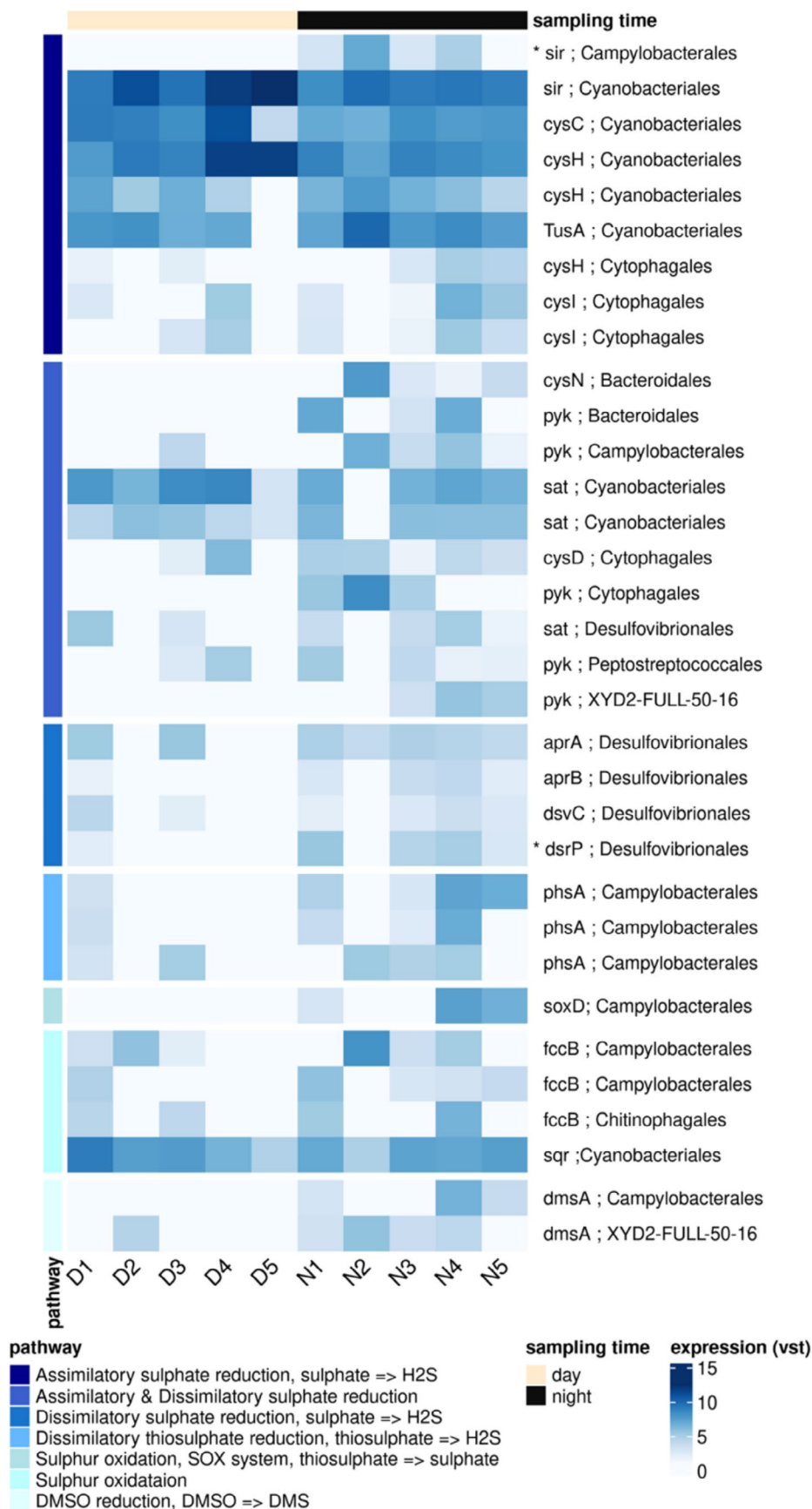


FIGURE 4 | Expression levels of the sulphur metabolism genes between day and night BBD lesion samples across five colonies. Each row represents a single gene and its affiliated bacteria. Columns indicate samples and are ordered with all daytime samples on the left and nighttime on the right. The blue colour scale shows the VST transformed counts of each gene in each sample. Asterisks (*) indicate genes with statistically significant differential expression between day and night (adjusted $p < 0.05$).

