Dynamics of Metabolic Activities and Gene Expression in the *Roseobacter* Clade Bacterium *Phaeobacter* sp. Strain MED193 during Growth with Thiosulfate

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Metagenomic analyses of surface seawater reveal that genes for sulfur oxidation are widespread in bacterioplankton communities. However, little is known about the metabolic processes used to exploit the energy potentially gained from inorganic sulfur oxidation in oxic seawater. We therefore studied the *sox* gene system containing *Roseobacter* clade isolate *Phaeobacter* sp. strain MED193 in acetate minimal medium with and without thiosulfate. The addition of thiosulfate enhanced the bacterial growth yields up to 40% in this strain. Concomitantly, *soxB* and *soxY* gene expression increased about 8-fold with thiosulfate and remained 11-fold higher than that in controls through stationary phase. At stationary phase, thiosulfate stimulated protein synthesis and anaerobic CO₂ fixation rates up to 5- and 35-fold, respectively. Several genes involved in anaerobic CO₂ fixation (i.e., pyruvate carboxylase, propionyl coenzyme A [CoA], and crotonyl-CoA carboxylase) were highly expressed during active growth, coinciding with high CO₂ fixation rates. The high expression of key genes in the ethylmalonyl-CoA pathway suggests that this is an important pathway for the utilization of two-carbon compounds in *Phaeobacter* sp. MED193. Overall, our findings imply that *Roseobacter* clade bacteria carrying *sox* genes can use their lithotrophic potential to gain additional energy from sulfur oxidation for both increasing their growth capacity and improving their long-term survival.

Heterotrophic bacteria in the surface ocean typically rely on dissolved organic carbon compounds for obtaining energy and cellular building blocks. Nevertheless, metagenomic inventories of the world's oceans reveal a substantial diversity and abundance of genes, allowing the use of alternative energy sources, such as hydrogen gas, CO, and inorganic sulfur, in oxic surface waters (1, 2). For example, using *soxB* as a marker gene, about 10% of the bacteria in the Sargasso Sea were identified to carry the sulfur oxidation (Sox) enzyme system (1). Also, community expression analysis in surface waters at the ALOHA Station in the North Pacific subtropical gyre showed that several of the genes in the *sox* cluster were actively expressed (3). Considering the abundance of *sox* genes and the fact that reduced inorganic sulfur compounds are usually found in very low concentrations in surface seawater, if at all, it remains unclear what the substrates for *sox*-encoded proteins are.

The sulfur-oxidizing enzyme system encoded by *sox* genes is widely present in heterotrophic, phototrophic, lithotrophic, and methylotrophic bacteria. The complete *sox* gene cluster contains 15 genes, which oxidize thiosulfate to two molecules of sulfate without any intermediates. The number of *sox* genes differs between organisms, from a complete set in, e.g., *Paracoccus pantotrophus* and *Rhodobacter palustris*, to partial sets in *Chlorobium tepidum* and *Allochrocatium vinosum* (4). The core set of genes that allows this process is *soxXYZABCD*, which encode the dimeric proteins SoxXA, SoxYZ, and SoxCD and the nondimeric protein SoxB, which are functional in the periplasm. The *sox* enzyme system was first reported in the alphaproteobacterium *P. pantotrophus* (5), after which individual components have been further genetically and biochemically characterized (4, 6). Mutational analysis of *soxB* and *soxY* in the phototrophic purple sulfur bacterium *A. vinosum* and *Bradyrhizobium japonicum* revealed that these genes are essential for thiosulfate oxidation (7, 8). The di-

meric SoxYZ does not hold metals or cofactors and covalently binds with sulfur of various oxidation states (9). The dinuclear manganese-containing SoxB, proposed to function as a sulfate thiol esterase or sulfate thiol hydrolase (9), indicates the presence of the *sox* enzyme system (10). The central importance of *soxB* and *soxY* in the *sox* gene system suggests that the study of the expression of these genes could be useful for monitoring the regulation of inorganic sulfur oxidation across different stages of growth in bacteria.

Thiosulfate oxidation plays a major role in the biological sulfur cycle, where it yields energy for bacterial cellular metabolism and growth in a variety of anaerobic marine environments (11–14), such as sediments or the interface between anoxic and oxic waters, where reduced sulfur compounds abound (7, 15–17). Still, few studies have investigated the ecological benefit and/or the potential function of *sox* genes of marine bacteria in aerobic seawater. In the *Roseobacter* clade member *Ruegeria pomeroyi*, thiosulfate oxidation increases the growth yield by up to 52% in acetate-grown aerobic batch culture compared to that in cultures without thiosulfate (1). Also, in *Pseudovibrio*, growth in aerobic cultures was stimulated by thiosulfate (16). In various contexts, it has been suggested that additional energy sources, such as reduced inor-

Received 19 June 2014 Accepted 27 August 2014

Published ahead of print 29 August 2014

Editor: H. Nojiri

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doi:10.1128/AEM.02038-14

ganic sulfur compounds, could benefit heterotrophic bacteria by supplying energy (1, 18–20). Thus, analysis of *sox* gene transcription patterns could potentially provide information on how this enzyme system is used by marine bacteria.

Although rather unexplored in the field of marine microbial ecology, anaplerotic reactions, including anaplerotic CO₂ fixation, are a conserved feature in the metabolism of all living organisms (21, 22). Thus, most heterotrophic bacteria assimilate CO₂ into biomass through various carboxylase reactions (23–25). These include, for example, anaplerotic enzymes, such as malic enzyme, phosphoenolpyruvate (PEP) carboxykinase, pyruvate carboxylase, and PEP carboxylase, that assimilate CO₂ and thereby replenish carbon molecules in the tricarboxylic acid cycle when cycle intermediates are used for biosynthesis. Such anaplerotic CO₂ fixation depends on the availability of substrate, the species composition in bacterial communities, and the metabolic state of the organisms (26–29). Still, in contrast to the fixation of inorganic carbon by ribulose-1,5-bisphosphate carboxylase/oxygenase or other autotrophic CO₂ fixation pathways, anaplerotic CO₂ fixation does not result in net CO₂ fixation in heterotrophic bacteria. Recent work on natural bacterioplankton communities from northwestern U.S. Pacific coastal waters, using stable isotope probing, shows that representatives of a very wide variety of major taxa have the ability to carry out CO₂ fixation through alternative pathways (26), including different members of the SAR11 and *Roseobacter* clades. These findings extend a previous report of significant bicarbonate uptake by prokaryotes at stationary phase in Arctic seawater cultures incubated in the dark (30).

Few studies have explored the potential for anaplerotic CO₂ fixation in model species of marine heterotrophic bacteria. In their genome analysis of *Roseobacter denitrificans*, Swingle et al. (19) showed the presence of a set of genes encoding anaplerotic enzymes that could be involved in anaplerotic CO₂ fixation. Thereafter, Tang et al. (20) revealed that *R. denitrificans* could fix CO₂ by pyruvate and/or phosphoenolpyruvate carboxylase. These studies thus support earlier suggestions of the CO₂-fixing role of enzymes, such as pyruvate carboxylase (1, 29). Also in the proteorhodopsin-containing organism *Polaribacter* sp. strain MED152, anaplerotic enzymes were suggested to account for the observed stimulation of CO₂ fixation in cultures exposed to light compared to the levels of CO₂ fixation of cultures in the dark (18). Another anaplerotic reaction to fix inorganic carbon is through reductive carboxylation in the ethylmalonyl coenzyme A (CoA) pathway, mediated by the enzymes crotonyl-CoA carboxylase/reductase and propionyl-CoA carboxylase, as originally described in the purple sulfur bacterium *Rhodobacter sphaeroides* (24). The genes encoding these carboxylases are present in all the organisms suggested to use the ethylmalonyl-CoA pathway for acetate assimilation (31). Taken together, these findings imply that experimental work establishing links between bacterial growth, anaplerotic CO₂ fixation, and the expression of genes involved in these processes in marine bacteria would be important.

