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A Single-Cell Genome for *Thiovulum* sp.

Ian P. G. Marshall,[a] Paul C. Blainey,[b]* Alfred M. Spormann,[a,c] and Stephen R. Quake[b,d]

Department of Civil and Environmental Engineering,[a] Department of Bioengineering,[b] and Department of Chemical Engineering,[c] Stanford University, Stanford, California, USA, and Howard Hughes Medical Institute, Stanford, California, USA[d]

We determined a significant fraction of the genome sequence of a representative of *Thiovulum*, the uncultivated genus of colorless sulfur *Epsilonproteobacteria*, by analyzing the genome sequences of four individual cells collected from phototrophic mats from Elkhorn Slough, California. These cells were isolated utilizing a microfluidic laser-tweezing system, and their genomes were amplified by multiple-displacement amplification prior to sequencing. *Thiovulum* is a gradient bacterium found at oxic-anoxic marine interfaces and noted for its distinctive morphology and rapid swimming motility. The genomic sequences of the four individual cells were assembled into a composite genome consisting of 221 contigs covering 2.083 Mb including 2,162 genes. This single-cell genome represents a genomic view of the physiological capabilities of isolated *Thiovulum* cells. *Thiovulum* is the second-fastest bacterium ever observed, swimming at 615 μm/s, and this genome shows that this rapid swimming motility is a result of a standard flagellar machinery that has been extensively characterized in other bacteria. This suggests that standard flagella are capable of propelling bacterial cells at speeds much faster than typically thought. Analysis of the genome suggests that naturally occurring *Thiovulum* populations are more diverse than previously recognized and that studies performed in the past probably address a wide range of unrecognized genotypic and phenotypic diversities of *Thiovulum*. The genome presented in this article provides a basis for future isolation-independent studies of *Thiovulum*, where single-cell and metagenomic tools can be used to differentiate between different *Thiovulum* genotypes.

Thiovulum is a genus of as-yet-uncultivated colorless sulfur bacteria thought to conserve energy through the aerobic oxidation of reduced sulfur compounds at the oxic-sulfidic interface in marine environments (47). The *Thiovulum* genus has primarily been defined observationally by its distinct large, egg-shaped cell morphology (Fig. 1) and unusual rapid swimming motility (60). A species within the genus *Thiovulum* was first described as *Volvox punctum* (39) and then renamed *Monas muelleri* (60). Two similar species were described in 1913 and given the names *Thiovulum majus* and *Thiovulum minus* (17, 47). *Monas muelleri* was then renamed *Thiovulum muelleri* after its similarities to Hinze's new species were recognized (31). These *Thiovulum* species were classified according to their size and motility, with *T. muelleri* 4.9 to 10.2 μm in diameter and moving in an undirected zigzag manner, while *T. minus* and *T. majus* cells revealed a more unidirectional motility and were 7.2 to 9 μm across and 9 to 17 μm across, respectively (5, 17). Despite many isolation attempts since its discovery (29, 59, 61), no *Thiovulum* pure culture has been reported so far.

In spite of the fact that *Thiovulum* has evaded isolation in pure culture, its distinctive morphology has enabled a number of important ecological and physiological studies in enrichments and laboratory microcosms. *Thiovulum* has been described as chemolithoautotrophic (61) and microaerophilic (13, 22). It is one of the fastest-swimming bacteria ever observed, swimming at 615 μm/s (14). Large numbers of *Thiovulum* cells naturally form conspicuous veils at marine oxic-anoxic interfaces (17). These veils consist of a polymeric matrix where *Thiovulum* cells are reversibly attached and can rapidly traverse within a 50- to 100-μm-wide, diffusion-controlled, overlapping sulfide-O_2 gradient (23). This steep sulfide-O_2 gradient is self-generated, as the calculated mean residence time of both substrates within this overlapping layer is 0.1 to 0.6 s. *Thiovulum* cells demonstrated chemotaxis toward O_2 in seeking out these anoxic-oxic interfaces (56), and veil-associated cells were postulated to use their flagella to generate advective water flow within the veil (57). *Thiovulum* is the only large-celled (cell diameter $>$ 5 μm) colorless sulfur bacterium classified as one of the *Epsilonproteobacteria*, while other large colorless sulfur bacteria such as *Beggiatoa*, *Thioploca*, and *Thiomargarita* are classified within a single cluster of the *Gammaproteobacteria* (Fig. 2) (24, 51).

Thiovulum's large cells, with their distinctive morphology, 226-year history of observations, and uncultivated status, make *Thiovulum* an ideal candidate for applying the recently developed method of single-cell genome sequencing using a microfluidic laser-tweezer system (6, 25, 35, 66). In this method, a mixture of microorganisms from an environmental sample (or an enrichment culture) is introduced into a poly(dimethylsiloxane) (PDMS) microfluidic device. Single cells are selected upon microscopic inspection and then guided with a laser trap into separate chambers, where each sorted cell is lysed individually and then subjected to whole-genome amplification by multiple-displacement amplification (MDA) (30). Amplified genomic DNA from each cell is then separately recovered from the chip and sequenced.

In 2007, students of the Hopkins Microbiology Course in Pacific Grove, California (http://hmc.stanford.edu), observed *Thi-*

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Address correspondence to Alfred M. Spormann, spormann@stanford.edu, or Stephen R. Quake, quake@stanford.edu.
* Present address: Paul C. Blainey, Broad Institute, Cambridge, Massachusetts, USA.
I.P.G.M. and P.C.B. contributed equally to this article.
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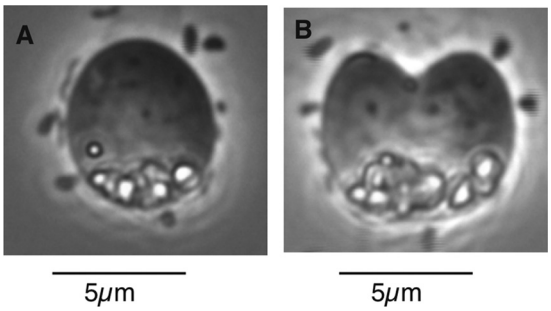

FIG 1 Phase-contrast micrographs of *Thiovulum* sp. cells from an enrichment from Elkhorn Slough, California. (A) Typical *Thiovulum* cell from this environment; (B) a cell undergoing division. Note the highly refractile polar sulfur granules in both cells. Micrographs are courtesy of Marie B. Lund.

ovulum veils in phototrophic microbial mats from Elkhorn Slough, California. In these mats, the circadian changes in flux of light result in well-oxygenated surface layers during the day and anoxic, fermenting cyanobacterial layers during the night (9, 62), thus pushing the oxic-anoxic transition zone of *Thiovulum*'s habitat into the mat during the day and out of the mat during night, respectively. In 2009, we set out to sequence the genome of cells from a *Thiovulum* population recovered from these mats in order to reveal the molecular basis of its known ecophysiological characteristics and to form hypotheses about as-yet-unknown physiological capabilities based on genomic data and comparisons with its closest sequenced relatives.

MATERIALS AND METHODS

***Thiovulum* collection and enrichment.** Phototrophic microbial mats were collected from Elkhorn Slough in California in fall 2009 as previously described (9, 62). A 10-cm by 10-cm section of mat overlaid with approximately 2 cm of seawater was incubated at room temperature overnight in the laboratory. By morning, a white cloudy veil had formed. This veil was confirmed by phase-contrast microscopy to contain cells matching the morphological and motile characteristics detailed in previous descriptions of *Thiovulum*.

Microfluidic sorting and whole-genome amplification. Sorting in a microfluidic laser-tweezer device and whole-genome amplification were carried out as previously described (6).

