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Draft Genomes Shed Light on the Dual Bacterial Symbiosis that Dominates the Microbiome of the Coral Reef Sponge *Amphimedon queenslandica*

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Amphimedon queenslandica is a coral reef demosponge that houses a low complexity and low abundance microbiota dominated by a proteobacterial duo for which draft genomes are presented here. The most prevalent symbiont, *AqS1,* is a sulfur-oxidizing gammaproteobacterium closely related to other demosponge symbionts and to free-living Ectothiorhodospiraceae (Chromatiales). The predicted gene repertoire of *AqS1* indicates that it is capable of sulfur oxidation, carbon monoxide oxidation and inorganic phosphate assimilation, and that some of its metabolic capabilities may have been acquired via horizontal gene transfer from alphaproteobacteria. The second most prevalent symbiont, *AqS2*, is a betaproteobacterium whose closest known relatives are other demosponge symbionts. *AqS1* has characteristic sponge symbiont features, including a versatile nutrient use with large number of transporters, ankyrin-repeat-containing proteins, and a CRISPR system. Based on the size of its genome assembly, *AqS2* is predicted to have a much smaller genome with many fewer symbiotic features than *AqS1.* The smaller is reflected in its more limited metabolic capabilities that include carbohydrate metabolism, but not sulfur oxidation or phosphorus metabolism. Within-pathway complementation and resource partitioning potentially occur between the two bacteria. The addition of these symbiont genomes to extensive genome and transcriptome resources already available for the sponge host now permits the development of mixed-species genome-scale metabolic models as a foundation for experimental investigations of resource partitioning between symbionts and host.

Keywords: bacterial symbionts, metagenomics, coral reef symbiosis, proteobacteria, porifera, Chromatiales

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INTRODUCTION

Marine sponges (Phylum Porifera) are abundant sessile, benthic filter feeders that have a major influence on biogeochemical fluxes in coral reef and coastal environments (Bell, 2008; Southwell et al., 2008b; de Goeij et al., 2013; Colman, 2015). Their ecosystem engineering roles are enabled by diverse communities of symbiotic prokaryotes that can account for up to 40% of the sponge body volume (Taylor et al., 2007a,b; Webster et al., 2010; Hentschel et al., 2012). The symbionts extend

the metabolic capabilities of the host by mediating processes, such as photosynthesis (Wilkinson, 1983; Arillo et al., 1993; Steindler et al., 2002), carbon (De Goeij et al., 2008a,b; de Goeij et al., 2013), nitrogen (Wilkinson and Fay, 2004; Mohamed et al., 2008; Southwell et al., 2008a; Hoffmann et al., 2009; Schläppy et al., 2010; Fiore et al., 2013), methane (Vacelet et al., 1995), sulfur (Hoffmann et al., 2005), and phosphorus (Sabarathnam et al., 2010; Zhang et al., 2015b) cycling. Metagenomic and transcriptomic studies of sponge microbiomes have provided independent molecular evidence of the contribution of symbiotic bacteria, in particular, to biogeochemical and nutrient cycling (Hentschel et al., 2012; Fiore et al., 2015; Rua et al., 2015), underscoring the significance of the holobiont perspective in understanding ecological roles of animal hosts in marine ecosystems.

More broadly, recent metatranscriptomic, and metaproteomic analyses of sponge-associated microbial consortia have begun to unveil their global expressed gene repertoire (Liu et al., 2012; Radax et al., 2012; Moitinho-Silva et al., 2013, 2014; Fiore et al., 2015; Rua et al., 2015). Single-cell genomic and metagenomics studies have contributed to correlating particular traits with specific symbiont lineages (Thomas et al., 2010; Siegl et al., 2011; Kamke et al., 2013; Gao et al., 2014; Tian et al., 2014; Wilson et al., 2014; Burgsdorf et al., 2015; Britstein et al., 2016), revealing functional gene convergence in phylogenetically-distinct microbial communities with core features that likely reflect adaptation of microorganisms to the sponge host environment (Fan et al., 2012; Hentschel et al., 2012). Traits that have repeatedly been reported in prokaryote symbionts of poriferans include versatile nutrient utilization, protection from environmental and host-specific stress, and eukaryotic-like proteins (Fan et al., 2012; Hentschel et al., 2012). Unsurprisingly, however, other traits are specific to particular symbionts, including variable carbon fixation and secondary metabolism processes, motility, and reduced genome size. A more complete picture will emerge only as more sponge and associated symbiont genomes become available.

The persistent presence of dominant bacterial phylotypes in several species of sponge indicates that many specific host-microbe associations are stable over time, likely facilitated by vertical inheritance (Taylor et al., 2007b; Erwin et al., 2012; Hentschel et al., 2012; Thomas et al., 2016). In non-sponge host-symbiont systems, the temporal stability of associations involving two or more unrelated bacterial symbionts (Takiya et al., 2006; Wu et al., 2006; Dubilier et al., 2008; Zimmermann et al., 2014) has been postulated to involve the use of different energy sources to reduce competition between the bacterial species (Duperron et al., 2006; Kleiner et al., 2012b). It has also been suggested that some symbionts might rely on one another for their metabolism, in a mutual dependency resulting from loss of metabolic redundancies that originally existed within symbiotic partners at the time of their acquisition by the host (Wu et al., 2006; Rao et al., 2015). The extent to which these phenomena occur in sponge symbionts is currently largely unknown.

Here we report draft genomes of the dominant bacterial symbionts of the genome- and transcriptome-enabled coral reef demosponge *Amphimedon queenslandica* (Srivastava et al., 2010; Conaco et al., 2012; Anavy et al., 2014; Fernandez-Valverde et al., 2015; Levin et al., 2016). In their native habitat on the Great Barrier Reef of Australia, adult *A. queenslandica* support a relatively low diversity bacterial symbiont community characterized by a core group that are inherited vertically from the maternal parent during early embryonic development (Fieth et al., in review). The metagenomics approach that we adopt herein reveals that the adult microbiome of this sponge is dominated by just two phylotypes that we name *AqS1* (*A. queenslandica* Symbiont 1) and *AqS2*, corresponding to OTU1 and OTU4, respectively, in Fieth et al. (in review). We successfully assemble draft genomes for both of these, which allows us to present the gene repertoire of the two species. We discuss potential metabolic interactions that might take place between the symbionts, and between the symbionts and their sponge host. We also report phylogenetic affinities of a third, lower abundance bacterial symbiont (*AqS3*, corresponding to OTU5 in Fieth et al., in review) for which we retrieve a draft genome of lesser quality.

MATERIALS AND METHODS

Acquisition of Bacterial gDNA

Six adult sponges (*Amphimedon queenslandica*) were collected from Shark Bay, Heron Island, Great Barrier Reef, QLD (23° 27′S, 151° 55′ E), three in September 2012 (HSep1-3), and three in December 2012 (HDec1-3). Each sponge was removed from the substrate with a sterile scalpel and pieces were stored in tubes of 0.22 μm filter sterilized seawater (FSW) half submerged in iced water and quickly transported back to the Heron Island Research Station for immediate processing. Enrichment of sponge-associated bacteria from sponge cells followed the protocol of Thomas et al. (2010) with slight modifications, and total genomic DNA was extracted from these cell preps using a standard phenol-chloroform method. Details are provided in text of the Supplementary Material.

Genome Sequencing and Assembly

Each of the six samples was multiplexed and sequenced by Macrogen (http://www.macrogen.com) in one lane using the HiSeq Illumina platform. Pair-end reads were 100 bp long and had an insert size ranging between 150 and 250 bp. Reads were filtered with Trimmomatic (Bolger et al., 2014), removing the first five bases from the start of the read, performing a sliding window trimming with a window of four bases and an average quality threshold of 15, and eliminating resulting reads that were less than 50 bp long. We also did not retain ambiguous reads with Ns, nor reads that aligned to contigs that unambiguously belonged to the genome of the host sponge, *Amphimedon queenslandica,* using Bowtie2 (Langmead and Salzberg, 2012). Metabinning and genome construction were performed following the protocol of Albertsen et al. (2013) with slight modifications (Supplementary Material text; Figure S1).

Small Subunit 16S rRNA Gene Reconstruction from Metagenomic Shotgun Data

Small subunit (i.e., 16S) rRNA gene-containing reads were identified from the filtered FASTQ files using Metaxa v. 2.0.1 (Bengtsson et al., 2011). Extracted reads were assembled with IDBA-UD (Peng et al., 2012) using a 99% overlap identity. We first performed assemblies on these reads keeping samples separate, and then another assembly pooling reads from all samples. We used CAP3 (Huang and Madan, 1999) on all assemblies to merge overlapping contigs, and checked manually the resulting singletons and collapsed contigs. These derived 16S rRNA sequences were broadly classified using BLASTN and the NR database, excluding uncultured and environmental samples. After confirming that we recovered most reads that had been initially extracted by Metaxa within our final list of assembled contigs, we then used the original FASTQ files and our derived 16S rRNA sequences as reference, and applied the closed-reference OTU picking strategy of the QIIME program (Caporaso et al., 2010b) to derive 16S rRNA gene abundance estimates for each sample.

Gene Annotation

We obtained gene annotation for the two bacterial genomes that we deemed had adequate coverage (*AqS1* and *AqS2*). Contigs for each genome were uploaded on the RAST server (Aziz et al., 2008; Overbeek et al., 2014) and annotated using the Classic annotation scheme, the RAST gene caller, and FIGfams v. 70. We opted for errors and frame shifts to be fixed automatically, and for the pipeline to BLAST large gaps for missing genes. We compared the gene content of *AqS1, AqS2, Thioalkalivibrio* sp. *HK1, three* of *AqS1* closest relatives and other known sulfur-oxidizing symbionts using the function based comparative tool available on the SEED viewer within the RAST platform. For each genome, we either used predicted protein-coding sequences that were publically available or obtained these ourselves using the RAST server. Although a gene annotation for *T.* sp. *HK1* is available on NCBI, we derived a new *one* via the RAST server for ease of comparison with the *AqS1* genome. This annotation can be made available upon request.

