UNIVERSITY OF CALIFORNIA, SAN DIEGO

# Molecular diversity and adaptations of microorganisms from the deep ocean

A dissertation submitted in partial satisfaction of the
Requirements for the degree Doctor of Philosophy

in

Marine Biology

by

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2011

The Dissertation of Emiley Ansley Eloe is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2011

DEDICATION

For my family – Mama, Dad, Tommy

And for my Doug

With all my love, always.

# EPIGRAPH

The reports of biologists are the measure, not of the science, but of the men themselves.

John Steinbeck
*The Log from the* Sea of Cortez

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| AABW | Antarctic bottom water |
| AT | atmospheric pressure |
| BLAST | basic local alignment search tool |
| Cm | chloramphenicol |
| COG | cluster of orthologous groups |
| DOC | dissolved organic carbon |
| DNA | deoxyribonucleic acid |
| EGS | estimated genome size |
| FACS | Fluorescence-activated cell sorting |
| HP | high pressure |
| HSP | high-scoring pair |
| HV | high viscosity |
| KEGG | Kyoto Encyclopedia of Genes and Genomes |
| Kn | kanamycin |
| MDA | Multiple Displacement Amplification |
| MPa | Megapascal (0.101 MPa = 1 atmosphere) |
| NADW | North Atlantic deep water |
| ORF | open reading frame |
| OTU | operational taxonomic unit |
| PCR | polymerase chain reaction |
| POM | particulate organic matter |
| Rif | rifampicin |
| RNA | ribonucleic acid |
| rRNA | ribosomal RNA |
| RT-PCR | reverse transcription PCR |
| tRNA | transfer RNA |

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Chapter 4 is an invited Perspective article in preparation for submission under the title "The elusive art of microbial cultivation", with permission from coauthor D. H. Bartlett.

Chapter 5 is a full-length manuscript in preparation for submission under the title 'Isolation and characterization of the first psychropiezophilic Alphaproteobacterium,' with permission from coauthor D. H. Bartlett.

Chapter 6 is a full reprint of the publication: Eloe E. A., F. M. Lauro, R. F. Vogel, and D. H. Bartlett. 2008. The deep-sea bacterium *Photobacterium profundum* SS9 utilizes separate flagellar systems for swimming and swarming under high-pressure conditions. *Applied and Environmental Microbiology*. 74(20): 6298-6305, with permission from all coauthors.

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ABSTRACT OF THE DISSERTATION

**Molecular diversity and adaptations of microorganisms from the deep ocean**

by

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Doctor of Philosophy in Marine Biology

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Professor Douglas H. Bartlett, Chair

The indigenous microbial members of deep oceanic environments mediate carbon fluxes in this realm and contribute to major biogeochemical cycles globally, yet their distribution, phylogenetic composition, and functional attributes are not yet well understood. Emerging concepts suggest the prevailing ecological processes and evolutionary constraints acting on these assemblages are more dynamic and

heterogeneous than previously thought. In this context, the research presented herein examines the composition and genomic repertoires of bacteria, archaea, and eukarya from an extreme deep ocean environment, 6,000 m depth within the Puerto Rico Trench. The results identify depth-specific taxonomic and functional trends, as well as expanded gene inventories indicative of unique lifestyle strategies divergent from their photic-zone counterparts. The findings indicate significantly different bacterial communities in particle-associated and free-living fractions at depth, which has implication for future sampling practices and diversity estimates from deep ocean habitats.

The disparity between the number of cultivated piezophilic ('high-pressure adapted') isolates and the expansive diversity identified using molecular techniques has limited further exploration of the physiological and biochemical properties of diverse piezophiles. This issue has been addressed through the application of dilution to extinction cultivation techniques at high-hydrostatic pressure and low temperature using a natural seawater medium. This work has led to the isolation and subsequent characterization of a unique piezophilic member of the Roseobacter lineage within the Alphaproteobacteria. The results provide further evidence for the temperature-pressure dependence of the growth rate for deep-ocean bacteria and substantiate hypotheses regarding piezophilic traits under nutritionally limiting conditions.

This research concludes with the detailed genetic characterization of the unique flagellar motility system of the model piezophilic bacterium *Photobacterium profundum* SS9. It is the first investigation of motility as a function of high hydrostatic pressure in a deep-sea microbial species and highlights the profound value of genetically tractable systems to test hypotheses regarding high-pressure adaptation.

# Chapter I

## Into the deep: An Introduction

The deep ocean constitutes the largest habitat in the biosphere, harboring the greatest abundance and diversity of aquatic microorganisms (53), and yet only an astonishing 5% of this immense environment has been explored (42). Exploration of the deep ocean, and its microbial inhabitants, has been riddled with both technological difficulties and continual scientific amendments to prevailing concepts. What follows is an abbreviated history of the developments in deep-sea microbiology, documentation of the unique properties of deep-sea microorganisms, and a discussion of the shifting paradigms in our understanding of microbial oceanography below the photic zone.

**From the Azoic Theory to astounding microbial diversity**

In 1844, Edward Forbes concluded that no life was present in the oceans below a depth of 600 m based on dredging observations in the Aegean Sea in what became known as the 'Azoic Theory' (42). While this theory was later discredited by the contributions of scientists aboard the H.M.S. *Challenger* (1872–1876) and culminated in the findings of the *Galathea* expedition (1950–1952) (6, 42), these first assertions regarding (microbial) life in the deep ocean seemingly set the stage for later misconceptions regarding metabolic activity and adaptation of autochthonous deep-sea bacteria. The pioneering work of Claude ZoBell and his contributions to the field of deep-sea microbiology provided the first evidence of high-pressure adapted bacteria (63), yet questions and criticisms of his initial findings, by both himself and others further delayed the definitive observation that deep ocean bacteria adapted to high hydrostatic pressure do exist and could be cultivated in the laboratory (59).

Yayanos and colleagues reported the isolation of the first piezophilic ('high-pressure adapted') bacterium from the cold, deep ocean and put to rest much of the controversy regarding the existence of high-pressure adapted bacteria (61). Since then, cultivation efforts have yielded multiple psychropiezophilic ('low-temperature and high-pressure adapted') isolates from a variety of oceanic locals (13, 15, 19, 21-23, 28, 34-38, 54, 56-58, 60, 61), as well as meso- and hyperthermophilic isolates displaying both piezophilic and piezotolerant physiologies (1, 2, 4, 8, 16, 18, 20, 29, 32, 40, 48, 49, 62). These isolates provide the basis for current understanding of the biochemistry, physiology, genetic and genomic adaptations of high-pressure adapted bacteria and archaea.

The advent of molecular techniques, including small-subunit ribosomal gene surveys, high-throughput ribosomal tag pyrosequencing, and metagenomic surveys, has significantly expanded our notions of taxonomic diversity from deep-sea environments in all three domains of life (12, 17, 39, 41, 43, 44, 46). Metagenomic inquiry of pelagic deep ocean microbial assemblages (14, 25, 30), deep-sea sediment (62), hydrothermal vents (11, 55), and the deep subsurface (9) indicates a much greater phylogenetic and putative metabolic diversity compared to what is currently available in piezophilic culture collections. It is increasingly evident that the piezophilic bacteria that are currently biochemically and physiologically characterized constitute only a minuscule fraction of the extant autochthonous microbial members. Thus, deep oceanic environments that were originally thought to be devoid of life are in fact teeming with unique bacterial, archaeal, and eukaryal communities.

**Characteristics of bacteria from the cold, deep ocean**

The current cultivated psychropiezophilic isolates are from a phylogenetically narrow grouping within the Gammaproteobacteria families *Colwelliaceae*, *Moritellaceae*, *Psychromonadaceae*, *Shewanellaceae*, and *Vibrionaceae*, and two gram-positive *Carnobacteriaceae* strains (13, 15, 19, 21-23, 28, 34-38, 54, 56-58, 60, 61) and all exhibit modifications advantageous to life at depth. The first observed physiological modulations included membrane phospholipid fatty acid unsaturation and cell division impairment in response to pressure (reviewed in (7, 27)). Since these initial studies, a greatly expanded view of high-pressure adaptation has emerged to include 'piezo-specific' gene regulation, membrane protein and transporter modification, mechanisms to deal with DNA damage and stress response, respiratory chain composition, alterations in ribosome structure, the accumulation of protein-stabilizing osmolytes, and changes in protein structure and composition, among others (reviewed in (45)). The development of genetic tools has enabled a genetically-tractable way to test hypotheses driving high-pressure adaptation in the model deep-sea bacterium *Photobacterium profundum* SS9, and more recently *Shewanella piezotolerans* WP3 (52). A wealth of data now exists for how piezophiles have adapted their cellular processes to life under high hydrostatic pressure conditions.

Data elucidating the genomic attributes of piezophilic deep-sea bacteria has come from the partial or whole genome sequences of *P. profundum* SS9, *Shewanella* sp. KT99, *Moritella* sp. PE36, *Psychromonas* sp. CNPT3, *Carnobacteirum* sp. AT7, *S. piezotolerans* WP3, and *Colwellia* sp. MT41 (5, 24, 27, 47, 50, 51). The sequence data from these isolates indicates the presence of a large number of transposable elements, a high ratio of rRNA operon copies per genome and larger-than-average intergenic regions

(27). Additionally, the genomes of these deep ocean isolates encode functions for the rapid response to environmental changes with a greater level of gene regulation and improved capacity for complex organic polymer utilization, which is indicative of an opportunistic (r-strategy) lifestyle (27).

Deep ocean metagenomic analyses have yielded many insights into the genomic characteristics and metabolic repertoire of deep-sea microbes. Metagenomic data from two currently available bathypelagic habitats, from 4,000 m depth at the Hawaii oceanographic time-series (HOT) station ALOHA in the North Pacific Subtropical Gyre (25) and 3,000 m depth from the Ionian Station Km3 in the Mediterranean Sea (30), provide evidence for expanded functions and further supports the hypothesis that deep ocean microbes maintain an opportunistic lifestyle (25, 30). Additionally, evidence for fundamentally different evolutionary constraints, particularly relaxed purifying (negative) selection, have been documented to act upon deep-water communities compared to photic-zone counterparts (14, 25, 26).

**Shifting paradigms in microbial oceanography of the deep ocean**

Pelagic deep ocean environments are distinguished from their shallow-water counterparts by a number of fundamental physiochemical characteristics, including the absence of sunlight, low temperature, high inorganic nutrient concentrations, refractory organic material, and increased pressure with depth. These conditions contribute to the ecological processes and evolutionary constraints that structure microbial assemblages in this realm. Recent work has led to a major reassessment of current perceptions regarding the distribution and functions of deep-sea microbial assemblages (reviewed in (3, 33)).

Enumeration of a subset of these issues serves as focal points for guiding the work embodied in this dissertation:

1. Particulate detrital material, marine snow, and other aggregates act as microenvironment "hot spots" for microbial assemblages to colonize and exploit as nutrient sources, contributing to heterogeneous diversity patterns in the meso- and bathypelagic realm (3, 10, 17, 33). This concept is contrary to the previous notion that deep-sea microbes are predominantly free-living and homogeneously distributed, and warrants further investigation of the composition, extent, and functional attributes of free-living and particle-associated microbial lifestyles.

2. A corollary to the idea that particle-associated modes are dominant, is a consideration of what mechanisms (eg. motility) facilitate these associations and how they are modulated by high hydrostatic pressure. Motility is one of the most pressure-sensitive processes in mesophilic microorganisms (31), yet the question remains whether piezophilic bacteria possess motility systems sufficiently divergent from their mesophilic counterparts enabling them to function at the pressures found in the deep ocean.

3. The disparity between the restricted phylogenetic groupings of confirmed piezophilic isolates and the expansive diversity identified using molecular techniques (46) limits current knowledge of the physiological and biochemical properties of diverse psychropiezophiles. It is necessary to corroborate the

putative high-diversity of microbes from deep ocean environments with detailed experiments from diverse psychropiezophilic isolates to assess if similar adaptive mechanisms exist in phylogenetically divergent lineages.

This dissertation seeks to explore the taxonomic, functional and metabolic properties of deep ocean microorganisms in light of the aforementioned conceptual shifts in deep-sea microbial oceanography. The research presented encompasses many of the tools environmental microbiologists have at their disposal, including small-subunit ribosomal gene sequencing surveys, environmental microbial metagenomics, and enrichment cultivation techniques, concluding in a directed genetic inquiry of the motility system of the model piezophilic bacterium *P. profundum* SS9.

Chapter 2 presents a comprehensive phylogenetic diversity survey of the size-fractionated (particle-associated, > 3 μm fraction and free-living, 3 μm – 0.22 μm fraction) microbial community from a hadopelagic environment to address whether there are taxonomic compositional differences between the two size fractions at a depth of 6,000 m within the Puerto Rico Trench (PRT). The results indicate significantly different bacterial communities in the particle-associated and free-living fractions and provide insight into the size-fractionated phylogenetic composition not previously appreciated.

Chapter 3 presents a metagenomic analysis complemented with partial whole-genome sequence data from four uncultivated single cells from the same extreme deep ocean environment within the PRT. This is the first metagenomic report from such an extreme deep ocean environment, that corroborates prevalent depth-specific trends

identified in deep ocean communities and provides insight into the lifestyle strategies employed by hadopelagic microbes. The single-cell data provided genomic context, as well as highly recruiting the metagenomic data.

Chapter 4 is an invited Perspective presenting a synthesis of different cultivation strategies, reasons why cultivation is tremendously important in the '-omics' era, and proposes a framework for how researchers can incorporate cultivation techniques to help bridge the gap between sequence data and characterized microbial species.

Chapter 5 documents the first isolation of a psychropiezophilic member of the Roseobacter lineage within the Alphaproteobacteria through dilution to extinction cultivation efforts using a natural seawater medium at high-hydrostatic pressure and low temperature. The subsequent growth characterization provided further evidence for the temperature-pressure dependence of the growth rate for deep-ocean bacteria.

