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ORIGINAL ARTICLE

# Cultivation of a chemoautotroph from the SUP05 clade of marine bacteria that produces nitrite and consumes ammonium

Vega Shah[1], Bonnie X Chang[2] and Robert M Morris[1]
[1]*School of Oceanography, University of Washington, Seattle, WA 98105, USA and* [2]*Joint Institute for the Study of the Atmosphere and Ocean, University of Washington, Seattle, WA 98105, USA*

**Marine oxygen minimum zones (OMZs) are expanding regions of intense nitrogen cycling. Up to half of the nitrogen available for marine organisms is removed from the ocean in these regions. Metagenomic studies have identified an abundant group of sulfur-oxidizing bacteria (SUP05) with the genetic potential for nitrogen cycling and loss in OMZs. However, SUP05 have defied cultivation and their physiology remains untested. We cultured, sequenced and tested the physiology of an isolate from the SUP05 clade. We describe a facultatively anaerobic sulfur-oxidizing chemolithoautotroph that produces nitrite and consumes ammonium under anaerobic conditions. Genetic evidence that closely related strains are abundant at nitrite maxima in OMZs suggests that sulfur-oxidizing chemoautotrophs from the SUP05 clade are a potential source of nitrite, fueling competing nitrogen removal processes in the ocean.**
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## Introduction

Nitrogen is a limiting nutrient in much of the world's ocean. Thirty to fifty percent of the fixed nitrogen available for marine organisms is lost from the ocean due to the biological production of dinitrogen gas ($N_2$) in oxygen minimum zones (OMZs) (Codispoti *et al.*, 2001; Galloway *et al.*, 2008). Nitrogen loss in these regions is attributed to two microbially mediated processes, heterotrophic denitrification (Jayakumar *et al.*, 2004; Castro-Gonzalez *et al.*, 2005; Ward *et al.*, 2009; Babbin *et al.*, 2014) and anaerobic ammonia oxidation (anammox) (Kuypers *et al.*, 2005; Thamdrup *et al.*, 2006; Hamersley *et al.*, 2007; Lam *et al.*, 2009). Genomic data have identified an abundant group of sulfur-oxidizing marine chemoautotrophs (SUP05) that are assumed to contribute to either denitrification or anammox (Walsh *et al.*, 2009; Canfield *et al.*, 2010; Zaikova *et al.*, 2010; Ulloa *et al.*, 2012; Wright *et al.*, 2012; Mattes *et al.*, 2013; Hawley *et al.*, 2014; Murillo *et al.*, 2014). Members of the SUP05 clade are hypothesized to contribute directly by sequential reduction of nitrate ($NO_3^-$) to nitrogenous gases ($N_2O$ or $N_2$), or indirectly by dissimilative $NO_3^-$ reduction to ammonia (DNRA), which can in turn fuel anammox (Walsh *et al.*, 2009; Canfield *et al.*, 2010; Hawley *et al.*, 2014; Murillo *et al.*, 2014).

SUP05 also have the genetic potential to produce nitrite ($NO_2^-$), which is a critical intermediate in denitrification and a necessary reductant for anammox. The sources of $NO_2^-$, and in particular the secondary $NO_2^-$ maximum, in OMZs are poorly understood (Lam *et al.*, 2009). Accumulation of $NO_2^-$ in OMZs has been attributed to heterotrophic denitrification leading to N-loss (Lam *et al.*, 2009). The current paradigm is that $NO_2^-$ in OMZs is produced at the oxycline by aerobic ammonia oxidizing archaea and bacteria (AOA, AOB) (Hawley *et al.*, 2014) and within the OMZ by heterotrophs (Codispoti *et al.*, 2001; Francis *et al.*, 2007; Lam *et al.*, 2009). Chemoautotrophic SUP05 also have the genetic potential to respire $NO_3^-$ and produce $NO_2^-$ (Walsh *et al.*, 2009; Hawley *et al.*, 2014; Murillo *et al.*, 2014). Here we test the hypothesis that a chemoautotrophic bacterium from the SUP05 clade respires $NO_3^-$ and produces $NO_2^-$.

We isolated a representative from the SUP05 clade to elucidate the effects of these sulfur-oxidizing bacteria on the marine nitrogen cycle. We describe the isolation, genetic potential and growth requirements of a sulfur-oxidizing chemolithoautotroph that produces $NO_2^-$ and is limited by ammonium ($NH_4^+$). We provide insights into the hypothesized roles of SUP05 in the marine nitrogen cycle that could not be inferred from environmental sequence data alone. Our primary finding suggests that SUP05 produce $NO_2^-$ in OMZs, a critical

Correspondence: RM Morris, School of Oceanography, University of Washington, Seattle, WA 98105, USA.
E-mail: morrisrm@uw.edu
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intermediate required for nitrogen removal processes (anammox and denitrification).

We propose the following name for the first isolate from the SUP05 clade:

Thioglobus gen. (Marshall and Morris, 2013).

"*Candidatus* Thioglobus autotrophicus" sp. nov

Etymology: au.to.tro'phi.cus. Gr. n. *autos* self; Gr. adj. *trophikos* nursing, tending or feeding; N.L. masc. adj. *autotrophicus* autotroph.

