Cryptic Carbon and Sulfur Cycling Between Surface Ocean Plankton

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Abstract

About half the carbon fixed by phytoplankton in the ocean is taken up and metabolized by marine bacteria, a transfer that is mediated through the seawater dissolved organic carbon (DOC) pool. The chemical complexity of marine DOC, along with a poor understanding of which compounds form the basis of trophic interactions between bacteria and phytoplankton, have impeded efforts to identify key currencies of this carbon cycle link. Here, we used transcriptional patterns in a bacterial-diatom model system based on vitamin B_{12} auxotrophy as a sensitive assay for metabolite exchange between marine plankton. The most highly upregulated genes (up to 374-fold) by a marine Roseobacter clade bacterium when co-cultured with the diatom *Thalassiosira pseudonana* were those encoding the transport and catabolism of 2,3-dihydroxypropane-1-sulfonate (DHPS). This compound has no currently recognized role in the marine microbial food web. As the genes for DHPS catabolism have limited distribution among bacterial taxa, *T. pseudonana* may use this novel sulfonate for targeted feeding of beneficial associates. Indeed, DHPS was both a major component of the *T. pseudonana* cytosol and an abundant microbial metabolite in a diatom bloom in the eastern North Pacific Ocean. Moreover, transcript analysis of the North Pacific samples provided evidence of DHPS catabolism by Roseobacter populations. Other such biogeochemically important metabolites may be common in the ocean but difficult to discriminate against the complex chemical background of seawater. Bacterial transformation of this diatom-derived sulfonate represents a new and likely sizeable link in both the marine carbon and sulfur cycles.

Significance

In the surface ocean, organic matter released by phytoplankton and degraded by heterotrophic

bacteria is a key step in the carbon cycle. Compounds important in this trophic link are poorly known, in part because of the thousands of chemicals making up marine dissolved organic matter. We co-cultured a Roseobacter clade bacterium with the diatom *Thalassiosira pseudonana* and used gene expression changes to assay for compounds passed to the bacterium. A C_3-sulfonate with no previously known role in the microbial food web was identified and subsequently shown to be an abundant diatom metabolite and actively cycling compound in seawater. This work identifies a missing component of the marine carbon and sulfur cycles.

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Main Text

Carbon-cycle-relevant interactions between heterotrophic marine bacteria and phytoplankton are mediated through compounds excreted from living phytoplankton cells or released when the cells are grazed or lysed [1]. Current views of these interactions are rooted in the idea of passive trophic transfer to heterotrophic bacteria, although recent findings of pathogen-like genes in bacterial genomes [2] and predictable associations between microbial taxa [3,4] suggest that more strategic interactions link autotrophic and heterotrophic plankton. Compounds serving as intermediates in bacterial-phytoplankton interactions are likely to be present in low concentration or to reach high concentration only within the cells' diffusive boundary layers [5,6] and in either case are difficult to discriminate over the complex chemical background of seawater. To the extent that these compounds remain unrecognized as currencies of trophic interactions, particularly when abundant plankton groups are involved, they represent gaps in our understanding of marine elemental cycles.

We established a model microbial system for exploring bacterial-phytoplankton exchanges in which the marine bacterium *Ruegeria pomeroyi* DSS-3 had an obligate dependency on the diatom *Thalassiosira pseudonana* CCMP1335 for fixed carbon while the diatom obtained an essential vitamin from the bacterium. As is common among marine eukaryotic phytoplankton, *T. pseudonana* harbors the B_{12}-requiring version of the methionine synthase gene (*metH*) yet cannot synthesize B_{12} [7] and must obtain it from an exogenous source. The >50 sequenced members of the Roseobacter lineage all carry genes for B_{12} biosynthesis [8; www.roseobase.org]. Both groups of organisms are important in the ocean, with diatoms responsible for up to 40% of

global marine primary productivity [9] and roseobacters ubiquitously distributed, metabolically active [10], and commonly associated with diatoms [3].