Chapter 6 presents the genetic characterization of the flagellar motility system of the model piezophilic bacterium *P. profundum* SS9 and is the first investigation of motility as a function of high hydrostatic pressure in a deep-sea microbial species. The results indicate that while motility is one of the most pressure-sensitive processes in mesophilic microorganisms, piezophilic bacteria possess uniquely adapted motility systems for optimal functionality at high pressure.

Lastly, the implications this body of work has for furthering our understanding of microorganisms adapted to the 'piezosphere' are discussed.

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# Chapter II

## Compositional differences in particle-associated and free-living microbial assemblages from an extreme deep-ocean environment

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# Compositional differences in particle-associated and free-living microbial assemblages from an extreme deep-ocean environment

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## Summary

**Relatively little information is available for the composition of microbial communities present in hadal environments, the deepest marine locations. Here we present a description of the phylogenetic diversity of particle-associated (> 3 μm) and free-living (3–0.22 μm) microorganisms present in a pelagic trench environment. Small subunit ribosomal RNA gene sequences were recovered from members of the *Bacteria*, *Archaea* and *Eukarya* obtained from a depth of 6000 m in the Puerto Rico Trench (PRT). Species richness estimates for the bacterial particle-associated fraction were greater compared with the free-living fraction and demonstrated statistically significant compositional differences, while the archaeal fractions were not found to be significantly different. The particle-associated fraction contained more *Rhodobacterales* and unclassified *Myxococcales* along with *Bacteroidetes*, *Planctomycetes* and chloroplast sequences, whereas the free-living fraction contained more *Caulobacterales*, *Xanthomonadales* and *Burkholderiales*, along with Marine Group A and *Gemmatimonadetes*. The *Eukarya* contained a high abundance of *Basidiomycota* Fungi 18S rRNA genes, as well as representatives from the super-groups *Rhizaria*, *Excavata* and *Chromalveolata*. A diverse clade of diplonemid flagellates was also identified from the eukaryotic phylotypes recovered, which was distinct from previously identified deep-sea pelagic diplonemid groups. The significance of these results to considerations of deep-sea microbial life and particle colonization is discussed in comparison to the few other deep-ocean phylogenetic surveys available.**

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## Introduction

Oceanic trenches are seafloor depressions formed at convergent margins where one lithospheric plate moves below another, and in extreme cases are the deepest locations on Earth (Stern, 2002). These unique topographic features are characterized by high hydrostatic pressure, cold, saline water masses (North Pacific Deep Water or Antarctic Bottom Water) and the absence of solar radiation. Within pelagic deep-sea settings, microbial communities are believed to largely subsist on photosynthetically derived organic matter in the form of sinking particulate organic matter (POM), as well as the flux of semi-labile dissolved organic carbon (DOC) (Reinthaler *et al.*, 2006; Hansell *et al.*, 2009). Additionally, recent isotopic measurements of carbon assimilation within free-living mesopelagic communities identified archaeal autotrophic carbon fixation as a significant source of carbon supporting microbial life at depth (Ingalls *et al.*, 2006; Hansman *et al.*, 2009). An emerging concept is the importance of macroscopic particles in the deep ocean as reservoirs of microbial heterogeneity and metabolism (Bochdansky *et al.*, 2010; Nagata *et al.*, 2010), yet few studies to date have systematically compared the diversity of particle-associated and free-living assemblages at depth.

Large-scale environmental genomic surveys, as well as massive ribosomal tag pyrosequencing efforts, have been used to examine the phylogenetic diversity and in some cases functional gene diversity of free-living microorganisms present in meso- and bathypelagic environments. The locations and maximum depths sampled include the Hawaii oceanographic time-series station ALOHA in the North Pacific Subtropical Gyre [4000 m (DeLong *et al.*, 2006; Pham *et al.*, 2008); 4400 m (Brown *et al.*, 2009)], the Ionian Station Km3 in the Mediterranean Sea [3000 m (Martín-Cuadrado *et al.*, 2007)], and six discrete depths

from the North Atlantic [4121 m (Sogin *et al.*, 2006)]. Taken together these studies suggest the presence of distinctive microbial communities and lifestyle strategies below the photic zone, which are separate from surface-water populations (Konstantinidis and DeLong, 2008; Konstantinidis *et al.*, 2009). Analyses of the genome content of microbial populations obtained at depth and from cultured deep-sea isolates indicate that deep-ocean microorganisms possess diverse adaptations for particle colonization, including polysaccharide and pilus synthesis (Vezzi *et al.*, 2005; DeLong *et al.*, 2006; Worden *et al.*, 2006; Lauro and Bartlett, 2008; Wang *et al.*, 2008; Konstantinidis *et al.*, 2009). Particulate detrital material, marine snow and other aggregates act as microenvironment 'hot spots' for colonization and nutrient acquisition in the meso- and bathypelagic realm (Arístegui *et al.*, 2009). However, there remains a major gap in knowledge of the extent to which deep-sea particle-associated and free-living microbial populations are distinct and which phylogenetic groups constitute this difference.

In this study, an analysis is presented of bacterial, archaeal and eukaryal diversity from a pelagic hadal environment, specifically at 6000 m depth within the Puerto Rico Trench (PRT), the deepest location in the Atlantic Ocean (ten Brink, 2005). In addition to examining the total microbial community composition present in the PRT, the bacterial and archaeal populations have been separated into free-living and particle-associated groupings. The results indicate diverse and significantly different assemblages in particle-associated and free-living trench communities, suggesting an important role for particulate matter in structuring phylogenetic diversity at depth.

## Results and discussion

### *Hydrographic characteristics of the Puerto Rico Trench*

Table 1 gives the mean inorganic nutrient concentrations and microbial and viral abundances for surface (1 m) and hadal seawater (6000 m), highlighting the oligotrophic nature of the overlying surface waters. Silicate concentration, salinity and oxygen values confirm that the 6000 m depth water mass sampled is Antarctic Bottom Water, which is consistent with previous samplings and values obtained from similar depths within the PRT (Joyce *et al.*, 1999; Schlitzer, 2002).

**Table 1.** Chemical and biological constituents of unfiltered surface (1 m) and hadal (6,000 m) seawater.

| | Depth (m) | |
|---|---|---|
| | 1 | 6000 |
| N + N ($\mu$mol $l^{-1}$) | 0.03 ± 0.026 | 24.18 ± 0.70 |
| $PO_4$ ($\mu$mol $l^{-1}$) | 0.013 ± 0.0058 | 1.67 ± 0.01 |
| Silicate ($\mu$mol $l^{-1}$) | 1.67 ± 0.058 | 63.77 ± 0.25 |
| $NO_2$ ($\mu$mol $l^{-1}$) | 0 | 0 |
| $NH_4$ ($\mu$mol $l^{-1}$) | 0.08 ± 0.02 | 0.043 ± 0.0058 |
| $NO_3$ ($\mu$mol $l^{-1}$) | 0.03 ± 0.026 | 24.18 ± 0.70 |
| $O_2$ ($\mu$mol $kg^{-1}$) | 195.7 | 240 |
| Salinity | 34.98 | 34.84 |
| TON ($\mu$M) | 5.7 ± 0.38 | 29.3 ± 0.76 |
| TOC ($\mu$M) | 78.1 ± 1.16 | 46.2 ± 2.06 |
| Viral abundance (particles $ml^{-1}$) | $3.92 \times 10^6$ | $4.50 \times 10^5$ |
| Microbial abundance (cells $ml^{-1}$) | $3.44 \times 10^5$ | $1.09 \times 10^4$ |

### *Differences between particle-associated and free-living fractions*

A total of 541 bacterial 16S rRNA gene sequences and 675 archaeal 16S rRNA gene sequences were obtained from particle-associated (> 3 $\mu$m) and free-living (3 $\mu$m–0.22 $\mu$m) size fractions, along with 339 eukaryal 18S rRNA gene sequences from the 3 $\mu$m fraction. Verified non-chimeric 16S and partial 18S rRNA gene sequences were deposited in GenBank under Accession Numbers HM798588–HM800142. Statistically significant differences in the bacterial libraries were found using the LibShuff algorithm (Singleton *et al.*, 2001), whereas difference in the archaeal libraries were not found to be statistically significant (Fig. S1). Similarity-based Operational Taxonomic Units (OTUs) and species richness estimates indicated that the particle-associated bacterial fraction hosted a greater diversity compared with the free-living fraction (Table 2, Fig. 1). This contrasts with two other reports documenting the relationships between free-living and particle-associated bacteria at depth (Moeseneder *et al.*, 2001; Ghiglione *et al.*, 2007). In those studies, a greater number of OTUs were present in the free-living fraction at depths down to 1600 m in the Mediterranean Sea. Prior investigations of the phylogenetic make-up of particle-associated and free-living communities from surface seawater have generally documented the presence of distinct communities (DeLong *et al.*, 1993; Crump *et al.*, 1999). However, comparison of species richness in particle-associated compared with free-living fractions is mostly limited to coastal marine settings, with little information regarding open ocean pelagic comparisons.

### *Taxonomic diversity of the Puerto Rico Trench*

*Alphaproteobacteria.* Approximately 40% of the bacterial sequences recovered from the two size fractions were classified as *Alphaproteobacteria*, consisting of six named orders (*Rhodobacterales*, *Rhizobiales*, *Sphingomondales*, *Caulobacterales*, *Rhodospirillales* and *Rickettsiales*) and one unclassified environmental grouping (Fig. 2A). The greatest numbers of sequences were classified within the order *Rickettsiales*, mainly within the ubiquitous SAR11 clade. Discrete groupings within the

**Table 2.** Similarity-based OTUs and species richness estimates.

| Library | Number of sequences | Cluster distance 0.01 OTU | 0.01 ACE | 0.01 Chaol | 0.03 OTU | 0.03 ACE | 0.03 Chaol | 0.05 OTU | 0.05 ACE | 0.05 Chaol |
|---|---|---|---|---|---|---|---|---|---|---|
| > 3 μm *Bacteria* (BB) | 292 | 211 | 786 | 744 | 174 | 473 | 420 | 156 | 351 | 336 |
| 3 μm–0.2 μm *Bacteria* (AB) | 249 | 131 | 370 | 327 | 107 | 239 | 208 | 93 | 170 | 160 |
| > 3 μm *Archaea* (BA) | 339 | 122 | 225 | 280 | 54 | 83 | 90 | 27 | 37 | 50 |
| 3 μm–0.2 μm *Archaea* (AA) | 336 | 114 | 196 | 187 | 46 | 68 | 68 | 30 | 44 | 39 |

In parentheses, the library code name is listed as follows: > 3 μm *Bacteria* (BB), 3 μm–0.2 μm *Bacteria* (AB), > 3 μm *Archaea* (BA) and 3 μm–0.2 μm *Archaea* (AA). Alignments for each library were created using MUSCLE (Edgar, 2004) and distance matrices were produced using the Dnadist program from PHYLIP (Felsenstein, 2005) for input into DOTUR (Schloss and Handelsman, 2005).

SAR11 clade were supported (Fig. S2A), further expanding the documented depth partitioning within this clade (Field *et al.*, 1997; Pham *et al.*, 2008), with an average intraclade sequence divergence (AD) of 4.6% (maximal intraclade sequence divergence, MD = 8.9%).

The particle-associated fraction hosted a large fraction of sequences within the Order *Rhizobiales*, that were most closely related to sequences derived from soil and sediments, a similar observation from tag-sequences retrieved from 800 and 4400 m depth in the North Pacific (Brown *et al.*, 2009). Close matches to characterized soil and sediment phylotypes could suggest similar degradation pathways inherent in these particular lineages for refractory compounds, in line with numerous pathways associated with xenobiotic degradation present in deep-ocean microbial genome fragments (Martín-Cuadrado *et al.*, 2007). Additionally, Martín-Cuadrado and colleagues found the greatest recruitment of sequences from a 3000 m depth in the Mediterranean to *Rhizobiales* genomes. Taken together with these previous observations, the discovery of abundant *Rhizobiales* 16S rRNA sequences in the PRT reinforces and expands a *Rhizobiales* connection to the deep ocean.

*Gammaproteobacteria.* Relatively few gammaproteobacterial sequences were recovered in either particle-associated or free-living fractions (6.2% and 15.3% of total respectively) (Fig. 2A, Fig. S2B). Five named orders (*Legionellales*, *Xanthomonadales*, *Alteromonadales*, *Chromatiales* and *Oceanospirillales*) were represented, with five additional groupings of uncultivated environmental organisms. Approximately half of the gammaproteobacterial sequences in the free-living fraction clustered within the order *Xanthomonadales* and were found to be significantly distinct between the two libraries (Fig. 2A). Surprisingly, the nine free-living and four

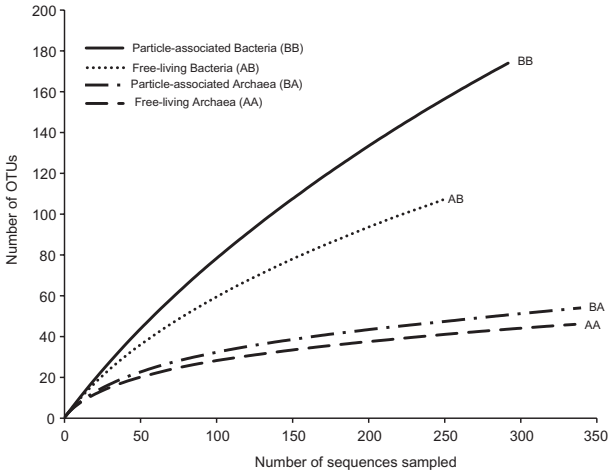


**Fig. 1.** Rarefaction analysis for bacterial and archaeal ribosomal small-subunit rRNA libraries shown for 97% similarity OTU bins.