Bacteria in the *Roseobacter* clade are widespread inhabitants of surface seawater, but they are also found in other marine environments, including the deep sea and anaerobic sediments (17, 32–34). While they are most abundant in coastal waters, *Roseobacter* clade bacteria are also frequent in open-ocean environments (35–37). The metabolism of roseobacters is highly versatile (21, 37, 38), with roseobacters encompassing bacteria with the capability for aerobic anoxygenic photosynthesis; anaplerotic CO₂ fixation;

aromatic compound degradation; and H₂, CO, and sulfur oxidation (1, 21, 39–42). Genes involved in sulfur and nutrient transformations and anaplerotic CO₂ fixation are among those highly expressed in oxic marine environments (3, 43, 44). Still, compared to the large amount of knowledge on the role of this group in organic sulfur compound transformations, e.g., dimethylsulfoniopropionate, that has been gained (35, 45), little or no information about the growth and transcriptional response of *Roseobacter* clade members upon exposure to reduced inorganic sulfur compounds in oxic marine environments is available.

The aim of the present study was to gain insights into how inorganic sulfur oxidation impacts the metabolism of marine bacteria. We therefore carried out experiments with the *Roseobacter* clade isolate *Phaeobacter* sp. MED193 in minimal medium with and without thiosulfate. *Phaeobacter* sp. MED193 has a *sox* gene cluster comprising 11 genes, including *soxXYZABCD*, essential for thiosulfate oxidation (*soxEFG* and *soxH* are absent). We investigated the temporal dynamics in growth responses, protein synthesis, and anaplerotic CO₂ fixation rates from early exponential phase to late stationary phase. Further, to characterize how these processes are regulated, we quantified the gene expression profiles of sulfur oxidation genes (*soxB* and *soxY*) and anaplerotic CO₂ fixation genes over time.

MATERIALS AND METHODS

Bacterial isolate and culture conditions. *Phaeobacter* sp. strain MED193 was originally isolated from surface water (0.5-m depth) in the northwestern Mediterranean Sea at the Blanes Bay Microbial Observatory (41°40'N, 2°48'E). Contextual information on the isolation of the strain in relation to other bacterioplankton has been reported by Lekunberri et al. (46). MED193 was obtained by cultivation on ZoBell agar plates and stored at –80°C in marine broth (Difco) with glycerol at a final concentration of 25%. The GenBank accession number of the MED193 genome sequence is [NZ_AANB000000000](#). The alignment used to construct the phylogenetic tree was generated using the MUSCLE program (47) and trimmed using trimAl software (48) to eliminate highly diverged regions (Fig. 1). The maximum likelihood tree was inferred with the RAxML program (49), using the GTR model with gamma distribution of rates (implemented as GTRGAMMA). Bootstrap analysis was performed with 100 replicates.

Isolate MED193 was grown in minimal medium with 5 mM acetate as the carbon source. The minimal medium contained 750 ml of artificial seawater (28.5 g of artificial sea salt per liter; Sigma Chemical Co., St. Louis, MO) and was supplemented with 150 ml of Tris-HCl solution (containing 1 M Tris-HCl with the pH adjusted to 7.5, 87 mg K₂HPO₄, and 1.5 g NH₄Cl). The medium was also supplemented with 50 ml of 0.1 mM FeEDTA solution (Sigma Chemical Co., St. Louis, MO), 0.1% vitamin solution (which contains 2 mg of biotin and folic acid, 10 mg pyridoxine-HCl, and 5 mg each of riboflavin, thiamine, nicotinic acid, pantothenic acid, and *p*-aminobenzoic acid with 0.1 mg cyanocobalamin per liter), and 0.1% mineral solution [3.92 mg CuSO₄·5H₂O, 13.63 mg ZnSO₄·7H₂O, 1.3 mg NiCl₂, 3.82 mg Co(NO₃)₂·6H₂O, 3.82 mg H₃BO₃ per liter]. Also, the vitamin levels of yeast extract were added (50 mg per liter; this concentration does not in itself support growth).

Bacterial growth experiments. Three sets of growth experiments were done with *Phaeobacter* sp. MED193. For each experiment, cells were first grown overnight in marine broth, after which for each treatment and replicate 1 ml of an overnight culture (optical density [OD] at 600 nm [OD₆₀₀], 1.13) was transferred into 300 ml of minimal medium with 5 mM acetate. All treatments and controls were done in three biological replicates, and the cultures were incubated in the dark at 22°C on shakers at 142 rpm. Growth was measured by determining the OD₆₀₀ (Beckman DU 640 spectrophotometer).

In experiment 1, the acetate medium was complemented with four

FIG 1 Maximum likelihood phylogenetic tree based on nearly complete 16S rRNA gene sequences depicting *Phaeobacter* sp. MED193 to be closely related to roseobacters. Grouping into clade 1 to 5 roseobacters follows the nomenclature by Newton et al. (38). For taxa with full Latin names, 16S rRNA gene sequences were from type strains of taxonomically validly described bacterial species. GenBank accession numbers are in parentheses. The sequence of *Rhodobacter sphaeroides* (GenBank accession number X53853) served as an outgroup (not shown). Numbers at the nodes indicate bootstrap values larger than 50%. The scale bar denotes the number of substitutions per nucleotide position.

concentrations of thiosulfate (10, 20, 40, and 100 mM), while the controls received no thiosulfate. Growth was measured through determination of the OD. In experiment 2, to determine thiosulfate utilization during bacterial growth, acetate medium with 10 mM thiosulfate was used (the controls received no thiosulfate). Growth was measured through determination of the OD, and the concentration of thiosulfate was monitored by the iodometric/thiosulfate titration method (50). Five-milliliter samples from each triplicate culture were collected at 0, 4, 6, 10, 14, 24, 30, and 36 h of incubation. Each sample received 500 μ l of formaldehyde (37%) and 1 ml of sodium acetate buffer (containing 120 g/liter of sodium acetate and 10 ml/liter concentrated acetic acid [1%, vol/vol]) with 600 μ l of iodine solution (0.05 M). Thereafter, the mixture was titrated with 0.01 M standard thiosulfate solution until the pale yellow color disappeared. The concentration of thiosulfate ($[S_2O_3^{2-}]$) in the sample was calculated by using the following equation: $[(2 \cdot D \cdot 0.05) - (F \cdot 0.01)]/C$, where D is the volume of iodine (in ml) added to sample, F is the volume of titrant (in ml) used in the sample, and C is the volume of sample (in ml). To control for the chemical stability of thiosulfate in the medium used, thiosulfate concentrations were also determined in separate triplicate bottles with medium only (no bacterial inoculum was added).

In experiment 3, the acetate medium was supplemented with 10 mM thiosulfate, while the controls received no thiosulfate. In this experiment, in addition to determination of the OD, we determined protein synthesis rates (through leucine incorporation rate measurements) and anaplerotic CO_2 fixation rates (through bicarbonate incorporation rate measurements) and collected RNA samples for gene expression analyses at five time points.

Leucine incorporation rates. Protein synthesis was estimated by determination of the level of incorporation of leucine into bacterial protein (51). Triplicate samples of 1.2-ml bacterial cultures were incubated with 40 nM [3H]leucine (specific activity, 172 Ci mmol $^{-1}$; Amersham). Samples were incubated in the dark at 22°C for 0.45 to 2.50 h, and controls

were killed with 120 μ l of 50% cold trichloroacetic acid (TCA). The incubation was stopped by adding 120 μ l of 50% cold TCA to each tube. Samples were stored at $-20^\circ C$ and then processed by the centrifugation method (52). The radioactivity in the samples was measured by liquid scintillation counting (Win spectral 1414; Wallac, Finland).

