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A novel Chromatiales bacterium is a potential sulfide oxidizer in multiple orders of marine sponges

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Summary

Sponges are benthic filter feeders that play pivotal roles in coupling benthic-pelagic processes in the oceans that involve transformation of dissolved and particulate organic carbon and nitrogen into biomass. While the contribution of sponge holobionts to the nitrogen cycle has been recognized in past years, their importance in the sulfur cycle, both oceanic and physiological, has only recently gained attention. Sponges in general, and *Theonella swinhoei* in particular, harbour a multitude of associated microorganisms that could affect sulfur cycling within the holobiont. We reconstructed the genome of a Chromatiales (class Gammaproteobacteria) bacterium from a metagenomic sequence dataset of a *T. swinhoei*-associated microbial community. This relatively abundant bacterium has the metabolic capability to oxidize sulfide yet displays reduced metabolic potential suggestive of its lifestyle as an obligatory symbiont. This bacterium was detected in multiple sponge orders, according to similarities in key genes such as 16S rRNA and polyketide synthase genes. Due to its sulfide oxidation metabolism and occurrence in many members of the Porifera phylum, we suggest naming the newly described taxon *Candidatus Porisulfidus*.

Introduction

Sulfur is assimilated into amino acids, vitamins, secondary metabolites and sulfo-lipid compounds, making it an essential element for all living organisms. Beyond its importance as a building block, many bacteria and archaea use reduced sulfur compounds as electron sources to generate energy and assimilate inorganic carbon (Vogler *et al*., 1942). Photoautotroph sulfur oxidizing bacteria (SOB) oxidize hydrogen sulfide and other forms of reduced sulfur under anoxic conditions, such as those that occur in stratified lakes (Jorgensen *et al*., 1979). Their counterparts, the chemolithotrophic SOB, can be found in a multitude of environments, from oxic to anoxic, terrestrial to deep-ocean hydrothermal vents [extensively reviewed by Ghosh and Dam (2009)]. In extreme environments, such as hydrothermal vents, sulfur-chemolitotrophs support communities of heterotrophic organisms by filling the role of primary producers (Stewart *et al*., 2005). In many of these cases SOB form intra- or extra-cellular symbioses with their

heterotrophic hosts (Bright and Giere, 2005; Taylor and Glover, 2006; Nakagawa *et al*., 2014).

Sponges are heterotrophic filter-feeders, some hosting up to 5×10^{10} bacteria (ml sponge)$^{-1}$. They inhabit marine and freshwater ecosystems (Taylor *et al*., 2007) and mostly feed by filtering up to 50,000 times their body volume per day (Weisz *et al*., 2008). Their evolutionary success may be ascribed to their complex association with diverse consortia of microorganisms (Schmitt *et al*., 2012), that provide them with various services, from chemical defense (Guo *et al*., 2011) to recycling metabolic waste (Mohamed *et al*., 2010). The role of sponge-associated microorganisms in biogeochemical cycles has been studied extensively over the past four decades (Maldonado *et al*., 2012; Webster and Thomas, 2016). However, most studies have focused on the carbon and nitrogen cycles. For example, it is now well established that ammonia excreted by the sponge cells serves as an energy and N source for its nitrifying symbionts (Southwell *et al*., 2008), and urea is used as a nitrogen source by sponge-associated bacteria (Su *et al*., 2013). Not only nitrification but also denitrification occurs within a sponge. The anoxic environment within the sponge body may provide the necessary conditions for bacteria and archaea responsible for such activities (Schläppy *et al*., 2010; Lavy *et al*., 2016).

While nitrogen metabolism in the sponge holobiont is widely studied, our knowledge of the sulfur cycle, and the role of sponge-associated bacteria in it, is very limited. The most thorough work on sulfur metabolism within sponges was conducted on the high microbial abundance (HMA) sponge *Geodia barretti* (Hoffmann *et al*., 2005). In that study, sulfate reduction rates were higher in areas close to filtration chambers compared to the sponge cortex. In accordance with the higher reduction rates, 5.3×10^{9} cells (ml sponge)$^{-1}$ of Deltaproteobacteria (of the *Desulfoarculus*/*Desulfomonile*/*Syntrophus* cluster) were found in the vicinity of filtration chambers using fluorescence *in-situ* hybridization (FISH) probes and none were found in the cortex.

Gene amplification studies have provided evidence of archaeal and bacterial sulfur reduction/oxidation processes in sponges. A recent work found that 87% of all amplified adenosine-5′-phosphosulfate reductase alpha subunit gene (*aprA*) sequences, which encodes a key enzyme in microbial sulfate reduction and sulfur oxidation, from *G. barretti*were affiliated with sulfur oxidizing Alphaproteobacteria and Gammaproteobacteria. (Jensen *et al*., 2016). The remaining 13% of sequences clustered with sulfate-reducing archaea of the phylum Euryarchaeota. Also, DGGE profiles of spatial samples taken at three locations within the sponge were indistinguishable, suggesting that the microbial community is similar in composition throughout the sponge body. Although these findings may seem to contradict those of Hoffmann *et al*. (2005), it should be noted that the two studies used different methods. In another study of the deep-sea sponge *Polymastia* cf. *corticata* (Meyer and Kuever, 2008), members of six identified sulfur-oxidizing and

sulfate reducing lineages containing the *aprA* gene were identified. Among them were the gammaproteobacterial SOB and non-sponge-specific alphaproteobacterial SOB that were present in the entire sponge body. However, the putative sponge-specific alphaproteobacterial sulfur-oxidizers and archaeal sulfate-reducing strains were restricted to the inner tissue sections. The co-occurrence of SOB and sulfate reducing bacteria (SRB) in both studies suggests the two processes take place in the same sponge.

DGGE and 16S rRNA gene amplicon sequencing studies are used to assess the structure of microbial communities in the environment in general and sponges in particular. Several such studies have identified Chromatiales bacteria in several sponges. Kennedy *et al*. (2014) have found Chromatiales related sequences in four deep water sponges (*Lissodendoryx diversichela*, *Poecillastra compressa*, *Inflatella pellicular* and *Stelletta normans*). Members of the Chroamtiales order were also detected in *Hymeniacidon sinapium* (Jeong *et al*., 2015), *Rhabdastrella globostellata* (Steinert *et al*., 2016), *Axinella corrugate* (White *et al*., 2012) and *Cinachyrella australiensis* (Cleary *et al*., 2013). In the latter, the 16S rRNA gene sequence clustered with sequences of other *Nitrosococcus* species. Chromatiales sequences were found in *Aplysina fulva* from Brazilian water (Hardoim *et al*., 2009) and in *Sarcotragus spinosulus* (Hardoim *et al*., 2012). Both studies suggested that these bacteria may carry out sulfide oxidation as other members of this order contains purple sulfur bacteria that are able to carry out anoxygenic photosynthesis using hydrogen sulfide as the electron donor. The sulfur-oxidizing bacteria of this order were found to be abundant in *Amphimedon queenslandica* and were further identified as members of the family *Ectothiorhodospiraceae*(Gauthier *et al*., 2016).The sequences clustered with other uncultivated clones, including some from a chemoautotrophic sulfur-oxidizing bacterium associated with the demosponge *Haliclona cymaeformis* detected by Tian *et al*. (2014). Using electron-microscopy, the authors found that the microbial cell lacks photosynthetic structures, but contain globules inside the cell membrane, hypothesized to be sulfur, as occurs in other purple-sulfur bacteria. While greatly improving our understanding of microbial community structure, amplicon studies are limited by the short reads length, thus can typically only identify microbes down to the family or genus level. Further, metabolic insights rely the assumption that the physiology of the organism with the most closely related 16S rRNA gene is similar to that of the organism of interest.