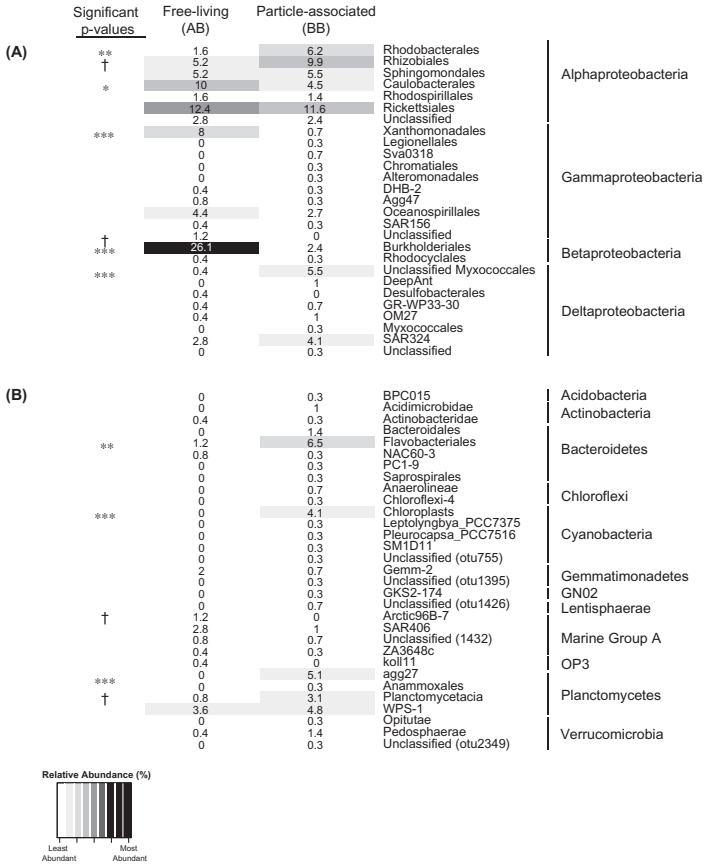


**Fig. 2.** Heatmap of classified bacterial orders from the free-living and particle-associated libraries. Values within the heatmap represent the relative abundance normalized to the library size with *P*-values listed for orders that were found to be significantly different between the two size fractions. (A) Proteobacterial orders and (B) non-proteobacterial order classifications. Significance codes for *P*-values are as follows: ***$P = 0–0.001$, **$P = 0.001–0.01$, *$P = 0.01–0.05$, †$P = 0.05–0.1$. The program Metastats (White *et al.*, 2009) was utilized to statistically test the differential abundances of order-level groupings between the two libraries.

particle-associated *Alcanivoracaceae* family sequences were identical to 16S rRNA fragments from nine separate fosmid clones collected from 500, 770 and 4000 m depth at Station ALOHA (Pham *et al.*, 2008). This phylotype appears to have a depth-related cosmopolitan distribution since it has now been retrieved from deep waters in two geographically distinct ocean basins.

*Betaproteobacteria.* Eight sequences (2.7% of the total) in the particle-associated fraction were classified as *Betaproteobacteria*, while 66 sequences (26.5% of the total) were found in the free-living fraction (Fig. 2A, Fig. S2B). The majority classified in the order *Burkholderiales*, within the three families *Alcaligenaceae*, *Comamonadaceae* and *Burkholderiaceae*. The number of identical sequences within the *Comamonadaceae* and *Burkholderiaceae* groupings was high, indicating that the same sequence was retrieved multiple times and could represent a clonal population.

The high percentage of betaproteobacterial sequences is curious, considering *Betaproteobacteria* have

not been found in significant abundances in marine bacterioplankton. The number of sequences retrieved with matches to other deep-ocean phylotypes or surface seawater leads us to believe the betaproteobacterial sequences are authentic rather than non-marine contamination during sampling or downstream processing. Furthermore, betaproteobacterial sequences have been recovered from other deep-ocean data sets (Martín-Cuadrado *et al*., 2007; Pham *et al*., 2008; Brown *et al*., 2009), unrelated to the presumed betaproteobacterial contaminant from the Sargasso Sea, *Burkholderia* SAR-1, which has been shown to be more closely related to clinical strains (Venter *et al*., 2004; Mahenthiralingam *et al*., 2006). The 17 free-living and three particle-associated sequences recovered from the PRT are almost identical to marine isolate *Limnobacter* sp. MED105 (NCBI Accession ABCT00000000), again suggesting a marine origin for the betaproteobacterial sequences in the PRT data set.

*Deltaproteobacteria.* Forty-nine sequences were classified as *Deltaproteobacteria*, 11 from the free-living fraction (4.4% of the total) and 38 particle-associated (13% of the total) (Fig. 2A, Fig. S1C). More than half of the sequences fell within the deep-water SAR324 clade (Wright *et al*., 1997; López-García *et al*., 2001), with the majority clustering within the ctg_NISA008 subclade comprising sequences from 500–4000 m depth (Penn *et al*., 2006; Pham *et al*., 2008) (AD = 3.5%, MD = 11.7%). A large fraction of sequences, almost exclusively from the particle-associated fraction, formed a distinct unclassified *Myxococcales* clade with the representative HF200-01L06 from 200 m depth at Station ALOHA, with an average sequence identity of 95.1% (Pham *et al*., 2008). The abundance of particle-associated *Myxococcales* retrieved from the PRT indicates this group might be an important player in particle colonization at depth.

*Non-proteobacterial sequences.* The remaining sequences not affiliated with the *Proteobacteria* consisted of 38 and 107 sequences from the free-living and particle-associated fractions respectively (Fig. 2B, Fig. S1D). The majority of the particle-associated sequences grouped within the phyla *Bacteroidetes* and *Planctomycetes*, forming numerous clades that were exclusively particle-associated. Four cyanobacterial-like and 12 plastid-related sequences were identified exclusively from the particle-associated fraction, indicating a portion of the particle-associated fraction is likely surface-derived.

The most dominant fraction from the free-living library was classified as SAR406 within the Marine Group A, a well-documented deep-dwelling group (Gordon and Giovannoni, 1996; Gallagher *et al*., 2004). The Marine Group A sequences were highly divergent with an AD of 10% and maximum divergence of 19.1%. *Gemmatimonadetes* were also more dominant in the free-living fraction compared with the particle-associated fraction (2% compared with 1% of the total), a group poorly characterized yet documented in other deep-ocean diversity surveys (DeLong *et al*., 2006; Martín-Cuadrado *et al*., 2007; Pham *et al*., 2008). *Actinobacteria*, *Acidobacteria*, *Chloroflexi* (within the environmental-only SAR202 clade), *Lentisphaerae* and *Verrucomicrobia* were additionally represented in the particle-associated fraction.

*Archaea.* Archaeal diversity consisted of sequences almost exclusively from the Group I Marine *Crenarchaeota* and Group II *Euryarchaeota* (Fig. S3). More than 80% of the sequences for both libraries were classified as Marine Group I *Crenarchaeota* with an average sequence identity of 97%. Euryarchaeal sequences were exclusively within the Marine Group II clade, with the exception of a single sequence (from the 3 μm fraction), which classified within the Marine Hydrothermal Vent Group (Fig. S3). The low archaeal diversity is consistent with the recent findings of Auguet and colleagues, who found the Marine Group 1.1a and *Thermoplasmatales* (Group II *Euryarchaeota*) dominated the phylotypes present in the marine water column (Auguet *et al*., 2009).

*Eukarya.* Eukaryotic sequences were classified into the four main super-groups *Opisthokonta*, *Rhizaria*, *Excavata* and *Chromalveolata* (Table 3), with the majority of sequences indentified as Fungi and *Metazoa* in the *Opisthokonta* super-group (87.6% of the total) (Adl *et al*., 2005). Fungi represented 52.2% of the total sequences recovered and were classified as *Basidiomycota* and

**Table 3.** Eukaryal classifications for partial 18S ribosomal gene sequences.

| Super-group | First-rank taxa | Absolute number | % Library |
|---|---|---|---|
| *Opisthokonta* | *Metazoa* | 120 | 35.4 |
| | Fungi | 177 | 52.2 |
| *Chromalveolata* | *Stramenopiles* | 4 | 1.2 |
| | *Alveolata* | 11 | 3.2 |
| *Excavata* | *Euglenozoa* | 18 | 5.3 |
| *Rhizaria* | *Radiolaria* | 8 | 2.4 |
| | *Cercozoa* | 1 | 0.3 |
| | Total | 339 | 100 |

| | Second-rank taxa | Absolute number | % Library | % Fungal sequences |
|---|---|---|---|---|
| Fungi | *Ascomycota* | 15 | 4.4 | 8.5 |
| | *Basidiomycota* | 162 | 47.8 | 91.5 |
| | Total | 177 | 52.2 | 100 |

Concatenated forward and reverse sequences were aligned using the SINA Webaligner (Pruesse *et al*., 2007), uploaded into the ARB program (Ludwig *et al*., 2004) and manually checked with the ARB_EDIT4 tool.

*Ascomycota* in accord with previous deep-sea fungal studies that suggest these taxa predominate at depth (Bass *et al.*, 2007) (Fig. S4A). Many of the sequences recovered from the PRT were most closely related to sequences associated with parasitic or pathogenic species, a recurring finding in recent deep-ocean eukaryotic surveys (Bass *et al.*, 2007; Guillou *et al.*, 2008; Brown *et al.*, 2009; Scheckenbach *et al.*, 2010). The similarity of many of the eukaryotic sequences recovered from the PRT to other deep-ocean phylotypes underscores a depth-associated evolutionary relationship for many deep-ocean eukaryotes that transcends ocean basin source.

The 42 non-*Opisthokonta* sequences recovered were classified into the five first-rank taxa *Stramenopiles*, *Alveolata*, *Euglenozoa*, *Radiolaria* and *Cercozoa* (Table 3, Fig. S4B). The *Excavata* super-group was the most abundant group after the *Opisthokonta*, with all first-rank *Euglenozoa* sequences classifying as Diplonema (18 sequences, 5.3% of the total). These sequences formed a divergent environmental clade distinct from the deep-sea pelagic diplonemid (DSPD) groups I and II, which contain phylotypes from the deep Pacific and deep Atlantic oceans (Lara *et al.*, 2009).

### *Comparison of Puerto Rico Trench bacterial assemblages to shallow and deep marine communities*

Bacterial assemblages from the PRT particle-associated and free-living bacterial libraries were compared with 16S rRNA sequences identified from fosmid libraries from seven depths at Station ALOHA (Pham *et al.*, 2008), 16S sequence fragments from the Global Ocean Survey (Rusch *et al.*, 2007), and two separate ribosomal tag pyrosequence studies from the deep North Atlantic (Sogin *et al.*, 2006) and three discrete depths at Station ALOHA (Brown *et al.*, 2009) (Fig. 3). When compared at the phylum level, the two PRT libraries clustered separately from the other deep-ocean samples (Fig. 3A). This is due to low abundances recovered within the phyla *Chloroflexi*, *Acidobacteria* and *Actinobacteria*, and the high abundances recovered of *Planctomycetes* and *Bacteroidetes* (Fig. S5). These differences could reflect the distinct location, depth, time of year and water mass of the PRT samples analysed. However, these differences could also reflect distinct aspects of sampling, the nature of the sequence information obtained and the degree to which the community profiles have been quantitative.

Similar considerations apply to the comparisons among the other deep-sea samples. This is especially germane when the two independent Station ALOHA data sets are examined. At both the phylum and OTU level of resolution, the two separate Station ALOHA data sets are not as similar to one another as the one Station ALOHA sample set obtained by 454-generated tag sequencing (Brown *et al.*, 2009) is with the North Atlantic Deepwater samples also obtained by tag sequencing (Sogin *et al.*, 2006). These caveats notwithstanding, the PRT differences noted between the PRT sequences and the other deep-sea samples are intriguing and suggest that the PRT bacterial communities might be distinguishable at a broad taxonomic level. Clearly, more work will be needed to definitively address this possibility in particular and to more fully explore pelagic deep-sea microbial populations in general.

At the highest taxonomic resolution examined (OTU level), the clustering pattern shifts to include the two PRT samples with all the other deep-ocean samples, while still excluding all of the shallow-water samples (Fig. 3B). This finding supports the more detailed phylogenetic comparisons presented previously (Fig. 2, Fig. S2), indicating that the phylotypes recovered from the PRT are most closely related to sequences obtained from other deep-ocean habitats.

The phylotypes from the particle-associated fraction of the PRT could be a mixture of microorganisms from the overlying surface waters that have sunk and survive at high pressure, in addition to autochthonous deep-water microorganisms colonizing and exploiting these niches. A recent study found that in a pressurized microcosm, a gradual pressure increase to 40 MPa led to differential effects on the abundance and viability of five shallow-water strains (Grossart and Gust, 2009). It seems reasonable to expect that the particle-associated microorganisms present at depth will reflect the magnitude and duration of exposure to elevated pressures and the relative pressure-resistance of the associated microbes. The fact that the PRT particle-associated library contained both eukaryotic and bacterial phytoplankton is evidence of a shallow-water microbial community connection. Nevertheless, the fraction of the surface-water microorganisms present must be relatively small given the high overall similarity of this community with other deep-sea communities at the OTU level, and with the free-living PRT sample under all conditions evaluated.