In co-culture, *R. pomeroyi* DSS-3 recovered B_{12}-limited *T. pseudonana*, restoring growth rates comparable to those of axenic cultures supplemented with exogenous B_{12} (Fig. 1). When bacterial transcriptomes collected after 8 h of co-growth with diatoms (n = 4) were compared to control incubations (f/2 medium, +Si, -B_{12}; n = 2), ten of the most highly upregulated genes (upregulated 56- to 374-fold) encoded proteins for the transport and catabolism of the C_3-sulfonate 2,3-dihydroxypropane-1-sulfonate (DHPS) (Table S1). Eight of these genes were co-located on the *R. pomeroyi* DSS-3 chromosome and included DHPS TRAP transporter components *hpsKLM* [11], dehydrogenases *hpsNOP* for converting DHPS to (*R*)-sulfolactate, and *slcD* for converting sulfolactate to sulfopyruvate (Fig. 2). Two genes were co-located on the bacterium's megaplasmid, including *cuyA*, which mediates conversion of cysteate to pyruvate and ammonium, and the bisulfite exporter *cuyZ*. RT-qPCR of *hpsN* confirmed that providing DHPS as a sole carbon source to *R. pomeroyi* DSS-3 resulted in the same pattern of gene regulation as growth with the diatom (Fig. 2). While the transcriptional response was strongest to DHPS, six co-located genes involved in transport and catabolism of C_2-sulfonate *N*-acetyltaurine were also upregulated by the bacterium. (Fig. S1). The enzymatic function of these bacterial genes was established previously [11,12], but their ecological role had remained unknown. Identification of both sulfonates as currencies in this model system suggests a diatom-derived oxidized organic sulfur pool that may be important in fueling the marine microbial food web.

Coincident changes in co-cultured *T. pseudonana* CCMP1335 were assessed with poly(A)-selected transcriptomes comparing cells cultured with *R. pomeroyi* (n = 2) to those that were axenic but B_{12}-replete (n = 2) during exponential growth (Table S2). As anticipated, the

gene encoding the B_{12}-binding cobalamin acquisition protein 1 (CBA1) [13] was one of the most highly upregulated diatom genes (11-fold) when bacteria served as the source of B_{12} compared to when the vitamin was added to the culture in excess. No other genes reported previously to be induced by B_{12} limitation [13] were differentially regulated in the co-culture. However, several sulfur-related genes were downregulated in the diatom when co-cultured with the bacterium, including *sqdB*, which mediates the first committed step in biosynthesis of the sulfolipid sulfoquinovosyl diacylglycercol (SQDG) (Tp269393; 6-fold; Fig. S2), two genes involved in the synthesis of diacylglycerol precursors for SQDG biosynthesis (Tp7093 and Tp263660; 4-fold), and a gene annotated as a sulfate transporter (Tp261147; 4-fold). These changes in gene expression suggest shifts in sulfur metabolism by the diatom in the presence of *R. pomeroyi*.

Some bacteria are capable of producing DHPS by degrading sulfoquinovose, the headgroup of SQDG [14]. In our model system, however, the upregulation of a dedicated DHPS transporter by *R. pomeroyi* and the previous identification of this compound in the cytosol of a freshwater diatom [15] argues that direct release of DHPS by the diatom formed the basis of the interaction. Diatoms might produce DHPS by a reductive deamination of cysteinolic acid, a potential cytosolic reservoir of reduced sulfur [16]. Despite significant changes in transcription patterns, indicators of the physiological state of *T. pseudonana* (i.e., growth rate, photosynthetic efficiency F_v/F_m) showed no differences between the axenic and co-cultured conditions.

The three known routes of bacterial DHPS degradation share an initial oxidation step mediated by HpsN, but carry out desulfonation via different enzymes (CuyA, SuyAB, or Xsc; Fig. 2, Table S3). Over half of *R. pomeroyi*'s sequenced relatives in the Roseobacter clade (26 of 41 genomes) harbor at least one of the DHPS degradation pathways (Table S3), as do some marine relatives in the *Rhodobacterales* (4 of 17 genomes) and *Rhizobiales* (4 of 15 genomes)

(Fig. 3, Table S3). HpsN sequences from these alphaproteobacterial taxa form a clade that includes proteins of experimentally verified function [11]. Other marine bacterial taxa that possibly harbor DHPS degradation pathways include alphaproteobacterial clade SAR116 and a limited number of marine Gammaproteobacteria (Fig. 3, Table S3). Members of the alphaproteobacterial SAR11 clade also have a possible HpsN protein, but the sequences have an unresolved evolutionary relationship with experimentally verified sequences even when accounting for the substantial compositional heterogeneity due to differences in G+C content of the corresponding genes (Figs. 3 & S3; Table S4); further, the SAR11 genomes lack orthologs to metabolically upstream genes *hpsO* and *hpsP* (Table S3). All sequenced members of the Roseobacter clade and most of the marine *Rhizobiales* and the other marine *Rhodobacterales* that harbor a DHPS degradation pathway also have a B_{12} biosynthetic pathway [8,17] (Table S3), while B_{12} biosynthesis is not nearly as widespread in the candidate DHPS-degrading SAR116 and Gammaproteobacteria taxa (<20%) and is absent in the SAR11 clade. Several taxa of soil bacteria, including strains associated with root nodules in plants, have DHPS pathway orthologs (Table S3), suggestive of similar sulfonate-based interactions between heterotrophic bacteria and terrestrial primary producers.