Anaplerotic CO_2 fixation. Bicarbonate uptake rates were measured essentially as previously described (18). At five time points, duplicate 20-ml samples of bacterial culture were collected. Controls received 2 ml of formaldehyde (final concentration, 2%). Twelve microliters of [^{14}C]bicarbonate (final concentration, 3 μ Ci; DHI, Denmark) was added to the controls and the samples, followed by incubation in the dark at 22°C for 3 to 4 h. Incubation was terminated by addition of 2 ml of formaldehyde solution to the samples. Subsequently, samples were filtered through 0.2- μ m-pore-size polycarbonate membrane filters (diameter, 25 mm; Pall), and the filters were exposed to fumes of concentrated HCl for 12 to 24 h. Then, the filters were placed in scintillation vials with 4 ml of scintillation cocktail (Perkin-Elmer), stored in the dark for at least 24 h, and counted by liquid scintillation. Bicarbonate uptake rates were calculated on the basis of the standard radioactive carbon assimilation technique (53). For both anaplerotic CO_2 fixation and leucine incorporation rates, statistical analyses were done by one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* honestly significant difference (HSD) test, using GraphPad Prism (v6.0) software (GraphPad Software, San Diego, CA). Differences with P values of <0.05 were regarded as significant.

RNA isolation. Samples for RNA isolation were collected from each replicate culture during experiment 2 after 4, 7, 10, 24, and 26 h of incubation. One milliliter of bacterial culture was stabilized by addition of 2 ml of RNeasy lysis reagent (Qiagen, Valencia, CA) to the culture. Cells were thereafter harvested by centrifugation at $15,000 \times g$ for 10 min, the supernatant was discarded, and the pellet was immediately frozen at $-80^\circ C$. Subsequently, the pellets were resuspended in RNA collection tubes and total RNA extraction was performed using an RNeasy minikit

TABLE 1 Nucleotide sequences of PCR primers used to assay gene expression by qRT-PCR

Protein	Gene	Locus tag	Sequence		Amplicon size (bp)
			Forward primer	Reverse primer	
Sulfur oxidation protein B	<i>soxB</i>	MED193_05734	5'-CCCACTCGGAATCAAAGATG-3'	5'-AACCTGATGCGATGACCTTC-3'	117
Sulfur oxidation protein Y	<i>soxY</i>	MED193_05749	5'-AACACCAGGGGTGGGTAC-3'	5'-CGTTGTTGATGATCGGATTG-3'	175
Pyruvate carboxylase	<i>pyc</i>	MED193_07548	5'-AGCTCTTCTCGGTTGAATGC-3'	5'-GCATCTGGGTCATCACATTG-3'	126
PEP carboxykinase	<i>pckA</i>	MED193_06644	5'-CTTTGACGGCTGGATTACTG-3'	5'-ATTGCGGTCTTTTCGACCTC-3'	87
Crotonyl-CoA carboxylase	<i>crr</i>	MED193_10433	5'-CATCCTGGTTGCAGTGAATG-3'	5'-ATGCCTCTGGTATCGTTTGG-3'	87
Propionyl-CoA carboxylase	<i>pcc</i>	MED193_21711	5'-TTGCTTACGGTGAAGCTACG-3'	5'-GCCAGGCATAGTTGAAATCAC-3'	118
DNA gyrase subunit A	<i>gyrA</i>	MED193_04431	5'-TGCGGAAGAGATCGAAGAAG-3'	5'-TAATCATGGGCCGAGGTTAG-3'	122
Recombinase A	<i>recA</i>	MED193_04366	5'-CAGGGCCGTAGATTCAATG-3'	5'-TCTCTGGGCTTGATATTGC-3'	73
RNA polymerase sigma 70 factor	<i>rpoD</i>	MED193_08543	5'-GCATCGACATCACATCTTCGA-3'	5'-GATCAGCTCAACCAAGTGC-3'	70

according to the manufacturer's instructions (Qiagen, Valencia, CA). In the final step, RNA was eluted in 50 μ l of nuclease-free water (Sigma). RNA was treated with DNase (Turbo DNA-free kit; Ambion) to remove genomic DNA. The total quantity of RNA and RNA purity were determined on a NanoDrop ND 2000 spectrophotometer (NanoDrop Technologies, Wilmington, MA), and samples were stored at -80°C .

Gene expression analysis by qRT-PCR. Quantitative real-time reverse transcription-PCR (qRT-PCR) was performed on an ABI StepOne real-time PCR system (Applied Biosystems). Prior to qRT-PCR, the total RNA was adjusted to a final concentration of 1 ng μ l $^{-1}$ with nuclease-free water. The primers listed in Table 1 were designed using the Primer3Plus web interface (54) on the basis of the genome sequence. The SYBR green-based qRT-PCR was carried out by using a one-step RT-PCR kit (Applied Biosystems), and each reaction was performed in a total volume of 10 μ l consisting of 1 μ l of RNA as the template, 0.08 μ l of reverse transcription reagents (which include MultiScribe reverse transcriptase [50 U/ml] and RNase inhibitor [20 U/ml]), and 5 μ l of 2 $\times$ SYBR green master mix with 0.45 μ M each primer. The one-step RT-PCRs were performed under the following conditions: 48 $^{\circ}\text{C}$ for 30 min and 95 $^{\circ}\text{C}$ for 10 min, followed by 40 cycles of 95 $^{\circ}\text{C}$ for 15 s and 60 $^{\circ}\text{C}$ for 1 min. Contamination of the reaction mixtures with genomic DNA and melting curve analyses were done to confirm RNA purity and target gene amplification in the reaction. All qRT-PCRs were performed in three technical replicates for each of three biological replicate samples per treatment along with negative control PCRs (in which the reaction mixtures did not contain RNA).

To obtain adequate reference genes for normalization of gene expression levels, the 16S rRNA, *rpoB*, *rpoD*, *recA*, and *gyrB* genes were evaluated. qRT-PCRs were carried out as described above. According to the minimum information for publication of quantitative real-time PCR experiment (MIQE) guidelines, three reference genes were selected for analysis (55). The expression stability of the reference genes was determined with the Normfinder (56) and Best Keeper (57) software tools. The expression levels of the genes encoding RNA polymerase subunit D (*rpoD*), recombinase A (*recA*), and DNA gyrase (*gyrB*) remained stable in the treatments mentioned above; therefore, these were selected as reference genes.

The relative gene expression ratios of different genes were calculated by the comparative threshold cycle ($2^{-\Delta\Delta CT}$) method, which combines gene quantification and normalization into a single calculation, incorporating the PCR efficiencies of the target and reference genes to correct for differences between the assays (58). Statistical analyses were done by one-way ANOVA followed by Tukey's *post hoc* HSD test using GraphPad Prism (v6.0) software. Differences with *P* values of <0.05 were regarded as significant.

RESULTS

Experiment 1: impact of thiosulfate on growth. To study the effect of thiosulfate on the growth of marine heterotrophic bacteria, the *Roseobacter* clade strain MED193 was grown in minimal acetate medium with and without thiosulfate. Phylogenetically, strain MED193 is a member of the genus *Phaeobacter* (Fig. 1), which, together with, for example, *Ruegeria pomeroyi* DSS-3, is part of the clade 1 roseobacters, as defined by Newton et al. (38). In *Phaeobacter* sp. MED193, the *sox* gene cluster consists of the 11 genes *soxXYZABCDVSRW*, and this set of genes is highly conserved among distinct *Roseobacter* clade members (Fig. 2). In addition, several roseobacters, including *Ruegeria pomeroyi* (clade 1), contain the *soxEF* genes downstream of *soxD*. The *Roseobacter litoralis* genome further contains the *soxHG* genes and thus contains the full complement of *sox* genes (Fig. 2).