PCR and quantitative PCR (qPCR). 16S rRNA genes were amplified from MDA-amplified genomic DNA using primers GM3F (5′-AGAGTTTGATCMTGGC-3′) (40) and Uni1392R (5′-ACGGGCGGTGTGTRC-3′) (28). PCR was performed with 0.5 μM each primer in 50-μl volumes using DreamTaq green master mix (Fermentas, Vilnius, Lithuania) with a

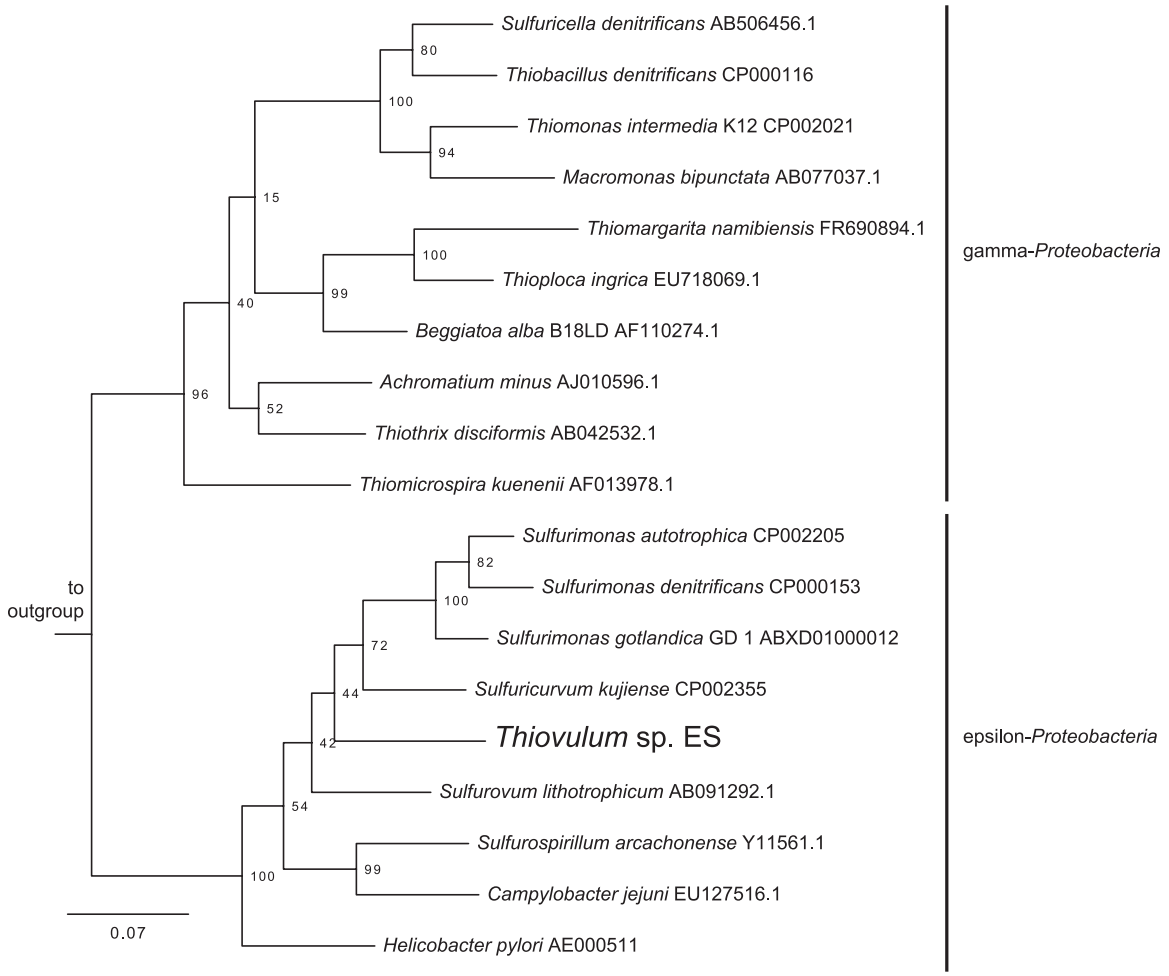

FIG 2 16S rRNA phylogenetic tree of *Thiovulum* sp. ES, other colorless sulfur bacteria, and other *Epsilonproteobacteria*. Shown is a maximum likelihood tree bootstrapped 100 times; branch labels show numbers of trees with displayed branching pattern.

5-min 95°C initial denaturation step, followed by 25 cycles of 1 min at 95°C, 1 min of annealing at 48°C, and then 1 min of extension at 72°C. PCR products were cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) and subsequently sequenced.

Quantitative PCR primers were designed using Geneious (Biomatters, Auckland, New Zealand) based on the 16S rRNA gene sequences obtained from the single cells. The forward primer was Thio_121F (5′-GGATAGT TACATGCCCCTTGGAG-3′), and the reverse primer was Thio_221R (5′-TAGGAGATAGCCCAATCCCTTGG-3′). Total bacterial DNA was quantified using universal bacterial primers Eub341F (5′-CTGCTGCCT CCCGTAGG-3′) and Eub534R (5′-ATTACCGCGGCTGCTGGC-3′) (40). The fraction of each MDA product that was from *Thiovulum* was estimated through qPCR using the method previously described (4), by quantifying total bacterial 16S rRNA gene copies and *Thiovulum* 16S rRNA gene copies with a plasmid standard created using the 16S rRNA gene cloning protocol described above.

Genome sequencing and assembly. 454 shotgun libraries were prepared from the amplified genome of each individual cell by a variation of the manufacturer's protocol (7). 454 sequencing was carried out according to the manufacturer's instructions for titanium chemistry (Roche, Branford, CT) to obtain 192 Mb raw sequence data. The data were filtered for read quality using the MOTHUR package (50) according to the following criteria: no homopolymers greater than 10, no ambiguous bases, and average quality score greater than 26. This yielded reads totaling 174 Mb. Individual assemblies were generated with the Newbler assembler (Roche; default parameters, except for specifying an increased expected read depth in excess of the actual value) and found to share large regions of homology exceeding 99% by BLASTn; thus, we took a coassembly approach, pooling the data from multiple single cells to generate a consensus coassembly. The raw reads were coassembled in an iterative "jackknife" procedure using Newbler (version 2.6, default parameters, except for specifying an increased expected read depth in excess of the actual value) to exclude the chimeric sequences generated by the MDA process without the aid of a reference sequence (for details, see methods described in the supplemental material). At this stage, the ~2-Mb coassembly was contained in approximately 240 contigs. Seeking to further improve the coassembly, we implemented two high-throughput gap-closing strategies, one based on PCR amplification of gap regions and the other taking advantage of a third-generation sequencing platform (for details, see methods described in the supplemental material).

GC content bias analysis. To check the correlation between GC content and coverage, the GC content for every assembled base more than 50 bases from a contig end was calculated based on a 100-bp window. Coverage was set equal to the "Total Depth" statistic generated by the Newbler assembler. The correlation was tested using the cor.test() function in R, version 2.13.1, with the Spearman method and other parameters set to the defaults. GC bias at contig ends was found by comparing the GC contents of the first and last 100 bp of each contig to the GC content of the entire contig for all contigs 1,000 bp or greater in length. The binomial test [binom.test() in R, version 2.13.1] was used to determine whether the results of this analysis differed from results expected through random changes at contig ends (25% high-GC ends, 25% low-GC ends, and 50% mixed low- and high-GC ends).