We searched for the presence of domains associated with proteins postulated to facilitate host-symbiont interactions in the predicted protein-coding sequences of *AqS1*, *AqS2*, other known sulfur-oxidizing symbionts and the three closest free-living relatives to *AqS1*. To do so, we scanned for specific PFAM domains using hmmsearch from the program hmmer 3.1 (hmmer.org), using the trusted cutoff bit score thresholding option (–cut_tc). We used Interpro (http://www.ebi.ac.uk/interpro/), NCBI conserved domains (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) to predict domains and transmembrane regions of the high-affinity branched-chain amino acid ABC transporter proteins. An example of putative 2D topology for one of the transmembrane protein complex was derived using TOPO2 (http://www.sacs.ucsf.edu/TOPO2/topo2-help.html). Finally, we used the CRISPRfinder program (http://crispr.u-psud.fr/Server/) to deduce the organization of the CRISPR in *AqS1*. The 16S rRNA gene sequences of *AqS1*, *AqS2*, and *AqS3* are available from the NCBI GenBank database under accession numbers KX772780-2. The draft genomes of *AqS1* and *AqS2* have been deposited as Whole Genome Shotgun projects at DDBJ/ENA/GenBank under the accession numbers MEID00000000 and MEIE00000000, respectively. The versions described in this paper are MEID01000000 and MEIE01000000, respectively. The genome browsers of *AqS1* and *AqS2* are hosted on the RAST server (http://rast.nmpdr.org/seedviewer.cgi) under genome IDs 6666666.207661 and 6666666.208689, respectively, and are accessible via username 'guest' with password 'guest').

Phylogenetic Analyses

Phylogenetic analyses were performed for the three most abundant 16S rRNA sequences in our metagenome data. Nearest related and described sequences were retrieved from the 2013 Greengene and NCBI NR databases. The partial sequence from *T.* sp. *HK1* reported in Tian et al. (2014) was kindly provided by Dr Ren-Mao Tian. A sequence alignment was derived with PyNAST (Caporaso et al., 2010a) using the template file "core_set_aligned.fasta.imputed" from QIIME v. 1.8.0 (Caporaso et al., 2010b) and manually checked using AliView (Larsson, 2014). A maximum-likelihood tree was constructed with RAxML v. 8 (Stamatakis, 2014) with 1000 bootstrap resamples, and visualized using FigTree v. 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/). For trees with low bootstrap support at given nodes, we checked whether some taxa had ambiguous or insufficient phylogenetic signal by deriving their leaf stability indices using the program RogueNaRok (Aberer et al., 2013).

Since a large proportion of genes that endow the *AqS1* symbiont with heterotrophic capabilities are most closely related to genes of several alphaproteobacteria, we wanted to test the hypothesis that some of these have been acquired horizontal gene transfers (HGT). To do so, we performed a phylogenetic analysis on the concatenated alignment of high-affinity branched-chain amino acid ABC transporter proteins. Nearest related and described sequences were retrieved from the NCBI NR database. We also included two species from the order Chromatiales as an outgroup. We derived alignments using AliView and checked for the presence of ambiguously aligned positions using Aliscore and Alicut (Kück et al., 2010). We used prottest3 (Darriba et al., 2011) to choose a model of evolution for each gene. We concatenated our sequence alignment into one supermatrix using FASconCAT (Kuck and Meusemann, 2010).

RESULTS

Genome Sequencing and Genome Assembly

We sequenced six bacterial-enriched samples from the sponge *Amphimedon queenslandica* and recovered between 23,906,452 and 27,938,004 pairs of Illumina reads from each library. Filtering out low quality reads and contaminating sponge host DNA resulted in 14,563,169 to 16,046,247 read pairs contributing to bacterial genome assemblies (Table S1). To isolate the symbiont

genomes of interest—*AqS1*, *AqS2*, and *AqS3*—we derived scaffold coverage plots with GC content and taxonomic information overlayed, using a slight modification of the protocol developed by Albertsen et al. (2013) (Supplementary Material; Figures S1, S2). The *AqS1* genome was assembled into 127 contigs (median length 13 Kbp, total length 4, 2 Mbp, with 59% average GC content) and the *AqS2* genome into 239 contigs (median length 3 Kbp, total length 1,6 Mbp, with 69% average GC content). We also recovered a third genome (*AqS3*) that we were able to assemble into 233 contigs (median length 5 Kbp, total length 3,17 Mbp, with 62% average GC content) (**Table 1**). To evaluate the quality of the assemblies, we checked for the presence of 105 conserved single-copy genes in each genome (Supplementary Material; Table S2). In general, we found that the total number of genes recovered in each of the three symbionts was on par with the unique essential gene list. Specifically, we estimate that the reconstructed *AqS1* genome is 99% complete (we recovered 104 out of 105 genes) and that of *AqS2* and *AqS3* are 85% and 94% complete, respectively. However, since the coverage of *AqS3* was low (<10X read coverage) and a principal component analysis on the tetranucleotide frequencies of its genome scaffolds indicated the presence of possible contamination that could not be removed, we elected to omit this genome from our downstream gene annotation, which is thus restricted to the two most abundant symbionts, *AqS1* and *AqS2*.

Phylogeny of the Three Main Phylotypes Present in *Amphimedon queenslandica*

Based on maximum-likelihood phylogenetic analysis of the 16S rRNA gene, the *AqS1* symbiont clusters admist symbionts of various other sponge species (identity range 95–99%); sister to these is a small clade of marine environmental samples (**Figure 1**; Figure S3). These clades fall at the base of the family Ectothiorhodospiraceae, order Chromatiales (commonly known as sulfur-oxidizing bacteria). The closest cultured bacterial relatives (identity range 90–92%) are from *Ectothiorhodospira* and *Thioalkalivibrio*, both in the family Ectothiorhodospiraceae; the top BLAST NR hit was to the free-living *Thioalkalivibrio paradoxus ARh 1*. The *AqS1* symbiont falls into the same sponge-specific clade as bacteria derived from the demosponges *Callyspongia vaginalis* and *Gelliodes carnosa*; also in this clade is a symbiont recently—but, we suggest, perhaps prematurely—named *Thioalkalivibrio* sp. *HK1,* whose genome was assembled from a metagenome, obtained from the sponge *Haliclona cymaeformis* (Tian et al., 2014). The other sponge-specific clade includes symbionts from a homoscleromorph (*Plakortis halichondrioides*) and several Demospongiae. For *AqS1*, we also performed phylogenetic analysis based on 30 concatenated essential marker genes, and including the genome of the close relative *T.* sp. *HK1* plus a selection of 11 other publically-available genomes from free-living Chromatiales bacteria. In agreement with the 16S rRNA phylogenetic analysis, both the *AqS1* and the *HK1* sponge symbionts were recovered in a cluster of bacterial species belonging mostly to the family Ectothiorhodospiraceae (data not shown).

The 16S rRNA sequence of the *AqS2* symbiont forms part of a large, well-supported cluster of demosponge and one coral-derived bacteria (identity range 84–99%), that is quite divergent from recognized families within the class Betaproteobacteria. Similar to *AqS1*, the *AqS2* sequence clades with bacteria whose genome was assembled from metagenomic data derived from *C. vaginalis* and *G. carnosa,* as well as another *Amphimedon* sp. from the Red Sea (**Figure 1**; Figure S4). The closest cultivated relatives of the 16S rRNA of *AqS2* are from the genera *Azoarcus* and *Thauera* (identity range 82–84%), both in the family Rhodocyclaceae; the top BLAST NR free-living hit was to the *Azoarcus* sp. CIB, but only with 84% identity.

The 16S rRNA gene sequence of symbiont *AqS3* shows highest percent sequence similarity to bacterial clones originating from several demosponges (identity range 84–99%). The closest described relative is the Chromatiales *Thioprofundum hispidum* strain gps61, but the identity score is low (86%). In our phylogenetic analysis, *AqS3* clustered separately with gammaproteobacteria derived from other demosponges, but this apparently novel clade has low bootstrap support, and thus its placement within the gammaproteobacteria is not yet resolved (**Figure 1**, Figure S3).

TABLE 1 | Summary of genomic features for the three main symbionts present in *Amphimedon queenslandica*.

	AqS1	*AqS2*	*AqS3*
Base pairs (Mbp)	4.2	1.61	3.2
No. contigs	127	239	233
Longest contig size	265,746	82,499	172,753
Median sequence size	13,046	3359	5488
G + C content (%)	58.9	68.8	61.9
No. of protein-coding genes	4778	1349	2418
Percent coding	86.5	81.2	81.2
No. of rRNA operons	1	1	1
No. of tRNA genes	47	42	59
No. of essential single copy genes	103	89	99

The Microbial Community of *Amphimedon queenslandica* Is Stable and Clearly Dominated by *AqS1* and *AqS2*

Since bacterial genomes can contain more than one copy of the 16S rRNA gene, the relative abundance of detected phylotypes from metagenomic DNA is potentially biased. We were interested in validating the binning strategy of our assembly by establishing whether the three assembled genomes would be also recovered as the most prevalent in the 16S rRNA pool, and whether any other prominent genome had not been accounted for. We also wanted to assess whether the overall composition of the microbial population found in the sponge *A. queenslandica* was broadly consistent across the six individual sponges that we sampled, and between the two different sampling seasons. It is also worth noting that we recovered the 16S rRNA gene in our single-copy gene list derived from our binned contigs for each of the three *AqS* genomes (Table S2).