## Materials and methods

*Cultivation*

Water was collected from Effingham Inlet (49°04.2685' N, 125°09.4270' W) during a research cruise aboard the R/V *Thomas G. Thompson* in February 2013 using a rosette with 10 liter Niskin bottles (General Oceanics, Miami, FL, USA) and equipped with a Seabird conductivity, temperature and density (CTD) device and dissolved oxygen (DO) sensors calibrated by Winkler titrations. Live cells were collected from the top of the suboxic zone (defined as the minimum value obtained by the Seabird DO sensor) (Supplementary Figure 1). Cell numbers were estimated based on prior results and diluted to extinction (5 cells/well) in two 96 well Teflon plates (SonomaTesting, Santa Rosa, CA, USA) using a high throughput culture method with on-site filter sterilized seawater (30 kD) used as media. One 96 well plate was enriched with 1 mM thiosulfate ($S_2O_3^{2-}$) while the other was used as a control and contained only filter sterilized seawater. Growth in each well was measured every 7 days in incubations at *in situ* temperatures (10 °C) by first transferring 150 µl aliquots of culture into 96 well polycarbonate plates (Millipore, Billerica, MA, USA) and then staining each well with SYBR Green I (Invitrogen, Carlsbad, CA, USA) diluted in TRIS buffer pH 7.4 at a final concentration of 1/2000. Cell concentrations in 96 well polycarbonate plates were measured using an Easyflow Guava flow cytometer equipped with a 96 well plate reader (Millipore, Billerica, MA, USA).

*Isolate identification*

Cultures were identified as previously described (Marshall and Morris, 2013), with the following modifications. Cells were transferred to 250 ml acid washed polycarbonate flasks (10% HCl) and grown to early stationary phase (~$2.0 \times 10^6$ cells/ml), then collected on sterile Supor-200 0.2 µm polyethersulfone filters (Pall, Port Washington, NY, USA). We identified cultures by amplifying and sequencing the 16S rRNA gene with bacterial primers (27F, 519F/R, 926F/R and 1492R). Sequences were subsequently aligned with nearly complete 16S rRNA gene sequences and a maximum likelihood tree was constructed using RAxML. The *Methylococcus capsulatus* (Texas strain) 16S rRNA gene was selected as an out-group and 100 bootstrap replicates were used to evaluate clusters (Figure 1a). Culture purity was verified using terminal restriction fragment length polymorphism (TRFLP) analysis. Briefly, universal primers 27F and 1492R were used to amplify 16S DNA that was then restricted with either MboI or HaeIII (New England Biolabs, Ipswich, MA, USA) as previously described (Marshall and Morris, 2013).

*Genome sequencing and annotation*

DNA was extracted as previously described (Marshall and Morris, 2013), with the following modifications. Genomic DNA was extracted from a total of 62 pure cultures grown in 100 ml aliquots. Cells were grown to early stationary phase (~$2.0 \times 10^6$ cells/ml) and then collected on sterile Supor-200 0.2 µm polyethersulfone filters (Pall, Port Washington, NY, USA). Clone library preparation for genome sequencing was performed at the University of Washington's Genome Sciences Department using Pacific Bioscience's Single Molecule Real Time (SMRT) sequencing technology. *De novo* assembly of the "*Ca.* T. autotrophicus" strain EF1 genome was conducted using the Hierarchical Genome Assembly Process (HGAP) as previously described (Koren *et al.*, 2012). This method has been found to produce highly accurate *de novo* assemblies of small prokaryotic genomes (Roberts *et al.*, 2013). HGAP assembly of the "*Ca.* T. autotrophicus" strain EF1 genome resulted in a single contig that was closed using one PCR reaction (Supplementary Methods). With an average coverage of 106X, the final assembly supported a single circular chromosome 1,512,449 bp in length (Figure 1b, Supplementary Table 1). Annotations obtained by NCBI's automatic Prokaryotic Genome Annotation Pipeline were checked and corrected by comparisons with RAST annotations, IMG annotations, and in some cases by phylogenetic inference. The complete genome sequence of strain EF1 was announced (Shah and Morris 2015), and is available under the GenBank nucleotide accession no. CP010552.

*Growth conditions*

*T. autotrophicus* EF1 cells were grown at *in situ* temperature (10 °C) in acid-washed (10% HCl) and autoclaved bottles containing copiotrophic seawater media from Puget Sound or oligotrophic seawater media from the Sargasso Sea (Supplementary Table 2). Aerobic cultures were maintained in acid washed polycarbonate bottles. Anaerobic cultures were maintained in acid washed and autoclaved 125 ml serum bottles, sealed with 20 mm butyl rubber stoppers (Wheaton, Millville, NJ, USA), then bubbled with an $N_2$:$CO_2$ gas mix (1000 ppm $CO_2$, Praxair, Danbury, CT, USA, Specialty Gas Mix) for 10 min, and headspace sparged for an additional 5 min. Complete removal of oxygen inside serum bottles was confirmed using BD (Franklin Lakes, NJ, USA)