Richardson et al. 2009; Sato et al. 2016), we have lacked evidence of changes in the specific metabolic activities of important taxa within the BBD lesion across light regimes, which contribute to disease virulence. Nanopore long-read sequencing improved the completeness of BBD-derived MAGs, particularly for low-abundance but functionally important taxa such as Campylobacteriales and *Desulfovibrionaceae*, aiding our ability to explore the metabolic responses among different bacterial groups within the BBD lesion. Previous BBD-retrieved MAGs were constructed with Illumina short-read sequencing, resulting in lower quality, low completeness and high contamination of reconstructed genomes for non-dominant but important BBD taxa (Meyer et al. 2017; Sato et al. 2017), including sulphide-oxidising and sulphur-reducing bacteria that have been implicated in BBD virulence. For example, the sulphur-reducing bacteria in the BBD MAGs were missing in Sato et al. (2017) and the sulphur-reducing *Desulfovibrio* only has 50%–73% genome completeness in Meyer et al. (2017) (Figure S4 and Table S2). Many of the resolved BBD-derived MAGs in this study are nearly complete with low contamination and collectively represent 81% of the BBD community on the basis of metagenomic reads, representing an important contribution to our genomic understanding of this coral disease. This includes members of key BBD bacterial functional groups such as the cyanobacterium *R. reptotaenium*, sulphide-oxidising bacteria (Campylobacteriales), and sulphur-reducing bacteria (*Desulfovibrionaceae*). We were able to reconstruct three Campylobacteriales MAGs, two affiliated with *Arcobacteraceae* and one affiliated with *Sulfurospirillaceae*, with 98.9%–100% genome completeness. In addition, a *Desulfovibrionaceae* MAG was reconstructed, with 100% genome completeness and 0.07% contamination. Comparative analyses between metagenomes recovered from Illumina short-reads and ONT long-reads have demonstrated the superiority of long-read sequencing approaches in generating higher quality MAGs, including the capability to capture strain-level variations (Chen, Zhao, et al. 2022; Orellana et al. 2023). The highly complete and contiguous MAGs derived from BBD lesions enabled investigation of bacterial gene expression patterns across day and night conditions.

Mapping our metatranscriptomic data to the 28 high- and medium-quality MAGs representing key functional groups within the BBD mat allowed profiling of the diurnal gene expression changes in cyanobacteria (*R. reptotaenium*), sulphur-oxidising bacteria (Campylobacteriales), sulphate-reducing bacteria (*Desulfovibrionaceae*) and other heterotrophic bacteria. Notably, the key functional groups central to this analysis were represented by complete or near-complete MAGs, including three Campylobacteriales genomes (98.9%–100% completeness, 0.2%–2.7% contamination) and one *Desulfovibrionaceae* genome (100% completeness, 0.07% contamination). To draw inferences about metabolic activity, our MAGs (derived from *A. cf. hyacinthus* BBD lesions) must adequately represent the metabolically significant taxa within our metatranscriptomic data, derived from BBD lesions on a different coral species, *P. duerdeni*. It is important to note that our objective was not to capture the transcriptional activity of all taxa within the BBD mat, but rather focus on the key microbial groups consistently implicated in BBD virulence. Accordingly, the MAGs recovered here provide sufficient genomic context for analysing diel gene expression dynamics in these functionally significant taxa, even though some transcripts from uncharacterised community members

remain unmapped. This assumption is supported by close similarities in major taxa (*R. reptotaenium*, Campylobacteriales, *Desulfovibrionaceae*) and functional groups (e.g., cyanobacteria, sulphate reducers and sulphur oxidisers) identified in our MAGs. Nevertheless, it is possible that unmapped transcriptomic reads are due to genetic differences between BBD-associated microbial members affecting different host species. Second, we assume that changes in gene expression between day and night are due to metabolic shifts within each taxon, rather than changes in relative abundance. Though total gene expression of each taxon, including the highly abundant taxa, *R. reptotaenium* and Cytophagales, shows no consistent change between day and night (Figure 1). Importantly, as no consistent increase or decrease in the highly abundant taxa was observed at night (Table S3), the observed differences in transcript abundance among lower-abundance taxa are unlikely to result from compositional masking or normalisation bias. Overall, the metabolic shifts identified in this study appear robust and are unlikely to be due to variations in relative abundance among different taxa. Many previous studies have profiled BBD lesions from different species and locations, highlighting consistent mat community members (Meyer et al. 2017; Miller and Richardson 2011; Sato et al. 2016, 2017; Wada et al. 2023) and our findings further support the stability of key functional groups across day and night and their metabolic shifts within the lesion.