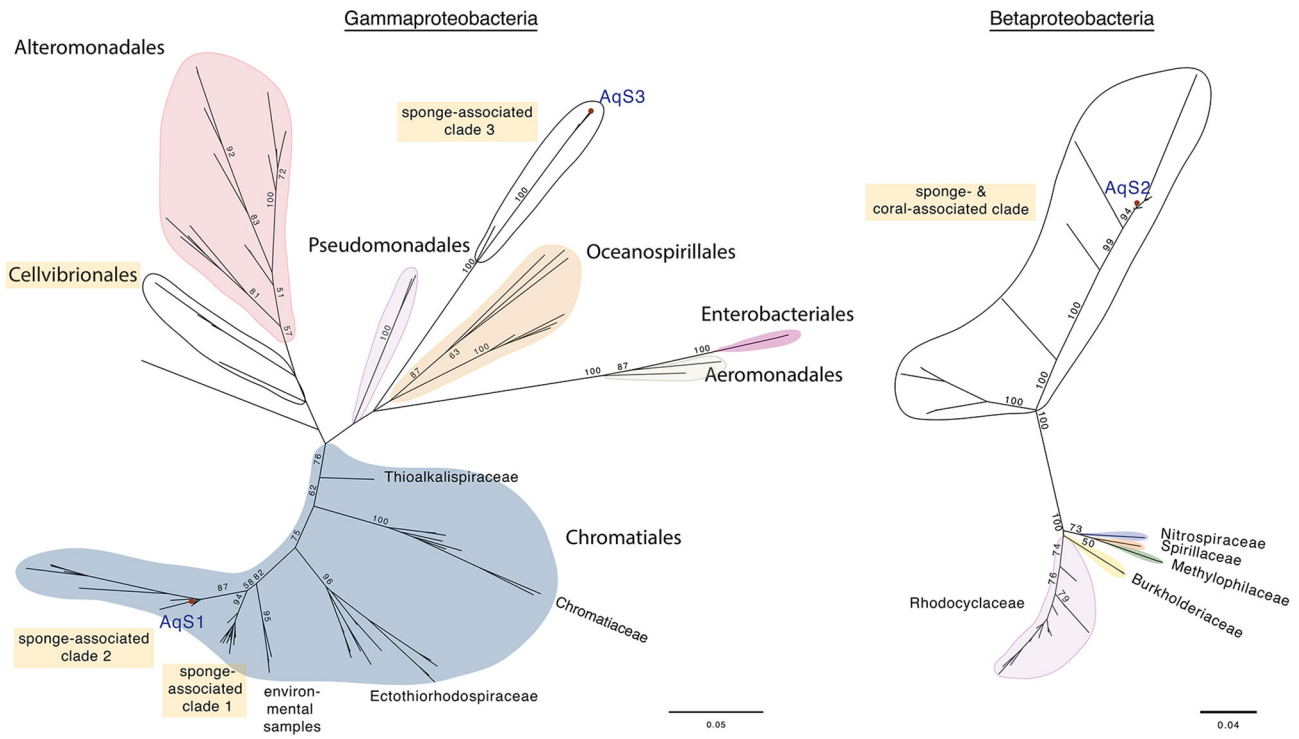

FIGURE 1 | Maximum-likelihood phylogenetic trees for *AqS1*, *AqS2*, and *AqS3*. (A) The symbionts *AqS1* and *AqS3* are affiliated with the class Gammaproteobacteria. The *AqS1* symbiont is recovered within the order Chromatiales in a sponge-specific clade; the nearest described species belong to the family Ectothiorhodospiraceae. The *AqS3* symbiont clusters separately with bacterial clones derived from other demosponges in a clade located between the orders Pseudomonadales and Alteromonodales, but its phylogenetic placement is not well resolved. The detailed tree is presented in Figure S3. **(B)** The *AqS2* symbiont sits within a large, well-supported sponge-specific cluster that is quite divergent from described families of the class Betaproteobacteria. The detailed tree is presented in Figure S4. The symbionts identified in *A. queenslandica* are indicated by a red dot and labeled as *AqS1-3*. Bootstrap values >50 are specified at major nodes.

Our 16S rRNA sequence reconstruction from the metagenomic Illumina data confirmed that the three symbionts for which we retrieved a genome assembly were also the most highly represented in the small subunit rRNA (ssrRNA) sequence pool derived from both separate and combined sponge metagenomic DNA samples (Table S3). In all of the individuals tested, and regardless of season, the microbiome was dominated either by the *AqS1* or the *AqS2* phylotype, which together represented as much as 90% of the overall microbial 16S rRNA pool. Reads matching to the ssrRNA sequence of the *AqS3* symbiont were detected across all samples but recovered on average 20-fold less than those matching to the *AqS1* phylotype. The remaining phylotypes that we detected all occurred at much lower abundance, with most displaying a relative abundance of <1% (Figure S5; Table S3), and could represent either rarer members of the *A. queenslandica* symbiont community or of the seawater, or contaminants introduced during sampling or processing. Because we could not discriminate between these scenarios, we do not discuss these further. Although we also recovered 16S rRNA sequences matching to members of the domain Archaea in three sponge individuals, these were from single pair-ended reads that could not be assembled into contigs.

We observed very little variation between the microbiome composition of individual sponges within a season; the two dominant phylotypes represented a similar proportion of the microbial community across sponge individuals. The *AqS1* phylotype was more prevalent than the *AqS2* phylotype in the samples collected in September, while the relative abundance of the two symbionts was more equivalent in those sponges collected in December, suggesting a slight seasonal variation (Figure S5; Table S3). Altogether, these data indicate that *A. queenslandica* individuals support a stable microbiome, the bulk of which comprises a coexisting gammaproteobacterium and betaproteobacterium. These observations are consistent with that reported in a companion paper, Fieth et al. (in review) in which OTU1 and OTU4 correspond to *AqS1* and *AqS2*, respectively. The genome contents of both of these symbionts are described below.

The *AqS1* Genome Encodes a Larger Gene Repertoire than the *AqS2* Genome

A total of 4778 protein-encoding genes are predicted in the draft genome of *AqS1*, 54% of which are hypothetical at time of writing, while 29% are incorporated into 347 subsystems derived from the RAST server. The genome contains one copy of the 5S rRNA,

the 16S rRNA, and the 23S rRNA genes – the latter bridging two scaffolds (scaffold 49 and 73), and 47 predicted transfer RNAs (tRNA). The gene repertoire of *AqS1* is very similar to that of *T.* sp. *HK1* (**Figure 2**; Table S4), and their contigs show high synteny (data not shown). Compared to *AqS1*, *AqS2* displays a much smaller gene repertoire that reflects its reduced genome assembly size. A total of 1349 protein-encoding genes are predicted in the genome of *AqS2*, but only 22% of these are hypothetical, while 50% are incorporated into 244 subsystems. For *AqS2*, we recovered a single copy each of the 5S rRNA, the 16S rRNA, and the 23S rRNA genes, plus 42 putative tRNAs.

The Symbiont *AqS1* Is a Putative Sulphur-Oxidising Bacterium

AqS1, along with its close relative *T.* sp. *HK1* (Tian et al., 2014), show the highest phylogenetic similarity to Ectothiorhodospiraceae and like most bacteria belonging to this order, the two bacteria appear capable of sulfur oxidation. The genome of *AqS1* encodes for a Sox enzyme complex (for periplasmic oxidation of various reduced sulfur compounds) coupled with a reverse dissimilatory sulfate reduction pathway (for sulfide oxidation in the cytoplasm) (**Figure 3**). The Sox pathway encoded by *AqS1* is truncated (that is, it includes a *soxBHXAYZ* but no *soxCD*), as is the case for *T.* sp. *HK1*, although a point of difference is that no *soxH* gene was recovered in the latter. The *Sox* genes are spatially separated in the genome of *AqS1*, with *soxB* and *soxH* not occurring contiguously with *soxAXYZ* (Table S5). In the absence of the *soxCD* genes, obligatory intermediate sulfur globules are predicted to form as pathway byproducts, and these can be subsequently oxidized to sulfate by the dissimilatory sulfite reductase (dsr) pathway (Pott and Dahl, 1998). Homologues to the DsrABC proteins, as well as associated proteins needed to build the DsrMKOJP complex, are present in both the *AqS1* and *T.* sp. *HK1* genomes. In the current *AqS1* assembly, the *dsr* genes are organized in a single polycistronic gene cassette (*dsrABEFHCMKLJOP*) that spans two contigs.

AqS1 and *T.* sp. *HK1* also encode a gene encoding for ATP sulphurylase (*sat*) and for adenosine-5′-phosphosulphate (APS) reductase (*aprAB*), both of which enable the conversion of sulfide to sulfate via APS. We did not detect the membrane-binding protein or the QmoABC complex that participate in the electron transfer during this conversion step in other bacteria (Frigaard and Bryant, 2008). However, the genomes of both these symbionts possess an operon-like gene cluster, adjacent to the *sat* gene, that comprises a *qmoA*—and a *qmoB*-like gene followed by a heterodisulphide reductase (*hdr*) *C* and *hdrB* gene (**Figure 3**; Table S5). This putative QmoAB-HdrCB protein complex could provide an alternative means of cytoplasmic electron transfer during sulfide oxidation, as described in another sulfur-oxidizing bacterium *Thiolapillus brandeum* (Nunoura et al., 2014).

Another enzyme involved in sulfur metabolism and that catalyzes the formation of sulfur globules is sulfide:quinone oxidoreductase, but we could not identify this gene in either of the *AqS1* or the *T.* sp. *HK1* genomes. However, we did detect the presence of a thiosulphate sulphurtransferase (*rhodanese*) gene and two rhodanese-related sulphurtransferases in both symbionts, with one copy located near the *soxH* gene in *AqS1.* The proteins encoded by these genes could participate in thiosulphate import to the cytoplasm (Aussignargues et al., 2012). Finally, both genomes include genes whose products can process extracellular taurine (i.e. *tauA*, *B and C*), alkanesulfonate (*ssuB*) and dimethylsulfoniopropionates (*dmdA* and *dmdB*), all of which can contribute to sulfide metabolism (**Figure 3**; Table S5).

AqS1 Has a Versatile, Heterotrophic Metabolism

Our RAST-based gene annotation analyses indicate that components of the Calvin-Benson-Bassham (CBB) pathway are present (Table S5), but both the *AqS1* and the *T.* sp. *HK1* genomes are missing homologs to key enzymes: (a) ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) form I and II, and (b) others known to be essential in alternative carbon fixation routes (Hügler and Sievert, 2011).