Whether the release of unique organic metabolites by *T. pseudonana* and other phytoplankton is used to enable mutualisms with vitamin-supplying or otherwise beneficial bacteria, or is simply excretion of excess materials that are subsequently scavenged, is a matter of debate [18,19]. In the case of DHPS, we asked whether the diatom might use a chemical signal based on the presence of a beneficial bacterium as a cue to initiate a mutualism [20]. The concentration of DHPS in the *T. pseudonana* cytosol was high (3.3 mM) and similar to concentrations of compounds known to be abundant in microbial cells [21,22], including the

nitrogen homeostasis metabolite glutamate and compatible solute proline (Fig. 4). Importantly, DHPS was present in similar concentrations in the axenic and co-cultured diatom cells, and present but slightly lower in B_{12}-limited diatom cells (Fig. 4). By comparison, concentrations of another ecologically important organic sulfur compound, osmolyte dimethylsulfoniopropionate (DMSP), ranged from 5-12 mM in cells of *T. pseudonana*, and also did not differ in the presence of bacteria. As for the bacterial side of the association, there was also no evidence of differential regulation of B_{12} pathway genes in *R. pomeroyi* DSS-3 during growth with the diatom. These findings suggest that neither of the co-cultured organisms regulates biosynthesis of the currencies exchanged based on a cue linked to the physical presence of the other. They do not rule out, however, the possibility of species-specific recognition systems in naturally occurring roseobacter-diatom associations. Alternatively, it is conceivable that these compounds do not serve as the basis of an explicit, taxonomically narrow symbiosis [23], but rather represent general metabolic linkages between broad taxonomic groups. The ubiquity of bacteria associated with the diatom phycosphere (the region surrounding the cell that is enriched in extracellular products [24]) under natural conditions assures that roseobacters or other DHPS-utilizing, B_{12}-releasing bacteria will be in close proximity in the ocean.

To determine whether DHPS metabolism occurs in marine surface waters, metatranscriptomic and metabolomic data were collected from four stations along a coastal-to-open-ocean transect in the eastern North Pacific during an active diatom bloom. Counts of bacterial *hpsN* transcripts (those mapping to the Roseobacter clade with experimentally verified function; Fig. 3) reached 2.5×10^7 L^{-1} in coastal regions of the transect (Station P1), decreasing 25-fold to 1.0×10^6 L^{-1} in the open ocean (Station P8; Fig. 5A) and tracking well with diatom cell counts. DHPS was identified in the metabolome of the eukaryotic plankton size fraction

(cells >1.6 μm), where it accounted for up to 27% of a suite of major metabolites (Fig. 5B). Maximum concentrations of DHPS were similar to those of DMSP (Fig. 5C), currently considered the central metabolite of the surface ocean sulfur cycle [25] and produced primarily by dinoflagellates and haptophytes. DHPS and DMSP concentrations were also similar in the seawater dissolved organic matter pool (1.5 $\pm$ 0.8 nM vs. 1.4 $\pm$ 1.1 nM; n = 4), and the low concentrations are consistent with efficient bacterial consumption of both metabolites. Given the importance of diatoms to carbon fixation in the global ocean, DHPS likely plays a major role in the flux of sulfur and carbon through marine food webs.

Biogeochemically important microbial metabolites may be cryptic because they have not yet been identified in the complex chemical milieu of seawater, or they are not readily targeted with existing analytical methods, or their concentrations are vanishingly low outside the phycosphere of phytoplankton. In waters of the eastern North Pacific, for example, chemical analysis of the organic carbon pools resolved >7,000 unique compounds. Unrecognized currencies in plankton interactions are highly likely to be represented among them. In this study, transcriptional responses in a two-member model organism system offered a sensitive methodology for identifying candidate metabolites of biogeochemical relevance. For DHPS, detection of this novel diatom metabolite suggests significant new links in both the carbon and sulfur cycles that are mediated by the specific bacterial lineages capable of sulfonate catabolism.

Methods Summary

T. pseudonana CCMP1335 was cultured in f/2 (+Si) medium at 18°C on a 16:8 h light:dark cycle at ~160 μmol photons m^{-2} sec^{-1}. *R. pomeroyi* DSS-3 cells were harvested from ½ YTSS medium in exponential phase and washed in f/2 medium. Four treatments were established

(n=2 or 4) in f/2 (+Si) medium: *T. pseudonana* + B_{12} (369 pM), *T. pseudonana* - B_{12}, *T. pseudonana* + *R. pomeroyi*, and *R. pomeroyi* alone. For RT-qPCR, *R. pomeroyi* DSS-3 was also grown in f/2 (+Si, -B_{12}) with either acetate or DHPS. Diatom and bacterial growth was tracked by chlorophyll fluorescence and/or flow cytometry.