Imhoff and Trüper (1976) isolated four Gammaproteobacterial phototrophic SOB (*Chromatium gracile*, *C. vinosum*, *C. minutissimum* and *Ectothiorhodospira mobilis*) and two Alphaproteobacteria (*Rhodopseudomonas sulfidophila* and *R. palustris)* SOB from four sponge species. The authors concluded that a special micro-environment is formed, allowing suitable growth conditions for the anaerobic phototrophic bacteria in the sponge. However, it was only in 2014 that a genome of a sponge-associated SOB was published (Tian *et al*., 2014). The genomic bin Gspo,

named *Thioalkalivibrio* sp. HK1 (order Chromatiales) in its NCBI GenBank entry, was obtained from a metagenome of the sponge *Haliclona cymaeformis*. HK1 displays genetic characteristics such as a lack of transposases and an abundance of ankyrin repeat protein domains, which are common in bacterial symbionts. The presence of *soxABXYZ* and *dsrAB* genes, as well as absence of *soxCD* suggest that this organism oxidizes sulfide or sulfite through the reverse sulfate reduction pathway (Friedrich *et al*., 2001), as do other Chromatiaceae. Interestingly, the bacterium was found to be a non-photosynthetic mixotroph. The addition of heterotrophic capability could be crucial in the oxidized environments that frequently occur in the sponge body throughout the day (Lavy *et al*., 2016). Heterotrophic metabolism could also support these bacteria if they are horizontally transferred between sponges via an oxidized seawater environment.

In the past three years, attention has focused on the role of sulfur metabolizing bacteria in sponges. Three new draft genomes of sponge-associated sulfur oxidizing bacteria were published. Gsub from *Suberites* sp. and SOB1 from *Lophophysema eversa*, both of the order Thiotrichales are closely related to *Candidatus Vesicomyosocius okutanii* and *Candidatus Ruthia maganifica*, endosymbionts of deep sea clams (Tian *et al*., 2016, 2017). These bacteria show the potential to oxidize sulfite to sulfate using the Sox operon. A third sulfur oxidizing sponge-associated Chromatiales named AqS1, closely related to HK1, was recently found in the sponge *Amphimedon queenslandica* (Gauthier *et al*., 2016). The genomic differences between AqS1 and HK1 suggest that the two bacteria form two distinct species and maybe even genera. Moreover, Gauthier *et al*. (2016) show that *Thioalkalivibrio* HK1 may in fact, not be a *Thioalkalivibrio* at all, and therefore will be named HK1 from now on.

The sponge *Theonella swinhoei* is common on Indo-Pacific coral reefs, including the Gulf of Aqaba, and Eilat northern Red Sea (Ilan *et al*., 2004). It harbors a dense consortium of photosynthetic and heterotrophic bacteria, with up to 10^{10} bacteria (ml sponge)$^{-1}$ (Magnino *et al*., 1999). So far, nine bacterial phyla (Gemmatimonadetes, Chloriflexi, Cyanobacteria, Nitrospira, Proteobacteria, Spirochaetes, Verrucomicrobia, Actinobacteria, Acidobacteria) and four candidate phyla (Poribacteria, Tectomicrobia, OP10 and OS-K) were detected by culture-independent methods in *T. swinhoei* (Schmitt *et al*., 2012). This study found 16S rRNA gene sequences related to sulfate reducing *Desulfobulbaceae* and *Desulfovibrio*. However, no sulfur oxidizing microbes were identified. Members of three additional phyla (Bacteriodetes, Firmicutes and Lentisphaerae) were recently detected in a culturing experiment (Lavy *et al*., 2014). In the latter experiment, two *Desulfovibrio* isolates were grown under microaerobic conditions. The two isolates (TSAR6 and TSAR16) are closely related to known SRB (964 bp 16S rRNA > 97% similar, 100% coverage). Moreover, a recent study reported barite ($BaSO_4$) formation, which is linked to sulfur oxidation, within *T. swinhoei* (Keren *et al*., 2017). Numerous secondary metabolites have been

extracted from *T. swinhoei*, some are novel antitumor active polyketides, whereas others have antifungal or antibacterial activity (Bewley *et al*., 1996; Schmidt *et al*., 2000; Okada *et al*., 2002; Piel, 2004). Some of these compounds have been attributed to extracellular bacterial symbionts (Bewley *et al*., 1996; Piel, 2004, 2009; Wilson *et al*., 2014). Similar to other high microbial abundance (HMA) sponges, *T. swinhoei* has dense tissue and slow water flow through its body (Yahel *et al*., 2003; Weisz *et al*., 2008). A recent study found that microaerobic conditions occur temporally and spatially within the sponge over long periods of time (Lavy *et al*., 2016).

As in the case of many other sponge species, very little is known about the sulfur cycle in *T. swinhoei* and its symbionts. This could be because sulfur chemical species such as sulfide are not stable and therefore hard to detect *in-situ*. In order to overcome this barrier, we studied the sulfur biogeochemical cycle within the sponge through analysis of the microbial community. Using genome resolved metagenomics we revealed the presence of a SOB in the sponge. In this study, we present the genome of the discovered bacterium and discuss its role in the sulfur cycle in *T. swinhoei* and in other sponge species.

Results and discussion

General genomic characteristics

Sponges are considered as holobionts, as their microbial counterparts undertake important roles in their host's survival. Studies in the past decade have shown that these symbionts provide numerous services for their host, from chemical protection (Wakimoto *et al*., 2014) to nitrogen cycling (Hoffmann *et al*., 2009). Genome resolved metagenomics, which provides an opportunity to investigate the vast microbial community of the coral reef sponge *T. swinhoei*, revealed a bacterium that seems to participate in the sponge's sulfur metabolism by oxidizing sulfide and sulfite to sulfate.

The recovered *T. swinhoei* SOB (TsSOB) draft genome consists of 21 contigs and has a total size of 1.59 Mbp (Supporting Information Fig. S1). The longest contig size is 444 Kbp and the draft genome N50 is 153 Kbp (Table 1). The genome has an average GC content of 59.4% and a coding density of 94%. TsSOB was found to be the 10th most abundant bacterium in *T. swinhoei*'s microbial community, based on rank abundance analysis using the rpS3 gene sequences (Supporting Information Fig. S2). The genome has genes coding for 19 tRNA types, 17 tRNA synthetases, 51 ribosomal proteins, and a total of 1567 genes (Supporting Information Table S1). Completeness and possible contamination were evaluated by CheckM (Parks *et al*., 2015) to be 92.9% and 0% respectively. The reads mapping to the contigs of TsSOB were also present in the metagenome sequenced from a second *T. swinhoei* sample (breadth 0.96, average coverage 7.3), and partially in the third sample (breadth 0.48, average coverage 1.7) but not in a seawater sample (breadth 0.01, average coverage 1.7).