To establish the heterotrophic capabilities of *AqS1*, we focussed on annotated genes found in the *AqS1* symbiont but absent in its three closest free-living relatives, all of which are known to be obligate autotrophs (**Figure 2B**). From this list of 285 genes (86% of which are also present in the genome of *T.* sp. *HK1*), we recovered several genes capable of utilizing a wide range of sugars (monosaccharides, polysaccharides, organic acids and sugar alcohol subcategories) as well as amino acids and derivatives (e.g., polyamine, aromatic compound and creatine metabolism) as energy sources. We also found multiple genes encoding membrane transporters. These include four tripartite adenosine triphosphate (ATP)-independent periplasmic (TRAP) type transporters that are required for dicarboxylic acid import, as well as 29 tripartite adenosine triphosphate (ATP) binding cassette (ABC) transporter genes responsible for carbohydrate, amino acid and peptide uptake (Table S6A). Focusing on the high-affinity branched-chain amino acid ABC transporters, the *AqS1* genome encodes multiple copies of the polycistronic *LivFGHMK* gene cassette, five of which are complete, and one of which is missing a *livM* homolog. Each cassette displays a different gene organization. Nonetheless, based on the transmembrane domain and protein domain predictions for each of the five complete cassettes, they all have the capacity to form functional transmembrane complexes (**Figures 4A,B**; Table S5). Finally, both D-octopine dehydrogenase and opine oxidases A, B and C are present in the genome of *AqS1* (Table S5; Table S6A), which could enable *AqS1* to use methyl-group donors like opines for carbon and nitrogen source. Therefore, as was observed for the symbiont *T.* sp. *HK1* (Tian et al., 2014), the symbiont bacterium *AqS1* harbors a large repertoire of genes that play a role in heterotrophic metabolism. This suggests that it might not need to rely on carbon fixation, as it is able to acquire and oxidize organic compounds.

As further evidence of the versatile metabolism of *AqS1*, we also found genes encoding small, medium and large subunits of the aerobic-type carbon monoxide (CO) dehydrogenase, a CO dehydrogenase F and a CO oxidation accessory protein (Table S5). These imply that the symbiont can generate reductive energy

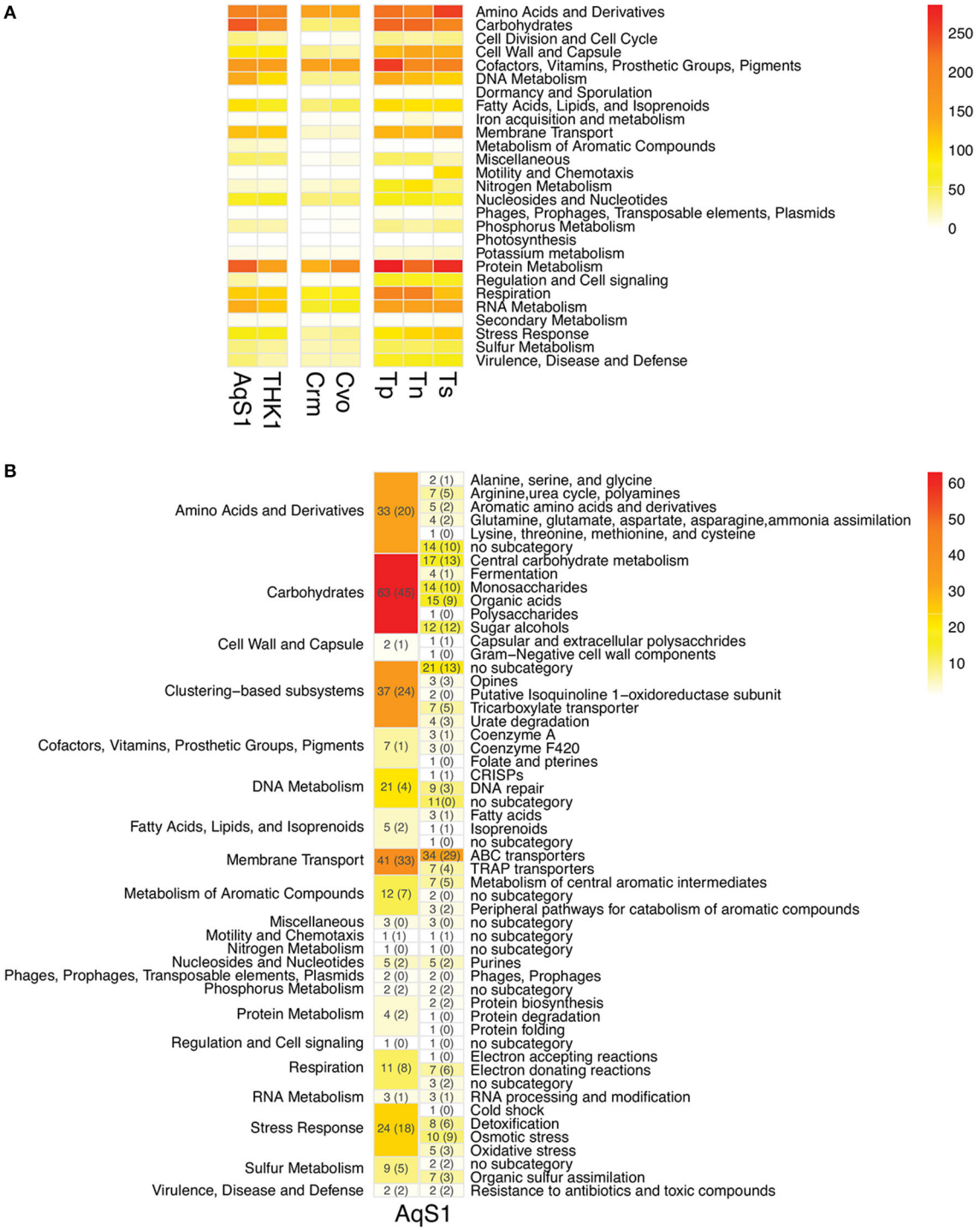

FIGURE 2 | (A) Functional comparison between *AqS1*, three closest free-living and two symbiont sulfur-oxidizing strains using FIGfam-based subsystems derived from RAST annotations. THK1 *Thioalkalivibrio* sp. *HK1* (isolated from the sponge *Haliclona cymaeformis)*; Crm *Candidatus* Ruthia magnifica (isolated from the clam *Calyptogena magnifica*); Cvo *Candidatus* Vesicomyosocius okutanii (isolated from the clam *Calyptogena okutanii*); Tp *Thioalkalivibrio paradoxus* ARh 1; Tn *Thioalkalivibrio nitroreducens*; Ts *Thioalkalivibrio sulfidophilus* HL-EbGr7. Genes might contribute to more than one category. The detailed gene counts can be found in Table S4. **(B)** Number and functional categories of annotated genes present in *AqS1* that are not recovered in *T. paradoxus* ARh 1, *T. nitroreducens* or *T. sulfidophilus* HL-EbGr7 (columns to the left). The FIGfam-based subcategories are further divided into subsystems (columns to the right). Numbers in brackets refer to the number of genes that had a top BLAST hit to an alphaproteobacterium.

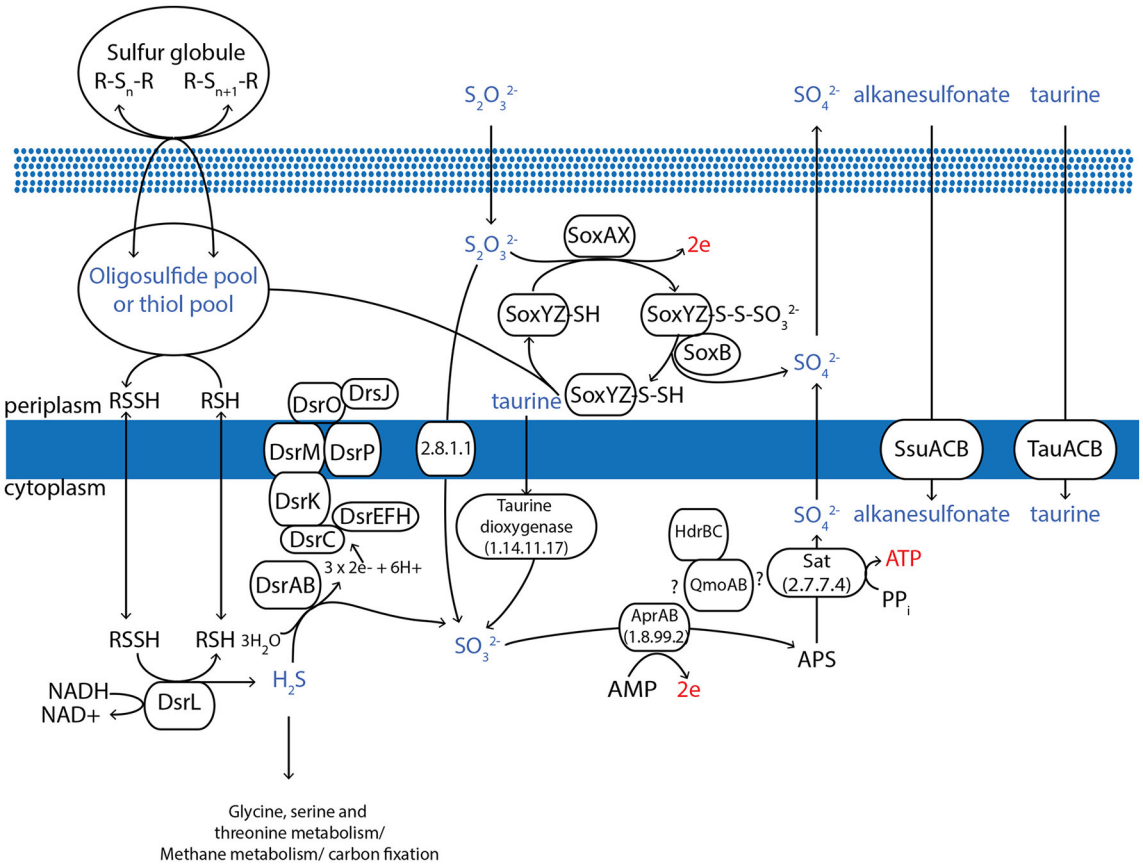

FIGURE 3 | Overview of the proposed pathways for the oxidative sulfur metabolism of *AqS1*. All of the genes depicted in the figure are identifiable in the genome assembly. Specifically, *AqS1* contains a Sox enzyme complex for periplasmic oxidation of reduced sulfur compounds, coupled with a reverse dissimilatory sulfate reduction pathway for sulfide oxidation in the cytoplasm. The *AqS1* genome also includes other genes that can process extracellular taurine, alkanesulfonate and dimethylsulfoniopropionate, all of which can contribute to sulfide incorporation into carbon. Figure adapted from Frigaard and Dahl (2008).

from the oxidation not only of sulfide, as described above, but also of CO. The RAST-based gene model predictions suggest that for catabolism, *AqS1* is equipped with a complete Embden-Meyerhof-Parnas (glycolysis) pathway for the metabolism of hexose sugar, and a near-complete TCA cycle for the production of intermediates in cofactors and amino acid biosynthesis. A near complete pentose phosphate pathway is also present, providing a mechanism for production of NADPH and ribose sugars for nucleotide biosynthesis.

