

Supplementary Information

Supplementary Methods, Figures S1-S8, Tables S1-S3

for

**Integrated metatranscriptomic and metagenomic analyses of stratified microbial
assemblages in the open ocean**

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Supplementary Methods

Sample Collection and DNA/RNA extraction

Bacterioplankton samples (size fraction 0.22 μm – 1.6 mm) from the photic zone (25m, 75m, 125m) and the mesopelagic zone (500m) were collected from the Hawaii Ocean Time-series (HOT) Station ALOHA site in March 2006, as described previously (Shi et al 2009). Briefly, four replicate 1-liter seawater samples were prefiltered through 1.6-mm GF/A filters (Whatman, Maidstone, U.K.) and then filtered onto 0.22- μm Durapore filters (25mm diameter, Millipore, Bedford, MA) using a four-head peristaltic pump system. Each Durapore filter was immediately transferred to screw-cap tubes containing 1 ml of RNAlater (Ambion Inc., Austin, TX), and frozen at -80°C aboard the R/V Kilo Moana. Samples were transported frozen to the laboratory in a dry shipper and stored at -80°C until RNA extraction. Total sampling time, from arrival on deck to fixation in RNAlater was less than 20 minutes.

Replicate filters were pooled for RNA extractions, which were performed as previously described (Shi et al 2009), using the *mirVana*TM RNA isolation kit (Ambion, Austin, TX). Samples were thawed on ice, and the 1 ml RNAlater was loaded onto two Microcon YM-50 columns (Millipore, Bedford, MA) to concentrate and desalt each sample. The resulting 50 μl of RNAlater was added back to the sample tubes, and total RNA extraction was performed following the *mirVana*TM manual. Genomic DNA was removed using a Turbo DNA-freeTM kit (Ambion, Austin, TX). Finally, extracted RNA (DNase-treated) from four replicate filters were combined, purified, and concentrated by

using the MinElute PCR Purification Kit (Qiagen, Valencia, CA).

Bacterioplankton sampling for DNA extraction and DNA extraction was performed as previously described (Frias-Lopez et al 2008).

RNA amplification and cDNA synthesis

Roughly 100 ng of total RNA was amplified using the MessageAmp II-Bacteria kit (Ambion) as described previously (Frias-Lopez et al 2008, Shi et al 2009). Briefly, total RNA was polyadenylated using Escherichia coli poly(A) polymerase. Polyadenylated RNA was converted to double-stranded cDNA via reverse transcription primed with an oligo(dT) primer containing a promoter sequence for T7 RNA polymerase and a recognition site for the restriction enzyme BpmI (T7-BpmI-(dT)¹⁶VN, GCCAGTGAATTGTAATACGACTCACTATAGGGGCGACTGGAGTTTTTTTTTT TTTTTTVN). cDNA was then transcribed in vitro at 37 °C for 6 hours, yielding large quantities (~100 ug) of antisense RNA. An aliquot of antisense RNA (~5 ug aliquot) was polyadenylated again and converted to double-stranded cDNA using first the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) with priming via oligo(dT) for first-strand synthesis, and then the SuperScript Double-Stranded cDNA synthesis kit (Invitrogen) for second-strand synthesis. cDNA was then purified with the QIAquick PCR purification kit (Qiagen), digested with BpmI for 2-3 hours at 37 °C to remove poly(A) tails, and purified again with the QIAquick PCR purification kit. Purified cDNA was used for the generation of single-stranded DNA libraries and emulsion PCR according to established protocols (454 Life Sciences, Roche). Clonally amplified library fragments were then sequenced on a Genome Sequencer GS20 System (Roche).

Bioinformatics analyses

Taxonomic classification of 16S rRNA sequences. Ribosomal RNA sequences were first identified by comparing the data sets to a combined 5S, 16S, 18S, 23S, and 28S rRNA database derived from available microbial genomes and sequences from the ARB SILVA LSU and SSU databases (www.arb-silva.de). 16S rRNA sequences were then selected by BLASTing (Altschul et al 1990) against SILVA SSU databases (bits score ≥ 50 , alignment length $\geq 80\%$ of the read length, and alignment length ≥ 100 bp), and classified using the online Greengenes classifier tools (<http://greengenes.lbl.gov/cgi-bin/nph-classify.cgi>), using the Hugenholtz taxonomy. The parameters used for classifying 16S rRNA were a minimum alignment length of 100bp, and a minimum sequence identity of 75%. For the shotgun sequences, 16S rRNA reads were chosen based on the cutoff of a bits score ≥ 50 and an alignment length ≥ 280 bp, and the parameters used for classifying 16S rRNA were a minimum alignment length of 280bp, and a minimum sequence identity of 75%.

Taxonomic classification of protein-coding sequences. Protein-coding sequences were identified by blasting against the NCBI non-redundant (NCBI-nr) protein database. The BLASTx output was parsed to analyze the taxonomic breakdown using MEGAN (Huson et al 2007), with bit scores > 40 within 10% of the top scoring hits.

Functional analyses using the SEED database and GOS protein cluster database. Non rRNA reads were assigned to SEED subsystems and GOS protein clusters based on top BLASTx hits with bits score ≥ 40 . A bootstrapping method (Rodriguez-Brito et al 2006), which takes care of the size difference among subsystems and looks for

statistically significant differences metagenomes, was applied to identify subsystems that were enriched in the cDNA libraries relative to the corresponding DNA libraries. GOS protein cluster-based analysis was performed as previously described (Frias-Lopez et al 2008). Briefly, cluster-based expression ratios were calculated as the number of reads found for each protein cluster in the cDNA library relative to that found in the DNA library, which was further normalized for the difference in DNA and cDNA library size. Functional annotations for GOS protein clusters, when available, were available from a study by Yooseph *et al* (Yooseph et al 2007). The cluster-based expression ratios were ranked from highest to lowest (Figure 3) to look at clusters being expressed at elevated levels.