4.2 | Cyanobacterial Photosynthesis Contributes to Major Diurnal Metabolic Shifts in BBD Lesions

Cyanobacteria photosynthesis was the primary contributor to the day/night differential gene expression responses within the BBD mat. Daytime expressed photosynthesis-related genes included the PSI and PSII (*psaACD*, *psbACDEO*), cytochrome b6/f complex and electron transport (*petABDEF*), ATP synthase (*atpBG*) and light-harvesting pigments (*cpcABG*, *cpeABC*). As the dominant bacterium in the BBD mat, it is not surprising that metabolic shifts in *R. reptotaenium* dominate the observed gene expression differences. Cyanobacteria undergo significant metabolic and physiological adaptations to optimise growth and survival in varying light conditions, including adjustments in their photosynthesis and chemotaxis activity (Buerger et al. 2016; Casamatta et al. 2012; Paerl 1996; Richardson et al. 2014; Stocker and Seymour 2012). Increased daytime expression of key Calvin–Benson cycle genes, including phosphoribulokinase (*prk*) and fructose-bisphosphate aldolase (*fbaA*), suggests increased carbon flux and biomass growth in *R. reptotaenium* (Liang and Lindblad 2016; Figure S3). The Calvin–Benson cycle is central to autotrophic carbon fixation in cyanobacteria, deriving sugars for metabolism from the fixation of carbon dioxide (Xiong et al. 2015), with its activity strongly influenced by light intensity through the light-dependent production of ATP and NADPH. Although fixation can occur both in the presence and absence of sunlight, the light intensity has a significant effect on cyanobacterial metabolism (Hudson 2024). The increased Calvin–Benson cycle expression in *R. reptotaenium* suggests that daylight enhances carbon fixation and metabolic activity, supporting increased growth and biomass during the day. These gene expression patterns underscore the pivotal role of cyanobacterial photosynthesis and carbon fixation in shaping microbial dynamics within BBD lesions across light regimes.

Cyanobacteria are generally sensitive to sulphide as it irreversibly blocks the electron transport across PSII, thereby preventing photosynthesis and energy generation (Cohen et al. 1986). In some cyanobacteria, the sulphide:quinone:reductase (*sqr*) enzyme facilitates sulphide-driven anoxygenic photosynthesis by oxidising H_2S and transferring electrons from sulphide to PSI (Arieli et al. 1994; Hamilton et al. 2018; Klatt et al. 2016, 2015). The *sqr*-encoded gene is also predicted to facilitate anoxygenic photosynthesis in *R. reptotaenium* under high sulphide conditions in the BBD mat (Den Uyl et al. 2016; Meyer et al. 2017; Sato et al. 2017). In our study, gene expression patterns showed that the *sqr* gene from *R. reptotaenium* was consistently expressed during both day and night, indicating that this pathway remains active across diurnal periods, potentially facilitating adaptation to the sulphide-rich micro-environment found in BBD lesions.

4.3 | Campylobacterales and Desulfovibrionaceae are Central to Sulphur Cycling and Virulence in BBD Lesions