AqS1 is well equipped for phosphorus metabolism, including mechanisms to regulate the assimilation of inorganic phosphate (Pi). Specifically, the *AqS1* genome encodes for components of the Phosphate (Pho) regulon, namely a Phosphate regulon sensor protein (PhoR) that detects levels of Pi in the environment, and downstream response genes (i.e., Phosphate regulon transcriptional regulatory protein [PhoB], Phosphate-starvation-inducible protein, predicted ATPase [PhoH], Phosphate transport system regulatory protein [PhoU], and two genes encoding response regulators in a two-component regulatory system) (Table S5). Also present is a gene encoding a polyphosphate kinase (EC 2.7.4.1) that catalyzes the formation of long-chain polyphosphate (polyP) (Table S5).

Finally, this symbiont has retained either partial or complete biosynthesis pathways for most essential amino acids (**Figure 5A**) and, like most bacteria, is capable of assimilating ammonia (genes encoding EC 1.7.1.4, EC 1.4.1.13, and EC 1.4.1.2 all are present) (Table S5). On the other hand, the absence of ureases and their associated proteins suggests that *AqS1* is unable to use urea to cover its nitrogen needs. *AqS1* also appears to lack a denitrification pathway; we could not detect genes encoding the intermediate enzymes of the anaerobic respiratory chain, nitrite reductase (NO forming) (EC 1.7.2.1) or nitric oxide reductase (EC 1.7.99.7).

There Is Evidence of Extensive Past Horizontal Gene Transfer in the *AqS1* Genome

The identification of 285 genes found in the *AqS1* symbiont, but not in its closest free-living relatives, led us to examine the origin of these genes. Interestingly, 136 of the 285 genes

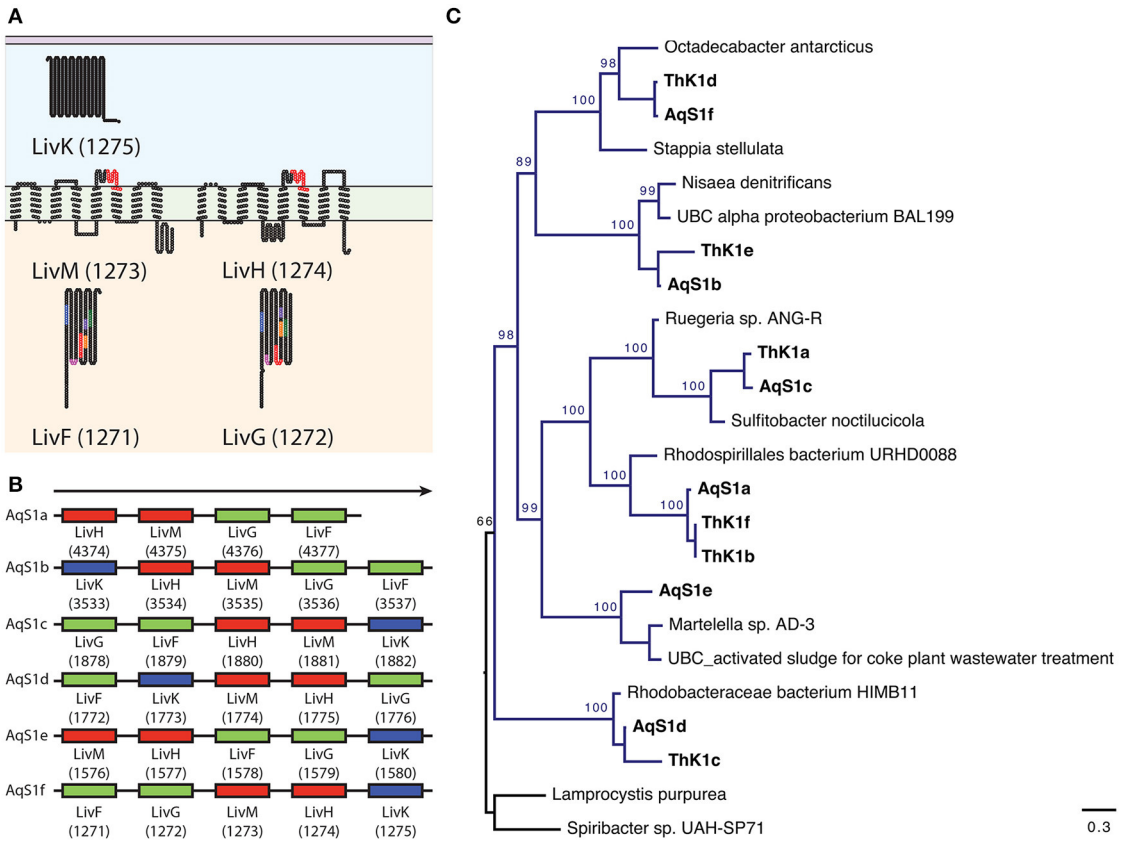

FIGURE 4 | Repertoire of high-affinity branched-chain amino acid ATP binding cassette (A,B,C) transporters present in *AqS1*. The symbiont encodes multiple copies of the polycistronic *LivFGHMK* gene cassettes that are ATP-dependent high-affinity branched-chain amino acid transport systems involved in leucine metabolism and transport. **(A)** An example of putative 2D topology for one of the *LivFGHMK* transmembrane protein complex, derived using the program TOPO2. Conserved motifs are represented in different colors on the diagram. For LivF and LivG: blue AP-loop, pink Q-loop, red ATP transporter signature motif, orange Walker B-loop, purple D-loop, green, H-loop/switch region; For LivM and LivH: red TM-ABC transporter signature motif. The number in brackets corresponds to the RAST gene model id. **(B)** The *AqS1* genome encodes six complete *LivFGHMK* gene cassettes; five of these cassettes are complete, while one – AqS1a – is missing a *LivK* homolog. Each cassette displays a different gene arrangement. **(C)** Maximum-likelihood phylogenetic tree for concatenated genes *LivF, LivG, LivH, LivM, and LivK* present within a polycistronic cassette for both *AqS1* and *T.* sp. *HK1*. We used two Chromatiales representatives as outgroup. All cassettes nested with different representatives of the class Alphaproteobacteria, within the orders Rhodobacterales and Rhodospirilales. Bootstrapping percentages greater than 50% are shown.

showed highest BLASTP similarity to various species in the class Alphaproteobacteria, with the order Rhodobacterales being the most represented (please refer to numbers that are reported in brackets in **Figure 2B**; Tables S7A,B). This suggests that the versatile metabolic capacity of the *AqS1* symbiont could be, at least in part, a result of horizontal gene transfer (HGT) from one or more alphaproteobacteria. To further explore this possibility, we performed a phylogenetic analysis focusing on the multiple high-affinity branched amino acid ABC transporter protein cassettes found in both *AqS1* and *T.* sp. *HK1* (see above). These all clade with different representatives of Alphaproteobacteria, and specifically within the orders Rhodobacterales and the Rhodospirilales. This same association was also discussed by Tian et al. (2014) for *T.* sp. *HK1* (**Figure 4C**).

Further support for an HGT origin, albeit not alone compelling, is that the *AqS1* genome encodes genes implicated in HGT mechanisms, including four copies of the ISSpo3 transposase, mobile element proteins and phage-associated proteins (i.e., adenine DNA methyltransferase, phage-associated; putative prophage protein ps3). Genetic systems for conjugation and transformation were not detected, but DNA recombination and repair enzymes are present (i.e., Rec F, N, O and R), as well as protein families involved in general DNA-modifying activities (i.e., HNH endonuclease), and restriction-modification (R-M) and toxin–antitoxin systems (Type I, Type II, and Type III R-M systems, proteins from the Doc/Phd family, and VapI of the HigAB system) (Table S5).

Interestingly, mechanisms to control excessive genetic exchange and to minimize the introduction of foreign DNA are also encoded by the *AqS1* genome. Single clustered, regularly interspaced, short, palindromic repeats (CRISPRs) and their associated proteins form adaptive immunity systems that occur in most archaea and many bacteria and act against invading genetic elements, such as viruses and plasmids (Makarova et al.,