This study provides new information on the phylogenetic characteristics of bacterial, archaeal and eukaryal hadal life, and the relationship of bacterial populations residing on particulates or present as unattached cells. Considering that most aquatic microorganisms exist below the photic zone and that their activities are of paramount importance to the cycles of many elements in the biosphere (Arístegui *et al.*, 2009), it is startling how little is known of their identities and distributions in the deep sea. The paucity of microbial community data for hadal environments, and the deep ocean in general, renders a limited view of deep-water communities and future efforts

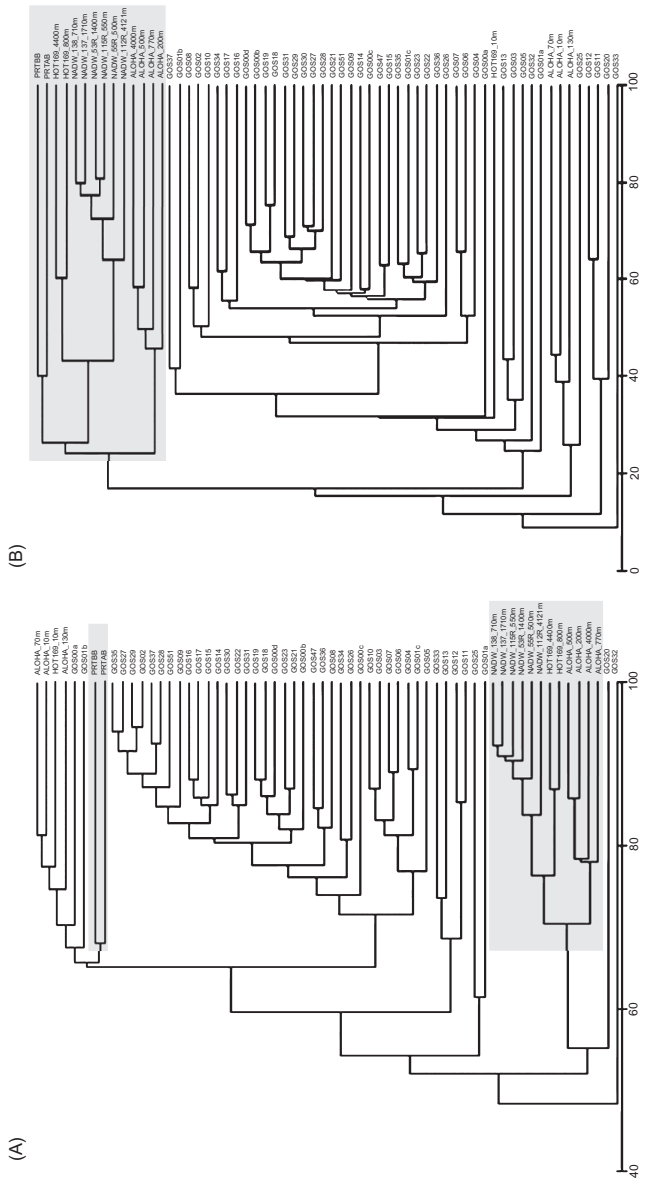


**Fig. 3.** UPGMA hierarchical clustering based on the bacterial abundance matrix of (A) phylum-level and (B) OTU-level groupings for the particle-associated and free-living PRT libraries, 16S rRNA gene sequence fragments from the forty-four Global Ocean Survey (GOS) sites (Rusch *et al.*, 2007), ribosomal tag sequences from six depths from the North Atlantic Deep Water (Sogin *et al.*, 2006), 16S rRNA sequences identified from fosmid libraries from seven depths at Station ALOHA (Pham *et al.*, 2008), and ribosomal tag sequences from three depths at Station ALOHA (Brown *et al.*, 2009). Taxonomic classifications were assigned by taking the best high-scoring pair (HSP) from the resultant BLAST search against the greengenes database (DeSantis *et al.*, 2006), then parsed using the Hugenholtz taxonomic outline (available at http://greengenes.lbl.gov/Download/Taxonomic_Outlines/) with custom perl scripts. The relative abundances for the highest resolution classifications (OTU-level) were used to generate a Bray–Curtis similarity matrix for UPGMA clustering. For the phylum-level classifications, the *Proteobacteria* were divided into the following groups *Alphaproteobacteria*, *Deltaproteobacteria*, *Desulfovibrionales*, *Gammaproteobacteria* and *Epsilonproteobacteria* according to the Hugenholtz taxonomic scheme (DeSantis *et al.*, 2006) and the square-root transformed relative abundances were used to generate a Bray–Curtis similarity matrix for cluster analysis using the Primer v5 package (Clark and Warwick, 2001).

to more intensely sample from numerous locations will enable global generalizations.

In addition to the need for basic information on the evolutionary history and stratification of these microorganisms, it will be useful in the future to distinguish the autochthonous, metabolically active members of the community. New sampling strategies are also needed to collect deep-ocean samples, with considerations for preserving particle and colloidal microenvironments as well as retaining the *in situ* high pressure and low temperature conditions. Analyses of RNA in addition to DNA will help to discriminate the active members (La Cono *et al*., 2009) of the community. In summary, this study represents an important step towards teasing apart the microbial community present in the water column and those members associated with particulates, yielding a more complete view of microbial diversity at depth.

**Acknowledgements**

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from the lower ocean surface layer. *Appl Environ Microbiol* **63:** 1441–1448.

### Supporting information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Coverage plots for the bacterial libraries (AB = 3 μm–0.22 μm; BB = 3 μm) and archaeal libraries (AA = 3 μm–0.22 μm; BA = 3 μm) generated by the LibShuff program (available at http://libshuff.mib.uga.edu/) as described by the authors (Singleton *et al*., 2001). The differences between the two bacterial libraries (A and B) are statistically significant at *P*-value of 0.001. The differences between the two archaeal libraries (C and D) are not statistically significant at *P*-values of 0.465 and 0.842.

**Fig. S2.** Bacterial phylogenetic trees depicting the relationship of the particle-associated and free-living sequences from the Puerto Rico Trench. Clones are represented by ovals, with open ovals representing the free-living fraction and black ovals representing sequences from the particle-associated fraction. Numbers in parentheses next to clone symbols represent the number of clones condensed in that branch. The rRNA gene sequences were aligned using the SINA Webaligner (Pruesse *et al*., 2007), uploaded into the ARB program (Ludwig *et al*., 2004) and manually checked with the ARB_EDIT4 tool. Aligned sequences were exported for bootstrap analysis using PHYLIP (Felsenstein, 2005) and FastTree (Price *et al*., 2009) for neighbour-joining and maximum likelihood methods respectively. Bootstrap support for nodes are indicated (PHYLIP NJ method/FastTree ML method; values > 50% are shown, values < 50% are marked with –). The outgroup used to calculate phylogeny was archaeaon *Nitrosopumilus maritimus* SCM1 (Accession number DQ085097). (A) *Alphaproteobacteria*; (B) *Gamma*- and *Betaproteobacteria*; (C) *Deltaproteobacteria*; (D) other non-*Proteobacteria*.

**Fig. S3.** Archaeal phylogenetic tree depicting the relationship of the particle-associated and free-living sequences from the Puerto Rico Trench. Clones are represented by ovals, with open ovals representing the free-living fraction and black ovals representing sequences from the particle-associated fraction. Numbers in parentheses next to clone symbols represent the number of clones condensed in that branch. The rRNA gene sequences were aligned using the SINA Webaligner (Pruesse *et al*., 2007), uploaded into the ARB program (Ludwig *et al*., 2004) and manually checked with the ARB_EDIT4 tool. Aligned sequences were exported for bootstrap analysis using PHYLIP (Felsenstein, 2005) and FastTree (Price *et al*., 2009) for neighbour-joining and maximum likelihood methods respectively. Bootstrap support for nodes are indicated (PHYLIP NJ method/FastTree ML method; values > 50% are shown, values < 50% are marked with –). The outgroups used to calculate phylogeny were *Bacillus subtilis* 168 (AL009126) and *Escherichia coli* K-12 (U00096).

**Fig. S4.** Eukaryal phylogenetic trees depicting the relationship of (A) *Opisthokonta* and (B) non-*Opisthokonta* fungal sequences from the Puerto Rico Trench. Clones are represented by black ovals, with numbers in parentheses representing the number of clones condensed in that branch. Partial 18S ribosomal gene sequences were concatenated and aligned using the SINA Webaligner (Pruesse *et al*., 2007), uploaded into the ARB program (Ludwig *et al*., 2004) and manually checked with the ARB_EDIT4 tool. Bootstrap support for nodes are indicated (PHYLIP NJ method/FastTree ML method; values > 50% are shown, values < 50% are marked with –). The outgroups used to calculate phylogeny were *Bacillus subtilis* 168 (AL009126) and *Escherichia coli* K-12 (U00096).

**Fig. S5.** Heatmap of relative abundances of bacterial phyla (and groupings with the *Proteobacteria*) for deep-ocean data sets. Taxonomic classifications were assigned by taking the best high-scoring pair (HSP) from the resultant BLAST search against the greengenes database as described in the main text.

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**Supplementary Material – Figures.**

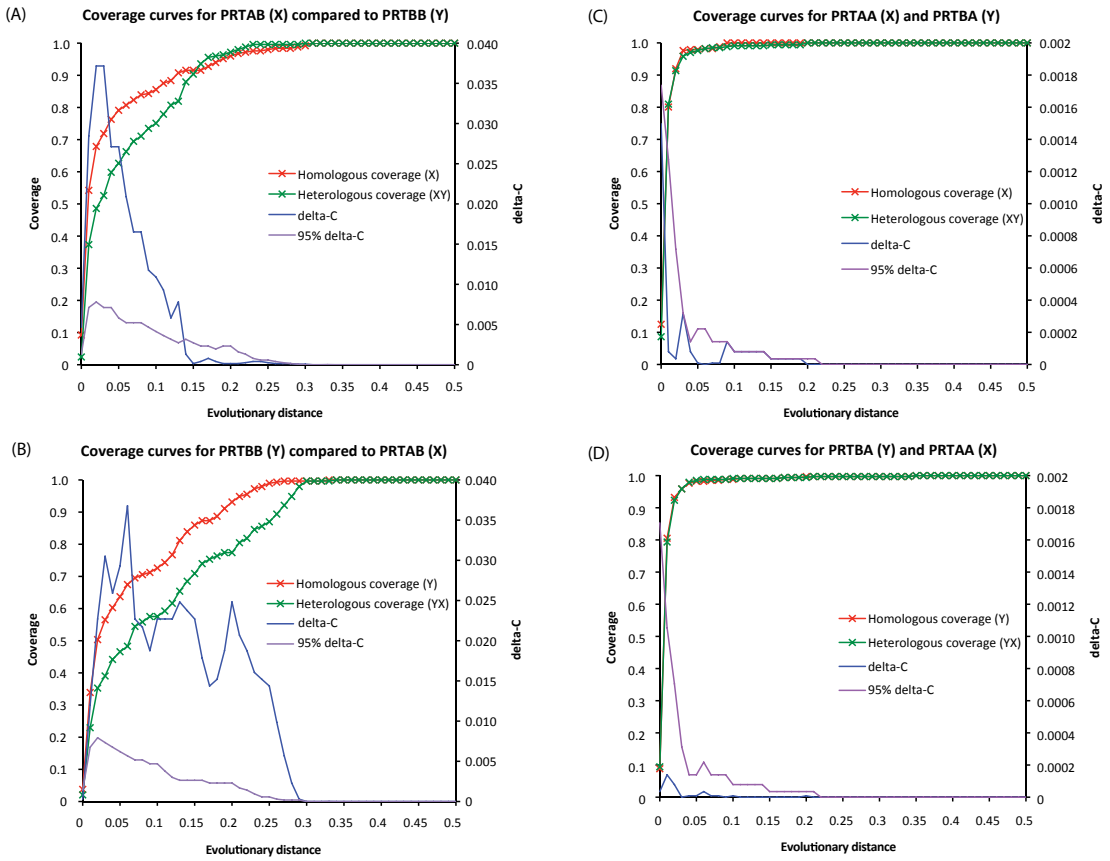


**Figure S1.** Coverage plots for the bacterial libraries (AB = 3 μm – 0.22 μm; BB = 3 μm) and archaeal libraries (AA = 3 μm – 0.22 μm; BA = 3 μm) generated by the LibShuff program (available at http://libshuff.mib.uga.edu/) as described by the authors (Singleton et al., 2001). The differences between the two bacterial libraries (A and B) are statistically significant at p-value 0.001. The differences between the two archaeal libraries (C and D) are not statistically significant at p-values 0.465 and 0.842.

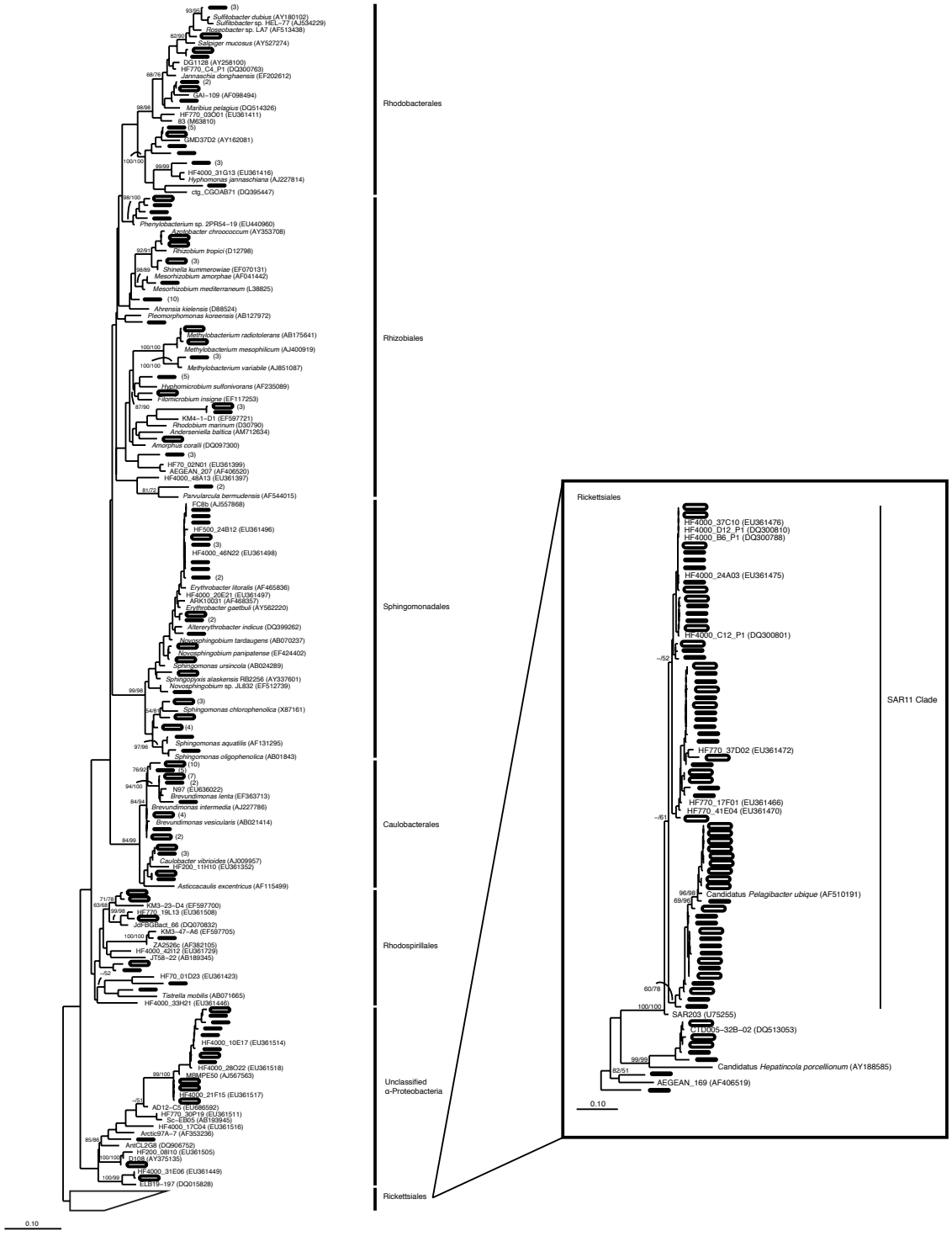


**Figure S2A.** Alphaproteobacterial phylogenetic trees depicting the relationship of the particle-associated and free-living sequences from the Puerto Rico Trench.