Cells were collected after 8 h (bacterial transcriptomics) or during exponential phase (bacterial RT-qPCR, diatom transcriptomics/metabolomics) and flash frozen in liquid N_2. RNA was extracted and processed as previously described [26], with slight modifications, and sequenced on either Illumina MiSeq (Illumina Inc., San Diego, CA) or SOLiD version 4 (Life Technologies, New York) platforms. Reads mapped using BWA (v.0.5.9) [27] were analyzed for differential expression using the baySeq package in R, with 0.2 – 4.4 x 10^6 mapped reads per sample for *R. pomeroyi* and ~8 x 10^6 mapped reads per sample for *T. pseudonana*. RT-qPCR analysis of *hpsN* transcripts was performed as previously described [28]. *T. pseudonana* metabolites were measured using ultra-performance liquid chromatography-high resolution mass spectrometry (UPLC-HRMS) on an Exactive Plus Orbitrap [29] using external calibration curves generated with pure metabolite standards. These data were converted to cytosolic concentrations based on an experimentally determined fluorescence:cell number relationship and assuming a cytosolic cell volume of 30 μm^3.

Experimentally verified HpsN sequences [11,12] were used as queries in BLASTP analysis to identify orthologs in marine bacterial genomes. Protein sequences were aligned and trimmed, and phylogenetic trees were created assuming three different evolutionary models using the P4 and PhyloBayes software packages [30].

Marine plankton consisting primarily of eukaryotic cells (retained on a 1.6 µm or 2.0 µm pore-size filter) and seawater dissolved organic matter concentrated on solid phase extraction

columns were collected in May 2012 along the North Pacific Line P transect. Analysis of cellular metabolites was carried out by liquid chromatography triple-stage quadrupole mass spectrometry (LC/TSQMS). Metatranscriptomic datasets were obtained as above, with some modifications, including addition of an internal standard to all samples immediately prior to cell lysis [26,31]. Reads representing *hpsN* transcripts were identified using a BLASTX search (bit score cutoff $\geq$40) and hits were confirmed by analysis of the positive reads against the RefSeq protein database. Further details of all methods are given in the Supplemental Methods.

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Figure Legends

Figure 1. Recovery of *Thalassiosira pseudonana* growth by addition of exogenous B_{12} (filled circles) or *Ruegeria pomeroyi* DSS-3 (open circles) compared to the B_{12}-limited control (filled triangles). The star indicates the growth stage at which diatom cells were collected for RNA and metabolite analyses. Inset: Cell counts for *T. pseudonana* following addition of exogenous B_{12} (filled circles) or *R. pomeroyi* (open circles) and for co-grown (open squares) or control (filled squares) *R. pomeroyi* over the first two days of the experiment. Error bars represent the standard deviation of duplicate cultures.

Figure 2. DHPS transport and degradation genes in marine roseobacter *R. pomeroyi* DSS-3 (blue font) that were upregulated (purple boxes, fold-change upregulation indicated underneath) during co-growth with *T. pseudonana*. Genes mediating metabolism of sulfopyruvate to cysteate (*coa* and *gdh*) were not differentially expressed, consistent with previous work [32]. Two alternate routes of DHPS degradation present in members of the Roseobacter clade are shown in gray (see Table S3). Bold arrows indicate transporters. Inset: RT-qPCR quantification of *hpsN* transcripts in *R. pomeroyi* in co-culture with *T. pseudonana* (Thps; see Fig. 1, open circles) and in sterile f/2 seawater medium (SW) after 8 h, and during exponential growth with acetate or DHPS as the sole carbon source. Error bars represent standard deviation of three technical replicates from duplicate cultures.