Table 1. Genome features.

Feature	Value
Genome size (Mb)	1.59
Number of contigs	21
N50 (scaffolds)	153662
GC (%)	59.48
Predicted genes	1567
Total rRNA	1
5S rRNA	1
16S rRNA	1
23S rRNA	1
tRNA	42
Bacterial single copy genes	51
Ribosomal proteins[a]	51
Coding density (%)	94
Completeness estimate (%)	92.98
Genome size estimate (Mb)	1.72

a. List of ribosomal proteins, tRNA and tRNA synthetases that are present in the genome is given in Supporting Information Table S1.

A phylogenetic analysis based on the protein sequences of 16 concatenated ribosomal proteins reveals that TsSOB is a Gammaproteobacteria from the order Chromatiales (Fig. 1and Supporting Information Fig. S3). TsSOB forms a long branching sister clade to *Halothiobacillus neapolitanus*, a non-photosynthetic, carbon fixing, sulfide oxidizing bacterium (Garrity *et al*., 2010). It is therefore suggested that TsSOB may be a member of *Halothiobacillaceae* family which currently has only one genus, *Halothiobaillus* or even represents a new family. Members of the *Halothiobacillaceae* family are known to be aerobic, halophilic, and considered to play an important role in global carbon and sulfur cycles (Pokorna and Zabranska, 2015). They depend entirely on inorganic compounds (CO_2 and reduced sulfur) for their carbon and energy needs (Garrity *et al*., 2010).The average nucleotide identity (ANI) between TsSOB and *Halothiobacillus neapolitanus* is 67.3%, suggesting that TsSOB is a distinct genus or family (Goris *et al*., 2007). The two other SOB sponge symbionts HK1 and AqS1, which are the symbionts of the sponges *Haliclona cymaeformis* and *Amphimedon queenslandica* respectively, form a cluster close to *Ectothiorhodospiraceae*, another class of Chromatiales.

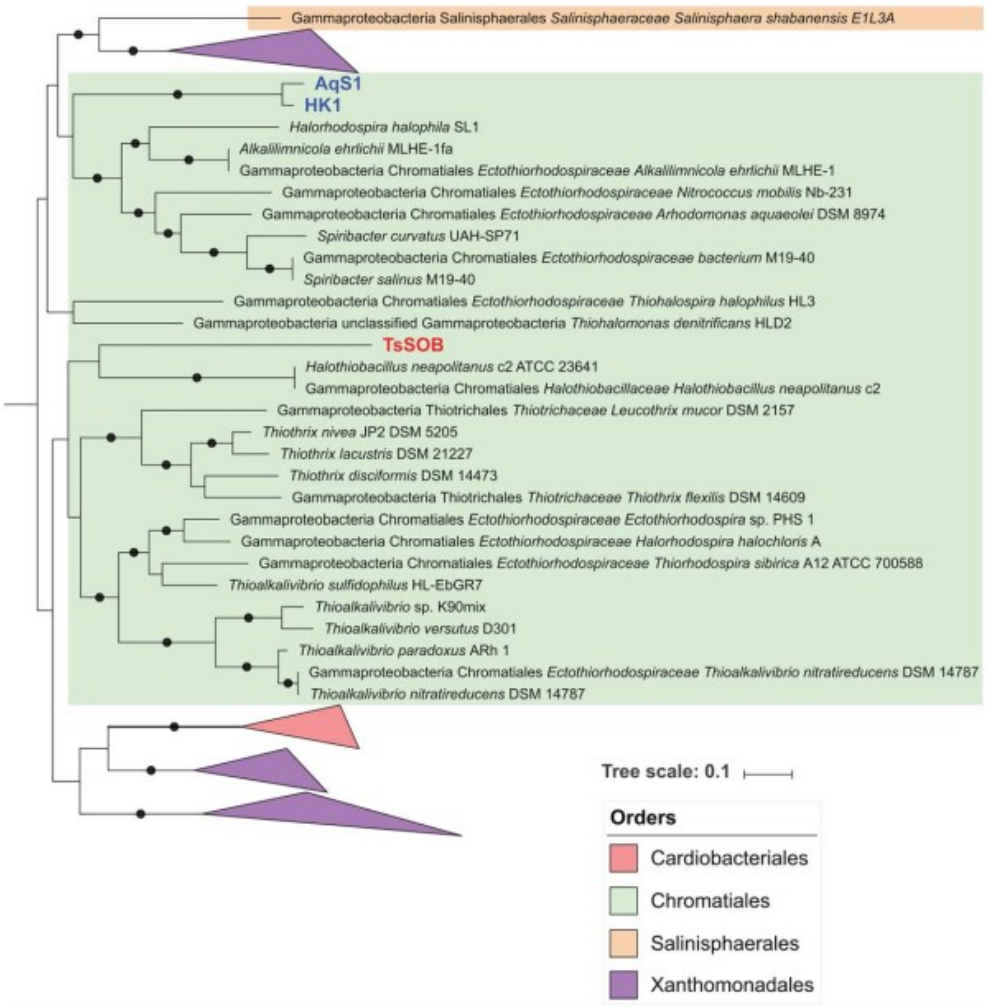

Figure 1

TsSOB is member of the Chromatiales order, most closely related to the sulfur-oxidizing *Halothiobacillus neapolitanus*. A subset of a Maximum-likelihood (ML) phylogenetic tree which includes taxons within the vicinity of TsSOB. The tree was calculated with RaXML with 100 bootstraps and based on sequences of 16 concatenated ribosomal proteins. Bootstrap values greater than 0.8 are shown as black dots on branches. Blue text indicates sulfur-oxidizing sponge symbionts reported in the literature, whereas TsSOB is marked in red.

Sulfide oxidation

The presence of dissimilatory (bi)sulfite reductase (*Dsr*) genes as well as sulfide dehydrogenase flavocytochrome-C (*FCC*) suggests that TsSOB is capable of oxidizing sulfide to be used as an electron source. The 13 *Dsr* genes are arranged in one operon that consists of *dsrABEFHCMKLJOPN* (Fig. 2 and Supporting Information Fig. S4). As expected, the additional DsrD, which is considered a marker for sulfate reduction (Sander *et al*., 2006), is not present. Sulfurtransferase *tusE*, which is a member of the dsrC/tusE and may function as a regulatory sulfur-related protein, is located immediately after the *dsr* operon (Venceslau *et al*., 2014). The two cytochrome subunits of *FCC* are found outside on another scaffold. The cytochrome complex allows