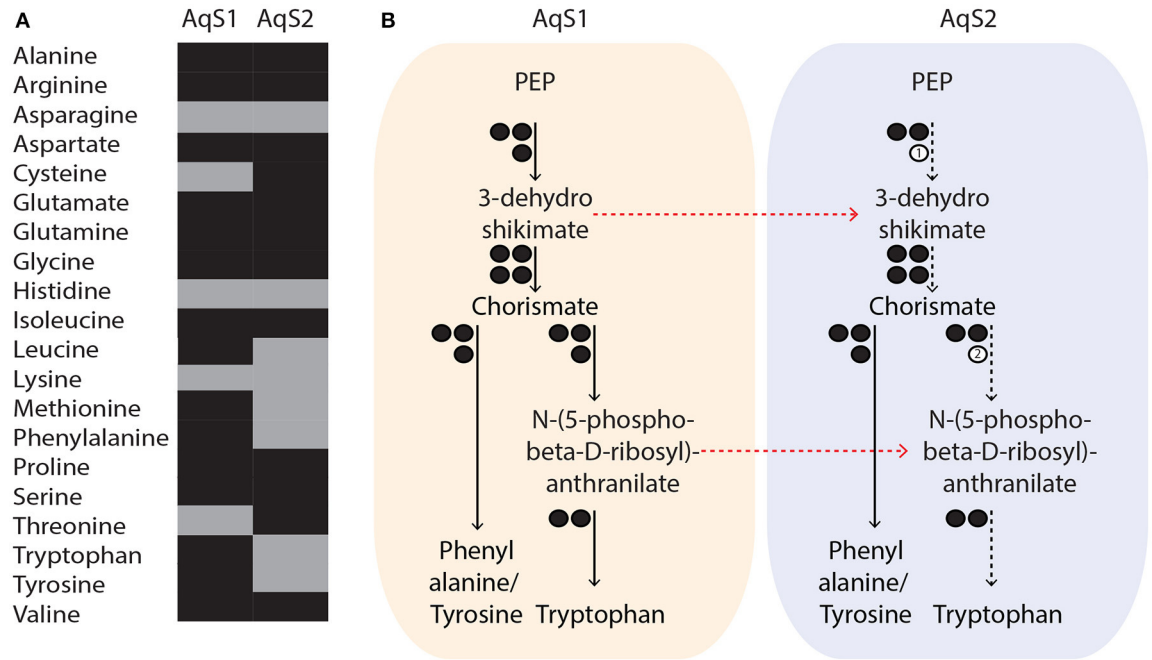

FIGURE 5 | (A) Putative metabolic capacity of amino acid biosynthesis in *AqS1* and *AqS2*. Amino acids are represented in different colors, depending on whether their biosynthesis pathway was predicted to be complete (black) or partially complete (gray) by the RAST server. **(B)** Example of possible metabolic complementation occurring between *AqS1* and *AqS2*. *AqS2* could rely on the metabolism of *AqS1* to supply intermediate substrates that are required for the synthesis of aromatic amino acids. The number of genes involved in each pathway is shown as circles. Presence or absence of enzymes is indicated by black or white circles, respectively. (1) 3-dehydroshikimate and (2) N-(5-phospho-beta-D-ribosyl)-anthranilate. All paths that are partial are represented by dashed instead of solid arrows and red arrows indicate the possible movement of intermediary metabolites between the two symbionts. PEP: phosphoenolpyruvate.

2011). A single CRISPR is found in the genome of *AqS1*. It is composed of at least fifteen direct repeat regions that are each 36 nucleotides in length, separated by at least thirteen spacers that are each 30 nucleotides in length. A gap present in our assembly, which bridges the first recovered spacer at the 5′ end of the predicted CRISPR, precludes the recovery of its leader sequence. The *AqS1* CRISPR system has a canonical genomic arrangement (Makarova et al., 2011), with CRISPR-associated (CAS) genes (*Csn1* and *Cas1*) located upstream of and directly adjacent to the CRISPR array (Figure S6; Table S5).

AqS2 Also Has a Versatile Metabolism

Our RAST-based gene annotation analyses recovered complete or near-complete key functional pathways in *AqS2,* despite its smaller genome assembly size. These included glycolysis, the TCA cycle and the pentose phosphate pathway. This symbiont does not seem to have nitrogen reduction, carbon fixation or sulfur oxidation capacity and, in stark contrast to the *AqS1* genome, there are no genes associated with phosphorus metabolism. However, *AqS2* does possess a gene operon that includes the three subunits of carbon monoxide (CO) dehydrogenase form I, D and F, and CO oxidation accessory protein (CoxG), suggesting that, like *AqS1*, it can generate reductive energy from the oxidation of CO. *AqS2* might also be capable of assimilating ammonia, as it encodes NADP-specific glutamate dehydrogenase (EC 1.4.1.4) (Table S5). It also (like *AqS1*) has retained partial or complete biosynthesis pathways for most essential amino acids (**Figure 5A**), and genes involved in carbohydrate metabolism and membrane transport are also present, indicating *AqS2* has heterotrophic abilities (Table S7). On the other hand, the genome of this symbiont does not show any evidence of past horizontal gene transfer (including no transposable insertion elements, or CRISPR-Cas resistance systems). We did recover genes involved in protection against foreign DNA (e.g., R-M systems) and cellular stress response (e.g., chaperones, membrane proteases) (Table S5).

Evidence of Resource Partitioning and Gene Repertoire Complementarity between *AqS1* and *AqS2*

It has been postulated that co-occurring symbionts avoid competition by utilizing different carbon and energy sources (Duperron et al., 2006; Kleiner et al., 2012a). To establish preliminary evidence of resource partitioning between *AqS1* and *AqS2*, we scanned for differences in their overall repertoire of predicted proteins involved in carbohydrate metabolism and transport. *AqS1* encodes a wider range of membrane transporters than *AqS2* (e.g., ABC transporter branched-chain amino acid, ABC transporter dipeptide, ABC transporter tungstate, copper transport system) (Table S7), and a genome-wide comparison clearly shows that each symbiont is equipped with a unique suite of proteins that would permit them to import or export different

substrates (**Figure 6**). These include sulfur-related compounds, ammonium, vitamin B12, putrescine, spermidine, glutamate, aspartate, L-proline, glycine betaine, benzoate and chromate in *AqS1;* and biotin, methionine and manganese in *AqS2*.

Differences also exist in the means by which the two bacteria exploit carbohydrates (Table S7). *AqS2* can utilize N-acetylglucosamine and chitin, both of which are important sources of carbon and nitrogen for marine organisms (Driscoll et al., 2007; Souza et al., 2011). It is also predicted to metabolize and catabolise a larger number of monosaccharides than *AqS1*, with subcategories, such as D-galactonate catabolism, D-gluconate and ketogluconates metabolism, deoxyribose and deoxynucleoside catabolism, mannose metabolism and xylose utilization being only recovered in *AqS2*. On the other hand, *AqS1* is able to use the organic acid tricarballylate, which is a citrate analog and a potential source of carbon and energy (Gutnick et al., 1969) (Table S7).

While both the *AqS1* and *AqS2* genomes have the capacity to independently synthesize *de novo* most amino acids, they also lack key enzymes required for the production of others (**Figure 5A**). For instance, 3-dehydroquinate dehydratase (EC 4.2.1.10), an enzyme-encoding gene that participates in the production of aromatic acids (phenylalanine, tyrosine and tryptophan), is not present in the *AqS2* genome. *AqS2* also lacks a homolog for phosphoribosylanthranilate isomerase (EC 5.3.1.24) that catalyzes the third step of tryptophan synthesis. Interestingly, however, these missing genes are present in the genome of *AqS1*, suggesting that *AqS2* could synthesize all three aromatic acids provided that *AqS1* metabolism supplies the intermediate substrates, 3-dehydroshikimate and N-(5-phospho-beta-D-ribosyl)-anthranilate (Table S5; **Figure 5B**). The alternative—that AqS2 could could directly take up the synthesized amino acid provided by AqS1—is not supported, because we are unable to detect transporters for phenalanine, tryptophan or tyrosine amongst the predicted proteins of AqS2. These observations suggest that both essential and non-essential amino acid synthesis could depend on within-pathway complementation between the two symbionts.

The Gene Repertoire Reflects a Symbiotic Lifestyle for Both *AqS1* and *AqS2*

Sponge-associated bacteria are predicted to use characteristic eukaryote-like domain-containing proteins to facilitate host-microbe interactions; these include ankyrin-repeats, tetratricopeptide-repeats, leucine-rich repeats, fibronectin domain IIIs, and cadherins (Thomas et al., 2010; Fan et al., 2012; Hentschel et al., 2012). Our comparison of *AqS1* with its free-living relatives provided support for this prediction in that the genome of *AqS1* is enriched in ankyrin-repeat containing proteins (**Figure 7**); a similar trend was reported for *T.* sp. *HK1* (Tian et al., 2014). On the other hand, the *AqS2* genome does not show enrichment for any of these domains (**Figure 7**). There is little evidence of virulence factors in either of the symbiont genomes; they share just a small number of predicted protein-coding genes conferring resistance to copper, cobalt-zinc-cadmium, and/or virulence. In *AqS1*, the predicted

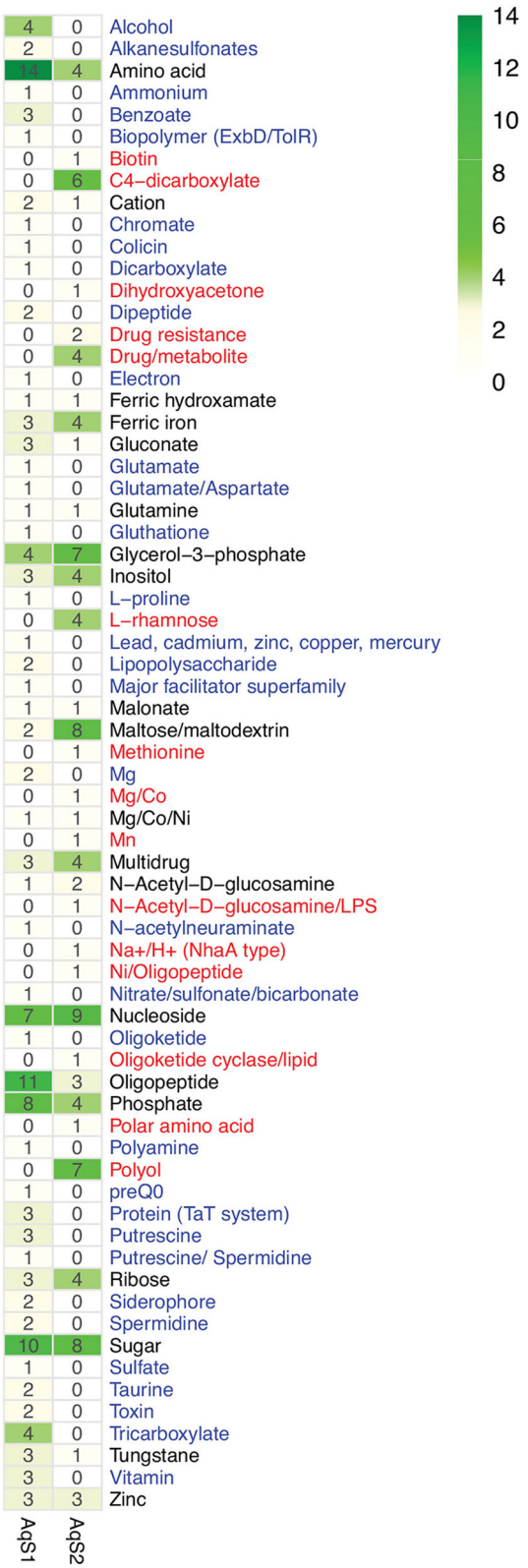

FIGURE 6 | Counts of putative substrates derived from predicted transporters present in the genome of *AqS1* only (blue), *AqS2* only (red), or both (black).