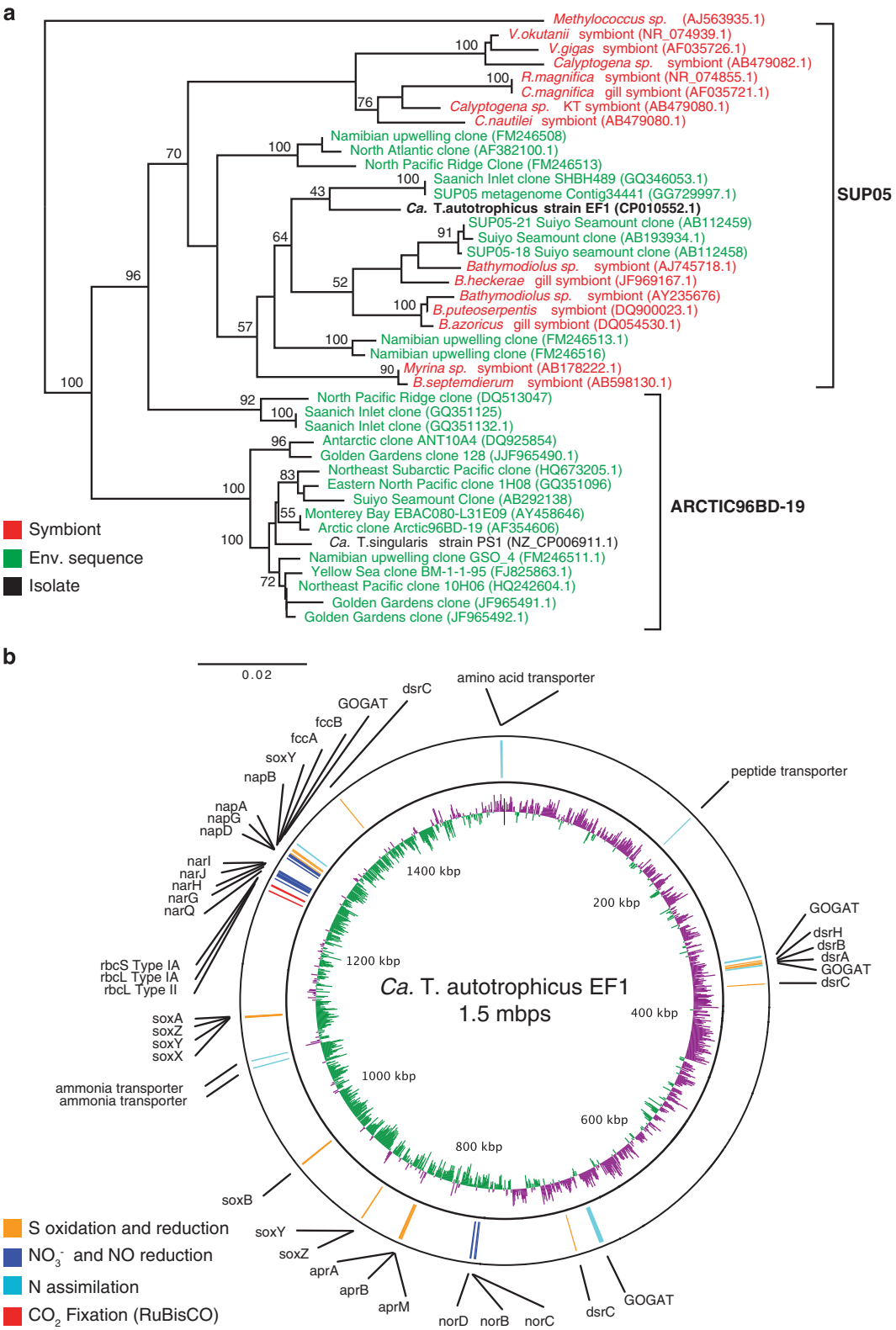


**Figure 1** Identity and genome of "*Ca.* T. autotrophicus" strain EF1. (**a**) Maximum likelihood tree of marine gamma sulfur-oxidizing bacteria 16S rRNA sequences. The tree was constructed using RAxML. Bootstrap values >40 are labeled at nodes (100 iterations). Red are symbionts, green are clones and black are isolates. (**b**) Circular representation of the strain EF1 genome. Innermost circle - GC skew (purple is negative skew and green is positive skew). Bars within solid lines indicate predicted coding regions colored by metabolic categories. Orange (S-oxidation and reduction genes): *sox* (sulfur oxidation), *apr* (adenosine-5-phosphate reductase), *fcc* (flavocytochrome c sulfide dehydrogenase), and *dsr* (dissimilatory sulfite reductase). Navy ($NO_3^-$ and NO reduction genes): *nap* (periplasmic nitrate reductase), *nar* (nitrate reductase), *nor* (nitric oxide reductase). Teal (N assimilation): GOGAT (glutamate synthase) and ammonia, amino acid, and peptide transporters. Red ($CO_2$ fixation): *rbcS* (RuBisCO small subunit), *rbcL* (RuBisCO large subunit).

GasPak Anaerobic strips added to an un-inoculated control serum bottle. Experiments (aerobic and anaerobic) were started by transferring 1000 cells in early exponential growth phase to new media. Culture purity was checked before and after each physiology study by sequence analysis and TRFLP.

## Results and Discussion

*Isolation of a representative from the SUP05 clade*

Six out of 192 culture wells inoculated with water from a redox gradient in Effingham Inlet were positive for growth after 21 days. Four of the cultures had identical 16S rRNA gene sequences and were identified as members of the SUP05 clade. One culture was identified as a closely related gamma-proteobacterium just outside the clade and one culture was identified as an epsilon-proteobacterium related to Arcobacter sp. associated with marine sponges. All are suspected sulfur-oxidizing bacteria. "*Ca.* T. autotrophicus" strain EF1 was selected for further study. The remaining cultures were cryopreserved.