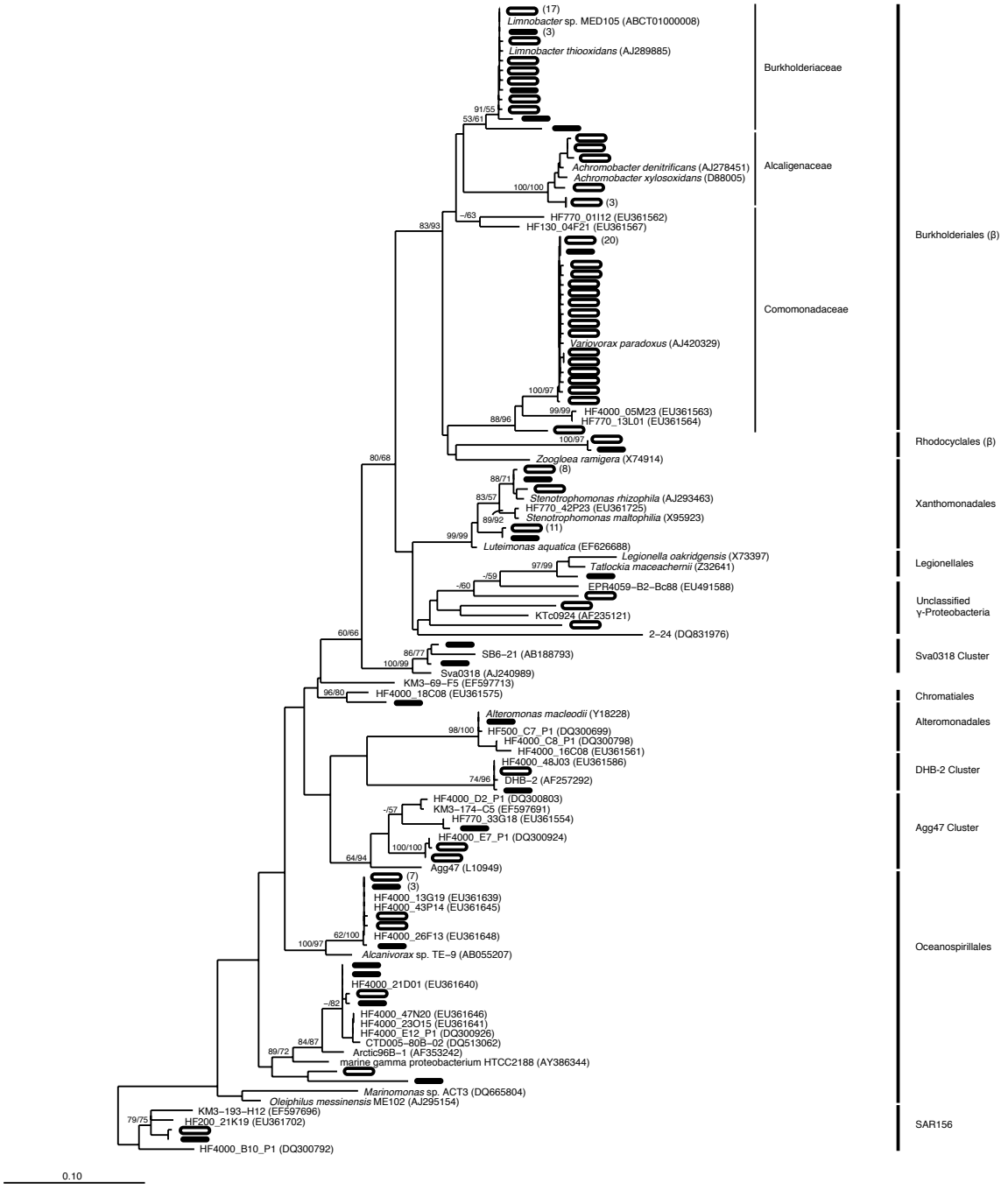


**Figure S2B.** Gamma- and Betaproteobacterial phylogenetic tree depicting the relationship of the particle-associated and free-living sequences from the Puerto Rico Trench.

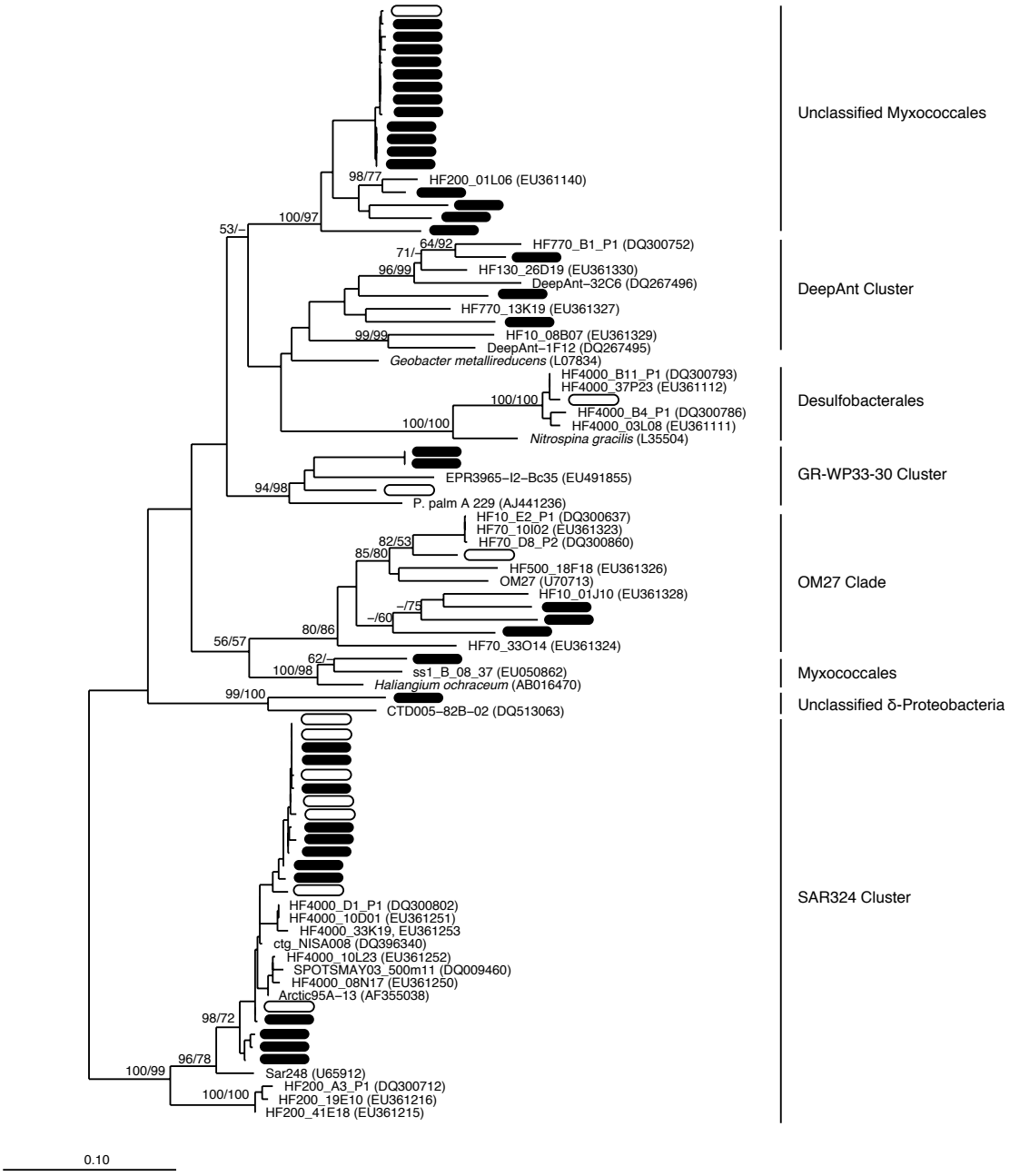


**Figure S2C.** Deltaproteobacterial phylogenetic tree depicting the relationship of the particle-associated and free-living sequences from the Puerto Rico Trench.

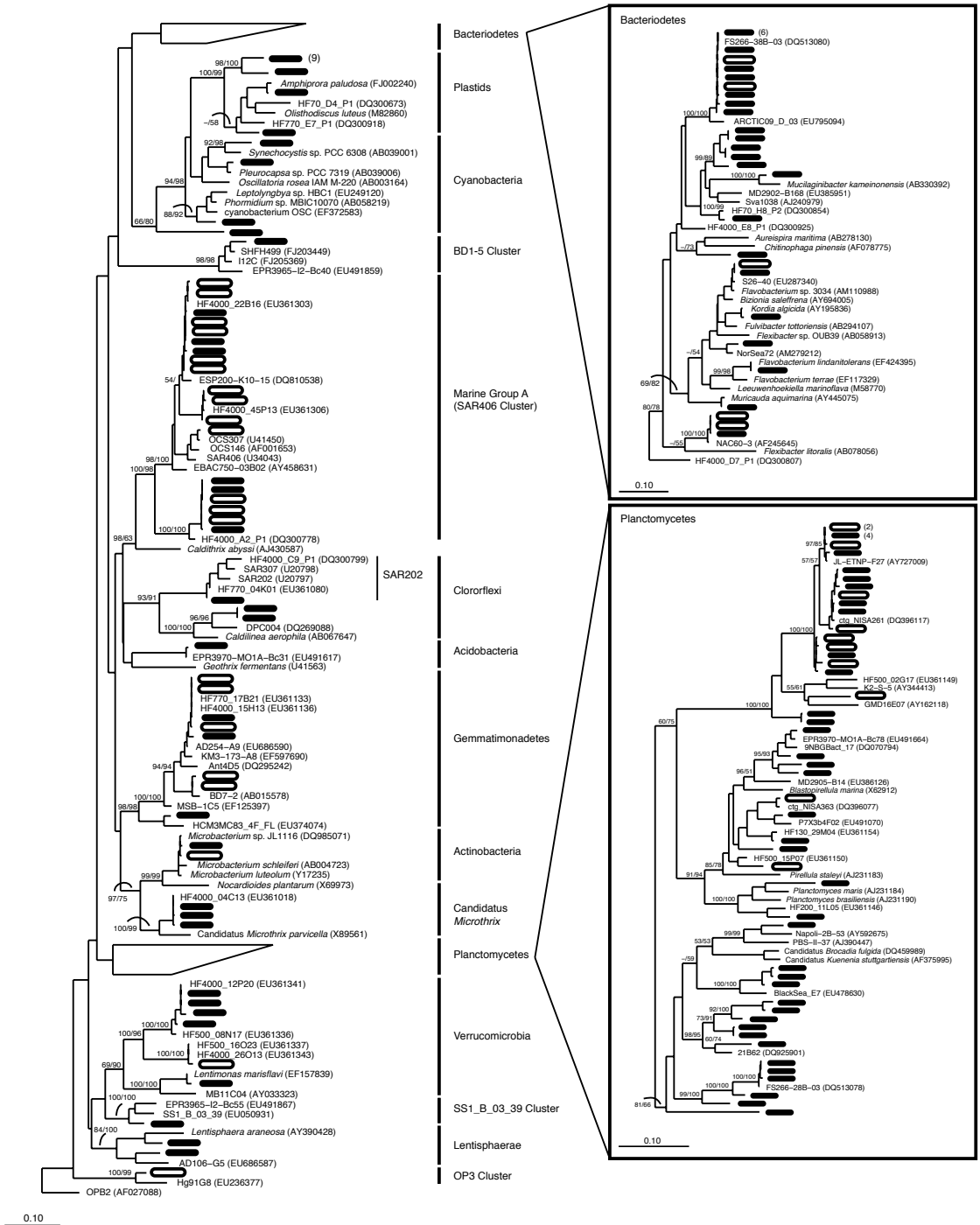


**Figure S2D.** Other non-Proteobacterial phylogenetic trees depicting the relationship of the particle-associated and free-living sequences from the Puerto Rico Trench.