Genome purity analysis. Nucleotide sequences of genes expected to be present in single copies (44) from the assembled four-cell genome were used with BLAST (2) as query sequences against four databases, each containing contigs from one of the four individual-cell assemblies. Results of this BLAST search were used to determine whether the genomes for all of the four cells were nonidentical.

Genome annotation. Assembled contigs were submitted to the Integrated Microbial Genomes database annotation pipeline (36) in late 2011 (IMG, version 3.4). Some computationally assigned annotations were manually changed based on inspection of evidence for the IMG-assigned annotations, orthologs in related genomes and gene neighborhoods. Pathways were predicted using the PathoLogic tool in Pathway Tools, version 15.5.

Phylogenetic analysis. A 16S rRNA gene tree was constructed by uploading the full *Thiovulum* 16S rRNA gene sequence from the assembled genome to the online Silva SINA aligner (http://www.arb-silva.de/) (43) and then downloading the aligned *Thiovulum* sp. ES sequence along with the closest relatives for which a genome exists, some representative *Epsilonproteobacteria* and other colorless sulfur bacteria from within the *Gammaproteobacteria*. This alignment was used to construct a 100×-bootstrapped phylogenetic tree in Geneious (Biomatters) using the PhyML tool with default settings (16).

A coding sequence phylogeny was created by taking single-copy genes from *Thiovulum* sp. ES and related genomes (see Table ST1 in the supplemental material), performing translation alignments of the nucleic acid sequences using the MUSCLE alignment feature within Geneious, and then concatenating those alignments together. The concatenated alignments were used as input for PhyML to generate a 1,000×-bootstrapped tree with other parameters at default settings. *Campylobacter jejuni* subsp. *jejuni* M1 was used as the outgroup genome.

Ortholog analysis. Orthologs were determined by constructing a BLAST (2) database containing protein sequences from the *Thiovulum* genome plus five related genomes obtained from IMG, version 3.4. All 6 genomes were used as query sequences against this database. BLAST hits with an E value of less than 10^{-5} and 35% or greater sequence identity were considered homologs, with only the lowest E value hit counting where multiple hits met this criterion for a single query sequence. These homolog pairs were then considered ortholog pairs if the same criterion applied for the reverse BLAST with query and subject sequences exchanged.

Nucleotide sequence accession numbers. This whole-genome shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession number AKKQ00000000. The version described in this article is the first version, AKKQ01000000.

RESULTS AND DISCUSSION

Genome completeness. Six single *Thiovulum* cells from Elkhorn Slough mat material were sorted into single chambers on a microfluidic device, and whole-genome amplification was performed subsequently on the lysed cells. 16S rRNA gene sequences obtained from the sorted *Thiovulum* DNA by PCR were identical to each other. Of the six *Thiovulum* whole-genome amplification products obtained, four were estimated to have 100% of their 16S rRNA gene copies representing *Thiovulum* by qPCR (data not shown). These four whole-genome amplification products were thus thought to contain minimal, if any, non-*Thiovulum* DNA contamination. A genome was reconstructed by assembling reads sequenced from the amplification of those four cells. Single-copy genes in the four-cell assembly almost exclusively shared 100% identity with each of the four single-cell assemblies, with the exceptions being either single-base insertions (and thus more likely to be a sequencing error than a frameshift mutation) or single nucleotide polymorphisms within 7 bases of the end of a contig (and thus probably a sequencing error) (see Table ST2 in the supplemental material). The cells used are pictured in Fig. SF1 in the supplemental material. For the purpose of convenience, we have named the strain represented by this assembled genome *Thiovulum* sp. ES, with ES being an abbreviation for Elkhorn Slough.

The genome was assembled into 221 contigs ranging in size from 100 bp to 81,617 bp. Eighty-two percent of the contigs were longer than 500 bp, 50% were longer than 2,592 bp, and 28% were longer than 10,000 bp. The N50 weighted median statistic of genome completeness (37) was calculated as 28,163 bp. GC content was investigated as a potential source of bias in MDA and sequenc-

TABLE 1 Statistics for the genome of *Thiovulum* sp. ES and of its five closest sequenced relatives as obtained from the Integrated Microbial Genomes Database in January 2012 (IMG, version 3.5)[a]

Parameter	*Thiovulum* sp. ES	*Sulfurimonas autotrophica* OK10	*Sulfurimonas denitrificans*	*Sulfuricurvum kujiense* YK-1	"*Sulfurimonas gotlandica*" GD 1	*Sulfurovum* sp. NBC37-1
Total genome size (Mb)	2.083	2.153	2.201	2.819	2.946	2.562
No. of contigs	221	1	1	5	12	1
Coding DNA (%)	91.83	94.88	93.72	93.03	90.38	90.70
GC content (%)	33	35	34	45	33	43
Total no. of genes	2,162	2,220	2,164	2,879	2,804	2,525
No. of protein-coding genes	2,120	2,165	2,104	2,818	2,745	2,465
No. of orthologs shared with *Thiovulum*	2,120	1,005	1,016	1,147	1,182	920
No. of protein-coding genes with functional prediction	1,438	1,525	1,499	1,954	1,908	1,333
No. of copies of 5S rRNA	1	4	4	3	4	3
No. of copies of 16S rRNA	1	4	4	3	4	3
No. of copies of 23S rRNA	1	4	4	3	4	3
No. of tRNA genes	36	43	44	50	47	44

[a] Ortholog numbers were calculated as described in Materials and Methods.

ing and found to be slightly positively correlated with coverage (Spearman's rho = 0.08; $P < 2.2e{-}16$). It appears that this bias affected where contigs were terminated, as contigs were more likely to have two 100-bp ends of low GC content (42% of contigs 1,000 bp or greater; $P = 5.054 \times 10^{-6}$) than two 100-bp ends of high GC content (12% of contigs 1,000 bp or greater; $P = 9.091 \times 10^{-8}$). A similar positive GC coverage correlation has been observed in the past while using the RepliG kit for MDA (65).

Despite being unfinished, the assembled genome appears to provide a reasonable representation of *Thiovulum* sp. ES gene content, with a complete rRNA gene operon, 36 tRNA genes enabling translational insertion of all amino acids from all codons (see Table ST3 in the supplemental material), and genes encoding all proteins necessary for replication, transcription, and translation, with the exception of two ribosomal proteins (S14 and L33) considered universal in bacteria (67) (see Table ST4 in the supplemental material). The total number of genes identified was 2,162 and the total genome length was 2.083 Mbp, which are comparable to values for its closest sequenced relatives from the genera *Sulfurimonas*, *Sulfurovum*, and *Sulfuricurvum* (Table 1). Although it cannot be ruled out that genes may be missing from the genome in its present partially finished state, we believe that coverage is sufficient to draw some significant yet cautious conclusions about the microorganism's genetic inventory and resultant physiology.