Phylogenetic analysis and genome sequencing further confirmed the identity and genetic potential of "Ca. T. autotrophicus" strain EF1 (Figure 1). Strain EF1 is most closely related to sequences from the original SUP05 clade described by Walsh *et al.* (2009). These include environmental clones recovered from a broad range of OMZs and symbionts of deep-sea mollusk Bathymodiolus sp. Related sequences derived from the whole genomes of symbionts, and from environmental clones from the northeast Pacific ridge (Huber *et al.*, 2006), the Namibian upwelling system (Lavik *et al.*, 2009), Suiyo Seamount (Sunamara *et al.*, 2004), Saanich Inlet (Walsh *et al.*, 2009), the Eastern North Pacific and Eastern South Pacific (Stevens and Ulloa, 2008), the South Atlantic and North Pacific Gyres (Swan *et al.*, 2011) and Puget Sound (Marshall and Morris, 2013). The complete 16S rRNA gene sequences obtained from the "*Ca.* T. singularis" strain PS1 and "*Ca.* T. autotrophicus" strain EF1 genomes were also analyzed using the SILVA high quality ribosomal RNA database (Quast *et al.*, 2013). "*Ca.* T. singularis" strain PS1 was most closely related to sequences in the Arctic96BD-19 subclade (ZD0405 in SILVA). "*Ca.* T. autotrophicus" strain EF1 was most closely related to sequences in the SUP05 subclade.

The purity of "*Ca.* T. autotrophicus" strain EF1 was confirmed by several methods. TRFLP analyses identified a single 266 bp fragment using the restriction enzyme MboI and a 193 bp fragment using the restriction enzyme HaeIII (Supplementary Figure 2). These exactly match the fragments predicted from the 16S rRNA gene sequence. No other fragments were observed. Purity was further confirmed by quantitative fluorescence *in situ* hybridization (FISH) analyses with a SUP05 specific probe (GSO-1032) that exactly matches the 16S rRNA (Glaubitz *et al.*, 2013) (Supplementary Figure 2). All of the DAPI stained objects observed in three images (117/115/118) also hybridized to the SUP05 probe (total = 350/350). Cultures were subsequently cryopreserved and revived from glycerol stocks several times and under both aerobic and anaerobic growth conditions. Sequence and restriction analyses were used to check purity every time a culture was revived from glycerol stocks and before and after every physiology experiment. In every case, these analyses produced the same 16S rRNA gene sequences and the same restriction patterns. Transmission electron microscopy images revealed a single morphology, indicating that strain EF1 is a small (~0.3–0.4 μm) cocci shaped bacterium that produces extracellular globules resembling those produced by "*Ca.* T. singularis" PS1 (Supplementary Figure 3).

*Genetic potential of "Ca. T. autotrophicus" strain EF1*

The complete genome of "*Ca.* T. autotrophicus" strain EF1 has a GC content of 39.1%. It codes for 1,506 proteins, 92 pseudogenes, 3 rRNAs (5S, 16S and 23S) and 35 tRNAs. It has the genetic potential to grow as a facultatively anaerobic chemolithoautotroph that oxidizes sulfur and can reduce $O_2$, $NO_3^-$ and NO (Figure 1b). The genome codes for key enzymes for carbon fixation via the Calvin-Benson-Bassham (CBB) cycle, including cbbYCOQR, carbonic anhydrase and cytochrome $cbb_3$, one copy of the small subunit of RuBisCO (form I) and two copies of the large subunit of RuBisCO (form I and form II) (Badger and Bek, 2008). RuBisCO form I is composed of large and small subunits and is present in most chemoautotrophic bacteria, cyanobacteria, red and brown algae and all plants. Form II is a dimer of large subunits and is present in purple non-sulfur bacteria, some chemoautotrophic bacteria and in dinoflagellates. Some non-sulfur phototrophic bacteria contain both forms of RuBisCO. Strain EF1 is facultatively anaerobic and has likely adapted to use form IA RuBisCO or form II RuBisCO, depending on the ratio of $CO_2$ to $O_2$. Strain EF1 codes for complete glycolytic and phosphogluconate pathways (nonoxidative), and has most genes encoding the tricarboxylic acid (TCA) cycle. "*Ca.* T. autotrophicus" strain EF1 does not code for α-ketoglutarate dehydrogenase, a key TCA enzyme that is also missing from closely related symbiont genomes and a planktonic SUP05 population genome from Saanich Inlet (Walsh *et al.*, 2009). The absence of α-ketoglutarate dehydrogenase suggests that "*Ca.* T. autotrophicus" strain EF1 is an obligate autotroph (Wood *et al.*, 2004). Cytochrome c oxidase was also identified, along with a suite of genes for oxidative phosphorylation, further indicating the potential for strain EF1 to grow under aerobic conditions.

Genes for chemoautotrophic energy conservation were also identified. These include genes for inorganic sulfur oxidation (*fccAB, dsrABCH, aprABM, soxABXYZ* and *rhodanese sulfurtransferase*) and for aerobic and anaerobic respiration on $O_2$, $NO_3^-$ and NO (*narQGHIJ, napABGD* and *norBCD*) (Figure 1b). Sulfur oxidation genes confer the ability to oxidize a broad range of reduced sulfur compounds, including hydrogen sulfide ($H_2S$), elemental sulfur ($S^0$) and thiosulfate ($S_2O_3^{2-}$). Similar to Saanich Inlet SUP05, strain EF1 is also missing *soxCD* sulfur dehydrogenase genes, suggesting that they store $S^0$. The absence of soxCD has coincided with the ability of a closely related symbiont, *Ruthia magnifica,* to store sulfur in the form of extracellular globules (Newton *et al*., 2007).