TsSOB to oxidize sulfide to elemental sulfur, which can then be further oxidized by the proteins coded by *dsr*. Upstream to the *dsr* operon are the sulfite oxidation genes *SoxABXYZ* (Fig. 2 and Supporting Information Fig. S4). While *dsr* and *FCC* allow sulfide oxidation, the presence of *sox* genes suggests that thiosulfate oxidation is another possible process carried out by the bacterium (Fig. 3). Previous studies showed that when *soxCD* are missing, as in the case of TsSOB, cytochrome c reduction rates decrease to 25% (Friedrich *et al*., 2001). In order to compensate for the loss of efficiency, sulfur oxidation may continue by the product of *dsr* genes, as in the case of *Thioploca ingrica* (Kojima *et al*., 2015). Sulfur oxidation is a trait not unique to *T. swinhoei's* associated bacteria. Previous studies showed that this process may be mediated by symbionts of *H. cymaeformis* (HK1) (Tian *et al*., 2014), *Suberites* sp. (Gsub) (Tian *et al*., 2016) *Lophophysema eversa* (SOB1) (Tian *et al*., 2017) and *A. queenslandica* (AqS1) (Gauthier *et al*., 2016). AqS1 and *Thialkalivibrio* sp. HK1 both possess a polycistronic cassette of 12 *dsr* genes. FCC, which is involved in catalyzing the formation of sulfur globules and enables sulfide oxidation, was not detected. It is likely that both bacteria do have FCC as they are closely related to members of the *Ectothiorhodospiraceae* family which are capable of sulfide oxidation.

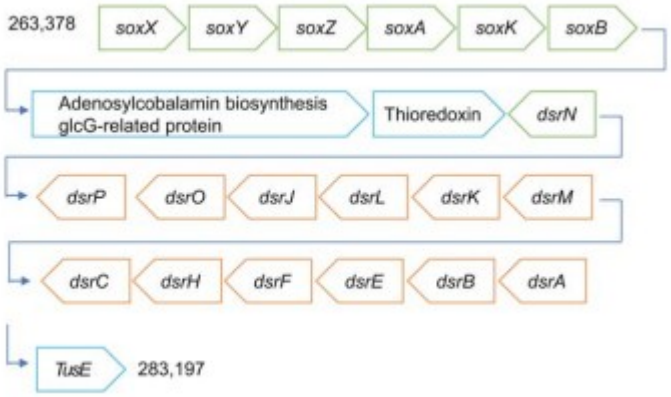

Figure 2

Schematic representation of sulfide oxidation operon structure found in the draft genome of TsSOB – The operon is present in scaffold_2 of the draft genome. The *soxXYZAKB* genes are found on the forward strand while *dsrABEFHCMKLJOP* are on the complement strand. As expected, the regulatory gene *dsrD*, which is considered a marker for sulfate reduction, is missing from the operon. Both cytochrome subunits of sulfide dehydrogenase Flavocytochrome-C (FCC) are present in another scaffold. Size-accurate representation of the genes is given in Figure S4. Numbers at the beginning and end of the sequence indicate the location of the structure along the scaffold. Green and orange colors denote genes of the Sox and Dsr families, respectively.

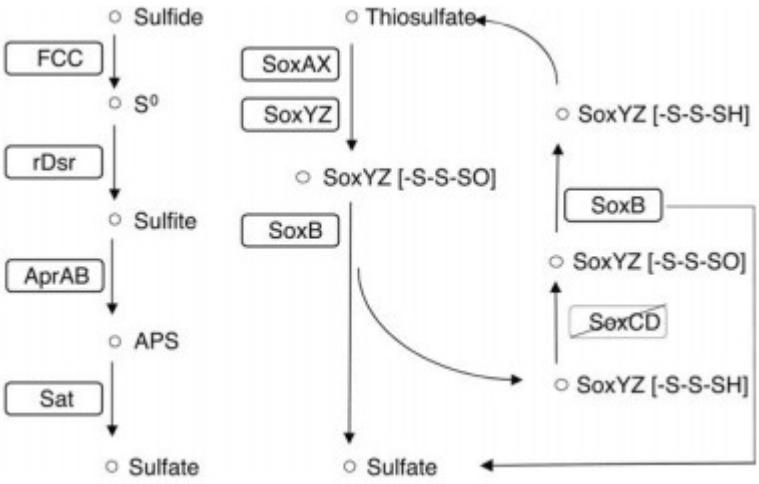

Figure 3

Sulfide and thiosulfate oxidation pathways present in the genome. TsSOB can oxidize sulfide to sulfate through the reverse-Dsr pathway. Both subunits of Flavocytochrome-C (FCC), which are required in order to oxidize sulfide to sulfur intracellularly, are present in the genome. Thiosulfate is oxidized to sulfate through the Sox pathway. Solid outline - genes present in the draft genome. Crossed-out genes are missing from the draft genome.

In the case of sulfur oxidizers and reducers, the phylogeny of these traits could be traced according to the accumulation of changes in the sequence of the *dsrAB* genes that code for dissimilatory (bi)sulfite reductase subunits *dsrA* and *dsrB* (Muller *et al*., 2015). A Maximum Likelihood (ML) phylogenetic tree based on the *dsrAB* amino-acid sequences of TsSOB and other sulfur oxidizing bacteria agrees with the 16 concatenated ribosomal proteins tree, as TsSOB forms a clade distinct from HK1 and AqS1 (Fig. 4). This evidence strengthens the finding that the sulfur oxidation property of TsSOB is not related to that of other sulfur oxidizing sponge symbionts such as HK1 and Gsub.

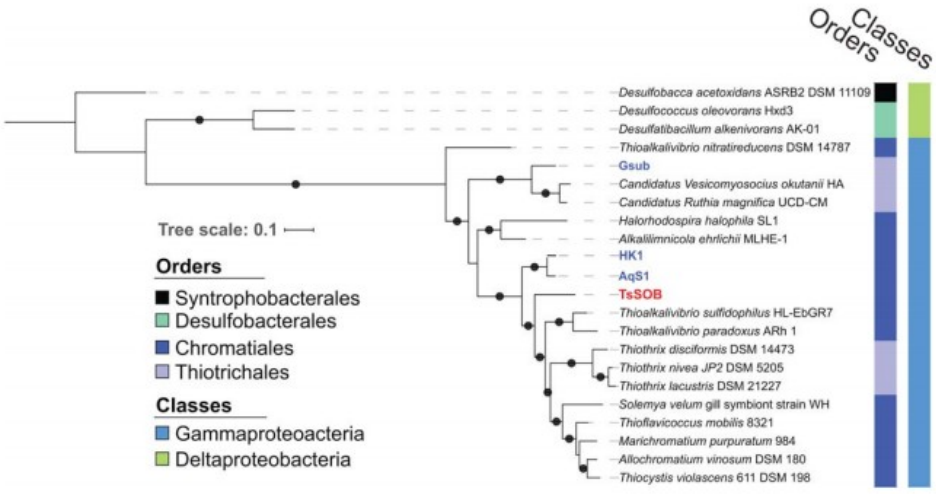

Figure 4

The dsrAB genes of TsSOB are distinct from those HK1 and AqS1 sponge symbionts. The Maximum-Likelihood phylogenetic tree of the amino-acid sequences of *dsrAB* depicts the evolutionary distance in dsrAB between TsSOB and other sulfur oxidizing sponge symbionts. The *dsrAB* sequences were identified with HMMs TIGR02064 and TIGR02066. No *dsrAB* sequence was found in SOB1 from *Lophophysema eversa*. Bootstrap values greater than 0.7 are marked as black dots on branches. Blue text indicates sulfur-oxidizing sponge symbionts reported in the literature, whereas TsSOB is marked in red.