The reconstructed Campylobacterales MAGs affiliated with *Arcobacteraceae* and *Sulfurospirillaceae*, two families that display similar functional traits in sulphur cycling. Comparative gene expression profiles suggested that Campylobacterales, that is, *Arcobacteraceae* and *Sulfurospirillaceae*, are central to sulphur cycling within the BBD lesions, as supported by the presence of sulphur oxidation, assimilatory sulphate reduction and thiosulphate reduction genes/pathways in the MAGs. The SOX pathway gene *soxD*, which converts thiosulphate into sulphate, has increased expression in Campylobacterales at night (though not statistically significant; adj. $p=0.065$). Meyer et al. (2017), reported that the sulphur oxidation SOX pathway genes were not detected in any of the five BBD metagenomes or associated MAGs, suggesting that sulphur oxidation via the SOX pathway may be rare or under-represented in BBD, noting that Campylobacterales MAGs were absent from this earlier study. In contrast, the sulphur oxidation genes *soxABCDY* were present in *Arcobacteraceae* and *soxCDY* were present in the *Sulfurospirillaceae* MAGs reconstructed in this study, indicating potential for sulphur oxidation within Campylobacterales members of the BBD mat. The sulphur oxidation *fccB* genes, often found in sulphur-oxidising bacteria to generate energy by catalysing oxidation of hydrogen sulphide to sulphur or sulphite under aerobic or anaerobic environments, also showed increased expression in Campylobacterales at night. Many of these sulphur oxidation pathways have been extensively characterised in other marine Campylobacterales taxa (Deng et al. 2023; van der Stel and Wösten 2019). Although gene presence alone does not confirm active metabolism, these findings are consistent with the expression of sulphur oxidation pathway genes detected at night and with previous reports of sulphur cycling activity in BBD microbial consortia.

Campylobacterales are known for their ability to utilise sulphur compounds and are primarily recognised as sulphide-oxidising bacteria, especially *Arcobacteraceae* and *Sulfurospirillaceae*, though species have demonstrated the capacity for sulphur compound reduction (Finster et al. 1997; Li et al. 2024; Miller et al. 2007; Roalkvam et al. 2015; Wirsén et al. 2002). The sulphite reductase [Ferredoxin] (*sir*) gene from Campylobacterales

was highly expressed at night, highlighting enhanced assimilatory sulphate reduction activities that reduce inorganic sulphite to sulphide for biosynthesis of amino acids like cysteine and methionine into cell biomass (Kushkevych et al. 2020). The thiosulfate reductase *phsA* gene, involved in the reduction of thiosulfate to sulphide which produces hydrogen sulphide under anaerobic conditions (Liu, Yang, et al. 2022; Liu, Shan, et al. 2022; Mo et al. 2023), also demonstrated increased expression in Campylobacterales at night (though not significant; adj. $p=0.060$). Wada et al. (2023) reported a positive correlation between Campylobacterales abundance and BBD lesions progression. While dissimilatory sulphur reduction is uncommon among Campylobacterales, the identification of thiosulfate reductase *phsA* homologues in *Arcobacteraceae* MAGs indicates potential reductive sulphur metabolism and contribution to the high sulphur environment in the BBD mat. However, further biochemical or expression-based validation would be required to confirm their activity, especially under local redox conditions.

The Campylobacterales increased expression of rTCA-related genes during the nighttime (Figure S3), highlighting the critical role of CO_2 fixation pathways, supported by energy generated from sulphide oxidation, in sustaining metabolism under low oxygen and high sulphide conditions (Li et al. 2018; Xu et al. 2022). The rTCA cycle may support autotrophic growth in Campylobacterales using reduced sulphur compounds as electron donors, consistent with the presence of assimilatory sulphate reduction pathways that contribute to biosynthetic demands rather than energy generation (Assié et al. 2020; Hüglér and Sievert 2011; Waite et al. 2017). Additionally, there was a significant increase in the expression of nitrate reduction-related genes, including nitrate reductase (*napB*) and nitrite reductase (*nrfA*), from Campylobacterales during the night. These enzymes facilitate nitrate reduction, allowing Campylobacterales to use nitrate and nitrite as terminal electron acceptors under oxygen-limited conditions, thus supporting their growth and energy requirements (Pittman et al. 2007; Pittman and Kelly 2005; Vetriani et al. 2014). While oxygen is the preferred electron acceptor and leads to a higher growth rate, Campylobacterales can also respire using a variety of other compounds, including sulphur and nitrogen species (van der Stel and Wösten 2019). For example, the dimethyl sulfoxide reductase (*dmsA*) gene, central to reducing dimethyl sulfoxide (DMSO) to dimethyl sulphide (DMS), showed increased expression at night. DMSO reduction may provide a competitive advantage for Campylobacterales in the environment characteristic of the BBD mat since it is essential for anaerobic energy generation, respiring DMSO to gain ATP when oxygen is scarce (McDevitt et al. 2002; Ray et al. 2003).