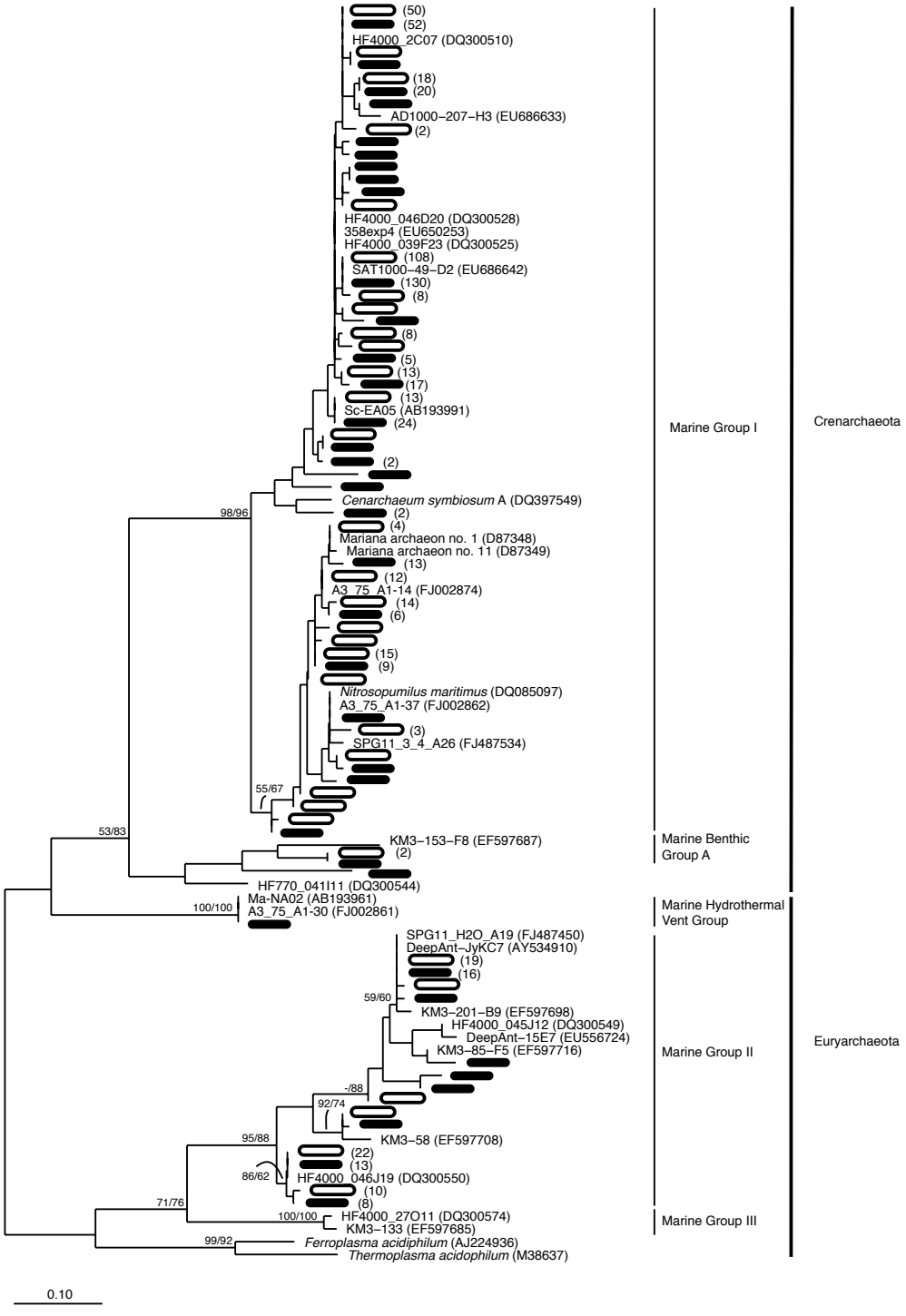


**Figure S3.** Archaeal phylogenetic tree depicting the relationship of the particle-associated and free-living sequences from the Puerto Rico Trench.

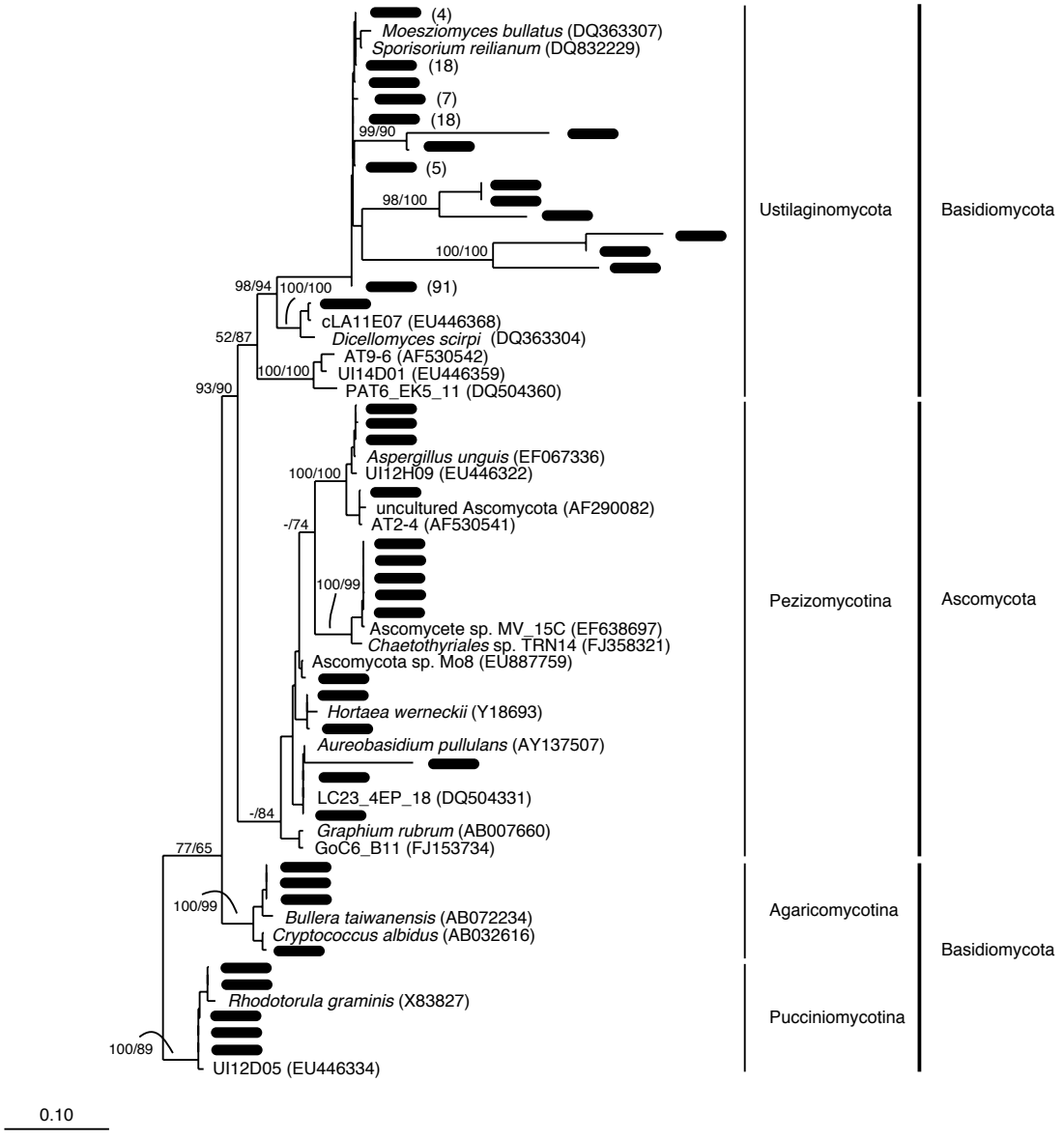


**Figure S4A.** Eukaryal phylogenetic tree depicting the relationship of Opisthokonta fungal sequences from the Puerto Rico Trench. Clones are represented by black ovals, with numbers in parentheses representing the number of clones condensed in that branch.

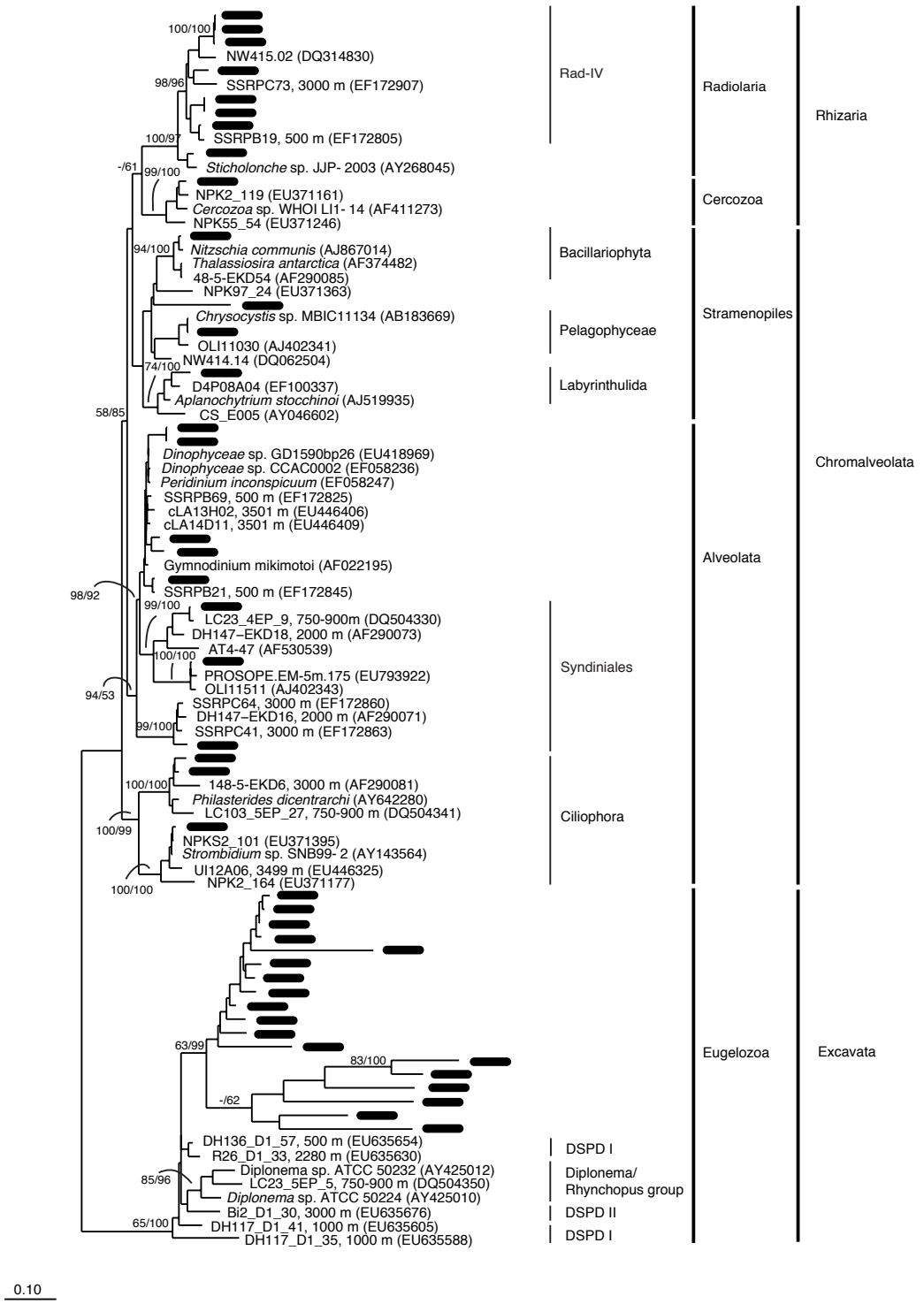


**Figure S4B.** Eukaryal phylogenetic tree depicting the relationship of non-Opisthokonta sequences from the Puerto Rico Trench. Clones are represented by black ovals, with numbers in parentheses representing the number of clones condensed in that branch.

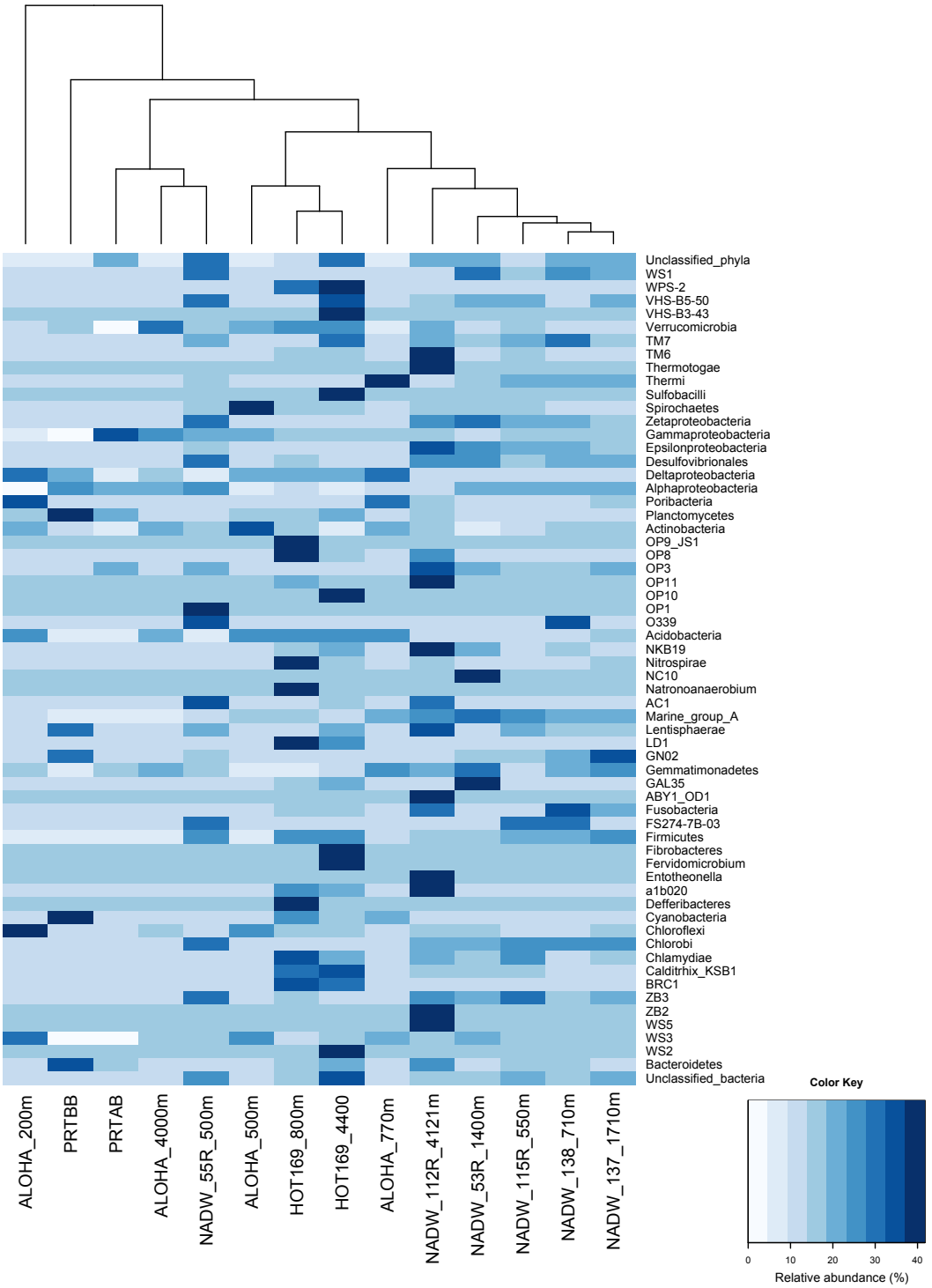


**Figure S5.** Heatmap of relative abundances of bacterial phyla (and groupings within the Proteobacteria) for deep ocean datasets. Taxonomic classifications were assigned by taking the best high-scoring pair (HSP) from the resultant BLAST search against the greengenes database as described in the main text.