The Red Sea is an oligotrophic body of water, poor in labile sources of carbon, nitrogen and phosphate (Yahel *et al*., 2003; Batayneh *et al*., 2014). One significant source of sulfur are corals, which release dimethylsulfoniopropionate (DMSP) into the surrounding water (Hill *et al*., 1995). Being the only known external source for reduced sulfur in coral reefs, it is surprising that TsSOB lacks the genes required to catabolize DMSP and dimethylsulfide (DMS). While it is possible that genes coding for enzymes responsible for these processes are present in the missing parts of the genome, they are absent from other genomes of *Halothiobacillus* currently available in the Integrated Microbial Genomes (IMG) database ($N = 3$). It is also possible that marine bacteria liberate sulfate from DMSP (Raina *et al*., 2010), and the sulfate is then reduced to sulfur by anaerobic *Desulfovibrio*, an SRB that was previously isolated from the same host sponge (Lavy *et al*., 2014). Sulfate concentrations in the Red Sea surface water are approximately 2.1 mM (El-Manharawy and Hafez, 2003). At a pumping rate of 6 ml min^{-1} (ml sponge)$^{-1}$, which fits the pumping rates of HMA sponges in general (Weisz *et al*., 2008) and *T. swinhoei* in particular (Yahel *et al*., 2003), 18.8 mmol sulfate would pass through 1 ml of sponge tissue daily. This sulfate could be reduced by SRB in the sponge, becoming available for TsSOB. A similar mechanism was suggested for the sulfur cycle in the sponge *G*. *barretti* (Jensen *et al*., 2016).

Carbon, nitrogen and amino-acids metabolism

Metabolic prediction shows that TsSOB is an auxotroph for amino acids other than alanine, glycine, asparagine and glutamine. While genes involved in these pathways might be found in the missing parts of the genome, auxotrophy for alanine, histidine, phenylalanine, tyrosine and tryptophan is shared with the three other *Halothiobacillus* species in the IMG database. However, a search for amino-acids synthesis-related genes using Hidden Markov models (HMMs) suggests that all genes required for the synthesis of valine, leucine and isoleucine are present in the genome of TsSOB (Supporting Information Fig. S5). While it has the potential for synthesizing branched amino acids, TsSOB also has genes encoding for branched amino-acid transporters (livFGHKM) in its genome. Interestingly, while it is capable of importing and synthesizing branched amino-acids, most of the genes associated with valine, leucine and isoleucine degradation are missing (Supporting Information Fig. S6). The ability to synthesize branched amino-acids and the presence of their transporters may indicate that the TsSOB can export these amino-acids to be used by the host or other host-associated microbes.

The genome of TsSOB may also provide clues as to the sources of carbon and nitrogen that the bacterium requires for growth. The *urtABCDE* and *ureDABCEFG* genes that code for urea transport proteins and urease respectively, were found in one operon (scaffold 6). Their occurrence suggests that urea, a valuable source of nitrogen and carbon, can be taken up and catabolized by the bacterium. Interestingly, the KEGG reference genome of its close relative, the free-living *Halothiobacillus neapolitanus*, does not have the genes coding for urease. Urea, along with creatinine were recently suggested to fuel the nitrogen cycle within sponges (Moitinho-Silva *et al*., 2017). Upon cleavage, urea breaks down to ammonia and CO_2. Although ammonia oxidation-related genes are absent from its genome, two other genes that are related to ammonia utilization are present. The first is cytidine triphosphate (CTP) synthase which takes ammonia and Uridine-5′-triphosphate (UTP) and converts them to CTP. The second is glutamine-synthetase adenylyltransferase which regulates the activity of glutamine-synthetase. While glutamine-synthetase is missing from the genome, the presence of its regulator suggests that it may be present in the full genome.

TsSOB is probably capable of fixing carbon through the reductive citric acid cycle, as its genome shows most of genes required for this metabolic pathway (Supporting Information Fig. S7). This trait is common for *Halothiobacillus*. By using urea, which is a metabolic waste product of the sponge, TsSOB gains CO_2 and ammonia from its breakdown. Interestingly, AqS1 and HK1, which are the also sulfur oxidizing sponge symbionts, both lack genes encoding for ureases (Gauthier *et al*., 2016).

All members of the family *Halothiobacillaceae* are aerobic bacteria, often found in environments rich in hydrogen sulfide (Garrity *et al*., 2010). TsSOB may be an aerobe or microaeorbe. Sponges are aerobic animals but microaerobic conditions can occur within their bodies for prolonged periods of time (Hoffmann *et al*., 2007, 2008). A recent study reports consistent suboxic and anoxic conditions in *T. swinhoei* (Lavy *et al*., 2016). Moreover, ribosomal protein S3 sequences of Deltaproteobacteria and Clostridia, which are likely to be anaerobes, were abundant (x25 and x9 coverage respectively) (Supporting Information Fig. S2). Previous studies have isolated anaerobic sulfate reducing *Desulfovibrio* from *T. swinhoei*(Lavy *et al*., 2014; Keren *et al*., 2015, 2016). A co-localization of sulfate reducing and sulfide oxidizing bacteria was recently found in the sponge *G. barretti* (Jensen *et al*., 2016). Similarly, alternating aeration conditions within the body of *T. swinhoei*, as was found by Lavy *et al*. (2016), could support both sulfate reduction by *Desulfovibrio* as well as sulfide oxidation by TsSOB.

Symbiont lifestyle

DNA repair-related enzymatic functions are known to deteriorate in host-associated bacteria, and therefore can be used to assess genome streamlining (Moran and Wernegreen, 2000). Out of 26 genes that are reported by Moran and Wernegreen (2000) to indicate genome streamlining,

10 are missing in TsSOB's draft genome (*alkA*, *mutH*, *mutT*, *nfo*, *phnW*, *phrB*, *recB*, *recC*, *recD* and *tag*). Their absence could be attributed to the incompleteness of the genome, but it could also be that as a symbiont, TsSOB genome has lost these functions. According to checkM estimate of completeness (92%), the full genome of TsSOB is predicted to be 1.7 Mbp in length. For comparison, the genome size of its most closely related free-living bacterium *Halothiobacillus neapolitanus* c2 ATCC 23641 (GenBank accession NC_013422.1) is 2.58 Mbp. Other free living Chromatiales have genomes that are larger than TsSOB. For example, *Thioalkalivibrio* sp. K90mix isolated from sediment samples has a 2.74 Mbp genome (Muyzer, *et al*., 2011), and the genome of *Thioalkalivibrio sulfidophilus* HL-EbGr7, isolated from a bioreactor removing sulfide from gas, is 3.46 Mbp (Muyzer, *et al*., 2011). Sponge-associated sulfur oxidizing bacteria have estimated genome sizes in the range of 1.37 Mbp (Gsub 98% complete) to 3.46 Mbp (HK1, 99.1% complete).