Increased expression of chemotaxis genes (*CheA*, *PctC*) was observed in Campylobacterales in night samples, suggesting the use of chemotaxis to locate optimal growth conditions, which links their motility directly to energy production. Campylobacterales migrate and colonise the oxic-anoxic interface to effectively compete with other sulphur-oxidising bacteria in high sulphide environments (Sievert et al. 2007). This behaviour is consistent with previous observations that sulphur-oxidising bacteria, including Campylobacterales, migrate to the top layer of the BBD mat during dark periods when the oxygen levels decrease, repositioning along diel

redox gradients to optimise their microaerophilic metabolic activity (Sato et al. 2017). Correspondingly, the flagellar assembly pathway was enriched in Campylobacteriales at night, suggesting increased motility during this time, as the characteristic spiral-shape movement of this taxa is driven by flagella (Cohen et al. 2020; Guerry 2007). Although no differential expression of *flaB* was observed between day and night samples, basal motility likely contributes to surface sensing and microenvironmental positioning, which can precede attachment events and colonisation (Hendrixson and DiRita 2003). Chemotaxis and motility have also been implicated in the early-stage colonisation and biofilm initiation in other systems, where directed movement enables surface approach and triggers EPS production (Merritt et al. 2007; Reuter et al. 2020). Campylobacteriales showed a notable increase in nighttime expression of the *rssB* and *rscC* genes, part of the Rcs phosphorelay system that regulates motility and biofilm formation (Dong and Schellhorn 2010; Ferrières et al. 2009; Howery et al. 2016; Latasa et al. 2012; Tsai et al. 2011; Wölflingseder et al. 2022). Despite the absence of the *rpoS* component of the Rcs phosphorelay system in the Campylobacteriales genomes (Parkhill et al. 2000), the *rssB* homologue (*hnr*, KO:K02485) is present, suggesting possible alternative regulatory roles such as polysaccharide synthesis or stress response that may support surface stability and localised microcolony development (McLennan et al. 2008; Okoli et al. 2007). A recent study also showed that lesion front migration rates correlate with the relative abundance of *Arcobacteraceae* within the mat (Wada et al. 2023), reflecting the cumulative outcome of chemotactic and flagellar-driven behaviours within the mat. Overall, the observed enhanced motility and chemotaxis response in Campylobacteriales likely facilitates dynamic repositioning within the mat in response to diel shifts in redox conditions, while also potentially supporting surface attachment, microcolony formation and early biofilm formation under suitable microenvironmental conditions (Chaban et al. 2015; Colin et al. 2021; Guerry 2007). The coordinated regulation of carbon and energy metabolism, nitrogen and sulphur cycling, motility and chemotaxis likely enables Campylobacteriales to sustain their metabolic activity within the BBD mat during periods of darkness, while the nighttime upregulation of genes linked to potential biofilm development may further enhance BBD virulence by promoting bacterial adhesion and aggregation, forming a physical barrier that could impede host immune defences (Figure 5).

Sulphate reduction related genes were highly expressed in *Desulfovibrionaceae*, known for their role in sulphate reduction within BBD lesions (Meyer et al. 2016; Viehman et al. 2006), during the nighttime. Our *Desulfovibrionaceae* MAG harbours the core dissimilatory sulphite reductase genes (*dsrAB*) that reduce sulphite to sulphide (Müller et al. 2015). While *dsrAB* transcripts were not detected in our day/night metatranscriptomic dataset, potentially due to their low abundance or masking by dominant cyanobacterial expression, we observed high nighttime expression of *dsrP*, which encodes an accessory protein that transfers electrons to the DsrAB complex (Grein et al. 2010). We also detected a higher nighttime expression of *aprAB*, which encodes adenylylsulfate reductase, the enzyme responsible for reducing activated sulphate (APS) to sulphite. In addition, *dsvC*, a gene encoding a critical sulphur carrier protein that interacts