**Supplementary Material – Experimental Procedures.**

*Study site and sample collection*. Hadal seawater was collected from 6,000 m (19.667°N, 65.966°W) aboard the R/V Atlantic Explorer (Bermuda Atlantic Time Series; BATS) with a 24, 12L CTD rosette in October 2008. 210 L were filtered first through a 142mm 3μm TSTP (Millipore) pre-filter to collect the particle-associated community and subsequently filtered onto a 142mm 0.22μm PES filter (Millipore) to collect the free-living microbial fraction. Filters were stored frozen until DNA extraction. Seawater for nutrient analyses was collected and processed accordingly (http://bats.bios.edu/bats_methods.html; SIO ODF Analytical Facility). TOC and TON measurements were obtained with a Shimadzu 500 VCSN/TNM-1 Total Organic Carbon/Total Nitrogen Analyzer. Viral and prokaryotic abundances were determined as described previously (Patel et al., 2007).

*DNA extraction and library production.* DNA extraction was performed as described previously (Rusch et al., 2007) with omission of the CTAB purification step. Instead, DNA was purified through a Purelink Genomic DNA Mini Kit column (Invitrogen) using the manufacturers specifications and eluted in 50ul of sterile 1x TE buffer. PCR amplicons were generated from DNA extracted from the 3μm and 0.22μm impact filters using Bacterial 16S-specific primers (27F (5'-AGAGTTTGATYMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACT-3')) and Archaeal 16S-specific primers (Arch21F (5'-TTCCGGTTGATCCYGCCGGA-3') and Arch958R (5'-YCCGGCGGTGAMTCCAATT-3')). Additionally, amplicons were generated from the larger filter size using eukaryotic 18S-specific primers (EukA (5'-ACCTGGTTGATCCTGCCAGT-3') and EukB (5'-TGATCCTTCTGCAGGTTCAC-3')).

Four reactions per sample containing 0.2mM of each dNTP, 2mM $MgCl_2$, 0.5mM of each primer, 1ul of 1:100 diluted template DNA (1-10ng), 1X PCR buffer and 0.1U of Taq Polymerase (Invitrogen) were carried out on a BioRad DNA Engine thermocycler using an initial 2 min denaturation at 94°C, followed by 30 cycles containing 1 min denaturation at 94°C, 30 sec primer annealing at 53°C (EukA/EukB), 55°C (27F/1492R), or 58°C (Arch 21F/Arch958R), and 2 min elongation at 72°C, with a final 10 min extension step at 72°C. Treatment with T4 Polymerase was used to render amplicon ends blunt. Library construction, template preparation of 384 clones per pooled reaction, and Sanger automated cycle sequencing was performed at the J. Craig Venter Institute in Rockville, MD as previously described (Rusch et al., 2007). 384 clones each were sequenced bidirectionally for the five libraries: [1] 3.0μm Bacteria (library code = BB), [2] 3.0μm Archaea (BA), [3] 3.0μm Eukarya (BE), [4] 0.22μm Bacteria (AB), and [5] 0.22μm Archaea (AA).

*16S and 18S rRNA sequence analyses and phylogeny.* Sequences were vector trimmed, mate-paired, and checked for chimeric sequences (Huber et al., 2004; DeSantis et al., 2006). Since the internal region of the 18S rRNA gene was not sequenced, the vector-trimmed forward and reverse reads were quality trimmed using CLC Genomics Workbench 4 (CLC bio, Cambridge, MA), concatenated, aligned, and checked for chimeras using the Bellerophon server (Huber et al., 2004) and the software package KeyDNATools (Guillou et al., 2008).

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# Chapter III

## Going deeper: Metagenome of a hadopelagic microbial community

**ABSTRACT**

The paucity of sequence data from pelagic deep-ocean microbial assemblages has severely restricted molecular exploration of the largest biome on Earth. In this study, an analysis is presented of a large-scale 454-pyrosequencing metagenomic dataset from a hadopelagic environment from 6,000 m depth within the Puerto Rico Trench (PRT). A total of 145 Mbp of assembled sequence data was generated and compared to two pelagic deep ocean metagenomes and two representative surface seawater datasets from the Sargasso Sea. In a number of instances, all three deep metagenomes displayed similar trends, but were most magnified in the PRT, including enrichment in functions for two-component signal transduction mechanisms and transcriptional regulation. Overrepresented transporters in the PRT metagenome included outer membrane porins, diverse cation transporters, and di- and tri-carboxylate transporters that matched well with the prevailing catabolic processes such as butanoate, glyoxylate and dicarboxylate metabolism. A surprisingly high abundance of sulfatases for the degradation of sulfated polysaccharides were also present in the PRT. The most dramatic adaptational feature of the PRT microbes appears to be heavy metal resistance, as reflected in the large numbers of transporters present for their removal. As a complement to the metagenome approach, single-cell genomic techniques were utilized to generate partial whole-genome sequence data from four uncultivated cells from members of the dominant phyla within the PRT, Alphaproteobacteria, Gammaproteobacteria, Bacteroidetes and Planctomycetes. The single-cell sequence data provided genomic context for many of the highly abundant functional attributes identified from the PRT metagenome, as well as recruiting heavily the PRT metagenomic sequence data compared to 172 available reference marine

genomes. Through these multifaceted sequence approaches, new insights have been provided into the unique functional attributes present in microbes residing in a deeper layer of the ocean far removed from the more productive sun-drenched zones above.

## INTRODUCTION

Although at one time deep oceanic environments were considered to be devoid of life, it is now well appreciated that such settings are part of the largest fraction of the biosphere, harboring the greatest numbers and diversity of aquatic microorganisms [1]. Yet despite their significance, deep ocean environments remain poorly sampled. One reflection of this is that the Global Ocean Sampling (GOS) Expedition alone has surveyed the metagenomes of 52 surface water locations [2,3,4], but only two pelagic deep-seawater metagenome studies have been performed to date [5,6,7].

Pelagic deep ocean environments are distinguished from their shallow-water counterparts in a number of fundamental physical characteristics, including the absence of sunlight, low temperature, and increased pressure with depth. Additionally, the chemical constituents of the deep ocean consist of high inorganic nutrient concentrations, such as nitrate and phosphate, and refractory dissolved organic material. The microbial biomass is largely supported by organic carbon availability, which is mainly distributed as either aggregated or dissolved sinking material exported to depth via the biological pump from the productive surface waters [8]. The microbial loop, which is well documented to exert a major influence on a variety of biogeochemical cycles in surface waters, is largely unknown in the dark ocean [9,10].

Current information on the genomic attributes of deep-sea microorganisms from non-reducing environments has come mostly from two sources. The first is the genome sequences obtained from piezophilic ('high-pressure adapted') bacterial species [11,12,13]. Whole-genome sequence data has indicated thus far an improved capacity for complex organic polymer utilization, large numbers of transposable elements, a high ratio of rRNA operon copies per genome and larger-than-average intergenic regions [12]. The cultivated deep ocean 'bathytypes' have an opportunistic (r-strategy) lifestyle, allowing rapid response to environmental changes and a greater level of gene regulation [12]. While these confirmed piezophilic isolates are restricted to only a narrow phylogenetic grouping, the lifestyle strategies observed could reflect similar adaptive mechanisms across a wide range of phylogenetic types. It has recently been suggested that deep ocean microbial communities harbor functional properties indicative of 'copiotrophs,' separate from the streamlined high recruiting genomes found to dominate in oligotrophic surface seawater [3].

The second source of information on the genomic characteristics of deep-sea microorganisms consists of metagenomic analyses from two bathypelagic environments [5,6]. Metagenomic approaches provide invaluable insights into the metabolic repertoire and putative functional profile of a microbial assemblage. The two bathypelagic metagenomic datasets include a 4,000 m whole-genome shotgun dataset from the Hawaii oceanographic time-series (HOT) station ALOHA (HOT4000) in the North Pacific Subtropical Gyre [6] and a 3,000 m fosmid library from the Ionian Station Km3 (DeepMed) in the Mediterranean Sea [5]. Evidence for expanded genomic repertoires in these two metagenomic datasets further supports the hypothesis that deep ocean microbes

maintain an opportunistic lifestyle [5,6]. Additionally, multiple lines of evidence suggest differential evolutionary constraints, particularly relaxed purifying (negative) selection, act upon deep-water communities compared to photic-zone counterparts [6,7,14]. However, these two published deep ocean metagenomes differ significantly in their particular physiochemical properties, notably in the temperature (~1.5°C and 13.9°C for the deep station ALOHA and the Km3 site, respectively), and oceanic regimes (open-ocean gyre versus an almost landlocked basin) the microbial assemblages experience. It is therefore important to obtain additional sequence data from microbial communities residing in diverse deep ocean pelagic environments to expand the coverage and further delineate community genomic components.

The Puerto Rico Trench (PRT) is the only hadal zone (depth in excess of 6,000 m) in the northwestern Atlantic Ocean and hosts an oligotrophic water column despite the proximity to the island-arc and periodic terrigenous inputs from the adjacent continental shelf [15]. The hydrographic characteristics of the PRT include high silicate and oxygen concentrations indicative of modified Antarctic Bottom Water (AABW) from the South Atlantic Ocean [16,17]. Our recent investigation of the PRT particle-associated and free-living microbial assemblages using small-subunit ribosomal gene libraries indicated a diverse composition of bacterial, archaeal, and eukaryal phylotypes [16].

In this study, we provide an analysis of a large-scale 454-pyrosequencing generated metagenomic dataset from the microbial community residing at 6,000 m depth within the Puerto Rico Trench (PRT). The PRT metagenome was quantitatively compared to the HOT4000 and DeepMed datasets, as well as to two datasets generated from surface seawater in the Sargasso Sea (GS00c and GS00d) [2], to identify unique

functional attributes in deep-ocean microbial communities compared to surface seawater communities. Additionally, we employed single-cell genomic techniques [18,19] to generate genomic sequence data from four uncultivated cells from the hadal sample. The results indicate that the PRT hadopelagic microbial community has high metabolic and functional versatility reflective of adaptive mechanisms to the extreme deep ocean environment.

## MATERIALS AND METHODS

**Sample collection, DNA extraction, and 454 pyrosequencing.** 210 l of hadal seawater was collected from 6,000 m (19.667°N, 65.966°W) aboard the R/V Atlantic Explorer (Bermuda Atlantic Time Series; BATS) and filtered serially through a 142 mm 3 μm TSTP (Millipore) pre-filter and a 142 mm 0.22 μm PES filter (Millipore). DNA was extracted from the 0.22 μm – 3 μm filter fraction as described previously [16], and was modified as in Andrews-Pfannkoch *et. al.* [20] to generate sufficient quantities of DNA for a half plate 454 Titanium pyrosequencing run (see Supplementary Methods).

**Assembly, functional annotation, genome size estimation, and nonredundant protein list.** 454-generated sequencing reads were assembled using the *de novo* Newbler assembler. Unassembled singleton reads were screened with the 454 Replicate filter with default parameters (available at http://microbiomes.msu.edu/replicates/) [21] and artificial replicate sequences were discarded. Open reading frames (orfs) greater than 90 nucleotides were called for the assembled contigs and nonredundant singleton reads using Metagene [22].

Small subunit (SSU) and large subunit (LSU) rRNAs were identified and taxonomically classified using blastn against the Silva reference database [23]. tRNAs and functional RNAs were identified using tRNAscan-SE1.23 [24] and by querying the Rfam database for non-coding and other structural RNA families [25], respectively. The taxonomic affiliations of predicted protein sequences were determined using the Automated Phylogenetic Inference System (APIS) [26], with additional functional classifications determined using the STRING v8.3 database for orthologous gene clusters (OGs) [27,28], KEGG orthologs [29], transporter classifications (TC IDs) for membrane transport proteins [30], and Pfams [31] (see Supplementary Methods for details). Estimated genome size (EGS) calculations were carried out for all metagenomes based on the method described by Raes *et. al.* [32].