While *Halothiobacilli* are motile via a single flagellum (Garrity *et al*., 2010), only *flhF*, a regulatory gene that affects the transcription of other flagellum biosynthesis genes, is present in the genome. The absence of genes might be related to the incomplete genome or indicate that the bacterium lacks a functional flagellum. It also possesses one copy of the gliding motility protein *gldG* and two twitching motility related proteins *pilT*. We calculated the iRep replication index to test whether TsSOB was actively replicating at the time of sampling. The index looks at the entire population, not at single cells, and its calculation assumes that more reads would be mapped to the origin of replication compared to the terminus in a replicating bacterium. The index is calculated as a ratio between the highest and lowest number of reads mapped along the genome at question, where a greater ratio value indicates initiation of more replication forks. Any value greater than 1 would mean that at least some part of the population is actively replicating. An iRep replication index of 1.23 was calculated for TsSOB (r^2=0.99, %windows = 100) suggesting it is actively replicating, and not merely being digested by the sponge.

TsSOB is a sponge-coral specific bacterium

The genome of TsSOB contains two polyketide synthase gene clusters *supA* and *supB*, which are common in sponge-specific bacteria (Supporting Information Fig. S8 and Table S2). The *supA* cluster is composed of ketosynthase, acyltransferase, dehydratase, methyl transferases, enoyl reductases and ketoreductases modules, whereas *supB* contains only one phosphopantetheine attachment site. The *supA* amino-acid sequences are 73% and 62% similar to genes of bacteria from the sponges *T. swinhoei* and *Aplysina aerophoba*respectively. The 76 amino-acid sequence of *supB*, was 56% and 39% similar to that of bacteria from *A. aerophoba* and *T. swinhoei*.

The presence of similar supA and supB genes in other sponge samples called for a broader search of TsSOB in microbial samples. Substantial effort has

been made in recent years to sequence 16S rRNA tags from sponge-associated microbial communities from around the world. This resulted in a detailed description of microbial communities of many sponges (Thomas *et al*., 2016). Searching NCBI's non-redundant nucleotide database for the 16S rRNA sequence of TsSOB shows that the bacteria with almost identical gene sequence are common in nine sponge and two coral orders (Fig. 5, Supporting Information Table S3 and Fig. S9). Sequences of 13 symbionts were ≥ 97% identical, suggesting they are of the same species and three are potentially from the same genus (> 94% identity). While TsSOB-related sequences were found in 13 sponge species from nine families, the next most common SOB, HK1, is found in 12 sponge species from nine families. It should be noted that the available 16S rRNA sequence of HK1 was only 324 bp, and therefore some the matches with identity over 97% could be due to size bias. The similarity between AqS1 and HK1, as inferred from their ribosomal proteins and *dsrAB* sequences, as well as the comparison that was conducted by Gauthier *et al*. (2016), suggest that they belong to the same genus. The occurrence of HK1 in every sponge species in which AqS1 was found (except for *Ancorina alata*) further strengthens this finding.

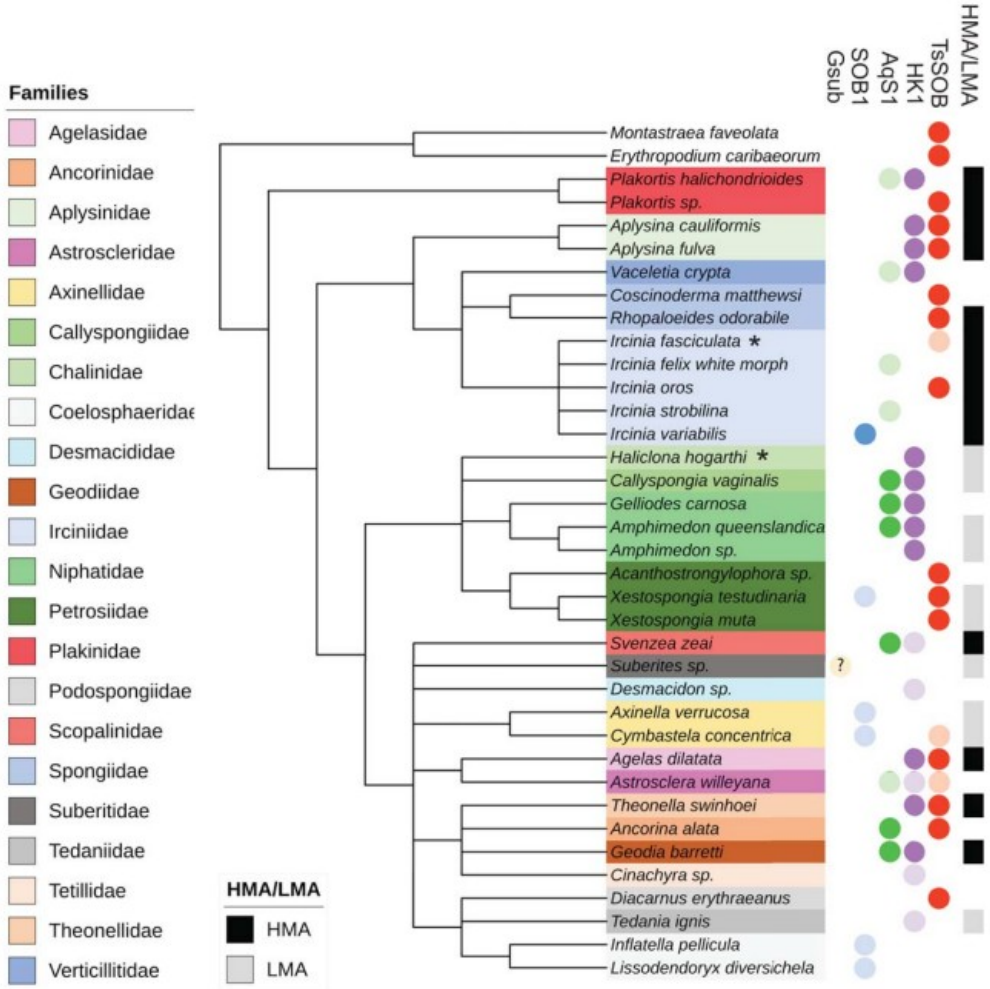

Figure 5

Association and grouping of sponge symbionts to their HMA/LMA hosts. 16S rRNA gene of TsSOB is present in multiple orders, and most often in HMA sponges. The 16S rRNA gene of other sulfide oxidizing sponge symbionts (SOB1, AqS1 and HK1, but not Gsub) were also in several sponges. The phylogenetic relations between sponges was adopted from Morrow and Cardenas (2015) and World Porifera Database (Van Soest *et al*., 2017). The 16S rRNA sequence of Gsub did not match any sponge associated bacterium in the database. The Gsub genome was originally reported from *Suberites* sp. and therefore it is marked in the tree with a question mark. Sponges were assigned as HMA or LMA according to the works of Gloeckner *et al.* (2014) and Moitinho-Silva *et al.* (2015). Lightly colored circles indicate matches that had less than 97% identity and therefor may represent another bacterial species. Two coral species, in which TsSOB was detected, are used as an outgroup. Asterisks denote species names which were updated since the sequences were submitted to NCBI database: *Haliclona hogarti* is now *Haliclona tubifera*, *Ircinica fasciculata* is now *Ircinica variabilis*, and *Ancorinca alata* is now *Ecionemia alata*. The full results of the NCBI nucleotide nr BLAST search are given in Table S3, and the phylogenetic relationship between the 16S rRNA sequences is presented in Figure S9.