with the DsrAB complex to complete the reduction of sulphite to sulphide (Venceslau et al. 2014), also showed increased expression at night. Both *aprAB* and *dsvC* have also been implicated in reverse dissimilatory sulphur oxidation pathways (Meyer and Kuever 2007; Venceslau et al. 2014; Watanabe et al. 2016). Together, the increased nighttime expression of these sulphur metabolism genes suggests that *Desulfovibrionaceae* facilitated the dissimilatory sulphur reduction pathway and contributed to elevated levels of hydrogen sulphide within the BBD mat, even in the absence of detectable *dsrAB* expression. The nighttime expression of sulphur reduction related genes in *Desulfovibrionaceae*, along with increased sulphur reduction and biofilm formation by Campylobacteriales, reflects synergistic microbial activity that likely intensifies BBD virulence in the coral host.

4.4 | Enhanced Nitrogen Metabolism and Vitamin Biosynthesis by Non-Cyanobacteria at Night Supports the Metabolism Requirement in BBD Community

The expression of nitrogenase-related genes (*nifH*) was elevated at night in Bacteroidales and *Acidaminobacteraceae*, conveying the ability to perform nitrogen fixation in the absence of oxygen (Minamisawa et al. 2004; Steppe and Pael 2002; Zehr et al. 1995). In addition, Campylobacteriales-affiliated genes encoding dissimilatory nitrate reduction (*napAB*, *nrjA*) were highly expressed at night, likely being important for the growth and survival of this taxon under an oxygen-limited environment, allowing the utilisation of nitrate and nitrite as alternative respiratory electron acceptors (Pittman et al. 2007; Saghai and Hallin 2024; Stewart et al. 2002). By producing ammonia or related compounds into the BBD mat, when *R. reptotaenium* photosynthesis is not the central metabolic process, these bacteria provide nitrogen sources for amino acid synthesis for cyanobacteria *R. reptotaenium* and/or other heterotrophic bacteria, further supporting the metabolic activities of the BBD bacterial consortium.

Most cyanobacteria fix nitrogen primarily during the night, since the nitrogenase enzyme complex is sensitive to oxygen and requires a near-to-anoxic environment (Cohen and Golden 2015; Zehr and Capone 2020). Interestingly, *R. reptotaenium*, a nonheterocyst-forming cyanobacterium, showed no significantly increased expression in nitrogen fixation genes at night relative to the day samples. While *R. reptotaenium* could potentially move to the base of the BBD lesion to access anoxic environments, aquatic cyanobacteria are known to have diverse strategies to protect nitrogenase from oxygen stress while meeting energy demand from photosynthesis (Stal 2015; Zehr and Capone 2020). For example, previous studies have suggested that filamentous nonheterocyst-forming cyanobacteria (such as *Trichodesmium* and *Schizothrix*) use enhanced oxygen scavenging mechanisms to restrict intracellular oxygen concentrations and thereby protect nitrogenase during nitrogen fixation (Berrendero et al. 2016; Chen, Rodriguez, et al. 2022; Chen, Zhao, et al. 2022; Gardner et al. 2023). The exact mechanism allowing cells to undertake photosynthesis and nitrogen fixation without temporal (diel cycle) or spatial (heterocyst) separation in *R. reptotaenium* remains unclear; however, aside from nitrogen