Genes for anaerobic respiration confer the ability to carry out two of the four steps associated with sequential denitrification ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$). Strain EF1 has the genetic potential to reduce nitrate to nitrite ($NO_3^- \rightarrow NO_2^-$) and to reduce nitric oxide to nitrous oxide ($NO \rightarrow N_2O$) (Figure 1b), but lacks genes to reduce nitrite to nitric oxide ($NO_2^- \rightarrow NO$) or to reduce nitrous oxide to nitrogen gas ($N_2O \rightarrow N_2$). Although the ability to use $NO_2^-$ and $N_2O$ as terminal electron acceptors has been observed in environmental datasets (Walsh *et al*., 2009; Hawley *et al*., 2014; Murillo *et al*., 2014). We also found that strain EF1 is missing key genes required to use hydrogen gas (H2) as an electron donor, as previously reported for environmental SUP05 in the Guaymas Basin (Anantharaman *et al*., 2013)

"*Ca.* T. autotrophicus" strain EF1 has key genes required to use either $NH_4^+$ or organic nitrogen for biosynthesis (Figure 1b). These include genes that confer the ability to regulate intracellular nitrogen and to assimilate $NH_4^+$ and amino acids. Strain EF1 codes for two ammonia transporters, NADPH-dependent glutamate synthase (GS) and glutamine oxoglutarate aminotransferase (GOGAT), as well as components of amino acid (AA) and peptide ABC-transporters.

*Growth requirements*

Physiology experiments confirmed that strain EF1 is a facultatively anaerobic chemolithoautotroph that requires inorganic sulfur as a source of electrons (Figure 2). Batch cultures grew to an average final cell density of $3.6 \times 10^6$ cells/ml under aerobic conditions in seawater media containing 1 mM $S_2O_3^{2-}$ and to an average final cell density of $2.8 \times 10^6$ cells/ml under anaerobic conditions in seawater media amended with 1 mM $S_2O_3^{2-}$, 100 μm of $NO_3^-$ and sparged with a mixture of $N_2$:$CO_2$ (Figures 2a and b, respectively). Cells were unable to grow in aerobic seawater media that lacked $S_2O_3^{2-}$ or in anaerobic media that lacked either $S_2O_3^{2-}$ or $CO_2$. This indicates that "*Ca.* T. autotrophicus" strain EF1 requires a reduced form of inorganic sulfur for electrons and $CO_2$ for biosynthesis. We have found that strain EF1 cells survive two transfers with no additional sulfur (Supplementary Figure 3). This is likely due to their potential to store sulfur in extracellular globules (Walsh *et al*., 2009; Marshall and Morris, 2013; Hawley *et al*., 2014). No growth was observed when "*Ca.* T. autotrophicus" strain EF1 was grown with $H_2$ as an electron donor (Supplementary Figure 4).

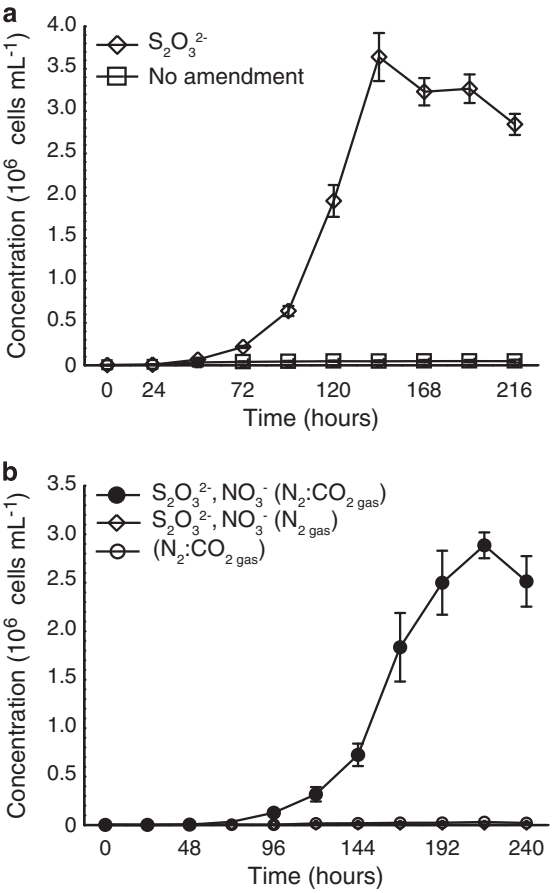


**Figure 2** Chemoautotrophic growth of "*Ca.* T. autotrophicus" strain EF1 under aerobic and anaerobic growth conditions. (**a**) Aerobic growth on copiotrophic seawater media from Puget Sound amended with 1mM $S_2O_3^{2-}$. (**b**) Anaerobic growth on seawater media bubbled with either $N_2$ only or $N_2$:$CO_2$ gas mix and amended with 1 mM $S_2O_3^{2-}$ and 100 μm $NO_3^-$. The controls were not amended with $S_2O_3^{2-}$, $NO_3^-$ or $CO_2$. Experimental treatments were conducted in triplicate.