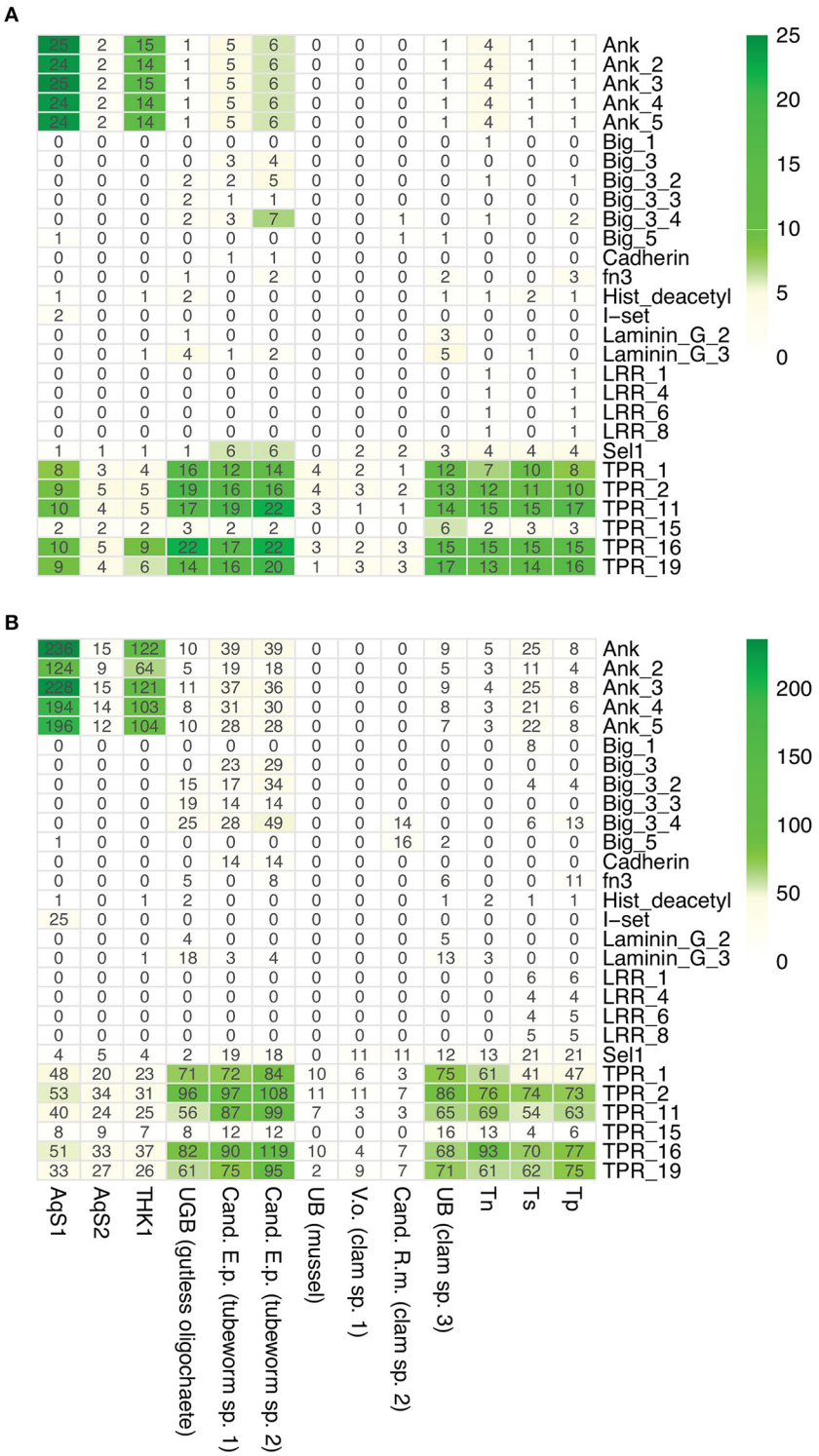

FIGURE 7 | Occurrence of eukaryotic-like containing proteins in *AqS1* and *AqS2*. (A) Counts of eukaryotic-like containing proteins. **(B)** Counts of eukaryotic domains. THK1 *Thioalkalivibrio* sp. HK1; UGB (gutless oligochaete*)* Gamma3 isolated from *Olavius algarvensis* JGI genome accession number 2004178004; Cand. E.p. (tubeworm sp. 1) *Candidatus* Endoriftia persephone isolated from *Riftia pachyptila*, accession number AFOC00000000; Cand. E.p. (tubeworm sp. 2) *Candidatus* E. persephone isolated from *Tevnia jerichonana*, accession number AFZB00000000; UB (mussel) Unknown thiotroph isolated from *Bathymodiolus* sp., accession number CAEB01000001; V.o. (clam sp. 1) *Candidatus* Vesicomyosocius okutanii isolated from *Calyptogena okutanii*, accession number NC_009465.1; Cand. R.m. (clam sp. 2) *Candidatus* Ruthia magnifica isolated from *Calyptogena magnifica*, accession number NC_008610.1; UB (clam sp. 3) Unknown symbiont isolated from *Solemya velum*, accession number NZ_JRAA00000000.1; Tn *Thioalkalivibrio nitroreducens;* Ts *Thioalkalivibrio sulfidophilus HL-EbGr7*; Tp *Thioalkalivibrio paradoxus ARh 1*.

proteins belonging to the virulence resistance category are involved in the production of bacteriocin Colicin V, universal stress proteins, such as heat shock protein UspA, as well as PotD, PotA and PotH. Resistance to chromium is predicted only in *AqS1* (Table S5).

Finally, *AqS1* and *AqS2* have the genomic capacity to produce products that could be beneficial to the host, although it must be noted that many non-symbiotic bacteria share the same capacity. For instance, the *AqS1* genome encodes genes associated with the synthesis of coenzyme A, as well as the essential pyrophosphate (vitamin B1), which animals must obtain from their diet (Begley et al., 2008) (Table S5). The ability to synthezise vitamin B1 has also been observed in other sponge associated bacteria and archaea (Fan et al., 2012). The two *Aq* symbionts also contain genes involved in pyridoxine (a form of vitamin B6), menaquinone/phylloquinone and folate biosynthesis. *AqS2* is additionally equipped with genes that are involved in biotin (vitamin B7) synthesis (Table S5).

DISCUSSION

Here we show that the coral reef demosponge *Amphimedon queenslandica* supports a microbiota dominated by two species of proteobacteria, and present the first draft genomes of both. These "core" bacterial symbionts are stable across individuals and across seasons, and represent the bulk of the microbiome, in sponge hosts sampled from Heron Island reef in the southern Great Barrier Reef of Australia (see Fieth et al., in review). The most prevalent symbiont, *AqS1,* is a putative sulfur-oxidizing gammaproteobacterium of the order Chromatiales, and the second most prevalent symbiont, *AqS2*, is an unknown betaproteobacterium whose closest relatives also include other sponge symbionts.

The co-occurrence of bacteria genetically related to *AqS1*, *AqS2,* and *AqS3* in *Calyspongia vaginalis* (Giles et al., 2012), and to *AqS1* and *AqS2* in *Haliclona simulans* (Kennedy et al., 2008), suggests that they could have undergone long-term codiversification with their hosts, as has been demonstrated in other systems involving multiple stable symbionts within a specific host (Dubilier et al., 2001, 2008; Takiya et al., 2006; Zimmermann et al., 2014). Because of limitations with our *AqS3* genome assembly, we choose not to discuss its gene repertoire; thus the remainder of this discussion focuses solely on *AqS1* and *AqS2*.

Sulfur-oxidizing symbioses are found in various marine habitats ranging from shallow water sediments, organic rich mud and mangrove peat, to deep-sea hydrothermal vents and cold seeps. These associations rely on sulfide produced either geothermally or biologically, and have evolved independently numerous times in phylogenetically diverse hosts (Dubilier et al., 2008). Several lines of evidence point to their widespread occurrence in marine sponges. Our own phylogenetic analysis confirm that bacteria belonging to the family Ectothiorhodospiraceae within the order Chromatiales, and closely related to *AqS1* and *T.* sp. *HK1,* have been reported in taxonomically distant sponges that live at varying latitudes and depths (Taylor et al., 2007b; Jensen et al., 2008; Kennedy et al., 2008; Giles et al., 2012; Radax et al., 2012; Karlinska-Batres and Wörheide, 2013; Pita et al., 2013; Olson et al., 2014; Della Sala et al., 2014). Furthermore, other studies on sponge symbionts have recorded the presence of both known and novel Chromatiales representatives belonging to either the Ectothiorhodospiraceae or Chromatiaceae family (Imhoff and Trüper, 1976; White et al., 2012), as well as sulfur-oxidizing bacteria from the Alphaproteobacteria class (Rhodospirillaceae, Rhodobacteraceae) and the phylum Chlorobi (Imhoff and Trüper, 1976; Bondarev et al., 2013).

The Chromatiales are generally lithotrophs, capable of synthesizing carbohydrates from the fixation of carbon dioxide using sulfur oxidation as an energy source (Grimm et al., 2008; Frigaard and Dahl, 2008; Ghosh and Dam, 2009). The presence of a putative Sox enzyme complex and a reverse dissimilatory sulfate reduction pathway in the genome of *AqS1* suggests that this symbiont, like its close relative *T.* sp. *HK1,* does indeed have the ability to oxidize sulfur. Further evidence of sulfur oxidation has been obtained via transmission electron microscopy, with the detection of pseudo-spherical, membrane-bound vacuoles in *AqS1* cells (Fieth et al., in review); these vacuoles resemble commonly-found bacterial organelles that contain sulfur globules (Lawry et al., 1981; Robertson and Kuenen, 2006; Docampo et al., 2010).