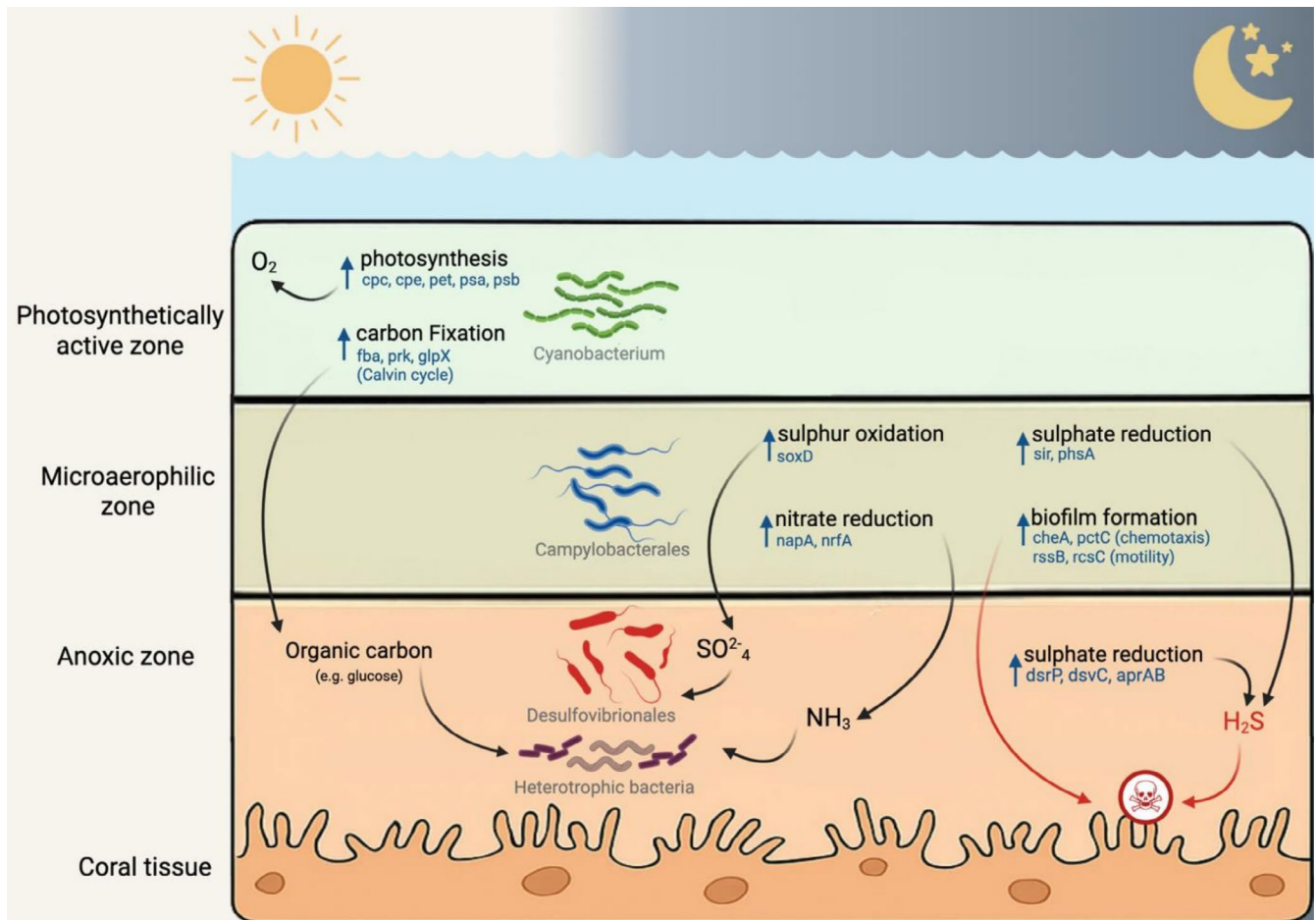


FIGURE 5 | Schematic figure of the microbial interactions contributing to BBD lesion progression during the day and at night. The diagram depicts major microbial groups and their metabolic activities across redox zones (photosynthetically active, microaerophilic, and anoxic). Blue arrows indicate genes showing differential expression between day and night, black arrows represent continuous metabolic fluxes, and red arrows denote processes leading to H_2S accumulation that may enhance BBD virulence. The sun-moon gradient represents the diel transition, and gene symbols (e.g., *psa*, *sox*, *dsr*, *nap*, *rssB*, *rcsC*) correspond to representative genes supporting these processes.

fixation, *R. reptotaenium* affiliated assimilatory nitrate reduction genes (*nirA*, *narB*), which reduce nitrate to ammonia for internal amino acid synthesis samples (Frias and Flores 2015; Unthan et al. 1996), also showed consistent expression patterns in both the day and night. Together, our findings reveal that while *R. reptotaenium* maintains consistent nitrogen-related gene expression throughout the diel cycle, other members of the BBD mat, particularly, Campylobacteriales, Bacteroidales and *Acidaminobacteraceae*, play critical roles in nighttime nitrogen cycling, collectively sustaining the reduced nitrogen exchange within the diverse microbial community of the BBD mat.