Genome phylogenetic identification. Prior to this study, there were only two 16S rRNA gene sequences from a *Thiovulum* enrichment in GenBank (accession numbers M92323.1 and M92324.1) in the form of two nonoverlapping fragments from the 5′ and 3′ ends of the gene (49), as well as a complete 5S rRNA gene sequence from the same source (accession number M35570.1). These sequences were derived from *Thiovulum* cells enriched from sediment from Eel Pond in Woods Hole, MA (D. Stahl, personal communication). These cells were about 9 to 10 μm in size (55). Interestingly, the 16S rRNA gene in the *Thiovulum* sp. ES genome (identical to the 16S rRNA sequences obtained from the MDA products by PCR) was 88.2% and 88.7% identical to the two 16S rRNA sequences in GenBank, and the 5S rRNA gene sequence was 81.9% identical. The top BLAST hit for the full gene sequence was from an uncultured bacterium (accession number EF467529.1) with 87.2% identity to the *Thiovulum* sp. ES 16S rRNA gene. Notably, these identities are well below the typical 94.9% ± 0.4% minimum identity observed between members of a genus and the type strain of a genus (64). This suggests that in nature, there may, in fact, be multiple genera within the group defined phenotypically as *Thiovulum*. However, the genus *Thiovulum* has been traditionally defined phenotypically rather than based on sequence identity, as there exists a paucity of available *Thiovulum* 16S rRNA gene sequences. For these reasons, we have chosen to call the microorganism whose genome was sequenced in this study a member of the genus *Thiovulum*. The phylogenetic positioning of the genus *Thiovulum* as a relative of other colorless sulfur bacteria within the *Epsilonproteobacteria* was confirmed by its position in phylogenetic trees based on 16S rRNA genes (Fig. 2) and a concatenated alignment of 35 single-copy genes highly conserved among bacteria (Fig. 3).

Catabolic-gene content. Physiological studies of *Thiovulum* have revealed that it is an aerobic colorless sulfur bacterium, capable of conserving energy by oxidizing H_2S to elemental sulfur with molecular oxygen as a terminal electron acceptor (11, 23, 61). This observation is supported by evidence from the genome, with genes encoding sulfide:quinone reductase (*sqr*), ubiquinol cytochrome bc_1 complex, and cytochrome *c* oxidase (Fig. 4; see also Table ST4 in the supplemental material). *sqr* has been shown to couple the oxidation of sulfide to sulfur to the reduction of quinones (53). There is also genomic evidence that *Thiovulum* may be able to use formate as an electron donor, with genes encoding a cytoplasmic formate dehydrogenase and cytochrome c_{553} (Fig. 4; see also Table ST4 in the supplemental material) that may enable the oxidation of formate in a manner similar to that in *Desulfovibrio vulgaris* (52).

Genes encoding enzymes for the reduction of various terminal electron acceptors were also identified. For aerobic respiration, a cytochrome *c* oxidase was identified (Fig. 4; see also Table ST4 in the supplemental material), which is of the high-affinity cbb_3 type, adapted for low O_2 concentrations (41, 42). This matches earlier observations that *Thiovulum* cells are typically found in microoxic (0 to 10 μM) environments (13, 22, 23). The genome also contains a gene predicted to encode a cytochrome *c* peroxidase and desulfoferredoxin, possibly involved in oxidative stress response.

Anaerobic growth using nitrate as an electron acceptor is a common trait among colorless sulfur bacteria (48), especially among the *Epsilonproteobacteria* (15, 18, 27). Other large colorless

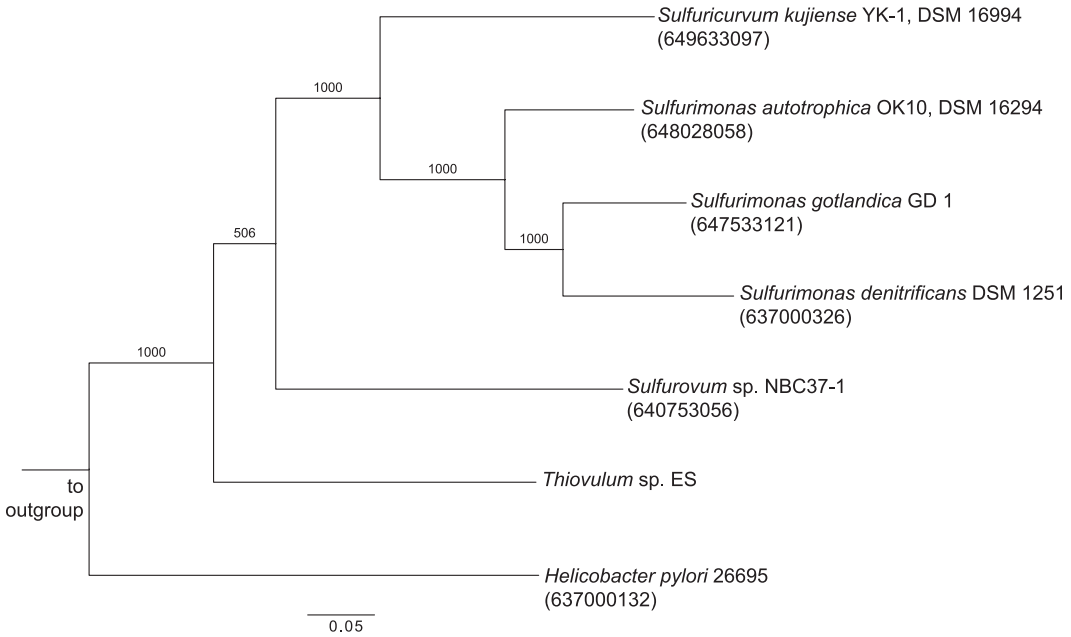

FIG 3 Phylogenetic tree of *Thiovulum* sp. ES and related *Epsilonproteobacteria* based on concatenated alignment of 35 single-copy genes (see Table ST1 in the supplemental material). Shown is a maximum likelihood tree bootstrapped 1,000 times; branch labels show numbers of trees with displayed branching pattern.

sulfur bacteria, including *Beggiatoa*, *Thiomargarita*, and *Thioploca*, profit from their large cell size by storing up to 0.3 M nitrate in vacuoles comprising the majority of the cell volume and then respiring that nitrate under anoxic conditions (24). Moreover, the epsilonproteobacterium *Wolinella succinogenes* has been shown to grow anaerobically by coupling the oxidation of formate to the reduction of elemental sulfur (21, 34). In the absence of a pure culture, the capacity for anaerobic growth by *Thiovulum* has been more difficult to evaluate. Wirsen and Jannasch (61) were unsuccessful in maintaining *Thiovulum* enrichments under anoxic conditions but hypothesized that *Thiovulum* could persist in a dormant state in anoxic environments. On the other hand, using an O_2 microsensor, Jørgensen and Revsbech (23) observed the majority of cells in *Thiovulum* veils to be on the anoxic side of the oxic-anoxic interface, which is possibly evidence of anaerobic energy conservation. Fenchel (13) refuted this finding, with observations of *Thiovulum* veils showing no cells present on the anoxic side of an oxic-anoxic interface.

The *Thiovulum* sp. ES genome contains two genes encoding the α- and β-subunits of periplasmic nitrate reductase (NarGH), as well as a membrane-associated polysulfide reductase (NrfD) (Fig. 2; see also Table ST4 in the supplemental material), suggesting that this particular population of *Thiovulum* may be capable of anaerobic growth either through the oxidation of reduced sulfur compounds coupled to the reduction of nitrate or through the oxidation of formate coupled to the reduction of sulfur. The above-mentioned contrasting conclusions on the capacity of *Thiovulum* for anaerobic growth over the last several decades could be explained in light of the genomic insights if multiple species of *Thiovulum* existed, including some facultative anaerobic species and some obligate aerobic species. A similar capacity for denitrification has been observed within the genus *Sulfurimonas*, with

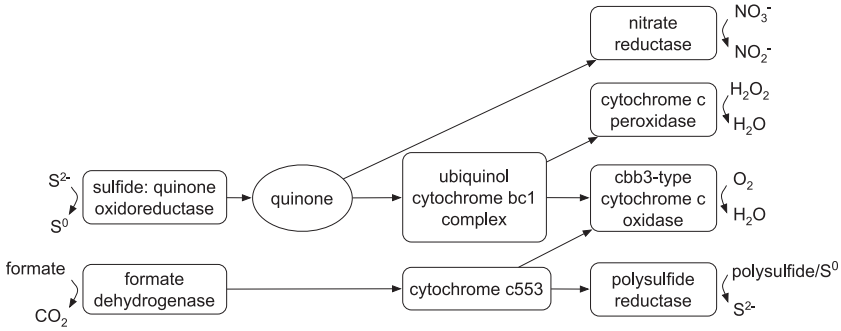

FIG 4 Genomically predicted catabolic electron flow network in *Thiovulum* sp. ES. Locus tags are sulfide:quinone oxidoreductase (ThvES_00003240), formate dehydrogenase (ThvES_00014980), ubiquinol cytochrome bc_1 complex (ThvES_00002990 to ThvES_00003010), cytochrome c_{553} (ThvES_00003480, ThvES_00009980, and ThvES_00017420), nitrate reductase (ThvES_00000060 to ThvES_00000070), cytochrome *c* peroxidase (ThvES_00008770 and ThvES_00012320), cbb_3-type cytochrome *c* oxidase (ThvES_00014560 to ThvES_00014590), and polysulfide reductase (ThvES_00006260).