The origin of the reduced sulfur compounds that would be required by *AqS1* for oxidation is unknown at this stage. Sulphur metabolism has been detected previously in several marine sponge species, and sulfate-reducing bacteria—sometimes co-occurring with sulfur oxidisers—have been isolated from sponge tissue (Imhoff and Trüper, 1976; Manz et al., 2000; Hoffmann et al., 2005; Taylor et al., 2007b; Zhang et al., 2015a). An endosymbiotic sulfur cycle, coupling sulfate-reducing with sulfide-oxidizing bacteria, has been reported in a marine oligochaete (Dubilier et al., 2001) and Hoffmann et al. (2005) proposed that a similar process might take place in some sponges. Sulphides can be toxic at high concentrations, but sulfide-oxidizing bacteria, such as *AqS1* could detoxify sulfides generated by sulfate-reducing bacteria in the sponge host body. Anoxic zones can form in sponge tissue, particularly during times of pumping inactivity, and would provide the right environment for sulfate-reducing bacteria (Hoffmann et al., 2005, 2008). Since *A. queenslandica* adults have the ability to close their oscula and thus stop pumping water for long period of times, both in natural and in laboratory environments, hypoxic pockets are also expected to form in its tissue; indeed, sites of reduced oxygen have been detected in *A. queenslandica* even while it continues to pump (J. Watson and J. Kroemer, personal observations). Reduced sulfur compounds might not be only symbiotic in origin, but could also be environmentally derived. The habitat of *A. queenslandica* on Heron Island reef is characterized by fine sand patches and often heavy siltation (Degnan et al., 2008). During times of high sand movement or high silt deposition, adult sponges can easily be partially buried to the extent that they may be subject to anoxic conditions with high sulfide concentrations. Together, these

observations indicate a functional role for *AqS1* in mediating sulfur nutrient cycling between host and symbiont.

Sulfur-oxidizing symbionts have varied metabolic capabilities, with some displaying obligate autotrophy while others can grow heterotrophically or mixotrophically (Kleiner et al., 2012a; Tian et al., 2014). We find no evidence for a complete carbon fixation mechanism in *AqS1*, although we acknowledge that this could be due to limitations of our current draft genome assembly. Nonetheless, it is also possible that the symbiont either fixes carbon using an unknown mechanism, or is a lithoheterotroph. Unlike its closest free-living relatives (*Thioalkalivibrio paradoxus ARh 1*, *T. nitratireducens* and *T. sulfidophilus*) that are obligate autotrophs, *AqS1* harbors a large repertoire of genes involved in heterotrophic metabolism (e.g., carbohydrate uptake and metabolism, membrane transport), which would allow the symbiont to import and utilize various substrates. Thus, *AqS1* might not need to rely on carbon fixation to acquire and oxidize organic compounds. For instance, the *AqS1* genome is particularly rich in amino acid and oligopeptide transporters and, in combination with peptidases and proteases, these could permit the acquisition and hydrolysis of complex organic compounds for nutrition, as has been proposed by Bondarev et al. (2013). Another example is the presence of both D-octopine dehydrogenase and opine oxidases A, B and C, which could enable *AqS1* to use methyl-group donors like opines for carbon and nitrogen source. The bacterium could produce this compound when maintaining redox balance under anaerobic conditions, and oxidize it when requiring energy (Strahl et al., 2011; van Os et al., 2012).

In oligotrophic marine habitats, such as coral reefs, phosphorous (P) can be one of the more limiting elements, particularly over longer time scales, and its biogeochemical cycle is intimately associated with the microbial community (Karl, 2014). Zhang et al. (2015b) recently reported a potentially important mode of P sequestration mediated by bacterial symbionts of marine sponges that likely use polyphosphate kinase (ppk) to catalyze the formation of long-chain polyphosphate (polyP). We find a ppk gene in the genome of *AqS1*, suggesting that it could have the same capability, and supporting the hypothesis of Zhang et al. (2015b) that oscillation between anoxic and oxic states inside sponges could select for polyP-accumulating organisms within the symbiotic community. In fact, *AqS1* is well equipped for phosphorus metabolism, with the ability to detect environmental levels of inorganic phosphate and accordingly regulate its assimilation as necessary, using a phosphate regulon system (Santos-Beneit, 2015).

Heterotrophy in general has been reported in several other sponge symbionts (Thomas et al., 2010; Siegl et al., 2011; Fan et al., 2012; Bondarev et al., 2013; Tian et al., 2014). Interestingly, a large proportion of genes that endow the *AqS1* symbiont with heterotrophic capabilities are most closely related to genes of several alphaproteobacteria. Our phylogenetic analysis of the high-affinity branched amino acid ABC transporter protein family present in both *AqS1* and *T.* sp. *HK1*, for example, strongly supports a hypothesis that these genes have been acquired via one or multiple horizontal gene transfers (HGT). Kleiner et al. (2012a) highlighted the metabolic similarities and differences between several sulfur oxidizing gammaproteobacterial symbionts and also speculated that HGT events might play an important role in the metabolic evolution of these microbes. However, it is unknown whether these events occurred before or after the establishment of the symbioses, and thus if the acquisition of certain gene sets by the microorganism was critical for the establishment of these associations and for their subsequent evolutionary success.

Based on its genome assembly, the betaproteobacterium *AqS2* is predicted to have a smaller genome compared to *AqS1,* and, as a result, a reduced functional toolkit. Genome streamlining reported in cyanobacterial symbionts of other sponges has been postulated to reflect adaptation to the mild intercellular environment present in the host (Gao et al., 2014; Burgsdorf et al., 2015). Despite its predicted reduced genome size, *AqS2* encodes genes required for heterotrophic metabolism, for transport of various substrates and for amino acid and vitamin biosynthesis. Thus, it seems to have retained genes that could be considered to play a major nutritional role for the sponge host, a feature that might be fundamental for its maintenance as a symbiont over evolutionary time.

Previous global metagenomic analyses of sponge microbiomes have provided evidence for functional genomic signatures that separate them from bacterial communities in the surrounding seawater (Thomas et al., 2010; Fan et al., 2012; Hentschel et al., 2012; Liu et al., 2012). Some of the signatures recovered in both *AqS1* and *AqS2* genomes are versatile nutrient utilization (e.g., carbon and nitrogen metabolism, amino acid biosynthesis, transporters), protection against foreign DNA (e.g., CRISPR in *AqS1*, restriction-modification systems in both microorganisms), response to cellular stress (e.g., chaperones, membrane proteases) and a deficiency in virulence factors. Other features recovered in *AqS1*, but not in *AqS2,* include evidence of past genetic transfer (e.g., transposases; mobile elements) and possible mechanisms for evading host detection (e.g., ankyrin-repeat proteins). While the predicted reduced size of its genome might be one of the reasons why these features were not detected in *AqS2,* this is inconsistent with these same features being present in other small-sized cyanobacterial genomes (Burgsdorf et al., 2015), and thus other factors are likely to play a role.

The existence of stable associations involving two or more symbionts has been reported in several non-sponge animal hosts (Takiya et al., 2006; Wu et al., 2006; Dubilier et al., 2008; Zimmermann et al., 2014). In such associations, the microbes are expected to use different carbon and energy sources to minimize competition (Duperron et al., 2006; Kleiner et al., 2012b). In *A. queenslandica*, the bacterium *AqS1* has a larger gene repertoire than *AqS2,* as well as a more versatile metabolism, and is therefore predicted to employ feeding strategies that differ from *AqS2* (e.g., sulfide oxidation, wider range of transporters). We also established that *AqS1* and *AqS2* differed in the ways they use carbohydrates. For example, *AqS2* can utilize N-acetylglucosamine or chitin, as well as a larger number of monosaccharide substrates than *AqS1*, while *AqS1* is able to use the organic acid tricarballylate. As yet another contrast, *AqS1* has extensive capabilities for phosphorus metabolism, while *AqS2* has

none at all. Together, these data strongly suggest that resource partitioning does indeed occur between the two most prevalent bacterial symbionts of *A. queenslandica*.

Finally, within-pathway complementation might exist between *AqS1* and *AqS2,* as has also been reported in other (non-sponge) symbionts (Wu et al., 2006; Rao et al., 2015). For example, enzyme-encoding genes that participate in the production of aromatic amino acids are present in the *AqS1*, but not in the *AqS2,* genome, suggesting that each symbiont could synthesize particular amino acids provided that the metabolism of the other supplies the intermediate substrates. It has been postulated that metabolic redundancies existing within symbiotic partners upon host initial acquisition might be lost over time, resulting in mutual dependence to produce compounds essential to their host (Wu et al., 2006; Rao et al., 2015). Alternatively, the function of the missing genes could be performed by other genes encoded in their respective genomes, either by a novel enzyme, or by an already-known enzyme that would have changed or extended its prior function by co-option (Charles et al., 2011; Sloan and Moran, 2012; Rao et al., 2015).

CONCLUSIONS AND FUTURE DIRECTIONS

Here we present draft genomes for the two dominant bacterial symbionts found in the coral reef demosponge *A. queenslandica*, and discuss evolutionary and functional insights based on predicted gene repertoires. These symbiont genomes complement extensive genomic and transcriptomic resources already existing for the sponge host. With these tools in hand, investigations are now underway to (i) construct genome-scale models to detail the metabolic interplay between sponge and symbionts, and (ii) characterize mechanisms of molecular crosstalk that regulate establishment and maintenance of the host-symbiont relationships (Hentschel et al., 2012, Fieth et al., in review). Thus, the *A. queenslandica* holobiont is poised to become a fruitful model for understanding the nature and consequences of interactions between animal hosts and their bacterial symbionts.

AUTHOR CONTRIBUTIONS

SD and MG designed the research. JW prepared the biological material and assisted in early stages of analysis. MG analyzed the data. MG and SD wrote the paper. All authors read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmars.2016.00196

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