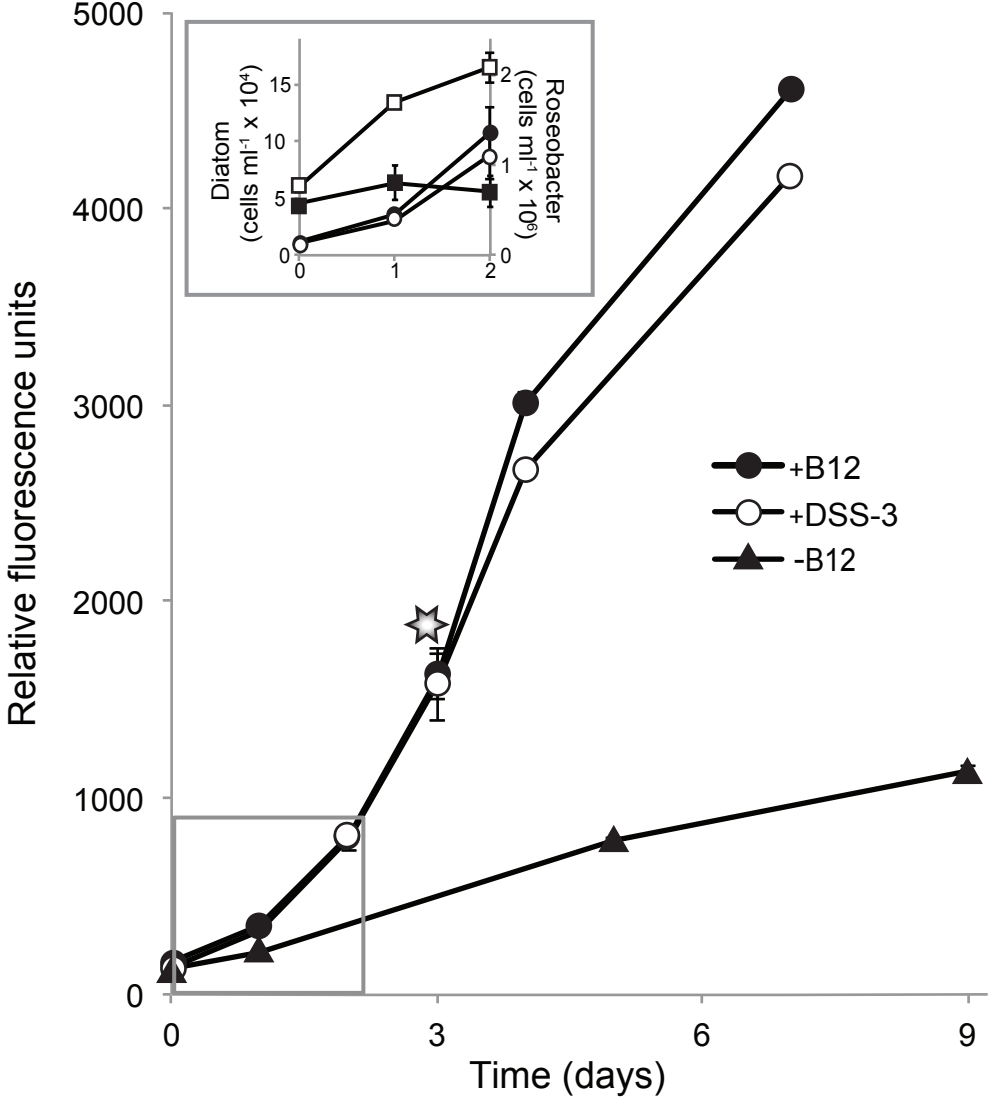
Figure 3. A Bayesian phylogenetic tree derived from reference HpsN sequences including experimentally verified sequences (bold) and orthologs in marine genomes available through the

Integrated Microbial Genomes database (http://img.jgi.doe.gov) (see Table S3 for additional information). The tree was constructed using the P4 software following a $\chi 2$ test on posterior distributed samples showing that a Bayesian composition-homogeneous model was adequate. Outgroups included a variety of histidinol dehydrogenase sequences. The value near each internal branch is the posterior probability, and the scale bar indicates the number of substitutions per site. Alphaproteobacterial HpsN clade sequences (and one gammaproteobacterial sequence from strain HIMB30) are marked with a bar, with Roseobacter clade members highlighted by the purple box and the *R. pomeroyi* DSS-3 sequence marked by a star. HpsN paralogs in Roseobacter genomes for which function has not been determined are marked with a dashed bar. The remaining sequences are candidate HpsN proteins with varying levels of bioinformatic support. Locus tags are given in parentheses. Tree topologies based on alternate tree-building algorithms are given in Fig. S3.

Figure 4. Concentrations of selected metabolites in the *T. pseudonana* cytosol during exponential growth in B_{12}-replete medium ($+B_{12}$), in co-culture with *R. pomeroyi* DSS-3 (+DSS-3), and in B_{12} limited medium ($-B_{12}$), as analyzed by ultra-performance liquid chromatography mass spectrometry. Error bars represent standard deviation of duplicate cultures.

Figure 5. DHPS cycling in the eastern North Pacific Ocean. A) chlorophyll *a* concentrations, diatom counts, and Roseobacter-clade *hpsN* transcript abundance at four stations along the Line P transect. B) Total ion current chromatogram for the Station P4 sample collected by triple-stage quadrupole mass spectrometry, with insets showing DHPS and DMSP peak details. R^2 values of standard curves with authentic standards were 0.98 for both compounds. Other identified