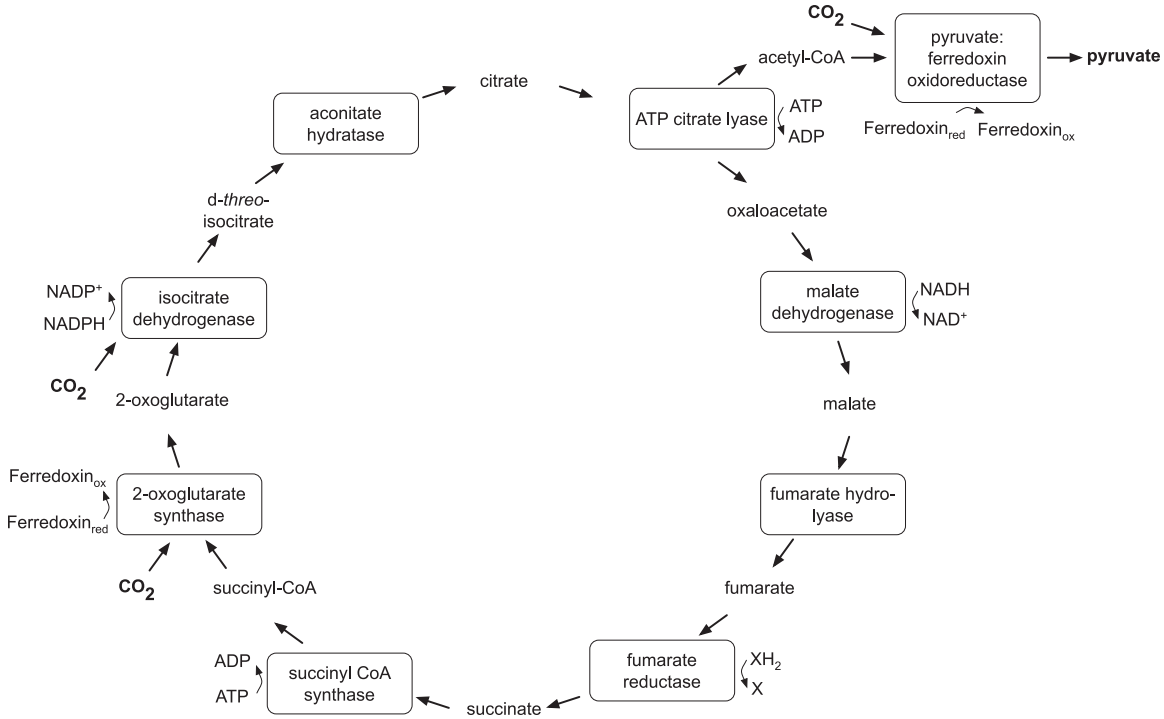

FIG 5 Genomically identified enzymes for the operation of a complete reverse TCA cycle and pyruvate synthase for autotrophic CO_2 fixation in *Thiovulum* sp. ES. The cofactors are indicated as predicted based on genome annotation and are not experimentally verified. Locus tags are isocitrate dehydrogenase (ThvES_00010990), aconitate hydratase (ThvES_00009840), ATP-citrate lyase (ThvES_00016940 and ThvES_00016970), malate dehydrogenase (locus tag no. ThvES_00001160), fumarate hydrolyase (ThvES_00001170 and ThvES_00007390), fumarate reductase (ThvES_00007350, ThvES_00007360, ThvES_00012790, ThvES_00017900, ThvES_00017910, and ThvES_00017920), succinyl coenzyme A synthase (ThvES_00013960 and ThvES_00014000), 2-oxoglutarate synthase (ThvES_00012430, ThvES_00012420, and ThvES_00012410), and pyruvate:ferredoxin oxidoreductase (ThvES_00006810, ThvES_00006820, ThvES_00006790, and ThvES_00006800).

species that are facultative anaerobes (18), obligate aerobes (20), and obligate anaerobes (15). An alternative explanation for the presence of genes encoding a nitrate reductase in the *Thiovulum* sp. ES genome is that the nitrate reductases are involved in anabolic nitrate reduction.

In contrast to nitrate reductase, no nitrite reductase was identified, indicating either that it is present but unsequenced or that *Thiovulum* sp. ES is an incomplete denitrifying bacterium. Also absent were homologs of the hydroxylamine oxidase and reductase hypothesized to play a role in nitrite reduction in the epsilonproteobacterium *Nautilia profundicola* (10). Other incompletely denitrifying colorless sulfur bacteria have been identified (27, 48), suggesting that the latter possibility may be true.

One puzzling finding from the genome was that no gene encoding an enzyme to further oxidize elemental sulfur was identified. While genes encoding enzymes for sulfite oxidation via adenosylphosphosulfate as an intermediate (26), including sulfate adenylyltransferase (ThvES_00004190-4200), were found to be present, these enzymes are also required for sulfate activation for sulfonation reactions or sulfate assimilation and are thus not evidence of the capacity to oxidize elemental sulfur to sulfate. Since elemental sulfur forms intracellularly in *Thiovulum* cells (12, 17, 29) and no obvious set of enzymes for the complete oxidation of elemental sulfur was found, *Thiovulum* cells somehow need to escape ever-accumulating intracellular sulfur. One possible mechanism is that *Thiovulum* has to oscillate between an aerobic mode of energy conservation in which elemental sulfur accumulates in the cell and an anaerobic mode of energy conservation in which intracellular sulfur serves as an electron acceptor, perhaps with formate acting as an electron donor or via anaerobic sulfur disproportionation. This explanation would be consistent with *Thiovulum*'s ecophysiology, as microbial mats go through diel oxic-anoxic fluctuations in which fermentation products, including formate, are formed during the night in the anoxic cyanobacterial layer (9). This would also explain why attempts at continuous aerobic or anaerobic *Thiovulum* cultivation have failed thus far, as obligatory oxic-anoxic cycling (possibly providing a second electron acceptor, such as formate, during the anoxic phase) would be critical for sustained cultivation. A second possible explanation is that the genes encoding an elemental sulfur oxidation pathway await discovery or further sequencing of *Thiovulum* sp. ES. Both explanations of how elemental sulfur leaves the cell are intriguing possibilities that warrant further investigation.