TsSOB is mostly found in HMA sponges. Out of nine sponge families in which TsSOB was detected (> 97% identity of 16S rRNA), only members of *Axinellidae* and *Petrosidae* are low microbial abundance (LMA) sponges (Fig. 4) (Gloeckner *et al*., 2014). The 16S rRNA sequence of TsSOB also matched sequences of bacteria derived from two corals, *Montastaraea faveolata* and *Erythropodium caribaeorum*, with 99% coverage and identity (Supporting Information Table S3). HK1 and AqS1 do not show a preference to either HMA or LMA sponges while the 16S rRNA of SOB1 is mostly found in LMA sponges. Although Gsub did not have any match to sponge-associated bacteria in NCBI's nucleotide database, Tian *et al*. (2017) reported its isolation from a *Suberites* sp. which is a LMA sponge, and therefore it is considered as an LMA-associated bacterium. The occurrence of TsSOB primarily in HMA sponges may indicate that it relies on interactions with other symbionts of the sponges or corals, for its survival. Another possibility, which seems more likely, would be that this pattern is related to the temporal microaerobic and anaerobic conditions that may occur in the body of the slow water pumping HMA sponges (Weisz *et al*., 2008).

With a 19/20 matches for the 16S rRNA sequence of TsSOB matching sponge- and coral-associated symbionts in NCBI nucleotide database, TsSOB might be considered a sponge-coral-associated bacterium (Simister *et al*., 2012). This is further supported by a 16S rRNA Maximum-Likelihood phylogenetic tree that shows that the TsSOB related sequences are distinct from those of other SOB symbionts (Supporting Information Fig. S9). The occurrence of the bacterium in both sponges and corals could suggest it has a pivotal role in sulfide and thiosulfate oxidation in the coral reef environment.

The occurrence of TsSOB in sponges from different orders and different locations around the globe, as well as it metabolic properties (i.e., ability to import and export amino acids and urea as well as ability for sulfide oxidation), may suggest that it is a true symbiont. The fact that the 16S rRNA sequence of TsSOB was not detected in seawater samples in NCBI's nr database is similar to the case of *Candidatus Poribacteria*, which are readily found in sponges but are rarely detected in seawater (Taylor *et al*., 2013).

This may indicate that the relationship between the bacterium and its host is an old one, potentially established by the last common ancestor of the nine sponge families, and that this bacterium is being vertically transferred (i.e., from parent to daughter). To date, no other *Halothiobacillaceae* is known to be sponge-associated.

Following the taxonomy guidelines for uncultivated prokaryotes (Konstantinidis *et al*., 2017), we suggest that TsSOB is a representative of a novel taxon, and propose naming it *Candidatus Porisulfidus*. The name indicates that this taxon mainly occurs in sponges (phylum Porifera), and is capable of sulfide oxidation.

Conclusions

Based on the metagenomic analysis of a sponge-associated microbial community, we identified a relatively abundant Chromatiales bacterium TsSOB with the capacity to oxidize sulfide and thiosulfate, for which we suggest the name '*Candidatus Porisulfidus*'. The bacterium, which is somewhat similar to *Halothiobacillus*, is predicted to use urea derived from its *Theonella swinhoei* sponge host, as a nitrogen and carbon source. The size of its genome, presence of urease genes, indication of active replication within the sponge and detection of its 16S rRNA sequence in several sponge orders but not seawater, suggest that TsSOB is a sponge-coral-associated, rather than a transient free-living bacterium. The only other organisms in the community that appear to be capable of sulfur compound transformations belong to the genus *Desulfovibrio*, the members of which can reduce sulfate. All of these bacteria may be responsible for sulfur cycling within *T. swinhoei*, regulating the amount of sulfate and sulfide available to the host and other symbionts residing in its mesohyl. Sulfate likely represents a significant resource for the sponge microbiome, given the concentration of sulfate in the seawater and the large volume of water that passes through the sponge on a daily basis. Its transformation to sulfide by *Desulfovibrio* and subsequently back to sulfate by TsSOB would form a complete cycle occurring within the holobiont.

Experimental procedures

Sampling and DNA preparation

Three *T. swinhoei* specimens were collected at Eilat, Red Sea (29°29'57.63″N/34°54'54.61″E) by SCUBA diving at 20 m depth, sealed in closed bags, and immediately processed on site at the Interuniversity Institute for Marine Sciences. All work henceforth was performed in a laminar flow hood under sterile conditions. Sponges were thoroughly rinsed in sterile calcium-magnesium-free artificial seawater (CMF-ASW) to remove transient bacteria and loosen cellular connections, and were cut to 1 cm layers. The outer-most layer of each sponge that contains cyanobacteria was removed, and cores were collected from each layer of each sponge into sterile tubes. Each sub-sample was homogenized in CMF_ASW and the resulting

homogenate was repetitively agitated by stirring, to separate clumps of cells. Sponge cells were separated by passive settling and centrifugation at 100 g (Wilson *et al*., 2014). Unicellular cells were collected by centrifugation at 3000 g and discarding of the supernatant.

Five litres of seawater were collected on site in a pre-bleached container. The seawater was serially filtered through 11 µm, 1.2 µm and finally a 0.22 µm Sterivex filter within 1 hour of sampling. A lysis buffer was added to the 0.22 µm Sterivex filter prior to DNA extraction as according to a protocol published by Wright *et al*. (2009).

DNA was extracted by following the CTAB extraction protocol. Briefly, the bacterial pellet from sponge samples and the seawater sample lysis buffer were suspended in 0.8 ml CTAB buffer and incubated at 60°C for one hour. Resulting lysate was mixed with an equal amount of chloroform:isoamylalchohol 24:1 (v/v), followed by centrifugation (15 min, 4°C). The aquatic phase was transferred into a new tube and treated with 1 µl RNase for 30 min at 37°C. For DNA precipitation, sodium acetate (1:10 v/v) and isopropanol (1:1 v/v) were added and the sample was incubated overnight (–20°C). Following incubation, DNA was pelleted (15 300 g, 15 min, 4°C), washed with cold ethanol and suspended in ddH_2O.

A metagenomic library was prepared for each of the three sponges and the seawater sample at the Tauber Bioinformatics Research Center of Haifa University after validating DNA concentration and integrity by using Qubit (Thermo Fisher Scientific) and gel electrophoresis respectively. The sample was sheared using Covaris E220 (settings: 40 s, 10% duty cycle, 200 cycles per burst, 175 peak incident power) and a library was prepared using NEB's Ultra DNA Library Prep kit for Illumina with AmpureXP bead selection aimed to give fragments of 250 bp according to the manufacturer's protocol. The library was sequenced at the Technion Institute using an Illumina Hiseq 2500, paired-end 150 base-pairs (bp) sequencing.