Addition of thiosulfate at 10, 20, and 40 mM concentrations improved the maximum growth yields about 30% after 24 h, while enrichment with 100 mM thiosulfate increased the growth yield nearly 40% compared to that for the unamended controls (Fig. 3A). Moreover, in the 100 mM treatment, the optical density did not decrease in the stationary phase like it did in the cultures with lower thiosulfate concentrations, but it remained stable for the last 48 h of the experiment (Fig. 3A).

Experiment 2: consumption of thiosulfate. To determine the ability for consumption of thiosulfate, we analyzed the concentration of thiosulfate in the medium during different time points of bacterial growth (Fig. 3B). The concentration of thiosulfate in the medium was stable at 10 mM during early stages of growth. The concentrations of thiosulfate started decreasing at 10 h and onwards, reaching 1 mM after 36 h of incubation and well into stationary phase.

Experiment 3: impact of thiosulfate on leucine incorporation and anaplerotic CO₂ fixation. A time course experiment to monitor the temporal development of metabolic activities was done by growing MED193 in cultures with 5 mM acetate with thiosulfate (10 mM) and without thiosulfate (Fig. 4A). In agreement with the findings in experiment 1, after 24 h of incubation, thiosulfate increased the growth yield 28% compared to that for the unamended control. As expected due to the active growth, leucine

FIG 2 Arrangement of *sox* cluster genes in *Phaeobacter* sp. MED193 compared with that in other roseobacters. Clade designations below the strain names denote the *Roseobacter* clades to which the strains were assigned by Newton et al. (38) (bacteria currently assigned to clade 4 do not have *sox* genes). Gene homologues are indicated by colors. Genes without color in *R. litoralis* are genes for two conserved hypothetical proteins. Sequence information for the strains was retrieved from GenBank.

incorporation rates in exponential phase were ca. 8-fold higher than those in stationary phase ($F = 16.95$, $P < 0.05$). Similarly, anaplerotic CO_2 fixation rates were >10-fold higher during exponential phase ($F = 20.57$, $P < 0.05$). Leucine incorporation rates during the exponential phase were, on average, 67% higher for cultures with thiosulfate than for the controls ($F = 18.32$, $P < 0.05$), while no differences in anaplerotic CO_2 fixation between cultures with thiosulfate and the controls were observed at these times ($F = 0.91$, $P > 0.05$). Although the overall metabolic activ-

ities were much lower in stationary phase (Fig. 4B), the presence of thiosulfate at this phase caused an approximately 5-fold stimulation of leucine incorporation compared to that for the controls ($F = 46.94$, $P < 0.05$). The thiosulfate-induced response of anaplerotic CO_2 fixation rates in stationary phase was even more pronounced, with an increase of up to 35-fold being detected in cultures with thiosulfate compared to that in control cultures ($F = 21.29$, $P < 0.05$) (Fig. 4C).

Experiment 3: impact of thiosulfate on gene expression patterns. The relative gene expression levels of the *soxB* and *soxY* genes were similar to those for the controls after 4 h. Then, from 7 h onwards, expression levels were nearly 5-fold higher in cultures with thiosulfate than in cultures without thiosulfate. The *soxB* gene was upregulated in cultures with thiosulfate after 7 h to 26 h, and relative gene expression levels peaked at 7 to 10 h and started declining toward stationary phase (one-way ANOVA, Tukey's *post hoc* test, $P < 0.05$) (Fig. 5A). The *soxY* gene expression level was even more strongly stimulated by thiosulfate from 7 h onwards, and transcript abundance declined only slowly into stationary phase ($F = 27.13$, $P < 0.05$) (Fig. 5B).

For anaplerotic enzyme (CO_2 fixation) genes, the relative expression level of the pyruvate carboxylase (*pyc*) (Fig. 5C) gene was about 1.0 in the exponential growth phase and decreased to about 0.4 at stationary phase. The phosphoenolpyruvate carboxykinase (*pckA*) expression level increased from a low of about 0.1 at 4 h to a peak of 1.5 at 10 h in the controls, while the peak was delayed until 24 h with thiosulfate (Fig. 5D). The highest level of crotonyl-CoA carboxylase/reductase (*ccr*) relative gene expression (nearly 1.5) was observed after 7 h in the controls, and it remained higher in the controls than in the cultures with thiosulfate throughout the experiment (Fig. 5E). Similarly, the expression level of propionyl-CoA carboxylase gene (Fig. 5F) peaked at about 1.5 after 7 h in the controls and then decreased to a level of about 0.2 in stationary phase.

DISCUSSION

We set out to study the impact of thiosulfate on growth, protein synthesis rates, and anaplerotic CO_2 fixation, together with anal-

FIG 3 Growth of MED193 in minimal medium with acetate and thiosulfate. (A) Growth in medium with 5 mM acetate and different concentrations of thiosulfate during experiment 1. (B) Bacterial growth and thiosulfate concentrations in medium with 5 mM acetate and 10 mM thiosulfate or without thiosulfate during experiment 2; white columns, thiosulfate concentrations in medium free of bacteria. Error bars denote the standard deviations from three biological replicates; if not visible, error bars are hidden in the symbols.

FIG 4 Dynamics of growth and metabolic activities of MED193 with and without thiosulfate during experiment 3. (A) Growth in acetate minimal medium in the presence (10 mM) and absence of thiosulfate. (B, C) Temporal development of leucine incorporation rates, i.e., protein synthesis (B) and CO₂ fixation (C), with thiosulfate (dark columns) and without thiosulfate (open columns). (Inset) The difference in anaplerotic CO₂ fixation levels between the cultures with and without thiosulfate after 24 and 30 h of growth. Error bars denote the standard errors from three biological replicates; if not visible, error bars are hidden in the symbols. Asterisks denote significance levels (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) by one-way ANOVA and Tukey's *post hoc* HSD test.

ysis of expression patterns of genes related to these processes, in *Phaeobacter* sp. MED193. The increase in growth yields of MED193 in response to thiosulfate enrichment (up to 40%) is in agreement with the finding that inorganic sulfur oxidation enhances biomass production up to 52% in the model *Roseobacter* clade species *R. pomeroyi* under similar experimental conditions (1). Consistent with these levels of growth stimulation, energy yields for thiosulfate oxidation calculated for *Thiobacillus* species growing under aerobic conditions range from 1.4 to 3.0 mol ATP per mol of thiosulfate (59), while energy yields from acetate are about 5 mol ATP per mol of acetate. Our finding that the most pronounced consumption of thiosulfate in MED193 occurred from mid-exponential phase onwards is highly consistent with the

detailed measures of thiosulfate consumption in *Thiobacillus* sp. strain A2 in a mixed acetate and thiosulfate batch culture (14). We did not measure the acetate concentrations in our cultures, but it could be noted that in the *Thiobacillus* cultures the consumption of thiosulfate was initiated when about half of the available acetate had been consumed. These findings indicate that the ability to efficiently use thiosulfate as an energy source for growth under oxic conditions is conserved among related members of the *Roseobacter* clade containing a similar complement of *sox* genes.