Anabolic-gene content. *Thiovulum* has been shown to grow autotrophically (61). Other autotrophic colorless sulfur bacteria, including *Epsilonproteobacteria* such as *Sulfurimonas denitrificans* and "*Candidatus* Arcobacter sulfidicus," fix carbon dioxide via the reverse tricarboxylic acid (TCA) cycle (19). We have identified genes encoding all enzymes necessary for the reverse TCA cycle, including ATP-citrate lyase, fumarate reductase, and 2-oxoglutarate synthase, specific to the reductive (reverse) rather than the oxidative TCA cycle (Fig. 5; see also Table ST4 in the supplemental material). No gene encoding citrate synthase has been identified, suggesting that the TCA cycle of the *Thiovulum* sp. ES is used only

for CO_2 fixation and anabolism. An exclusively reductive TCA cycle is consistent with observations by Wirsen and Jannasch (61), who did not observe any significant uptake of acetate or other organic compounds.

It is, however, unclear how *Thiovulum* sp. ES obtains the reduced ferredoxin the microbe needs as a reductant for the 2-oxoglutarate synthase reaction in the reverse TCA cycle as well as for the pyruvate synthase reaction. The oxidation of sulfide to elemental sulfur has a redox potential of only −270 mV under standard-state conditions (58), which is insufficient for the reduction of ferredoxin at ~−500 mV. One possible explanation is that electrons are bifurcated via the flavin-containing EtfBC complex in a similar process to that which occurs in *Clostridium kluyveri* (8). However, no genes homologous to the *C. kluyveri* genes encoding EtfBC or matching the "ETF" pfam family were identified in the *Thiovulum* sp. ES genome. Indeed, these genes are also absent from the genome of *Sulfurimonas denitrificans*, the first colorless sulfur bacterium in the *Epsilonproteobacteria* shown to fix CO_2 through the reverse TCA cycle (19). An inspection of pfam (http://pfam.sanger.ac.uk) reveals that of the 665 proteobacterial species shown to have genes encoding proteins in the ETF family, all are in the *Alpha-*, *Beta-*, *Gamma-*, and *Deltaproteobacteria*, with no ETF proteins in epsilonproteobacterial genomes. It appears that there may be an as-yet-undiscovered mechanism in *Thiovulum* sp. ES and other *Epsilonproteobacteria* that enables the reduction of ferredoxin coupled to sulfide oxidation.

In addition to genes encoding CO_2 fixation, genes encoding enzymes necessary for the biosynthesis of amino acids (tryptophan, leucine, isoleucine, histidine, alanine, tyrosine, lysine, glutamate, cysteine, methionine, arginine, threonine, proline, and glycine), purine and pyrimidine nucleotides, peptidoglycan, lipids, and various cofactors (tetrahydrofolate, NAD, flavins, coenzyme A, and thiamine) and glucose-6-phosphate (gluconeogenesis) were identified (see Table ST5 in the supplemental material; the pathway hole report is provided as Table ST6 in the supplemental material). This relatively complete repertoire of biosynthetic capabilities supports the hypothesis that *Thiovulum* is capable of autotrophic growth, as well as that this genome is reasonably complete.

No gene encoding nitrogenase was identified in the *Thiovulum* sp. ES genome, but a gene encoding the ammonium transporter (AmtB) was identified adjacent to a gene encoding the nitrogen regulatory protein PII (see Table ST4 in the supplemental material), implicated in the regulation of the ammonium transporter in *Azospirillum brasilense* and *Synechococcus* (3). This suggests that *Thiovulum* sp. ES is unable to fix N_2 but is capable of importing ammonia from the environment.

Chemotaxis and motility. *Thiovulum* has been noted to be highly motile (14, 23) and chemotactic (13, 56). Flagella have been observed via staining (17) and electron microscopy (11, 46). The *Thiovulum* sp. ES genome contains all genes necessary for flagellum biosynthesis (see Table ST4 in the supplemental material), including two genes encoding FliC (flagellin); these correspond to *flaA* and *flaB* in *Helicobacter pylori* (33). However, sequence identity with the *H. pylori* orthologs is not sufficiently high to determine which gene encodes FlaA and which encodes FlaB. There are also two paralogs each of *flgE* and *flgG*, which encode structural proteins of the flagellar hook and proximal rod, respectively. One interesting property of the *Thiovulum* strain ES genes encoding the flagellar motor proteins, MotA and MotB, is that in the related *Sulfurimonas* and *Sulfuricurvum* genomes, these genes are positioned adjacent to one another, suggesting that they are part of a single operon, while in *Thiovulum* sp. ES, *motA* and *motB* appear to be genetically unlinked and are present on different contigs (see Fig. SF2 in the supplemental material). Apart from this anomaly, at the genetic level, the *Thiovulum* flagellar machinery appears to be similar to that of well-characterized flagella in other bacteria, including *H. pylori* and *Escherichia coli*.

The genome also contains genes encoding the core chemotaxis proteins CheA, CheW, and CheY plus 13 genes encoding methyl-accepting chemotaxis proteins (MCPs) (see Table ST4 in the supplemental material). Of the auxiliary chemotaxis genes that have been defined (63), *Thiovulum* sp. ES was found to possess genes coding for CheC, CheD, CheV, and the *Helicobacter pylori*-type CheZ (32). However, the *Thiovulum* sp. ES genome does not contain clear homologs for the CheX, CheB, or CheR gene. No bacterial genome containing *cheCD* but missing both *cheB* and *cheR* has been identified to date (63), suggesting that genes enabling methylation-dependent chemotaxis have not yet been sequenced in this unfinished genome. On the other hand, methylation-independent chemotaxis is not unprecedented in knockout strains (5). As studies with *Bacillus subtilis* have shown, CheC, CheD, and CheV are involved in sensory adaptation to a wide range of substrate concentrations by a feedback mechanism from phosphorylated CheY to the MCP (CheC and CheD) (38, 45) or by modulating the efficiency of CheY phosphorylation by CheA (CheV) (1). CheZ accelerates the dephosphorylation of CheY, enabling a rapid response to changing environmental conditions (32).

Thirteen MCPs can be reasonably considered to constitute a complete suite of MCP proteins given the number of MCPs found in genomes of closely related sequenced organisms (15). Of the 13 MCPs, putative sensory domains could be identified in 5, with nitrate/nitrite sensory domains (54) in 3, a CACHE domain (associated with small-molecule sensing) in 1, and a PAS domain (associated with O_2 sensing as well as other substrates) in another. O_2 is the only chemotaxis substrate reported for *Thiovulum* to date, but the genes thought to encode MCPs in the genome suggest that *Thiovulum* may be capable of chemotaxis toward substrates other than O_2, possibly including nitrate/nitrite and small organic molecules.

Comparative genomics. Comparisons between the *Thiovulum* genome and its five closest sequenced relatives revealed the 16S rRNA gene identity, as described above, and the percentage of orthologs shared between each genome pair (Table 1). *Thiovulum* is more distantly related from all of its sequenced close relatives than any of its closest sequenced relatives are from each other (Fig. 2; Table 2). This means that although *Thiovulum* sp. ES is phylogenetically a member of the *Epsilonproteobacteria*, it is only distantly related to *Epsilonproteobacteria* with sequenced genomes, with less than half of its genes having orthologs in the most closely related genomes.