Reference genome-centric analysis. Two custom databases (one nucleotide database and one amino acid database) were constructed from 2067 publicly available microbial genome sequences and annotations (fully sequenced and draft genomes as of January 2009). Non-rRNA cDNA and DNA reads from all four depths were compared against the custom nucleotide database, and reads with top hit bits score ≥ 40 were assigned to the corresponding genome. In order to compensate for likely uneven phylogenetic representation in the databases, we allowed any read to map to several reference reads with the same alignment score. Recruitment of protein-coding cDNA and DNA reads onto reference genomes were performed by assigning reads to top amino acid sequences with bits score ≥ 40 . For each ORF, recruited cDNA abundance was divided by the recruited DNA abundance, to give an indication of per-copy cDNA level. If there were cDNA hits but no DNA hits for a given ORF, the number of DNA hits was

considered as 1.

To examine the expression of *Pelagibacter* strain HTCC7211-specific ORFs, putative *Pelagibacter* reads were first identified as reads with top BLASTx hit (against NCBI-nr) to *Pelagibacter* and with a bit score >40. Each of these putative *Pelagibacter* reads then was searched against a custom database of *Pelagibacter* ORFs derived from 3 fully sequenced *Pelagibacter* strains (HTCC1062, HTCC1002, HTCC7211) using BLASTx, and assigned to the best hit ORF. The HTCC7211-specific ORFs were identified as ORFs with no best reciprocal hit, based on the cutoff of a minimum sequence identity of 30%, and a minimum alignment length fraction of 75%, in the genomes of HTCC1062 or HTCC1002.

Figure Legends

Figure S1. Biogeochemical data of the sampling station collected on the cruise. Dashed lines indicate four sampling depths. Data source: <http://hahana.soest.hawaii.edu/hot/hot-dogs/interface.html>.

Figure S2. Taxonomic classification based on 16S rRNA-bearing shotgun sequences. The shotgun libraries and pyrosequencing libraries were constructed from identical DNA samples. Taxonomic assignments were binned at the Order level, using the Hugenholtz taxonomy of Greengenes (see Supplementary Methods). 16S rRNA sequences that could not be classified were excluded from the analysis. Y-axis scale represents the percentage of the total classified 16S rRNA reads. Only taxa that

represented $\geq 1\%$ of all classified reads are displayed.

Figure S3. Stacked area plot showing taxonomic classification of protein-coding sequences. Taxonomic assignments were based on BLASTx against NCBI-nr protein database, using MEGAN (Huson et al 2007), with default settings. Upper panel represents DNA samples, and lower panel represents cDNA samples.

Figure S4. Abundance and normalized expression levels of genes involved in nitrogen metabolism. The abundance of 16S rRNA genes was used to indicate taxon abundance, and was compared to detected abundance of a suite of functional genes (listed in figure legends). Normalized gene expression was calculated as described in Supplementary Methods. (A) Functional genes putatively originated from *Prochlorococcus* populations, in the three euphotic zone samples. (B) Functional genes putatively originated from marine group I crenarchaeota populations in the deep euphotic zone and the mesoplegic samples.

Figure S5. Abundance, expression and taxonomic origins of Proteorhodopsin (PR)-encoding reads. (A). Representation of PR-encoding reads in the DNA and cDNA data sets, and their normalized expression levels in the four depths. (B) Putative taxonomic breakdown of PR sequence reads. PR sequences were first identified by BLASTx against NCBI-nr database, then aligned to a custom PR sequence database (McCarren and DeLong 2007), and finally added to the backbone PR phylogenetic tree

using ARB's "parsimony insertion" feature. The taxonomic origin of a PR-encoding sequence was assumed the same as that of the most related sequence in the PR phylogenetic tree.

Figure S6. Expression of genes involved in aerobic anoxygenic phototrophy (AAP), using a *Roseobacter*-like BAC clone insert as a reference. The BAC clone is eBACred25D05 with an accession number of AY671989. *puf*: light-harvesting and reaction center genes; *bch*: bacteriochlorophyll biosynthesis genes; *crt*, carotenoid biosynthesis genes. Y-axis scale represents normalized cDNA to DNA ratio (normalized expression level; see Supplementary Methods).

Figure S7. Gene expression of *Pelagibacter* HTCC7211-specific ORFs. The HTCC7211-specific ORFs are denoted by the black dots on top the panel, and were identified as ORFs lack of apparent homology to ORFs in the two coastal *Pelagibacter* strains HTCC1062 and HTCC1002 (see Supplementary Methods).

Figure S8. Genome-wide expression profiles of marine crenarchaea-related populations, in all four depths. The x-axis, y-axis, and figure legend are the same as those in Figure 5.

Supplementary information is available at the ISME journal's website.