Consistent with the observed stimulation of growth by thiosulfate, our results showed that the expression levels of the *soxY* and *soxB* genes were high from mid-exponential phase onwards. The expression patterns of these genes merit some further examination. First, in agreement with their close location in the genome (*soxB* is three genes upstream of *soxY*), the overall *soxB* and *soxY* expression patterns were similar, although *soxY* expression was slightly more dynamic. Second, we note that both genes were expressed at low but detectable levels through all growth phases even when thiosulfate was not added to the medium; i.e., they showed constitutive expression at low levels. These expression patterns are consistent with previous observations that thiosulfate stimulated sulfur oxidation gene expression in the phototrophic purple sulfur bacterium *A. vinosum* during anaerobic growth (60). Third, in the cultures with thiosulfate, *soxB* and *soxY* expression was as low as that in the control cultures in early exponential phase (4 h; OD, 0.07), while expression levels increased nearly 3- and 21-fold until late exponential phase (10 h; OD, about 0.20). It is possible that these low values at early exponential phase were due to a temporal delay in the sensing of thiosulfate in the medium. Still, considering that the biomass at this early time point had already doubled and that, for example, the genes for anaplerotic CO₂ fixation were highly expressed at this time, we find it more likely that at this early time the cell metabolism of MED193 was entirely directed toward organoheterotrophy, and only when acetate consumption had progressed did the cells sense a change in resource availability that triggered an upregulation of lithoheterotrophy through the sulfur oxidation system. Interestingly, since the upregulation occurred only in the cultures with thiosulfate, this implies that the cells have multiple levels of regulation of the *sox* system, allowing them to integrate the sensing of general depletion of the medium and the presence of thiosulfate. Fourth, surprisingly, *sox* gene expression remained high well into stationary phase, potentially explaining the differential activity responses at this time (see below).

Metabolic activities and gene expression at exponential phase. During exponential growth, addition of thiosulfate did not influence anaplerotic CO₂ fixation and gene expression rates, although it did affect the rate of bacterial leucine incorporation, which is a standard measure of protein synthesis. Moreover, our study showed that not only bacterial leucine incorporation rates but also anaplerotic CO₂ fixation rates were directly dependent on the growth phase, with the rates being up to 20-fold higher during exponential growth compared to those during stationary phase. Very few studies have so far explored the relation between growth and anaplerotic CO₂ fixation in heterotrophic marine bacteria (18, 29). Working with Black Sea isolates, Sorokin (29) likewise found the highest bicarbonate uptake rates at exponential growth phase. Moreover, incubation experiments with natural seawater showed elevated rates of anaplerotic CO₂ fixation in stationary-phase cells, as detected by microautoradiography coupled with catalyzed reporter deposition-fluorescent *in situ* hybridization

FIG 5 Relative gene expression patterns in MED193 grown in the presence (dark columns) and absence (open columns) of thiosulfate during experiment 3. The relative expression profiles of genes involved in sulfur oxidation (A, B) and anaplerotic CO₂ fixation (C to F) are shown. Quantitative real-time PCR was performed using RNA from cultures in different growth phases from the experiment whose results are shown in Fig. 4. Relative levels of gene expression were obtained after normalization to the levels of expression for the housekeeping genes *rpoD*, *recA*, and *gyrB*. Error bars denote the standard deviations from three biological replicates. Asterisks denote significance levels (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) by one-way ANOVA and Tukey's *post hoc* HSD test.

(fixation rates were not measured during the active growth phase) (30). Heterotrophic bacteria could assimilate significant amounts of CO₂ into cell material using anaplerotic CO₂ fixation pathways via various carboxylases (28, 61). Roseobacters are known to contain genes encoding anaplerotic enzymes that could be involved in anaplerotic CO₂ fixation, such as pyruvate carboxylase (19, 38). Experimental work with *R. denitrificans* showed that while a majority of the cellular carbon during late exponential phase was derived from organic matter, between 10 and 15% came from CO₂ fixed by anaplerotic enzymes (20). We did not directly measure how much the anaplerotic CO₂ fixation in MED193 contributed to its cellular carbon budget. However, a calculation from the increase in abundance and the measured anaplerotic CO₂ fixation rates during active growth would result in at least 2 to 5% of the cell carbon in MED193 being provided by anaplerotic CO₂ fixation. These findings suggest that anaplerotic CO₂ fixation could be more important than has hitherto been recognized during active growth of heterotrophic marine bacteria.

Considering the large changes in anaplerotic CO₂ fixation from exponential to stationary phase in our experiments and the recognition of pyruvate and PEP carboxylases as key anaplerotic enzymes (19, 20), we expected to observe a shift in the expression levels of pyruvate carboxylase in isolate MED193 across growth phases (the genome of MED193 does not encode PEP carboxylase). However, pyruvate carboxylase gene expression remained relatively stable over time (although there was a tendency to somewhat higher levels of expression in exponential phase). Another anaplerotic enzyme encoded by the MED193 genome and potentially involved in anaplerotic CO₂ fixation was PEP carboxykinase. This enzyme is involved in either gluconeogenic or anaplerotic reactions, depending on the organism (25, 62–65). Tang et al. (20) suggested that PEP carboxykinase in *R. denitrificans* OCh 114 was involved in anaplerotic CO₂ fixation. The highly varied patterns of expression of this enzyme in our experiment gave no direct clues to its role in MED193. Nevertheless, the lack of other enzymes in this bacte-

rium carrying out the initial step in gluconeogenesis suggests a gluconeogenic role of PEP carboxykinase in this bacterium.

In addition to pyruvate carboxylase and PEP carboxykinase, genome analysis of MED193 showed the presence of genes involved in the ethylmalonyl-CoA pathway, which allows bacteria lacking the glyoxylate shunt to grow on carbon compounds like acetate (22, 31, 66, 67). An important step in this pathway is the fixation of 1 molecule of CO₂ and 1 molecule of bicarbonate per 3 molecules of acetate, whereby it fulfills an important anaplerotic reaction (24). In our study, the gene expression level of the two main genes in the ethylmalonyl-CoA pathway, i.e., those encoding crotonyl-CoA carboxylase/reductase and propionyl-CoA carboxylase, was up to 5-fold higher in the exponential phase than in stationary phase, coinciding with elevated levels of anaplerotic CO₂ fixation during active growth. This suggests that MED193 uses the ethylmalonyl-CoA pathway for growth on acetate and, further, that the activity of the carboxylases in this pathway could account for important parts of the increased level of anaplerotic CO₂ fixation during active growth.

Metabolic activity and gene expression at stationary phase.

Although the rates of leucine incorporation and anaplerotic CO₂ fixation were much lower in stationary phase than in exponential phase, we found a very strong positive impact of thiosulfate on the metabolic activities of *Phaeobacter* sp. MED193 in stationary phase. Ultimately, the maintenance of higher levels of metabolism during starvation could lead to prolonged survival, which was also indicated by the more stable OD values in stationary phase with 100 mM thiosulfate treatment than with treatments with lower concentrations. The ecological significance of such prolonged survival could be expressed either through the provision of additional energy to all cells by thiosulfate or through the selection of surviving cells and manifested through the resulting high per cell activity. Our gene expression analysis showed that the *sox* genes were highly expressed across growth phases from mid-exponential phase and well into stationary phase. We are unaware of studies detailing the progression from transcription of the *sox* genes to the maturation of the Sox enzyme system and how long it remains functional. Nonetheless, the continued *sox* gene expression indicates that the cells continued the synthesis of the Sox enzyme system, even though it should become progressively more costly the further that the cells advanced into stationary phase. Thus, our results suggest that, in addition to the importance of thiosulfate for providing energy for increased growth yields in marine bacteria, as observed here and elsewhere (1, 68), thiosulfate could play a largely unrecognized role in providing energy for maintaining the activity of cell metabolism under nutrient-depleted conditions and thereby lead to prolonged survival.