We grew strain EF1 on copiotrophic media (Supplementary Table 2) enriched with $NO_3^-$ to further evaluate its potential for dissimilatory $NO_3^-$ reduction under anaerobic conditions (Figure 3). Cultures grew to similar cell densities at *in situ* concentrations of $NO_3^-$ (32 μm) and when 100 μm of additional $NO_3^-$ was added to the media (Figure 3a). Strain EF1 was not limited by $NO_3^-$ on copiotrophic seawater media. In both cases there was strong evidence for dissimilatory $NO_3^-$ reduction, as indicated by a 1:1 conversion of $NO_3^-$ to $NO_2^-$ (Figure 3b), as well as uptake of $NH_4^+$ (Figure 3c) and an increase

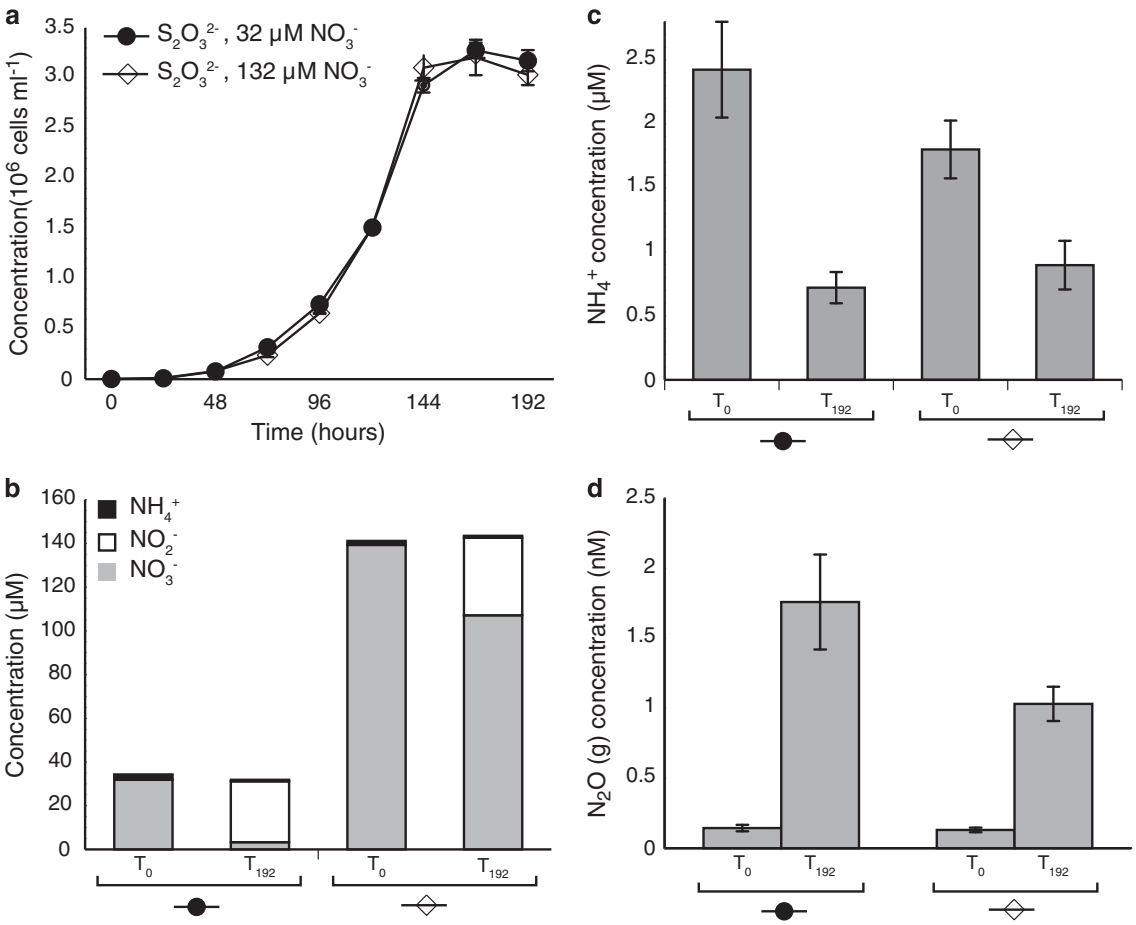


**Figure 3** Nitrogen utilization by "*Ca.* T. autotrophicus" strain EF1 on copiotrophic media. (**a**) Anaerobic growth on seawater media from Puget Sound with *in situ* concentrations of $NO_3^-$ (32 μM) and amended with 100 μM of additional $NO_3^-$ (total = 132 μM). (**b**) Concentrations of $NO_3^-$, $NO_2^-$, and $NH_4^+$. (**c**) Concentrations of only $NH_4^+$. (**d**) Concentrations of $N_2O$ measured by gas chromatography (Supplementary Materials and Methods). Experimental treatments were conducted in triplicate and nitrogen was measured at initial ($T_{0\ hour}$) and final ($T_{192\ hour}$) time points.

in the production of $N_2O$ (Figure 3d). Although NO was not added to the media, some NO may have been present in the seawater used to make the media or introduced into as a contaminant via the $N_2$:$CO_2$ gas mix used to sparge the media. Regardless, growth experiments support genomic predictions and previously published results from the field indicating that SUP05 produce $N_2O$ (Walsh *et al.*, 2009; Hawley *et al.*, 2014). EF1 cells did not produce $N_2$ gas (Supplementary Figure 4).