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Figure S1

Depth profile metadata

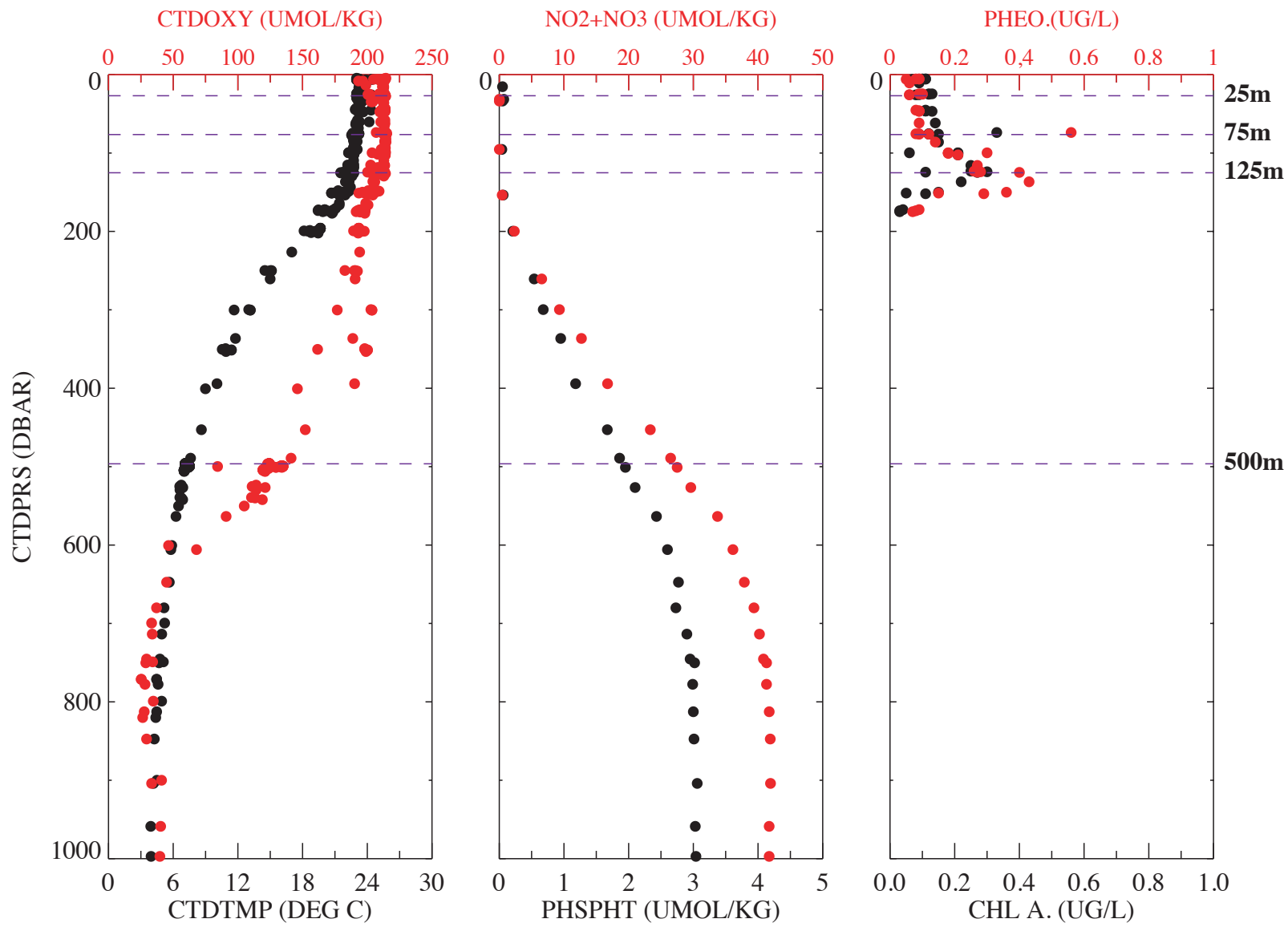


Figure S2