With regard to how energy from thiosulfate oxidation is used in stationary phase, the present data do not allow detailing of whether the pathway for anaplerotic CO₂ fixation in stationary phase was the same as that in exponential phase. However, the gene expression levels of the ethylmalonyl-CoA pathway decreased, while those of the pyruvate carboxylase pathway remained stable, suggesting that anaplerotic CO₂ fixation through the latter pathway may have increased in relative importance over time. In general, fatty acid and amino acid biosyntheses in heterotrophic bacteria are among the processes that require anaplerotic reactions. In *Escherichia coli*, the entry into stationary phase implies major rearrangements in protein and lipid composition, and lipid synthesis at this stage has been shown to require an increased

amount of anaplerotic CO₂ fixation (69). This supports our contention that thiosulfate could provide energy for cell metabolism in stationary-phase cells that require complementary anaplerotic CO₂ fixation.

Conclusion. The present study showed that the utilization of thiosulfate influenced bacterial metabolism during the active growth phase as well as in stationary phase. This implies that lithotrophy in this bacterium positively affects the two major fitness components, reproduction and survival. From an ecological perspective, it remains an intriguing issue to determine under which environmental conditions either of the two is more beneficial to the bacteria. The relative benefit of using thiosulfate for obtaining higher abundances or improved survival is likely to vary depending on the stability of the resource supply, the availability of organic matter compared to that of reduced inorganic substrates, and maybe also the inorganic nutrient concentrations. Overall, these results suggest that the genetic potential for sulfur oxidation via the *sox* enzyme system improves the metabolic versatility of marine bacteria facing ever-changing environmental conditions, potentially allowing a more efficient utilization of organic carbon to enhance growth and to contribute to cell energetics during starvation.

ACKNOWLEDGMENTS

We thank Jeremy Forsberg, Johanna Sjöstedt, Joakim Palovaara, Mark Dopson, Christopher Abin, and Sabina Arnautovic for skillful technical assistance and helpful advice during the experiments and for providing important food for thought.

This research was supported by the Crafoord Foundation, European Science Foundation/Swedish Research Council EuroEEFG project MOCA, BONUS project BLUEPRINT, and the research program Eco-Change (to J.P.) and by grant MarineGems (CTM2010-20361) from the Spanish Ministry of Science and Innovation (to J.M.G.).

REFERENCES

- Moran MA, Buchan A, Gonzalez JM, Heidelberg JF, Whitman WB, Kiene RP, Henriksen JR, King GM, Belas R, Fuqua C, Brinkac L, Lewis M, Johri S, Weaver B, Pai G, Eisen JA, Rahe E, Sheldon WM, Ye WY, Miller TR, Carlton J, Rasko DA, Paulsen IT, Ren QH, Daugherty SC, Deboy RT, Dodson RJ, Durkin AS, Madupu R, Nelson WC, Sullivan SA, Rosovitz MJ, Haft DH, Selengut J, Ward N. 2004. Genome sequence of *Silicibacter pomeroyi* reveals adaptations to the marine environment. *Nature* 432:910–913. <http://dx.doi.org/10.1038/nature03170>.
- Labrenz M, Jost G, Pohl C, Beckmann S, Martens-Habben W, Jürgens K. 2005. Impact of different in vitro electron donor/acceptor conditions on potential chemolithoautotrophic communities from marine pelagic redoxclines. *Appl. Environ. Microbiol.* 71:6664–6672. <http://dx.doi.org/10.1128/AEM.71.11.6664-6672.2005>.
- Poretsky RS, Hewson I, Sun SL, Allen AE, Zehr JP, Moran MA. 2009. Comparative day/night metatranscriptomic analysis of microbial communities in the North Pacific subtropical gyre. *Environ. Microbiol.* 11: 1358–1375. <http://dx.doi.org/10.1111/j.1462-2920.2008.01863.x>.
- Friedrich CG, Rother D, Bardischewsky F, Quentmeier A, Fischer J. 2001. Oxidation of reduced inorganic sulfur compounds by bacteria: emergence of a common mechanism? *Appl. Environ. Microbiol.* 67:2873–2882. <http://dx.doi.org/10.1128/AEM.67.7.2873-2882.2001>.
- Friedrich CG, Quentmeier A, Bardischewsky F, Rother D, Kraft R, Kostka S, Prinz H. 2000. Novel genes coding for lithotrophic sulfur oxidation of *Paracoccus pantotrophus* GB17. *J. Bacteriol.* 182:4677–4687. <http://dx.doi.org/10.1128/JB.182.17.4677-4687.2000>.
- Kappler U, Friedrich CG, Trüper HG, Dahl C. 2001. Evidence for two pathways of thiosulfate oxidation in *Starkeya novella* (formerly *Thiobacillus novellus*). *Arch. Microbiol.* 175:102–111. <http://dx.doi.org/10.1007/s002030000241>.
- Hensen D, Sperling D, Trüper HG, Brune DC, Dahl C. 2006. Thiosulfate oxidation in the phototrophic sulphur bacterium *Allochromatium*