Sequence analysis, annotation and assembly

Illumina adapters were removed and raw sequences were quality-trimmed with Sickle (Joshi and Fass, 2011). Sequences of one sponge sample were then matched and assembled using IDBA-UD (Peng *et al*., 2012). Thereafter reads were mapped with bowtie2 (Langmead and Salzberg, 2012) to calculate reads coverage. Genes were predicted by Prodigal (Hyatt *et al*., 2010) and predicted protein sequences were annotated using usearch (Edgar, 2010) against KEGG, UniRef100 and UniProt databases.

Initial binning of scaffolds was done based on similarity in GC content and coverage of scaffolds as well as taxonomic identity of genes in each scaffold. The putative genomes were identified using the ggKbase binning tools (http://ggkbase.berkeley.edu). Bins were assessed partly based on the number of Bacterial Single Copy Genes (BSCG), and Ribosomal Proteins (RP) found in each bin. Each putative genome bin was downloaded along with

other bins that share similar GC content and coverage. Scaffolds were split into 2500 bp chunks and the tetramer frequency of each segment was calculated. Scaffolds were clustered according to their tetramer frequency based on Emergent Self-Organizing Maps (ESOM) with Databionic ESOM Analyzer (Ultsch and Moerchen, 2005; Dick *et al*., 2009). Scaffolds that had less than 50% of their segments present inside an ESOM cluster, were omitted from the bin. The updated bins were loaded back into ggKbase for further inspection. Scaffolds present in the original bin that were < 2000 bp in length were retained so long as their gene taxonomy profile matched that of the rest of the bin. Scaffolding errors introduced by idba_ud were corrected using an in-house script as described by Brown *et al*. (2015), and fusion of overlapping scaffolds was done with Geneious version 8.0 [https://www.geneious.com, (Kearse *et al*., 2012)].

CheckM v1.0.7 (Parks *et al*., 2015) was used to verify the genome completeness and percentage of possible contamination, with the expected single copy gene sets defined based on the genomes of other Gammaproteobacteria. A phylogenetic tree was created using a standard set of 16 ribosomal proteins sequences (S2, S8, S10, S17, S19, L2, L3, L4, L5, L6, L14, L15, L16, L18, L22, L24) (Hug *et al*., 2013), with references from 52 other sulfur oxidizing bacteria. The amino acid sequences were obtained by predicted open reading frames (ORFs) for each genome using Prodigal v2.6.3, then searching these predictions for the 16 ribosomal proteins using an in-house script (Probst *et al*., 2017). Proteins were aligned with MUSCLE (Edgar, 2004) and non-informative ends were removed. The sequences were then concatenated, and columns with more than 95% gaps were stripped, resulting in a total of 2379 informative positions (i.e., positions along an alignment which at which at least 5% of the sequences have a non-gap information). The total number of informative position for each genome was verified, keeping genomes which spanned over 50% of the total length of the alignment. A Maximum-Likelihood tree was constructed with PhyML algorithm (v3.0) (Guindon *et al*., 2010) using LG+α + γ substitution model, and 20 substitution rate categories.

Functional profile of the genome was evaluated using ggKbase, KEGG KAAS (Moriya *et al*., 2007), the IMG system (Chen *et al*., 2017), and Hidden Markov Models for of shared KEGG orthologies (KOs) as follows. Predicted proteins of TsSOB were searched against a database of HMMs representing all the KOs (Kanehisa, 2000). The HMM database was compiled using the HMMER suite (Finn *et al*., 2015), based on assignment of proteins to KOs according to KEGG FTP Release 2015-06-22. Individual trusted thresholds were calculated by running HMM search of all the proteins with assigned KOs against the HMM database. Polyketide Synthase sequences were sought for by AntiSmash antibiotics & Secondary Metabolite Analysis Shell (Weber *et al*., 2015).

The taxonomy of sulfur oxidizing bacteria can be often assisted by observing the phylogeny of their *dsrAB* genes (Muller *et al*., 2015). The amino acid

sequences of *dsrA* and *dsrB* were sought for in the 52 genomes that were used to construct the phylogenetic tree (Fig. 1) using TIGRFAM HMMs for *dsrA* (TIGR02064) and *dsrB* (TIGR02066). Briefly, the HMMs were downloaded from TIGRFAM database and HMMER (v 3.0) (HMMER; Eddy, 1998) was used with default cutoffs to search for sequences within the protein sequences of the genomes. Amino acid sequences for *dsrAB* were found in 22 genomes, including TsSOB. The sequences were aligned and non-informative positions, as well non-informative ends were stripped, as described above. The 838 informative positions were aligned using phyml, and a ML tree with 100 bootstraps was constructed.

The 16S rRNA sequence of *Thioalkalivibrio* sp. HK1 was downloaded from JGI's IMG web site (Chen *et al*., 2017), while the sequences of Gsub, AqS1, and SOB were downloaded from NCBI Genebank database. The sequences were used for a BLAST search against NCBI nucleotide nr database. The results were filtered so that the top 20 unique hits were retained. Hits were considered unique if they originate from different studies or if they are from the same study but from different host/isolation source. The full results of this search are given in Supporting Information Table S2. The sequences of those hits were downloaded and aligned with MUSCLE and positions with > 95% gaps were removed (as was previously described). A Maximum-Likelihood phylogenetic tree was then calculated with PHYML using GTR model and estimated transition/transversion ratio, proportion of invariable sites and gamma-shape parameter. Invertebrates that were found to host any of the five bacteria were placed on a taxonomic dendrogram which relies on the latest Porifera taxonomy found in the World Porifera Database (Van Soest *et al*., 2017) and the taxonomy of demosponges published by Morrow and Cárdenas (2015). Sponges were identified as either LMA or HMA based on the two most recent publications by Gloeckner *et al*. (2014) and Moitinho-Silva *et al*. (2017).

Post processing of all phylogenetic trees was done in iTol (Letunic and Bork, 2016).

A rank abundance curve was calculated using sequencing coverage of scaffolds that encode ribosomal protein S3 (rpS3) as a proxy for genome abundance. All unique rpS3 sequences in the sample were aligned against 2887 known rpS3 sequences using MUSCLE. A Neighbor-Joining tree was then constructed to determine the identity of each rpS3 sequence to the class or phylum level.

The *in-situ* replication rate was inferred based on the sequencing coverage trend that results from bi-directional genome replication from a single origin of replication using iRep (Brown *et al*., 2016). The presence of TsSOB in the two un-binned sponge samples as well as in the seawater sample was determined by calculate_breadth.py, a python script which calculates the breadth and coverage of the genome according to the mapped reads from each of the metagenomic samples (Olm *et al*., 2017).

Nucleotide sequence accession number

TsSOB draft genome is publically available at Genbank, with the accession number: MRSX00000000. WGS raw sequences are available at NCBI's Sequence Read Archive (SRA) under BioSample accession number SAMN06111390

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