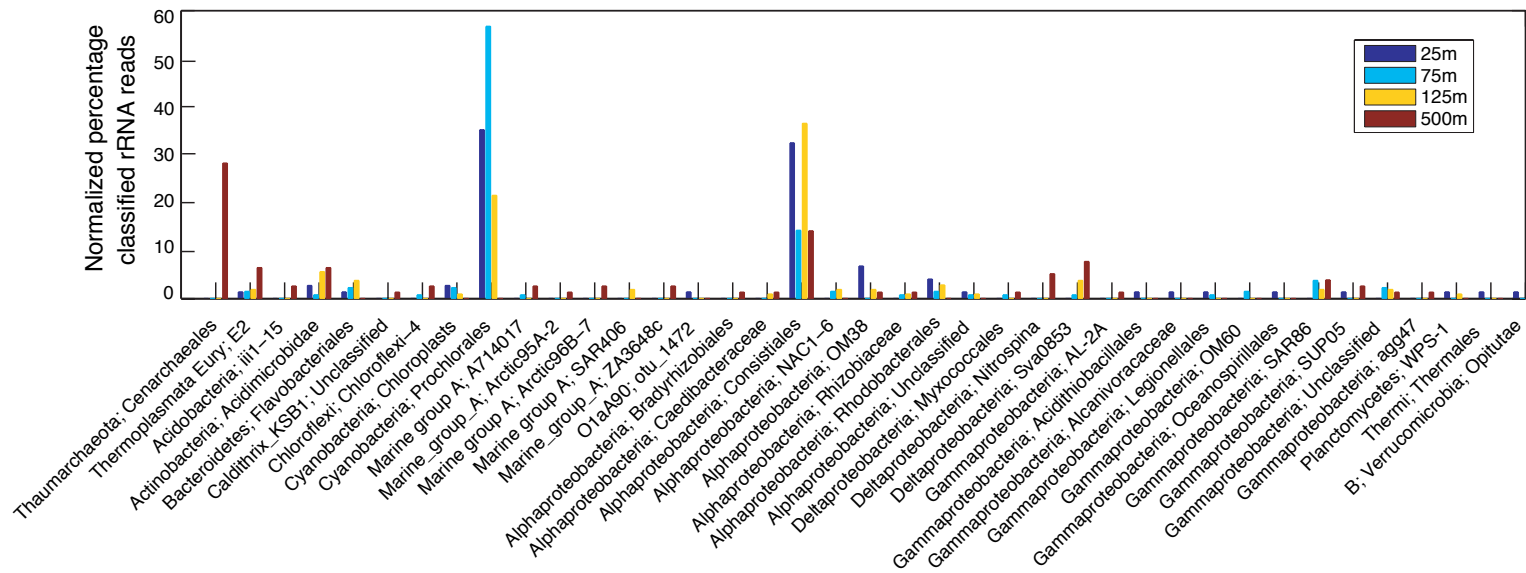


Figure S3

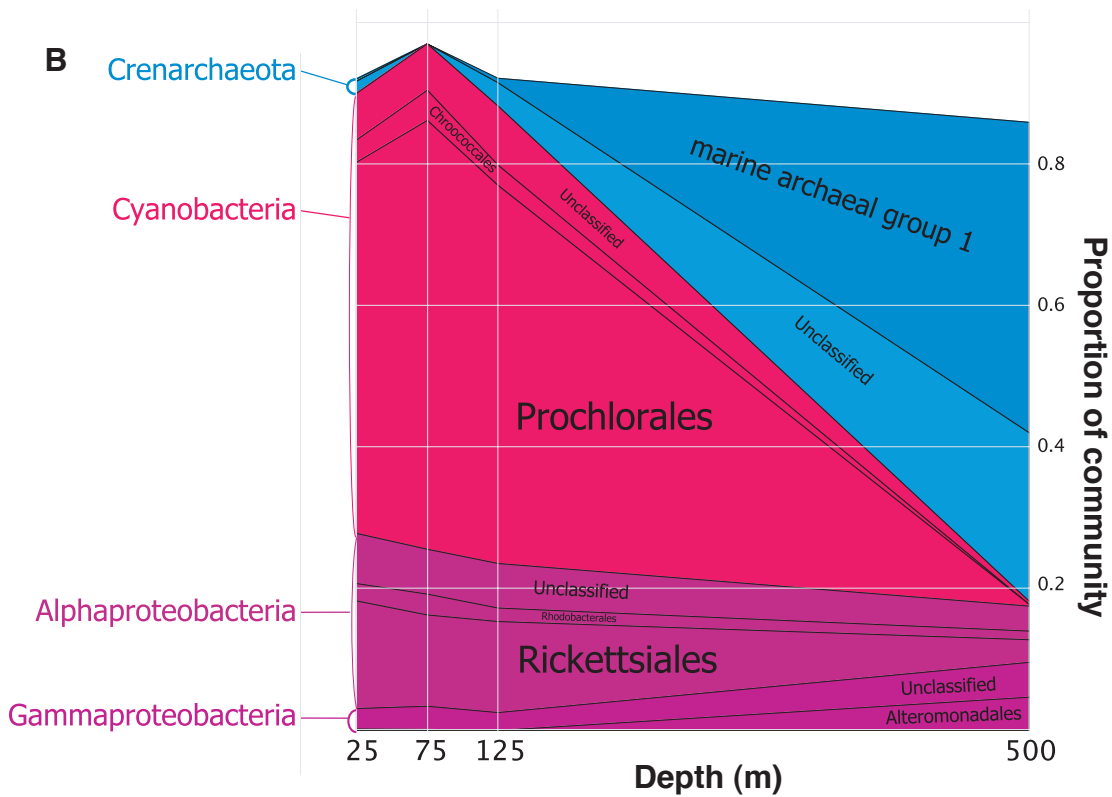
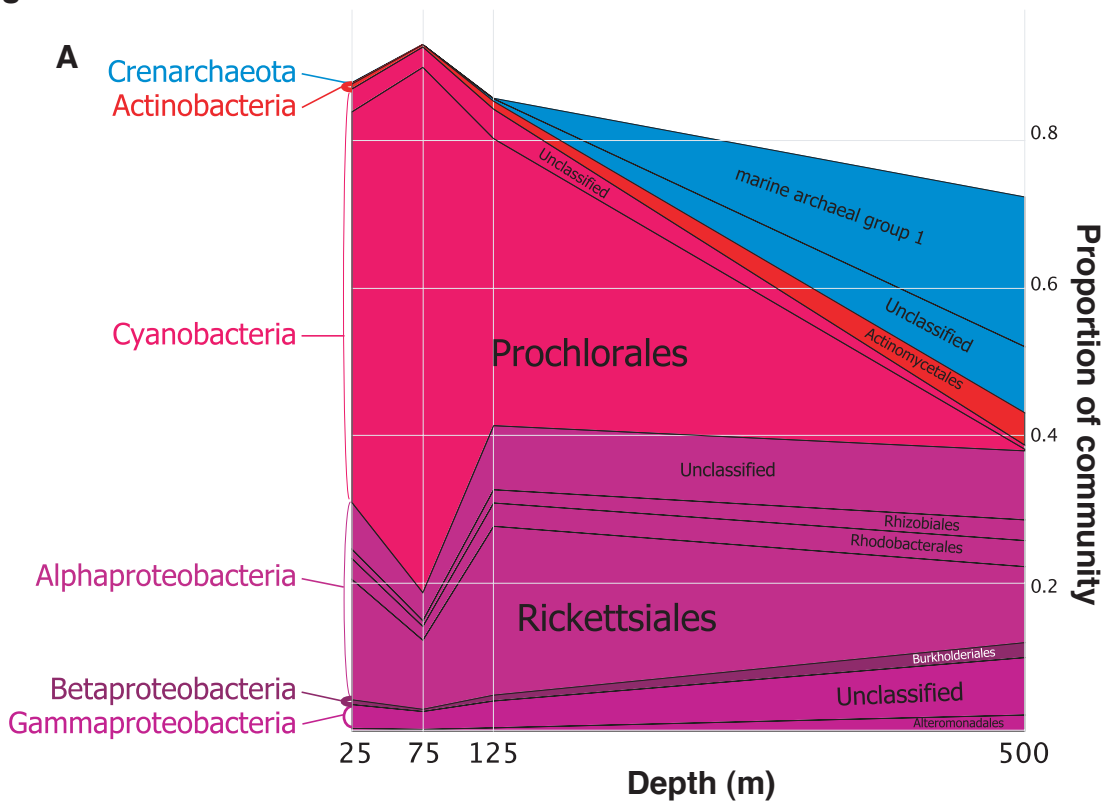