- vinosum*. Mol. Microbiol. 62:794–810. <http://dx.doi.org/10.1111/j.1365-2958.2006.05408.x>.
8. Masuda S, Eda S, Ikeda S, Mitsui H, Minamisawa K. 2010. Thiosulfate-dependent chemolithoautotrophic growth of *Bradyrhizobium japonicum*. Appl. Environ. Microbiol. 76:2402–2409. <http://dx.doi.org/10.1128/AEM.02783-09>.
 9. Friedrich CG, Bardischewsky F, Rother D, Quentmeier A, Fischer J. 2005. Prokaryotic sulfur oxidation. Curr. Opin. Microbiol. 8:253–259. <http://dx.doi.org/10.1016/j.mib.2005.04.005>.
 10. Meyer B, Imhoff JF, Kuever J. 2007. Molecular analysis of the distribution and phylogeny of the *soxB* gene among sulfur-oxidizing bacteria—evolution of the Sox sulfur oxidation enzyme system. Environ. Microbiol. 9:2957–2977. <http://dx.doi.org/10.1111/j.1462-2920.2007.01407.x>.
 11. Bamford VA, Bruno S, Rasmussen T, Appia-Ayme C, Cheesman MR, Berks BC, Hemmings AM. 2002. Structural basis for the oxidation of thiosulfate by a sulfur cycle enzyme. EMBO J. 21:5599–5610. <http://dx.doi.org/10.1093/emboj/cdf566>.
 12. Tuttle JH, Holmes PE, Jannasch HW. 1974. Growth rate stimulation of marine pseudomonads by thiosulfate. Arch. Microbiol. 99:1–14. <http://dx.doi.org/10.1007/BF00696218>.
 13. Sorokin DY, Robertson LA, Kuenen JG. 1996. Sulfur cycling in *Catenococcus thiocyclus*. FEMS Microbiol. Ecol. 19:117–125. <http://dx.doi.org/10.1111/j.1574-6941.1996.tb00204.x>.
 14. Gottschal JC, Kuenen JG. 1980. Mixotrophic growth of *Thiobacillus A2* on acetate and thiosulfate as growth limiting substrates in the chemostat. Arch. Microbiol. 126:33–42. <http://dx.doi.org/10.1007/BF00421888>.
 15. Sorokin DY, Banciu H, van Loosdrecht M, Kuenen JG. 2003. Growth physiology and competitive interaction of obligately chemolithoautotrophic, haloalkaliphilic, sulfur-oxidizing bacteria from soda lakes. Extremophiles 7:195–203. <http://dx.doi.org/10.1007/s00792-002-0313-4>.
 16. Bondarev V, Richter M, Romano S, Piel J, Schwedt A, Schulz-Vogt HN. 2013. The genus *Pseudovibrio* contains metabolically versatile bacteria adapted for symbiosis. Environ. Microbiol. 15:2095–2113. <http://dx.doi.org/10.1111/1462-2920.12123>.
 17. Lenk S, Moraru C, Hahnke S, Arnds J, Richter M, Kube M, Reinhardt R, Brinkhoff T, Harder J, Amann R. 2012. *Roseobacter* clade bacteria are abundant in coastal sediments and encode a novel combination of sulfur oxidation genes. ISME J. 6:2178–2187. <http://dx.doi.org/10.1038/ismej.2012.66>.
 18. González JM, Fernandez-Gomez B, Fernandez-Guerra A, Gómez-Consarnau L, Sanchez O, Coll-Llado M, del Campo J, Escudero L, Rodriguez-Martinez R, Alonso-Sáez L, Latasa M, Paulsen I, Nedashkovskaya O, Lekunberri I, Pinhassi J, Pedrós-Alió C. 2008. Genome analysis of the proteorhodopsin-containing marine bacterium *Polaribacter* sp. MED152 (Flavobacteria). Proc. Natl. Acad. Sci. U. S. A. 105:8724–8729. <http://dx.doi.org/10.1073/pnas.0712071105>.
 19. Swingle WD, Sadekar S, Mastrian SD, Matthies HJ, Hao J, Ramos H, Acharya CR, Conrad AL, Taylor HL, Dejesa LC. 2007. The complete genome sequence of *Roseobacter denitrificans* reveals a mixotrophic rather than photosynthetic metabolism. J. Bacteriol. 189:683–690. <http://dx.doi.org/10.1128/JB.01390-06>.
 20. Tang KH, Feng XY, Tang YJJ, Blankenship RE. 2009. Carbohydrate metabolism and carbon fixation in *Roseobacter denitrificans* OCh114. PLoS One 4:e7233. <http://dx.doi.org/10.1371/journal.pone.0007233>.
 21. Moran M, Belas R, Schell M, Gonzalez J, Sun F, Sun S, Binder B, Edmonds J, Ye W, Orcutt B. 2007. Ecological genomics of marine roseobacters. Appl. Environ. Microbiol. 73:4559–4569. <http://dx.doi.org/10.1128/AEM.02580-06>.
 22. Erb TJ. 2011. Carboxylases in natural and synthetic microbial pathways. Appl. Environ. Microbiol. 77:8466–8477. <http://dx.doi.org/10.1128/AEM.05702-11>.
 23. Dijkhuizen L, Harder W. 1984. Current views on the regulation of autotrophic carbon dioxide fixation via the Calvin cycle in bacteria. Antonie Van Leeuwenhoek 50:473–487. <http://dx.doi.org/10.1007/BF02386221>.
 24. Erb TJ, Berg IA, Brecht V, Muller M, Fuchs G, Alber BE. 2007. Synthesis of C₅-dicarboxylic acids from C₂-units involving crotonyl-CoA carboxylase/reductase: the ethylmalonyl-CoA pathway. Proc. Natl. Acad. Sci. U. S. A. 104:10631–10636. <http://dx.doi.org/10.1073/pnas.0702791104>.
 25. Sauer U, Eikmanns BJ. 2005. The PEP-pyruvate-oxaloacetate node as the switch point for carbon flux distribution in bacteria. FEMS Microbiol. Rev. 29:765–794. <http://dx.doi.org/10.1016/j.femsre.2004.11.002>.
 26. DeLorenzo S, Bräuer SL, Edgmont CA, Herfort I, Tebo BM, Zuber P. 2012. Ubiquitous dissolved inorganic carbon assimilation by marine bacteria in the Pacific Northwest coastal ocean as determined by stable isotope probing. PLoS One 7:e46695. <http://dx.doi.org/10.1371/journal.pone.0046695>.
 27. Hesselsoe M, Nielsen JL, Roslev P, Nielsen PH. 2005. Isotope labeling and microautoradiography of active heterotrophic bacteria on the basis of assimilation of ¹⁴CO₂. Appl. Environ. Microbiol. 71:646–655. <http://dx.doi.org/10.1128/AEM.71.2.646-655.2005>.
 28. Feisthauer S, Wick LY, Kästner M, Kaschabek SR, Schlömann M, Richnow HH. 2008. Differences of heterotrophic ¹³CO₂ assimilation by *Pseudomonas knackmussii* strain B13 and *Rhodococcus opacus* 1CP and potential impact on biomarker stable isotope probing. Environ. Microbiol. 10:1641–1651. <http://dx.doi.org/10.1111/j.1462-2920.2008.01573.x>.
 29. Sorokin DY. 1993. Influence of thiosulfate on activity of carbon dioxide assimilation by marine heterotrophic thiosulfate-oxidizing bacteria. Microbiology 62:488–493.
 30. Alonso-Sáez L, Galand PE, Casamayor EO, Pedrós-Alió C, Bertilsson S. 2010. High bicarbonate assimilation in the dark by Arctic bacteria. ISME J. 4:1581–1590. <http://dx.doi.org/10.1038/ismej.2010.69>.
 31. Alber BE, Spanheimer R, Ebenau-Jehle C, Fuchs G. 2006. Study of an alternate glyoxylate cycle for acetate assimilation by *Rhodobacter sphaeroides*. Mol. Microbiol. 61:297–309. <http://dx.doi.org/10.1111/j.1365-2958.2006.05238.x>.
 32. Morris RM, Frazar CD, Carlson CA. 2012. Basin-scale patterns in the abundance of SAR11 subclades, marine *Actinobacteria* (OM1), members of the *Roseobacter* clade and OCS116 in the South Atlantic. Environ. Microbiol. 14:1133–1144. <http://dx.doi.org/10.1111/j.1462-2920.2011.02694.x>.
 33. Selje N, Simon M, Brinkhoff T. 2004. A newly discovered *Roseobacter* cluster in temperate and polar oceans. Nature 427:445–448. <http://dx.doi.org/10.1038/nature02272>.
 34. González JM, Simo R, Massana R, Covert JS, Casamayor EO, Pedrós-Alió C, Moran MA. 2000. Bacterial community structure associated with a dimethylsulfoniopropionate-producing North Atlantic algal bloom. Appl. Environ. Microbiol. 66:4237–4246. <http://dx.doi.org/10.1128/AEM.66.10.4237-4246.2000>.
 35. González JM, Kiene RP, Moran MA. 1999. Transformation of sulfur compounds by an abundant lineage of marine bacteria in the α-subclass of the class *Proteobacteria*. Appl. Environ. Microbiol. 65:3810–3819.
 36. Wagner-Dobler I, Biehl H. 2006. Environmental biology of the marine *Roseobacter* lineage. Annu. Rev. Microbiol. 60:255–280. <http://dx.doi.org/10.1146/annurev.micro.60.080805.142115>.
 37. Buchan A, Gonzalez JM, Moran MA. 2005. Overview of the marine *Roseobacter* lineage. Appl. Environ. Microbiol. 71:5665–5677. <http://dx.doi.org/10.1128/AEM.71.10.5665-5677.2005>.
 38. Newton RJ, Griffin LE, Bowles KM, Meile C, Gifford S, Givens CE, Howard EC, King E, Oakley CA, Reisch CR, Rinta-Kanto JM, Sharma S, Sun SL, Varaljay V, Vila-Costa M, Westrich JR, Moran MA. 2010. Genome characteristics of a generalist marine bacterial lineage. ISME J. 4:784–798. <http://dx.doi.org/10.1038/ismej.2009.150>.
 39. Tolli JD, Sievert SM, Taylor CD. 2006. Unexpected diversity of bacteria capable of carbon monoxide oxidation in a coastal marine environment, and contribution of the *Roseobacter*-associated clade to total CO oxidation. Appl. Environ. Microbiol. 72:1966–1973. <http://dx.doi.org/10.1128/AEM.72.3.1966-1973.2006>.
 40. Cunliffe M. 2011. Correlating carbon monoxide oxidation with *cox* genes in the abundant marine *Roseobacter* clade. ISME J. 5:685–691. <http://dx.doi.org/10.1038/ismej.2010.170>.
 41. Allgaier M, Uphoff H, Felske A, Wagner-Döbler I. 2003. Aerobic anoxygenic photosynthesis in *Roseobacter* clade bacteria from diverse marine habitats. Appl. Environ. Microbiol. 69:5051–5059. <http://dx.doi.org/10.1128/AEM.69.9.5051-5059.2003>.
 42. Oz A, Sabehi G, Kobizek M, Massana R, Béjà O. 2005. *Roseobacter*-like bacteria in Red and Mediterranean Sea aerobic anoxygenic photosynthetic populations. Appl. Environ. Microbiol. 71:344–353. <http://dx.doi.org/10.1128/AEM.71.1.344-353.2005>.
 43. Shi YM, Tyson GW, Eppley JM, DeLong EF. 2011. Integrated metatranscriptomic and metagenomic analyses of stratified microbial assemblages in the open ocean. ISME J. 5:999–1013. <http://dx.doi.org/10.1038/ismej.2010.189>.
 44. Luo H, Loytynoja A, Moran MA. 2012. Genome content of uncultivated marine *Roseobacters* in the surface ocean. Environ. Microbiol. 14:41–51. <http://dx.doi.org/10.1111/j.1462-2920.2011.02528.x>.
 45. Rinta-Kanto JM, Burgmann H, Gifford SM, Sun SL, Sharma S, del Valle