compounds include glycine betaine (1.66 min; co-eluting with additional small organic acids), phenylalanine (3.63 min), a mixture of thymidine and 5-methylthioadenosine (6.49 min), and the deuterated biotin injection standard (9.36 min; co-eluting with lesser amounts of riboflavin and biotin). C) DHPS and DMSP concentrations as a percent (by mass) of 30 measured metabolites in the eukaryotic plankton size fraction (>1.6 μm diameter) at four Line P stations.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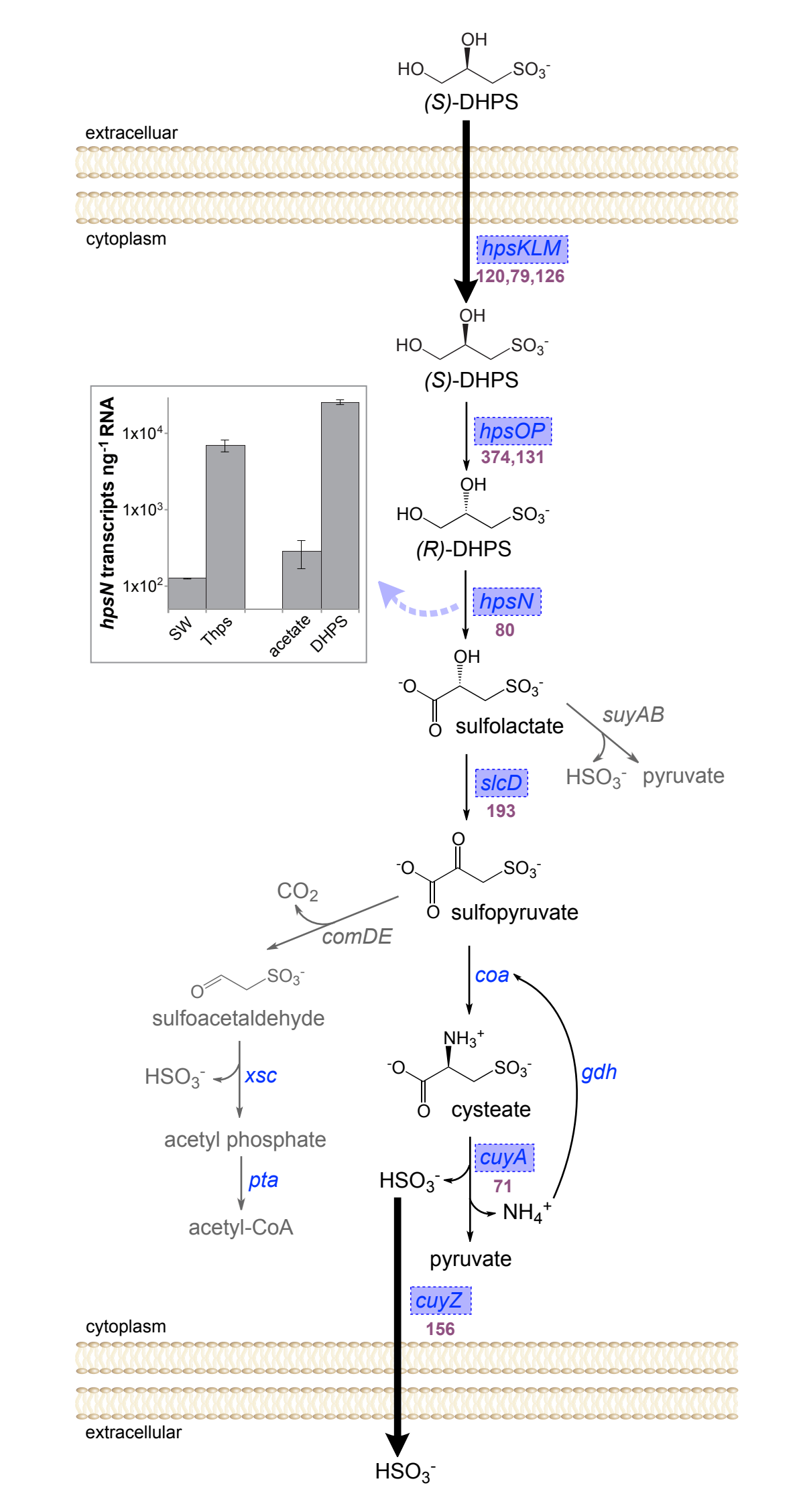
1
2


(S)-DHPS
extracelluar
cytoplasm
hpsKLM
120,79,126
(S)-DHPS
hpsOP
374,131
(R)-DHPS
hpsN
80
hpsN transcripts ng-1 RNA
1x10^4
1x10^3
1x10^2
SW
Thps
acetate
DHPS
sulfolactate
suyAB
HSO3- pyruvate
slcD
193
sulfopyruvate
CO2
comDE
sulfoacetaldehyde
HSO3-
xsc
acetyl phosphate
pta
acetyl-CoA
coa
cysteate
gdh
cuyA
71
HSO3-
NH4+
pyruvate
cuyZ
156
cytoplasm
extracellular
HSO3-