Conclusions. The sequencing and annotation of a genome from four single *Thiovulum* cells revealed genes encoding many traits previously observed in *Thiovulum*, like chemolithoautotrophy, motility and chemotaxis, and unusual cell shape. However, the 88% sequence identity between the genome's 16S rRNA gene and the only other *Thiovulum* 16S rRNA gene existing in public databases, the presence of genes encoding nitrate and polysulfide reduction while the literature record is ambivalent about *Thiovulum*'s capacity to grow anaerobically, and previous observations of

TABLE 2 16S rRNA gene identity and ortholog fraction for each genome pair for *Thiovulum* sp. ES and its five closest relatives[a]

Organism	% 16S rRNA gene identity with organism/total % protein-encoding genes					
	Thiovulum sp. ES	*Sulfurimonas autotrophica* OK10	*Sulfurimonas denitrificans*	*Sulfuricurvum kujiense* YK-1	"*Sulfurimonas gotlandica*" GD 1	*Sulfurovum* sp. NBC37-1
Thiovulum sp. ES	100.0/100.0	85.1/40.1	85.1/41.3	85.0/32.0	86.2/31.5	85.2/32.2
Sulfurimonas autotrophica OK10	85.1/41.0	100.0/100.0	93.2/68.9	93.2/48.8	88.6/56.6	87.1/50.6
Sulfurimonas denitrificans	85.1/41.0	93.2/66.9	100.0/100.0	93.9/48.9	88.0/56.0	85.9/45.9
Sulfuricurvum kujiense YK-1	85.0/42.5	93.2/63.5	93.9/65.5	100.0/100.0	87.7/52.1	86.4/46.6
"*Sulfurimonas gotlandica*" GD 1	86.2/40.8	88.6/71.8	88.0/73.1	87.7/50.7	100.0/100.0	86.7/48.0
Sulfurovum sp. NBC37-1	85.2/37.4	87.1/57.6	85.9/53.8	86.4/40.7	86.7/43.1	100.0/100.0

[a] The denominator always corresponds to the genome of the organism listed along the horizontal axis of the table.

different *Thiovulum* populations having different cell sizes all suggest that the *Thiovulum* species are more genotypically and phenotypically diverse than previously thought. We believe that future use of a combination of metagenomic sequencing and single-cell sequencing from *Thiovulum* populations containing different cell sizes and from different geographic locations will lead to a better understanding to the true extent of *Thiovulum*'s genotypic diversity.

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REFERENCES

1. **Alexander RP, Lowenthal AC, Ottemann KM.** 2010. CheV: CheW-like coupling proteins at the core of the chemotaxis signaling network. Trends Microbiol. **18**:494–503.
2. **Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ.** 1990. Basic local alignment search tool. J. Mol. Biol. **215**:403–410.
3. **Arcondéguy T, Jack R, Merrick M.** 2001. P(II) signal transduction proteins, pivotal players in microbial nitrogen control. Microbiol. Mol. Biol. Rev. **65**:80–105.
4. **Behrens S, et al.** 2008. Monitoring abundance and expression of "Dehalococcoides" species chloroethene-reductive dehalogenases in a tetrachloroethene-dechlorinating flow column. Appl. Environ. Microbiol. **74**: 5695–5703.
5. **Bibikov SI, Miller AC, Parkinson JS.** 2004. Methylation-independent aerotaxis mediated by the Escherichia coli Aer protein. J. Bacteriol. **186**: 3730–3737.
6. **Blainey PC, Mosier AC, Potanina A, Francis CA, Quake SR.** 2011. Genome of a low-salinity ammonia-oxidizing archaeon determined by single-cell and metagenomic analysis. PLoS One **6**:e16626. doi:10.1371/journal.pone.0016626.
7. **Blainey PC, Quake SR.** 2011. Digital MDA for enumeration of total nucleic acid contamination. Nucleic Acids Res. **39**:e19. doi:10.1093/nar/gkq1074.
8. **Buckel W, Thauer RK.** 15 July 2012. Energy conservation via electron bifurcating ferredoxin reduction and proton/Na^+ translocating ferredoxin oxidation. Biochim. Biophys. Acta. http://dx.doi.org/10.1016/j.bbabio.2012.07.002.
9. **Burow LC, et al.** 2012. Hydrogen production in photosynthetic microbial mats in the Elkhorn Slough estuary, Monterey Bay. ISME J. **6**:863–874.
10. **Campbell BJ, et al.** 2009. Adaptations to submarine hydrothermal environments exemplified by the genome of Nautilia profundicola. PLoS Genet. **5**:e1000362. doi:10.1371/journal.pgen.1000362.
11. **de Boer W, La Riviere JW, Houwink AL.** 1961. Observations on the morphology of Thiovulum majus Hinze. Antonie Van Leeuwenhoek **27**: 447–456.
12. **Fauré-Fremiet E, Rouiller C.** 1958. Etude au microscope electronique d'une bactérie sulfureuse, Thiovulum majus Hinze. Exp. Cell Res. **14**: 29–46.
13. **Fenchel T.** 1994. Motility and chemosensory behaviour of the sulphur bacterium Thiovulum majus. Microbiology **140**:3109.
14. **Garcia-Pichel F.** 1989. Rapid bacterial swimming measured in swarming cells of Thiovulum majus. J. Bacteriol. **171**:3560–3563.
15. **Grote J, et al.** 2012. Genome and physiology of a model Epsilonproteobacterium responsible for sulfide detoxification in marine oxygen depletion zones. Proc. Natl. Acad. Sci. U. S. A. **109**:506–510.
16. **Guindon S, Gascuel O.** 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. **52**:696–704.
17. **Hinze G.** 1913. Beiträge zur Kenntnis der farblosen Schwefelbakterien. Ber. Dtsch. Bot. Ges. **31**:189–202.
18. **Hoor A.** 1975. A new type of thiosulphate oxidizing, nitrate reducing microorganism: Thiomicrospira denitrificans sp. nov. Neth. J. Sea Res. **9**:344–350.
19. **Hügler M, Wirsen CO, Fuchs G, Taylor CD, Sievert SM.** 2005. Evidence for autotrophic CO_2 fixation via the reductive tricarboxylic acid cycle by members of the epsilon subdivision of proteobacteria. J. Bacteriol. **187**: 3020–3027.
20. **Inagaki F, Takai K, Kobayashi H, Nealson KH, Horikoshi K.** 2003. Sulfurimonas autotrophica gen. nov., sp. nov., a novel sulfur-oxidizing epsilon-proteobacterium isolated from hydrothermal sediments in the Mid-Okinawa Trough. Int. J. Syst. Evol. Microbiol. **53**:1801–1805.
21. **Jankielewicz A, Klimmek O, Kröger A.** 1995. The electron transfer from hydrogenase and formate dehydrogenase to polysulfide reductase in the membrane of Wolinella succinogenes. Biochim. Biophys. Acta **1231**:157–162.
22. **Jørgensen BB, Postgate JR.** 1982. Ecology of the bacteria of the sulphur cycle with special reference to anoxic-oxic interface environments. Philos. Trans. R. Soc. Lond. B Biol. Sci. **298**:543–561.
23. **Jørgensen BB, Revsbech NP.** 1983. Colorless sulfur bacteria, Beggiatoa spp. and Thiovulum spp., in O_2 and H_2S microgradients. Appl. Environ. Microbiol. **45**:1261–1270.
24. **Jørgensen BB.** 2010. Big sulfur bacteria. ISME J. **4**:1083–1084.
25. **Kalisky T, Blainey P, Quake SR.** 2011. Genomic analysis at the single-cell level. Annu. Rev. Genet. **45**:431–445.
26. **Kappler U, Dahl C.** 2001. Enzymology and molecular biology of prokaryotic sulfite oxidation. FEMS Microbiol. Lett. **203**:1–9.
27. **Kodama Y, Watanabe K.** 2004. Sulfuricurvum kujiense gen. nov., sp. nov., a facultatively anaerobic, chemolithoautotrophic, sulfur-oxidizing bacterium isolated from an underground crude-oil storage cavity. Int. J. Syst. Evol. Microbiol. **54**:2297–2300.
28. **Lane DJ, et al.** 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc. Natl. Acad. Sci. U. S. A. **82**:6955–6959.
29. **La Rivière J.** 1963. Cultivation and properties of *Thiovulum majus* Hinze, p 61–73. *In* Oppenheimer CH (ed), Symposium on marine microbiology. Charles C Thomas, Springfield, IL.
30. **Lasken RS.** 2009. Genomic DNA amplification by the multiple displacement amplification (MDA) method. Biochem. Soc. Trans. **37**:450–453.