Figure S4

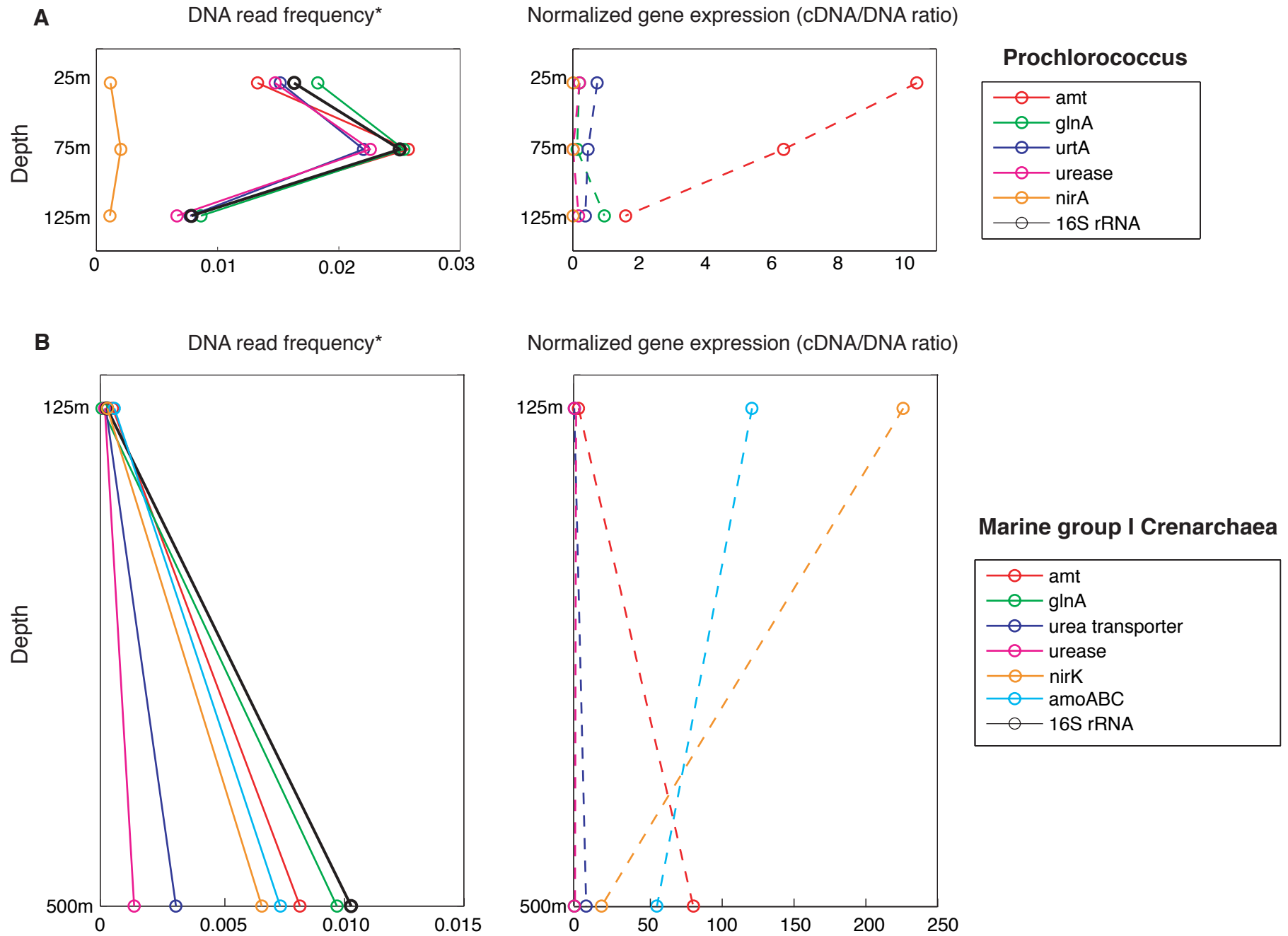
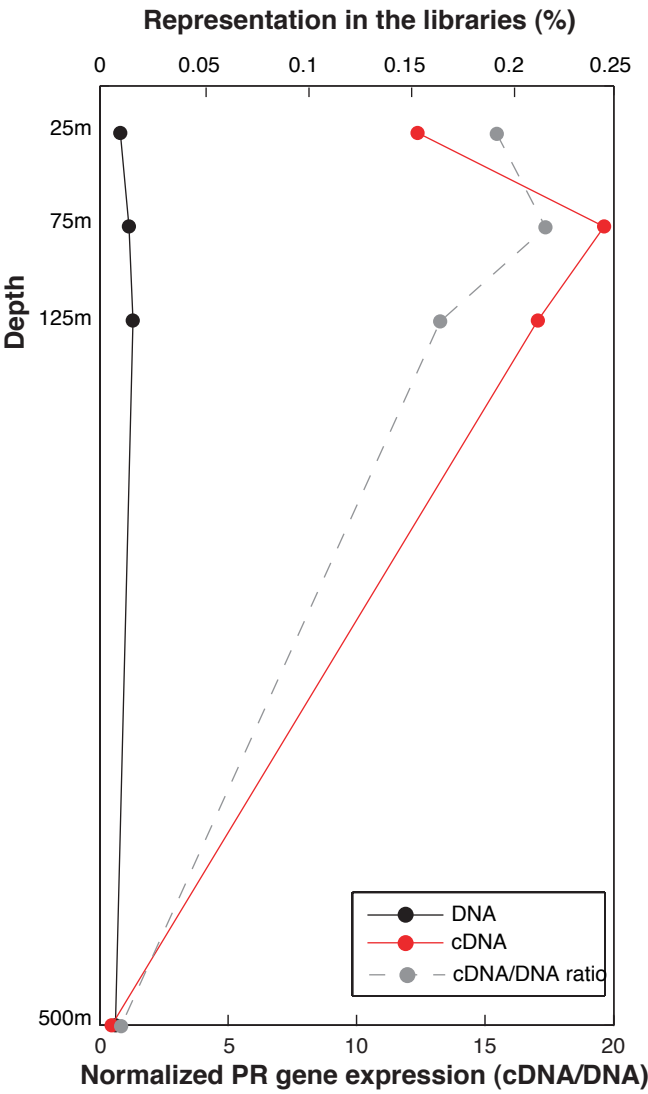