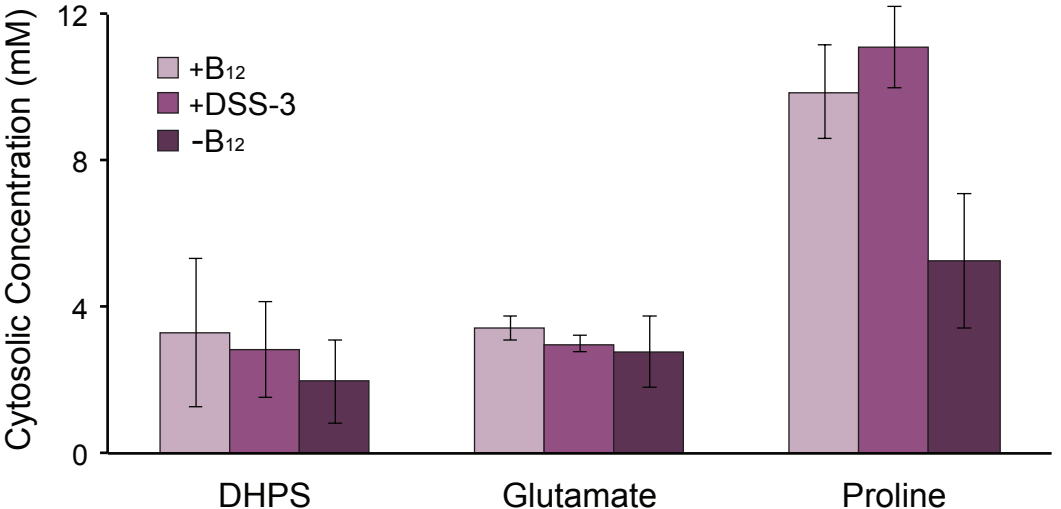
Ca. Pelagibacter-like HIMB083 (Pelub83DRAFT_0413)
Ca. Pelagibacter ubique SAR11 HTCC8051 (HTC8051_00486)
Ca. Pelagibacter sp. HTCC7211 (HTCC7211_00007440)
Alphaproteobacterium sp. SAR11 HIMB5 (HIMB5b_00003860)
Ca. Pelagibacter ubique SAR11 HTCC1062 (SAR11_0801)
Neptunomonas japonica DSM 18939 (G511DRAFT_00685)
Leucothrix mucor DSM2157 (LeumuDRAFT_1346)
SAR116 cluster Alphaproteobacterium sp. HIMB100 (SAR116_HIMB100_00017160)
Alphaproteobacterium SCGC AAA536-B06 (A536B6DRAFT_00013890)
Ca. Puniceispirillum marinum IMCC1322 (SAR116_2138)
Enterovibrio sp. AK16 (D515_03411)
Enterovibrio calviensis FF-85 (A1OSDRAFT_00057)
Enterovibrio norvegicus FF-162 (A1OQDRAFT_02463)
Vibrio nigripulchritudo ATCC 27043 (VINI7043_03893)
Gammaproteobacterium SCGC AAA076-F14 (A76F14DRAFT_00011990)
Gammaproteobacterium SCGC AAA076-D02 (A76D2DRAFT_00000640)
***Cupriavidus pinatubonensis* JMP134 (Reut_C6092)**
Ruegeria pomeroyi DSS-3 (SPO0981)
Sagittula stellata E-37 (SSE37 12946)
Roseovarius nubinhibens ISM (ISM_01480)
Roseovarius sp. 217 (ROS217_19722)
Phaeobacter gallaeciensis BS107 (PGA1_c25490)
Martelella mediterranea DSM 17316 (B038DRAFT_01720)
***Hoeflea phototrophica* DLF-43 (HPDFL43_21037)**
Nitratireductor indicus C115 (NA8A_22466)
Gammaproteobacterium HIMB30 (HIMB30_00018260)
Amorphus coralii DSM 19760 (C507DRAFT_02834)
Alphaproteobacterium SCGC AAA076-C03 (A76C3DRAFT_00007880)
Rhodobacterales bacterium HTCC2255 (OM2255_05970)
***Roseobacter denitrificans* OCh114 (RD1_3808)**
Ahrensia sp. R2A130 (R2A130_2002)
Phaeobacter gallaeciensis BS107 (PGA1_c07750)
Roseobacter sp. CCS2 (RCCS2_12634)
Jannaschia sp. CCS1 (Jann_1398)
***Roseovarius nubinhibens* ISM (ISM_05010)**
***Roseobacter* sp. MED193 (MED193_17064)**
***Ruegeria pomeroyi* DSS-3 (SPO0594)**
***Roseovarius* sp. 217 (ROS217_11221)**
Dinoroseobacter shibae DFL 12 (Dshi_2034)
Roseobacter HIMB11 (HIMB11_01024)
Labrenzia sp. DG1229 (DG122900690)
Roseibium sp. TrichSKD4 (TRICHSKD4_1929)
Labrenzia alexandrii DFL-11 (ladfl_03842)