- DA, Kiene RP, Moran MA. 2011. Analysis of sulfur-related transcription by *Roseobacter* communities using a taxon-specific functional gene microarray. *Environ. Microbiol.* 13:453–467. <http://dx.doi.org/10.1111/j.1462-2920.2010.02350.x>.
46. Lekunberri I, Gasol JM, Acinas SG, Gómez-Consarnau L, Crespo BG, Casamayor EO, Massana R, Pedrós-Alió C, Pinhassi J. 2014. The phylogenetic and ecological context of cultured and whole genome-sequenced planktonic bacteria from the coastal NW Mediterranean Sea. *Syst. Appl. Microbiol.* 37:216–228. <http://dx.doi.org/10.1016/j.syapm.2013.11.005>.
 47. Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32:1792–1797. <http://dx.doi.org/10.1093/nar/gkh340>.
 48. Capella-Gutiérrez S, Silla-Martínez JM, Gabaldón T. 2009. trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. *Bioinformatics* 25:1972–1973. <http://dx.doi.org/10.1093/bioinformatics/btp348>.
 49. Stamatakis A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22:2688–2690. <http://dx.doi.org/10.1093/bioinformatics/btl446>.
 50. Karchmer JH. 1972. The analytical chemistry of sulfur and its compounds. Wiley-Interscience, New York, NY.
 51. Kirchman D, Ducklow H. 1993. Estimating conversion factors for the thymidine and leucine methods for measuring bacterial production, p 513–517. In Kemp PF, Sherr BF, Sherr EB, Cole JJ (ed), *Handbook of methods in aquatic microbial ecology*. Lewis Publishers, Boca Raton, FL.
 52. Smith DC, Azam F. 1992. A simple, economical method for measuring bacterial protein synthesis rates in seawater using ^3H -leucine. *Mar. Microb. Food Webs* 6:107–114.
 53. Parsons TR, Maita Y, Lalli CM. 1984. *Manual of chemical and biological methods for seawater analysis*. Pergamon Press, Oxford, United Kingdom.
 54. Untergasser A, Nijveen H, Rao X, Bisseling T, Geurts R, Leunissen JAM. 2007. Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Res.* 35:W71–W74. <http://dx.doi.org/10.1093/nar/gkm306>.
 55. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* 55:611–622. <http://dx.doi.org/10.1373/clinchem.2008.112797>.
 56. Andersen CL, Jensen JL, Orntoft TF. 2004. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res.* 64:5245–5250. <http://dx.doi.org/10.1158/0008-5472.CAN-04-0496>.
 57. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper-Excel-based tool using pair-wise correlations. *Biotechnol. Lett.* 26:509–515. <http://dx.doi.org/10.1023/B:BILE.0000019559.84305.47>.
 58. Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29:e45. <http://dx.doi.org/10.1093/nar/29.9.e45>.
 59. Kelly DP. 1982. Biochemistry of the chemolithotrophic oxidation of inorganic sulfur. *Philos. Trans. R. Soc. B Biol. Sci.* 298:499–528. <http://dx.doi.org/10.1098/rstb.1982.0094>.
 60. Grimm F, Franz B, Dahl C. 2011. Regulation of dissimilatory sulfur oxidation in the purple sulfur bacterium *Allochrochromatium vinosum*. *Front. Microbiol.* 2:51. <http://dx.doi.org/10.3389/fmicb.2011.00051>.
 61. Roslev P, Larsen MB, Jørgensen D, Hesselsoe M. 2004. Use of heterotrophic CO_2 assimilation as a measure of metabolic activity in planktonic and sessile bacteria. *J. Microbiol. Methods* 59:381–393. <http://dx.doi.org/10.1016/j.mimet.2004.08.002>.
 62. Inui M, Nakata KO, Roh JH, Zahn K, Yukawa H. 1999. Molecular and functional characterization of the *Rhodospseudomonas palustris* no. 7 phosphoenolpyruvate carboxykinase gene. *J. Bacteriol.* 181:2689–2696.
 63. Willison JC. 1988. Pyruvate and acetate metabolism in the photosynthetic bacterium *Rhodobacter capsulatus*. *J. Gen. Microbiol.* 134:2429–2439.
 64. Goldie H. 1984. Regulation of transcription of the *Escherichia coli* phosphoenolpyruvate carboxykinase locus: studies with Pck-LacZ operon fusions. *J. Bacteriol.* 159:832–836.
 65. Østerås M, Finan TM, Stanley J. 1991. Site-directed mutagenesis and DNA sequence of *pckA* of *Rhizobium* NGR234, encoding phosphoenolpyruvate carboxykinase: gluconeogenesis and host-dependent symbiotic phenotype. *Mol. Gen. Genet.* 230:257–269. <http://dx.doi.org/10.1007/BF00290676>.
 66. Korotkova N, Chistoserdova L, Kuksa V, Lidstrom ME. 2002. Glyoxylate regeneration pathway in the methylotroph *Methylobacterium extorquens* AM1. *J. Bacteriol.* 184:1750–1758. <http://dx.doi.org/10.1128/JB.184.6.1750-1758.2002>.
 67. Meister M, Saum S, Alber BE, Fuchs G. 2005. L-Malyl-coenzyme A/beta-methylmalyl-coenzyme A lyase is involved in acetate assimilation of the isocitrate lyase-negative bacterium *Rhodobacter capsulatus*. *J. Bacteriol.* 187:1415–1425. <http://dx.doi.org/10.1128/JB.187.4.1415-1425.2005>.
 68. Sorokin DY. 2003. Oxidation of inorganic sulfur compounds by obligately organotrophic bacteria. *Microbiology* 72:641–653. <http://dx.doi.org/10.1023/B:MICL.0000008363.24128.e5>.
 69. Merlin C, Masters M, McAteer S, Coulson A. 2003. Why is carbonic anhydrase essential to *Escherichia coli*? *J. Bacteriol.* 185:6415–6424. <http://dx.doi.org/10.1128/JB.185.21.6415-6424.2003>.