Figure S5

A



B

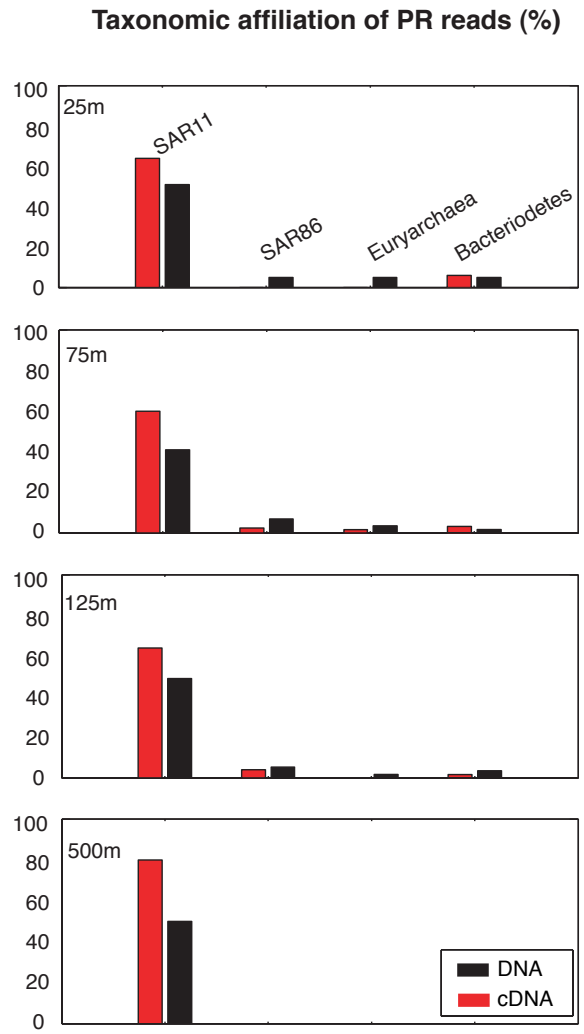


Figure S6

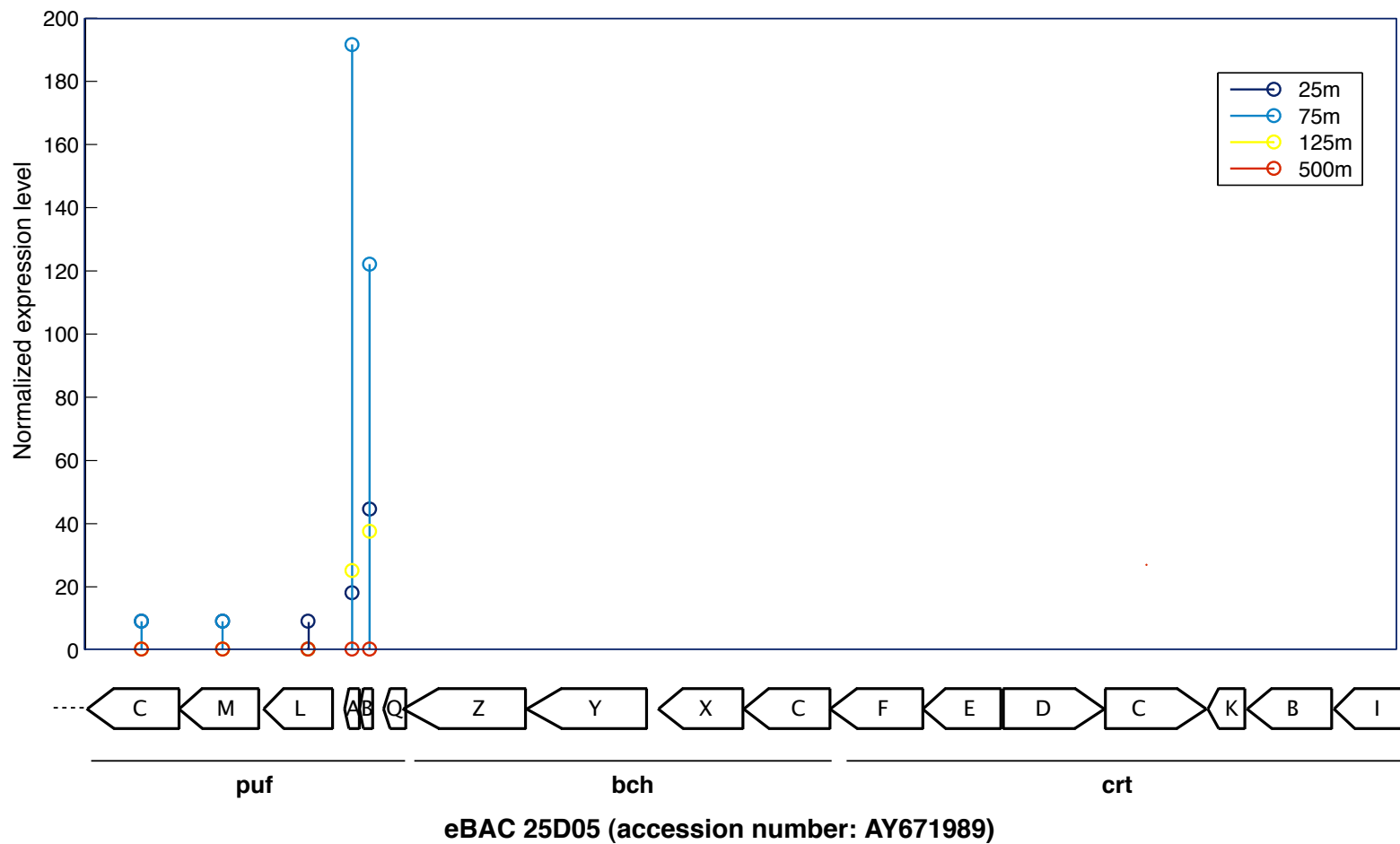


Figure S8

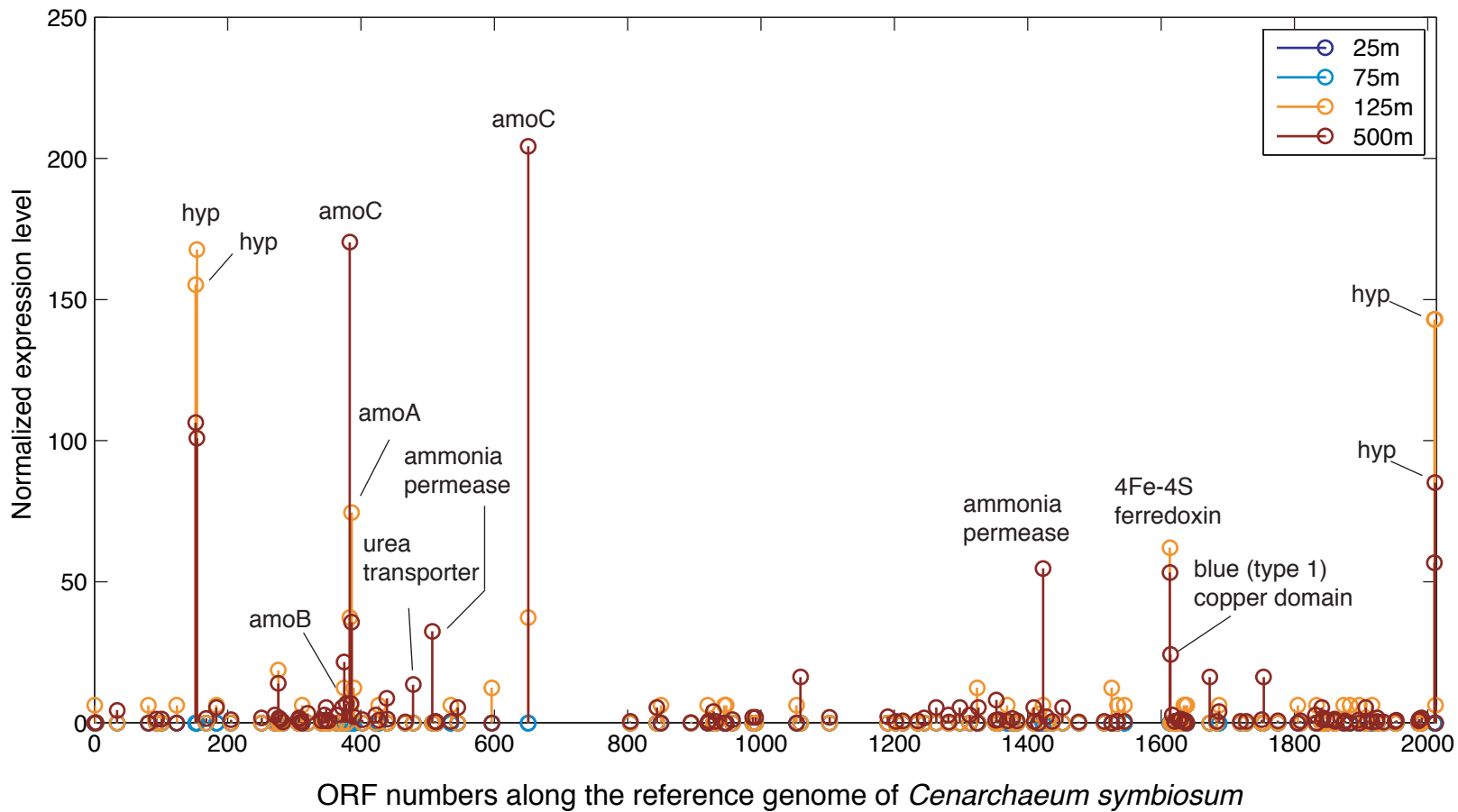


Table S1. Comparison of Prochlorales representation in HF (DeLong et al, 2006) and HOT 179 fosmid clone libraries.

Fosmid library	Sampling depth	# reads assigned to a taxon[*]	# (%) reads assigned to Prochlorales
HF	10 m	5165	341 (6.6%)
	75 m	5953	124 (2.1%)
	130 m	4530	169 (3.7%)
	500 m	6777	6 (0.09%)
HOT179	25 m	8196	820 (10%)
	75 m	10120	1502 (14.8%)
	125 m	15375	1300 (8.5%)
	500 m	16544	22 (0.13%)

^{*} Taxon breakdown was performed with MEGAN (Huson *et al*, 2007), using the following LCA parameters: min support = 1, min score = 70, top percent = 0.

Table S2. Recruitment of cDNA and DNA reads to abundant reference genomes.

Reference genomes	# of DNA reads assigned to a reference genome				# of cDNA reads assigned to a reference genome			
	25m	75m	125m	500m	25m	75m	125m	500m
<i>Prochlorococcus marinus</i> AS9601	28682	43034	10311	23	1656	1900	1926	4
<i>Prochlorococcus marinus</i> MIT 9301	24272	37042	8733	19	1683	2081	1887	7
<i>Prochlorococcus marinus</i> MIT 9312	14405	22578	5805	12	926	1125	1043	2
<i>Prochlorococcus marinus</i> MIT 9215	14354	21886	5193	21	5039	1902	2275	18
<i>Prochlorococcus marinus</i> MED4	1277	2737	644	5	197	269	163	0
Candidatus <i>Pelagibacter ubique</i> HTCC1062	1137	1241	1642	612	238	204	291	84
Candidatus <i>Pelagibacter ubique</i> B HTCC1002	1102	1242	1616	628	232	196	262	102
<i>Psychroflexus torquis</i> ATCC 700755 ATCC700755	1383	1287	1436	181	170	195	187	30
<i>Prochlorococcus marinus</i> NATL1A	126	847	2571	2	13	43	569	0
<i>Prochlorococcus marinus</i> NATL2A	111	786	2511	5	15	51	595	2