31. **Lauterborn R.** 1915. Die sapropelische Lebewelt. Ein Beitrag zur Biologie des Faulschlamms natürlicher Gewässer. Verh. Naturhist. Med. Ver. Heidelb. **13**:395–481.
32. **Lertsethtakarn P, Ottemann KM.** 2010. A remote CheZ orthologue retains phosphatase function. Mol. Microbiol. **77**:225–235.
33. **Lertsethtakarn P, Ottemann KM, Hendrixson DR.** 2011. Motility and chemotaxis in Campylobacter and Helicobacter. Annu. Rev. Microbiol. **65**:389–410.
34. **Macy J, Schröder I, Thauer R, Kröger A.** 1986. Growth the Wolinella succinogenes on H_2S plus fumarate and on formate plus sulfur as energy sources. Arch. Microbiol. **144**:147–150.
35. **Marcy Y, et al.** 2007. Dissecting biological "dark matter" with single-cell genetic analysis of rare and uncultivated TM7 microbes from the human mouth. Proc. Natl. Acad. Sci. U. S. A. **104**:11889–11894.
36. **Markowitz VM, et al.** 2009. IMG ER: a system for microbial genome annotation expert review and curation. Bioinformatics **25**:2271–2278.
37. **Miller JR, Koren S, Sutton G.** 2010. Assembly algorithms for next-generation sequencing data. Genomics **95**:315–327.
38. **Muff TJT, Ordal GWG.** 2007. The CheC phosphatase regulates chemotactic adaptation through CheD. J. Biol. Chem. **282**:34120–34128.
39. **Müller OF.** 1786. Animalcula infusoria fluvia tilia et marina. Nicolai Möller, Copenhagen, Denmark.
40. **Muyzer G, de Waal EC, Uitterlinden AG.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59**:695–700.
41. **Pitcher RS, Brittain T, Watmough NJ.** 2002. Cytochrome cbb(3) oxidase and bacterial microaerobic metabolism. Biochem. Soc. Trans. **30**:653–658.
42. **Preisig O, Zufferey R, Thony-Meyer L, Appleby CA, Hennecke H.** 1996. A high-affinity cbb3-type cytochrome oxidase terminates the symbiosis-specific respiratory chain of Bradyrhizobium japonicum. J. Bacteriol. **178**:1532–1538.
43. **Pruesse E, et al.** 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res. **35**:7188–7196.
44. **Raes J, Korbel JO, Lercher MJ, von Mering C, Bork P.** 2007. Prediction of effective genome size in metagenomic samples. Genome Biol. **8**:R10. doi:10.1186/gb-2007-8-1-r10.
45. **Rao CV, Glekas GD, Ordal GW.** 2008. The three adaptation systems of Bacillus subtilis chemotaxis. Trends Microbiol. **16**:480–487.
46. **Remsen CC, Watson SW.** 1972. Freeze-etching of bacteria. Int. Rev. Cytol. **33**:253–296.
47. **Rivière JWM, Schmidt K.** 2006. Morphologically conspicuous sulfur-oxidizing eubacteria, p 941–954. *In* Dworkin M, Falkow S, Rosenberg E, Schleifer K, Stackebrandt E (ed), The prokaryotes, 3rd ed. Springer, New York, NY.
48. **Robertson LA, Kuenen JG.** 2006. The colorless sulfur bacteria, p 985–1011. *In* Dworkin M, Falkow S, Rosenberg E, Schleifer K, Stackebrandt E (ed), The prokaryotes, 3rd ed. Springer, New York, NY.
49. **Romaniuk PJ, et al.** 1987. Campylobacter pylori, the spiral bacterium associated with human gastritis, is not a true Campylobacter sp. J. Bacteriol. **169**:2137–2141.
50. **Schloss PD, et al.** 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. **75**:7537–7541.
51. **Schulz HN, Jørgensen BB.** 2001. Big bacteria. Annu. Rev. Microbiol. **55**:105–137.
52. **Sebban-Kreuzer C, Dolla A, Guerlesquin F.** 1998. The formate dehydrogenase-cytochrome c553 complex from Desulfovibrio vulgaris Hildenborough. Eur. J. Biochem. **253**:645–652.
53. **Shibata H, Kobayashi S.** 2001. Sulfide oxidation in gram-negative bacteria by expression of the sulfide-quinone reductase gene of Rhodobacter capsulatus and by electron transport to ubiquinone. Can. J. Microbiol. **47**:855–860.
54. **Shu CJ, Ulrich LE, Zhulin IB.** 2003. The NIT domain: a predicted nitrate-responsive module in bacterial sensory receptors. Trends Biochem. Sci. **28**:121–124.
55. **Stahl D, et al.** 1987. Phylogenetic analysis of certain sulfide-oxidizing and related morphologically conspicuous bacteria by 5S ribosomal ribonucleic acid sequences. Int. J. Syst. Bacteriol. **37**:1–7.
56. **Thar R, Fenchel T.** 2001. True chemotaxis in oxygen gradients of the sulfur-oxidizing bacterium Thiovulum majus. Appl. Environ. Microbiol. **67**:3299–3303.
57. **Thar R, Kühl M.** 2005. Complex pattern formation of marine gradient bacteria explained by a simple computer model. FEMS Microbiol. Lett. **246**:75–79.
58. **Thauer RK, Jungermann K, Decker K.** 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. **41**:100–180.
59. **van Niel CB.** 1955. Natural selection in the microbial world. J. Gen. Microbiol. **13**:201–217.
60. **Warming E.** 1876. Om nogle ved Danmarks Kyster levende Bakterier. Bianco Lunos Bogtrykkeri, Copenhagen, Denmark.
61. **Wirsen CO, Jannasch HW.** 1978. Physiological and morphological observations on Thiovulum sp. J. Bacteriol. **136**:765–774.
62. **Woebken D, et al.** 2012. Identification of a novel cyanobacterial group as active diazotrophs in a coastal microbial mat using NanoSIMS analysis. ISME J. **6**:1427–1439.
63. **Wuichet K, Zhulin IB.** 2010. Origins and diversification of a complex signal transduction system in prokaryotes. Sci. Signal. **3**:ra50. doi:10.1126/scisignal.2000724.
64. **Yarza P, et al.** 2008. The All-Species Living Tree project: a 16S rRNA-based phylogenetic tree of all sequenced type strains. Syst. Appl. Microbiol. **31**:241–250.
65. **Yilmaz S, Allgaier M, Hugenholtz P.** 2010. Multiple displacement amplification compromises quantitative analysis of metagenomes. Nat. Methods **7**:943–944.
66. **Youssef NH, Blainey PC, Quake SR, Elshahed MS.** 2011. Partial genome assembly for a candidate division OP11 single cell from an anoxic spring (Zodletone Spring, Oklahoma). Appl. Environ. Microbiol. **77**:7804–7814.
67. **Yutin N, Puigbò P, Koonin EV, Wolf YI.** 2012. Phylogenomics of prokaryotic ribosomal proteins. PLoS One **7**:e36972. doi:10.1371/journal.pone.0036972.