The potential for "*Ca.* T. autotrophicus" strain EF1 to respire $NO_3^-$ and assimilate $NH_4^+$ was evaluated further under nitrogen limitation using oligotrophic seawater media (Figure 4, Supplementary Table 2). Cultures grew to the highest final cell densities (average $1.2 \times 10^6$ cells/ml) in media that was amended with 1 mM $S_2O_3^{2-}$, 100 μm $NO_3^-$ and 5 μm $NH_4^+$ (Figure 4a). Cells grew to lower cell densities (average $6.2 \times 10^5$ cells/ml) and had slower growth rates when only $S_2O_3^{2-}$ and $NO_3^-$ were added to the media. There was a 1:1 conversion of $NO_3^-$ to $NO_2^-$ in treatments amended with $S_2O_3^{2-}$ and $NO_3^-$, or with $S_2O_3^{2-}$, $NO_3^-$ and $NH_4^+$ (Figure 4b). The amount of $NO_3^-$ converted to $NO_2^-$ increased four-fold (average increase from 5 μm to 22 μm) when $NH_4^+$ was added to the media and there was a decrease in $NH_4^+$ concentration over time (Figure 4b). Some growth was observed in $S_2O_3^{2-}$ only controls. This is likely due to the low concentrations of $NO_3^-$ (0.15 μM) and $NH_4^+$ (0.04 μm) present in oligotrophic seawater media (Supplementary Table 2). This experiment further confirmed that "*Ca.* T. autotrophicus" strain EF1 was unable to use $NO_2^-$ as a terminal electron acceptor (Figures 4c and d). When cells were amended with $NO_2^-$ instead of $NO_3^-$, no difference in growth was observed between amendments and controls. $NO_2^-$ and $NH_4^+$ concentrations remained constant throughout the experiment. The potential to assimilate amino acids (Promega, Madison, WI, USA, Amino Acid Mixture) in oligotrophic seawater was also tested. Cultures were grown in media amended with 1 mM $S_2O_3^2$, 100 μM $NO_3^-$ and an amino acid mixture (5 μM of N) (Supplementary Figure 4). There was no discernable difference

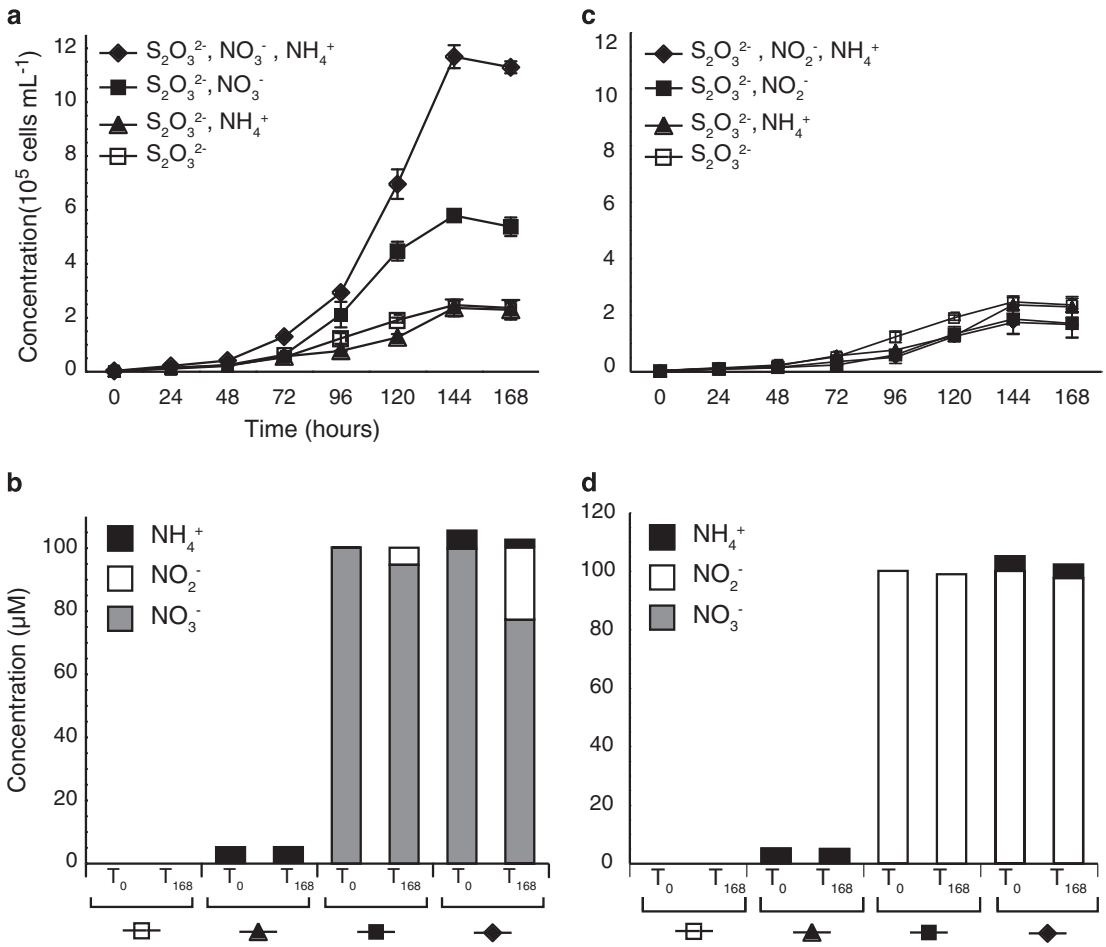


**Figure 4** Nitrogen utilization of "*Ca.* T. autotrophicus" strain EF1 on oligotrophic media. Anaerobic growth on seawater media from the Sargasso Sea amended with (**a**) $NO_3^-$ (100 μM), $NH_4^+$ (5 μM) and with only $S_2O_3^{2-}$ (1 mM) and (**b**) $NO_2^-$ (100 μM), $NH_4^+$ (5 μM) and with only $S_2O_3^{2-}$ (1 mM). (**c** and **d**) Concentrations of $NO_3^-$, $NO_2^-$ and $NH_4^+$ measured at initial ($T_{0\ hour}$) and final ($T_{168\ hour}$) time points in (**a**) and (**b**), respectively. All experimental treatments and nutrient measurements were conducted in triplicate.

between cultures amended with amino acids and unamended controls, indicating that strain EF1 prefers $NH_4^+$ for biogenic nitrogen.