<i>Synechococcus</i> CC9605	1421	1485	335	2	64	80	54	2
<i>Prochlorococcus marinus</i> MIT 9515	540	1042	243	4	59	86	120	0
<i>Synechococcus</i> sp. WH8102	146	272	35	0	16	29	16	1
<i>Alteromonas macleodii</i> Deep ecotype	10	2	2	426	4	3	5	406
<i>Prochlorococcus marinus</i> phi P-SSM4	179	104	55	0	18	9	4	0
<i>Nitrosopumilus maritimus</i> SCM1	1	2	44	260	0	2	188	1728
<i>Prochlorococcus marinus</i> phi P-SSM2	135	74	51	0	4	3	1	0
<i>Prochlorococcus marinus</i> CCMP1375	19	24	126	3	0	1	58	3
OM42 clade HTCC2255	36	45	50	24	6	8	11	10
<i>Erythrobacter</i> sp. SD-21	69	7	13	55	2	0	2	5
<i>Acinetobacter baumannii</i> SDF	101	9	2	27	0	0	0	2
<i>Prochlorococcus marinus</i> str. MIT 9211 MIT9211	13	25	91	2	0	3	34	0
<i>Tenacibaculum</i> sp. MED152	35	31	31	17	9	3	12	1
<i>Prochlorococcus marinus</i> MIT9313	2	4	101	1	2	1	21	24
<i>Prochlorococcus marinus</i> MIT 9303	9	6	87	0	1	0	12	2
<i>Synechococcus</i> RCC307	37	31	14	8	1	4	12	0
<i>Synechococcus</i> sp. RS9916 RS9917	28	35	15	2	6	2	12	0
Flavobacteriales bacterium ALC-1	17	23	23	12	3	9	8	1
<i>Kordia algicida</i> OT-1	22	27	14	11	5	2	3	0
<i>Acinetobacter baumannii</i> ACICU	44	2	11	11	0	0	0	1
<i>Rhodospirillales</i> sp. BAL199	15	13	8	33	3	6	10	7
Candidatus <i>Vesicomycosocius okutanii</i> HA	2	4	8	54	0	3	5	7
<i>Pseudomonas syringae</i> phaseolicola 1448A	38	5	4	19	2	1	1	4
Candidatus <i>Ruthia magnifica</i>	7	4	3	51	1	2	6	78
<i>Xanthomonas campestris</i> B100	36	6	3	15	0	0	0	15
marine gamma proteobacterium HTCC2080	24	14	11	7	18	11	8	5
Flavobacteriales sp. SCB49	25	13	9	5	2	5	3	3
Flavobacteriales sp. BAL38	23	12	13	4	1	3	7	2
<i>Synechococcus</i> sp. WH5701	14	14	13	11	2	4	7	3
<i>Staphylococcus aureus</i> phi G1	45	4	1	1	0	0	0	0
<i>Brevundimonas</i> sp. BAL3	27	6	2	16	1	0	2	0