Biosynthesis of cofactors, such as vitamins, was highly expressed in night time samples across many non-cyanobacteria BBD taxa. The Bacteroidales-affiliated thiamine-phosphate synthase (*thiE*) gene, essential for vitamin B1 (thiamine) biosynthesis, showed increased expression while biotin (vitamin B7) metabolism was also upregulated in *Acidaminobacteraceae*. Vitamin B1 serves as a cofactor for enzymes involved in glucose breakdown and is vital for bacterial energy generation and growth (Park et al. 2022), while biotin is a key cofactor in various carboxylation reactions that underpin fundamental metabolic processes such

as fatty acid synthesis, gluconeogenesis and amino acid catabolism (Satiaputra et al. 2016; Sirithanakorn and Cronan 2021). Genes related to vitamin B12 (cobalamin) import and transport (*btuB*, *btuC* and *btuF*), affiliated with SAR324, Bacteroidales and *Acidaminobacteraceae* and critical for DNA synthesis and cellular energy production (Rodionov et al. 2003), also exhibited increased expression during the nighttime. Vitamin B2 (riboflavin) is the key cofactor for metabolism of iron and the transporter gene (*ribU*) from *Acidaminobacteraceae* also displayed significantly higher expression at night (Duurkens et al. 2007; Gutiérrez-Preciado et al. 2015). The presence of biosynthesis, transport, uptake of vitamins, along with nitrogen metabolism, within microbial mats shapes the metabolic capabilities and interactions among community members (Kost et al. 2023), influencing the structure and function of the BBD microbial consortium.

5 | Conclusion

This study presents novel insights into the pathogenicity of BBD in corals by identifying light-dependent metabolic processes at

the level of individual microbes using long-read based MAGs and metatranscriptomic mapping. Metatranscriptomic analysis revealed significant variation in gene expression between day and night, with *R. reptotaenium* photosynthesis being the primary driver of differential expression. Campylobacteriales were found to be central to the sulphur cycle in the BBD mat at night, with increased activity in nitrogen and carbon cycles, potential expression of motility and surface adhesion related genes, thereby facilitating important metabolic roles in promoting the virulence of the disease. This study improves our understanding of the metabolic activity of key bacterial groups, including cyanobacteria, sulphide-oxidising bacteria, sulphide-reducing bacteria and heterotrophic bacteria linked to the microenvironmental dynamics within the BBD lesion. Together, these findings provide the first diel-resolution view of microbial gene regulation within the BBD mat and offer new insights into the complex ecological interactions underpinning coral disease virulence.

Author Contributions

Julia Y. Hung: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, visualisation, writing – original draft preparation, writing – review and editing. **Ira Cooke:** conceptualization, methodology, resources, funding acquisition, supervision, writing – review and editing. **Yui Sato:** conceptualization, data curation, resources, investigation, writing – review and editing. **David J. Miller:** supervision, writing – review and editing. **David G. Bourne:** conceptualization, funding acquisition, supervision, writing – review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Raw data generated in this study have been submitted to NCBI under BioProject PRJNA1155222. The 28 BBD-derived MAGs are available on Figshare (<https://doi.org/10.6084/m9.figshare.27611787>) and bioinformatics scripts on GitHub (https://github.com/JuliaHung1/BBD_metagenome_metatranscriptome).

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** emi70219-sup-0001-Supinfo.docx. **Table S1:** Table of all the significantly differentially expressed (DE) genes of the BBD community metatranscriptomic analysis. This table lists all DE genes (FDR < 5%) identified in the BBD mat, including

statistical outputs (log₂ fold change, *p*-value, adjusted *p*-value), associated MAG and taxonomy and functional annotations from Prokka, GhostKOALA, EggNOG. Columns include gene IDs, expression metrics, feature type, gene length, annotations (e.g., EC, COG, GO terms) and pathway or functional category assignments. **Table S2:** Comparison of the BBD-derived MAGs from this study to the Sato et al. 2017 and Meyer et al. 2017. **Table S3:** Proportional transcript contributions of dominant taxa (*Roseofilum reptotaenium* and Cytophagales) to total aligned reads across day and night BBD samples.