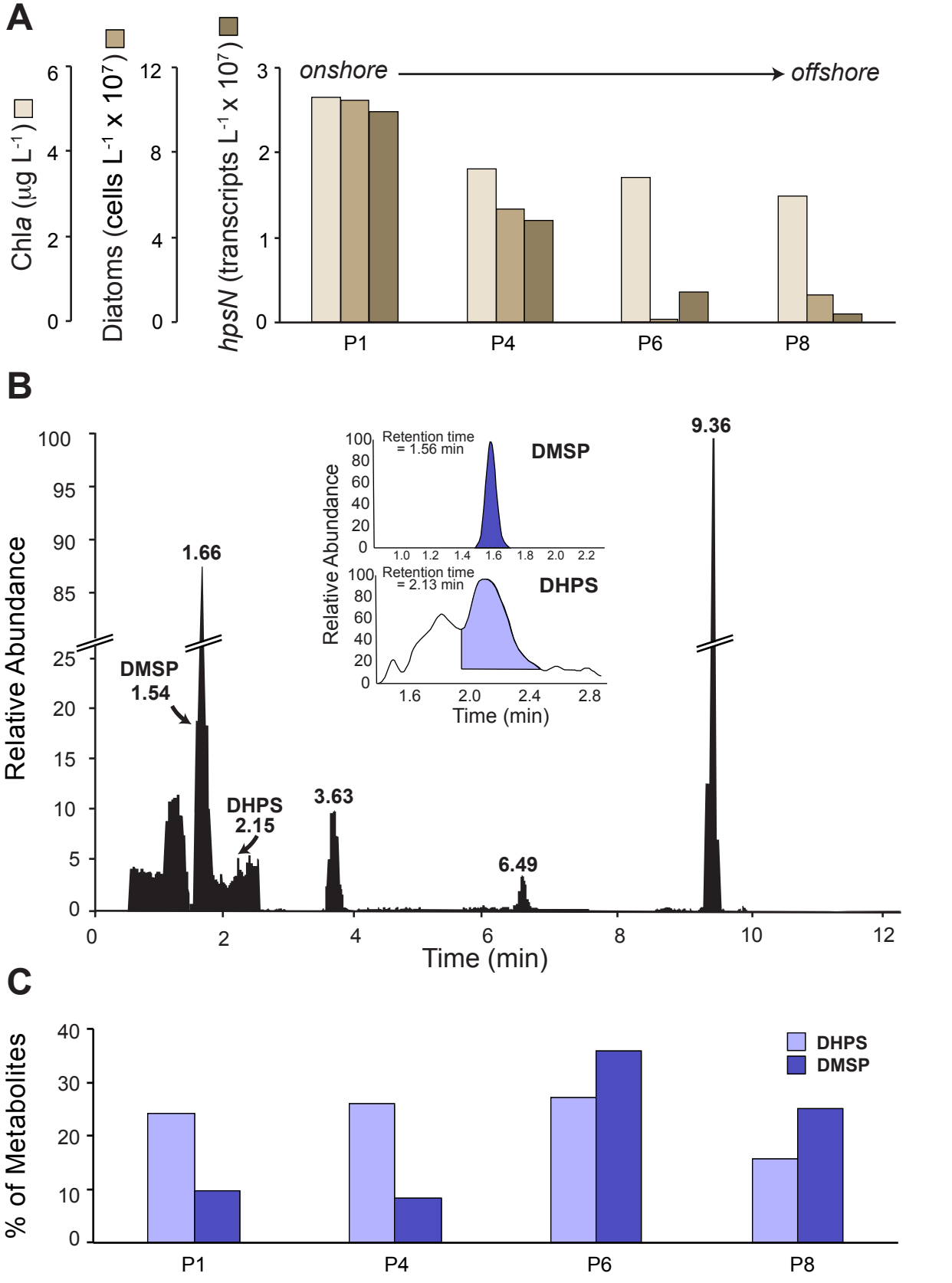
Roseobacter HpsN clade

Roseobacter paralogs

HpsN clade

0.2


Cytosolic Concentration (mM)
12
8
4
0
+B12
+DSS-3
-B12
DHPS
Glutamate
Proline


A
Chla (μg L-1)
Diatoms (cells L-1 x 107)
hpsN (transcripts L-1 x 107)
onshore
offshore
P1
P4
P6
P8
B
Relative Abundance
Time (min)
1.66
DMSP
1.54
DHPS
2.15
3.63
6.49
9.36
Retention time
= 1.56 min
DMSP
Retention time
= 2.13 min
DHPS
C
% of Metabolites
DHPS
DMSP