Table S3. Normalized gene expression of *Pelagibacter* strain HTCC7211 (top 60 highly expressed).

ORF number	25m	75m	125m	500m	annotation
1207	19.9	43.3	0.9	0.0	extracellular solute-binding protein, family 1
1263	3.2	14.9	1.8	37.8	spermidine/putrescine-binding periplasmic protein
507	22.1	28.6	18.6	1.8	bacteriorhodopsin
244	26.5	2.9	0.0	0.0	protein of unknown function
623	8.8	26.0	2.1	0.0	conserved hypothetical protein
631	22.7	8.7	9.9	0.0	acetaldehyde dehydrogenase II (acdH-ii)
1226	0.0	2.9	18.6	21.6	conserved hypothetical protein
664	0.0	0.0	18.6	0.0	hypothetical protein
1019	3.2	3.9	3.1	16.2	ABC transporter
1094	1.7	13.6	2.3	3.2	Bacterial extracellular solute-binding protein, family 7
371	13.2	1.4	1.2	0.0	heAt shock protein a
1170	13.2	0.0	0.0	0.0	selenium binding protein
914	5.3	13.0	4.1	0.0	ribosomal protein L13
1328	0.0	4.3	12.4	0.0	pilin (bacterial filament)
954	12.4	3.3	0.9	0.0	conserved hypothetical protein
1159	11.8	0.6	0.5	0.5	GTP cyclohydrolase I
243	10.1	11.0	3.2	5.4	Na ⁺ /solute symporter (Ssf family)
989	0.0	0.0	0.0	10.8	hdig domain protein
544	0.0	0.0	3.1	10.8	trap dicarboxylate transporter, dctp subunit
292	8.8	5.8	5.0	10.8	trap dicarboxylate transporter - dctp subunit
286	1.8	0.0	0.9	10.8	conserved hypothetical protein
195	0.5	0.0	0.0	10.8	chaperone protein DnaJ
823	0.0	1.1	9.3	0.0	transcription termination/antitermination factor NusG
961	8.8	0.0	2.1	5.4	mttA/Hcf106 family, putative
899	8.8	0.0	0.0	0.0	riboflavin biosynthesis protein RibD
878	8.8	0.0	0.0	0.0	ABC transporter permease component
836	8.8	0.0	0.0	0.0	ribosomal protein L23
743	8.8	8.7	0.0	0.0	conserved hypothetical protein
707	8.8	0.0	0.0	0.0	conserved hypothetical protein
674	8.8	5.8	0.0	1.1	ABC transporter, quaternary amine uptake transporter (QAT) family, substrate-binding protein, putative
595	8.8	0.0	0.0	0.0	conserved hypothetical protein
589	8.8	0.0	0.4	0.0	3-oxoacyl-[acyl-carrier-protein] reductase
472	8.8	0.0	0.0	0.0	modification methylase
377	8.8	0.0	0.0	0.0	conserved hypothetical protein
1337	8.8	5.8	0.0	0.0	type II Secretion PilT
1316	8.8	0.0	0.0	0.0	glutaredoxin 3
1133	8.8	0.0	0.0	0.0	glutathione-dependent formaldehyde-activating, GFA, putative
1126	8.8	0.0	0.0	0.0	sulfide dehydrogenase
920	0.0	8.7	0.0	2.7	6-O-methylguanine DNA methyltransferase
90	2.9	8.7	1.5	0.0	translation initiation factor IF-1
42	0.0	8.7	0.0	0.0	3-oxoacyl-[acyl-carrier-protein] reductase, putative
349	0.0	8.7	0.9	0.0	acid tolerance regulatory protein actr
30	0.0	8.7	0.0	0.0	UDP-glucose 4-epimerase
293	5.9	8.7	0.0	0.0	mannitol transporter
205	0.0	8.7	0.0	0.0	putative porin
1227	0.0	8.7	0.0	0.0	conserved hypothetical protein
1214	7.4	8.7	1.7	8.6	substrate-binding region of ABC-type glycine betaine transport system
1074	4.4	8.7	2.1	0.0	serine--glyoxylate aminotransferase
821	7.9	2.8	4.3	0.9	translation elongation factor Tu
687	7.6	7.8	4.6	2.7	taurine transport system periplasmic protein
1225	4.4	7.6	1.3	3.9	ammonium transporter
420	5.3	7.2	2.8	0.9	ATP synthase subunit C, putative
1129	6.6	7.1	7.0	3.6	non-specific DNA-binding protein HBSu
737	7.1	1.7	0.0	0.0	trap dicarboxylate transporter- dctp subunit
1269	2.2	2.9	0.4	7.0	ABC proline/glycine betaine transporter, periplasmic substrate-binding protein
855	6.6	2.5	0.0	0.0	ribosomal protein S13p/S18e
480	6.6	0.0	0.0	0.5	cell division protein FtsZ
96	0.0	0.0	6.2	0.0	ribosomal protein L34
815	0.0	0.0	6.2	0.0	prepilin-type N-terminal cleavage/methylation domain protein
637	0.0	0.0	6.2	0.9	molybdenum cofactor biosynthesis protein C