*Roles in the marine nitrogen cycle*

Evidence that cultured SUP05 produce $NO_2^-$ suggests that related strains are a potential source of $NO_2^-$ in OMZs. There is strong molecular evidence indicating that environmental SUP05 are capable of mediating sequential steps in denitrification. Because these environmental sequence data provide a fragmented view of a population of cells, it is also possible that different SUP05 cells carry out different steps in denitrification, depending on the diversity of SUP05 in a population, on the concentrations of substrates, and on the range of interactions within a community. Genetic and physiology data from this study suggest that a single strain of SUP05 carries out two non-sequential steps in denitrification. Both cultivation-dependent and cultivation-independent data indicate that the first step in denitrification ($NO_3^-$ reduction to $NO_2^-$) is highly conserved (Canfield *et al.*, 2010; Walsh *et al.*, 2009; Hawley *et al.*, 2014; Murillo *et al.*, 2014), while the potential to carry out subsequent steps in denitrification are not always identified (Murillo *et al.*, 2014). In addition, SUP05 are often most abundant in areas where $NO_2^-$ accumulates (Canfield *et al.*, 2010; Hawley *et al.*, 2014; Murillo *et al.*, 2014). Glaubitz *et al.* (2013) reported a positive correlation between SUP05 and nitrite concentrations in the Black Sea. Although heterotrophic $NO_3^-$ respiration is currently considered the primary process leading to $NO_2^-$ accumulation within OMZs, there is ample genetic and physiological data suggesting that sulfur-oxidizing chemoautotrophs from the SUP05 clade are a potential source of $NO_2^-$, fueling competing nitrogen removal processes in the ocean.

"*Ca.* T. autotrophicus" strain EF1 growth was also limited by $NH_4^+$. In the mid 1950s, Baalsrud and Baalsrud, and van Niel found that the S-oxidizing and $NO_3^-$-reducing bacterium *Thiobacillus denitrificans* required $NH_4^+$ or amino acids for biosynthesis (Baalsrud and Baalsrud, 1954; van Niel, 1955). Genes for $NH_4^+$ transport and amino acid assimilation were identified in the strain EF1 genome and in a SUP05 metagenome assembled from Saanich Inlet (Walsh

*et al.*, 2009). They were also expressed by SUP05 in Saanich Inlet and the Southern Ocean (Wilkins *et al.*, 2013; Hawley *et al.*, 2014). The biogenic nitrogen requirement of strain EF1 is low. For example, if "*Ca.* T. autotrophicus" strain EF1 cells have 10 fg of carbon/cell and are at Redfield ratios for carbon and nitrogen (106:16), we estimate that they required ~0.22 μM of nitrogen for biosynthesis in cultures grown on natural seawater media. By comparison, they reduced ~30 μM of $NO_3^-$ to ~30 μM $NO_2^-$ during respiration. There was no evidence that they respired $NO_2^-$ or that they used amino acids instead of $NH_4^+$ for biosynthesis (Supplementary Figure 4B). These data support the conclusion that strain EF1 requires relatively high concentrations of $NO_3^-$ for respiration and relatively low concentrations of $NH_4^+$ for biosynthesis.

"*Ca.* T. autotrophicus" strain EF1 also produced $N_2O$. Members of the SUP05 bacteria expressed genes to produce $N_2O$ in regions of significant $N_2O$ cycling and emission (Codispoti *et al.*, 2001). A recent study by Babbin and colleagues (Babbin *et al.*, 2015) suggests that rapid $N_2O$ cycling in the suboxic ocean could lead to future increases in $N_2O$ emissions. Environmental sequence data indicate that SUP05 are broadly distributed and abundant in these regions. Growth experiments support field expression data, suggesting that $N_2O$ producing chemoautotrophic SUP05 have important roles in biologically driven nitrogen loss from the ocean (Walsh *et al.*, 2009; Hawley *et al.*, 2014; Murillo *et al.*, 2014).

## Conclusions

Data resulting from the cultivation of "*Ca.* T. autotrophicus" strain EF1 have expanded the roles of SUP05 in the marine nitrogen cycle. They suggest that SUP05 are a potential source of $NO_2^-$ and sink for $NH_4^+$ in anoxic marine waters. In the conceptual metabolic coupling model of an OMZ proposed by Hawley *et al.* (2014), ammonia-oxidizing archaea are the source of $NO_2^-$ in the upper and lower oxycline and SUP05 are the hypothesized source of $NH_4^+$ at and below the lower oxycline. If under the right conditions SUP05 produce $NO_2^-$, then elevated concentrations of $NH_4^+$ are not required to account for the elevated $NO_2^-$ concentrations at and below the lower oxycline. There is strong evidence indicating that $NO_3^-$ reduction is an independent process that can account for a significant fraction of $NO_2^-$ accumulation in OMZs (Lam *et al.*, 2009). We hypothesize that SUP05 contribute to the secondary $NO_2^-$ maxima when $NO_3^-$ fluxes are relatively high, ammonia concentrations are relatively low and oxygen concentrations fall below 4 μM. Our physiological data suggest that SUP05 cells require between 0.03 to 0.06 μM of $NH_4^+$ for every μM of $NO_2^-$ produced (Figures 3 and 4). This is in stark contrast to ammonia-oxidation, which is 1:1. It suggests that chemoautotrophic members of the SUP05 clade are an important source of $NO_2^-$ in OMZs where $NH_4^+$ is limiting.

## Conflict of Interest

The authors declare no conflict of interest.

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