A nonredundant protein dataset was generated using the blastclust algorithm as described by Konstantinidis *et. al.* [6] to quantitatively compare the gene stoichiometries in each metagenome through reducing the effect of uneven species abundances. In order to directly compare the PRT, DeepMed, HOT4000, GS00c, and GS00d datasets, sequencing reads were annotated and clustered to generate nonredundant protein datasets, then functionally classified as described above.

**Metagenome comparisons.** Absolute abundances for the particular KOs, Pfams, and OGs were analyzed using the program ShotgunFunctionalizeR to test whether the normalized relative frequency of a particular gene family was statistically different from one metagenome compared to the other [33] (see Supplemental Methods).

For bacterial and archaeal OGs, the statistical techniques available within the program STAMP were utilized to assess the functional profiles annotated in the PRT metagenome compared to the surface metagenomes (GS00c and GS00d) taking into account effect size and the difference between proportions [34]. The statistical hypothesis test implemented was Fisher's exact test using the Newcombe-Wilson method for calculating confidence intervals (CIs) at the 95% nominal coverage and a Bonferroni multiple test correction. Results from these statistical methods were compared to the results obtained using the methods implemented in ShotgunFunctionalizeR and were found to be congruent in assessing the major differences in functional profiles between the PRT and surface seawater metagenomes.

**Fluorescence-activated cell sorting (FACS), whole-genome amplification, and screening of phylogenetically novel single cells.** Hadal seawater collected as described previously was returned to *in situ* temperature and pressure conditions upon CTD recovery using stainless steel pressure vessels [35]. Seawater samples were maintained in 15 ml polyethylene transfer pipet bulbs (Samco) and heat-sealed with a handheld heat-sealing clamp (Nalgene) until further processing at the JCVI. High-throughput single-cell sorting was performed using a FACS-Aria II flow cytometer (BD Biosciences) equipped with a modified cooling chamber to maintain the sample at 4ºC. Seawater samples were decompressed, stained for 15 min on ice with SYBR-Green I (Invitrogen), and loaded into the sample chamber with minimal exposure to fluorescent lighting. Individual cells were sorted into single wells of 384-well plates containing 4 µl TE (Tris-EDTA, pH 8.0) buffer. After sorting, plates were placed immediately at -80°C until further processing.

Cell lysis was performed using an alkaline lysis solution (645 mM KOH, 265 mM DTT, 2.65 mM EDTA pH 8.0) for 10 min on ice followed by neutralization (1290 mM Tris-HCl, pH 4.5). Handling of lysis and neutralization reagents was performed using an automated epMotion pipetting system (Eppendorf). Multiple Displacement Amplification (MDA) [36,37] was carried out according to the manufacturer's instructions (Illustra GenomiPhi HY kit; GE Healthcare) except that reactions were incubated at 30°C for 16 h, then heat inactivated at 65°C for 3 min in a total volume of 25 µL. MDA reactions were diluted 20-fold with Tris-EDTA buffer and used as template for bacterial and archaeal 16S rRNA screening [Bacterial 16S-specific primers: 27F (5'-AGAGTTTGATYMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACT-3') and Archaeal 16S-specific primers: Arch21F (5'-TTCCGGTTGATCCYGCCGGA-3') and Arch958R (5'-YCCGGCGGTGAMTCCAATT-3')]. 16S rRNA screening was performed in a PCR workstation with high efficiency particulate filtered air supply. 3 µl of 1:20 diluted template DNA was added to 17 µl Platinum Taq Supermix (Invitrogen), and PCRs were carried out on a BioRad DNA Engine thermocycler using an initial 2 min denaturation at 94°C, followed by 35 cycles of 1 min denaturation at 94°C, 30 sec primer annealing at 55°C (27F/1492R) or 50°C (Arch 21F/Arch958R), and 1 min 30 sec elongation at 72°C, with a final 10 min extension step at 72°C. Positive 16S rRNA PCR reactions were sequenced using Sanger automated cycle sequencing at the Joint Technology Center (JTC) of JCVI (Rockville, MD) as previously described [4].

**Pyrosequencing of MDA reactions and assembly.** A second round of amplification was performed on MDA reactions from four phylogenetically unique single cells. Briefly, 20 ng of the original MDA DNA was used as template in a second MDA reaction using heat denaturation and cycled at 30°C for 4 hrs. Reactions were extracted using phenol-chloroform, precipitated with ethanol, and diluted to ~ 50 ng/µl. 20 µg total product was used for paired-end 3kb library construction and sequencing using the Genome Sequencer FLX System (454 Life Sciences) at the JTC. Sequence reads were screened for contamination, artificial overrepresentation and chimera formation as described in the Supplemental Material. High quality reads were assembled using Newbler and processed through the JCVI metagenomic pipeline [38].

**Fragment recruitment to marine genomes and Puerto Rico Trench single-cell genomes.** The program FR-HIT [Niu and Li, unpublished; available at http://weizhong-lab.ucsd.edu/public/?q=softwares/fr-hit] was used to assess the relative number of recruited metagenomic reads from the Sargasso Sea and PRT to a reference genome database consisting of 170 sequenced genomes from the Marine Microbial Genome Sequencing Project (MMGSP; https://moore.jcvi.org/moore/), the genomes *Nitrosopumilus maritimus* SCM1 [39], Candidatus *Pelagibacter ubique* HTCC1062 [40], and the four partial genomes from the PRT single cells. The default parameters were used for FR-HIT, with a sequence identity threshold of 80%, and the output was parsed to tally the number of recruited hits to an individual genome. The unassembled reads were used after the 454-redundancy filter as described above for the PRT metagenome recruitment.

**Nucleotide sequence accession numbers.** The PRT 454 metagenome has been deposited in the GenBank Sequence Read Archive under accession number SRA029331. The single-cell genomic datasets are similarly under accession numbers as follows, Rhodospirillales bacterium JCVI-SC AAA001, SRA029317; Oceanospirillales bacterium JCVI-SC AAA002, SRA029318; Flavobacteriales bacterium JCVI-SC AAA003, SRA029319; and Planctomycetes bacterium JCVI-SC AAA004, SRA029320.

## RESULTS AND DISCUSSION

**Characteristics of a hadopelagic deep ocean metagenome.** A detailed analysis of the microbial metabolic potential within the Puerto Rico Trench is presented, providing the first view of the genetic repertoire of a hadal microbial assemblage. A total of ~ 145 Mbp of unique sequence data was generated with the majority consisting of unassembled singleton reads (Table 1). The average G+C content of the PRT metagenome (52.2%) was similar to that of the HOT4000 (52.1%) and DeepMed (50.1%) datasets, and distinct from the generally lower average content of surface seawater (~ 36%) [6]. The estimated genome size (EGS) for the PRT was approximately 3.57 Mbp (3.07 Mbp with the exclusion of eukaryote-like sequences) (Table 2). This is in contrast to 1.75 – 1.85 Mbp (1.51 – 1.60 Mbp excluding eukaryotic sequences) for surface seawater (GS00c and GS00d). The calculated EGS for the PRT metagenome lends further support to the hypothesis that deep ocean microbial assemblages harbor, on average, larger genome sizes than their surface seawater counterparts. This could reflect the need for additional genes to cope with reduced and altered nutrients, which are more chemically diverse and biologically recalcitrant [6]. Curiously, the HOT4000 metagenome has a slightly lower

EGS (2.52 Mbp excluding eukaryote-like sequences) compared to the PRT and the DeepMed metagenome (both estimated to be 3.07 Mbp excluding eukaryote-like sequences), which might be indicative of different selective pressures acting on this deep open-ocean gyre microbial assemblage.

**Taxonomic composition of the hadal microbial community.** Small-subunit ribosomal gene sequences recovered from the PRT metagenome are summarized in Figure 1. Proteobacteria, largely Alphaproteobacteria (~40% of the total SSU and LSU ribosomal genes), dominated the PRT microbial community, which is consistent with PCR-based 16S rRNA gene sequence analyses that were conducted previously [16]. Of the large and small ribosomal subunits recovered, a strikingly small number of sequences (16), representing less than one percent of the total, were classified as belonging to the experimentally verified piezophilic Gammaproteobacterial families *Colwelliaceae* (0), *Moritellaceae* (1), *Psychromonadaceae* (6), *Shewanellaceae* (5), and *Vibrionaceae* (4). This observation suggests that the cultivated piezophiles represent only a minuscule fraction of the total autochthonous pelagic deep trench community. Members from the phyla Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Deferribacteres, and Planctomycetes constituted the major remaining rRNA classifications outside of the Proteobacteria, consistent with the distribution of these groups within the HOT4000 metagenome (Fig. 1B).

Archaeal ribosomal genes were found sparingly (~1.5% of the total), with a surprising dominance of Euryarchaeota (Marine Group II Euryarchaeota) compared to Thaumarchaeota phylotypes. These findings are in contrast to our previous archaeal 16S

rRNA gene survey of the PRT, which found a dominance of Thaumarchaeota compared to the Euryarchaeota [16]. While Thaumarchaeota have generally been found to increase in abundance with depth [41,42,43], more recent reports suggest archaeal abundances are correlated with the particular water mass sampled [44,45], and that Thaumarchaeota display latitudinal decline in abundances toward the equator in the North Atlantic [46]. The recovery of eukaryotic SSU and LSU ribotypes (28 sequences, 1.5% of the total), the majority of which were Basidiomycota Fungi, were similar to our prior 18S rRNA gene analyses of the PRT [16].

Taxonomic affiliation of protein sequences using the Automated Phylogenetic Inference System (APIS) [26] closely mirrored the phylogenetic distribution from the recovered ribosomal fragments. Of the proteins with phylogenomic assignments (141,722 proteins, 37.3% of the total), 91.6% were bacterial-affiliated, with the remaining 3.2% archaeal, 1.3% eukaryal, 0.5% viral-associated (3.4% could not be definitively assigned) (Fig. 2).

**Overview of the functional attributes of the PRT metagenome.** The relative abundances of COG categories and KEGG pathways for the nonredundant protein comparisons clustered the deep-sea apart from the surface-water datasets, with the PRT most closely related to the HOT4000 metagenome (Fig. 3). In an effort to identify significantly different abundances of proteins and functional profiles, the nonredundant protein datasets annotated using the KEGG orthologs [29], orthologous groups (OGs) in the STRING database [27,28], and Pfam models [31] were rigorously tested using the statistical programs ShotgunFunctionalizeR [33] and STAMP [34]. A quantitative

comparison of the PRT nonredundant protein set with the two Sargasso Sea nonredundant proteins resulted in 375 and 532 orthologous groups (OGs) differentially represented in the GS00c and GS00d Sargasso Sea metagenomes, respectively. As shown in Fig. 4, the metagenomic profile comparisons for the PRT and Sargasso Sea identified the most differential orthologous groups overrepresented in the PRT as falling within signal transduction mechanisms (category T), replication, recombination and repair (category L), transcription (category K) and inorganic ion transport and metabolism (category P). These results are in contrast to a pairwise comparison of the PRT and HOT4000 metagenomes, where only 31 OGs were differentially abundant ($p < 0.05$ cutoff, Fig. S1) and no OGs were differentially abundant after taking into account metagenome size differences, as described in the Materials and Methods.

In line with the major genomic features reported for the other two deep-ocean metagenomes, the PRT contained an over-abundance of transposable elements, a diverse complement of transporters, components for aerobic carbon monoxide (CO) oxidation, as well as oxidative carbohydrate metabolic components for butanoate, glyoxylate and dicarboxylate metabolism [5,6,7]. As has been observed in the HOT4000 and DeepMed metagenomes, the PRT lacked genes whose products are associated with light-driven processes, including photosynthesis, rhodopsin photoproteins, and photorepair of DNA damage. The overrepresentation of CO dehydrogenase subunits (CoxS, CoxM, CoxL), indicative of aerobic CO oxidation, further substantiates previous studies indicating that these proteins are highly represented in deep-ocean environments [5,47]. Although the source for CO in deep ocean environments is unclear, one hypothesis proposes that CO could be produced via incomplete respiration of biologically labile organic matter [47].

In general, the enzymatic components for the main autotrophic $CO_2$ fixation pathways including the reductive pentose phosphate cycle (Calvin-Benson-Bassham, CBB cycle), the reductive TCA (rTCA) cycle, the 3-hydroxypropionate (3-HP) cycle, the reductive acetyl coenzyme A (acetyl-CoA) pathway (Wood–Ljungdahl pathway), the 3-hydroxypropionate/4-hydroxybutyrate (3-HP/4-HB) cycle, and the dicarboxylate/4-hydroxybutyrate cycle were represented in the PRT metagenome with comparable abundances in both the deep and surface seawater metagenomes. However, key enzymes from some of these pathways were either poorly represented or missing in the PRT. Only two low-identity matches to ATP-citrate lyase (EC 2.3.3.8), the key enzyme for the rTCA cycle, were identified, as well as sixteen hits to ribulose-bisphosphate carboxylase (EC 4.1.1.39) and four hits to phosphoribulokinase (EC 2.7.1.19), the key enzymes of the CBB cycle. Of the matches to ribulose-bisphosphate carboxylase (RubisCO), the majority of the proteins resembled the archaeal type III or type IV RubisCO-like protein (RLP) homologs, which have alternative functions for sulfur metabolism [48,49]. This in contrast to the type I and II RubisCO homologs identified in the surface seawater nonredundant proteins, as well as from reducing environments like hydrothermal vent chimneys that are predominantly fueled by autotrophic carbon fixation via the CBB pathway [50]. Importantly, the major enzymes necessary for the 3-HP and 3-HP/4-HB pathways, 3-hydroxypropionate dehydrogenase (EC 1.1.1.298) and malonyl-CoA reductase (EC 1.2.1.75), were absent from the PRT dataset. As a result of the absence or limited abundance of sequences encoding these enzymes, it is possible that these autotrophic carbon fixation pathways play a minor role compared to heterotrophic metabolic strategies.

**Features overrepresented in the deep ocean, and in particular the hadopelagic, identified through comparative metagenomics.**

*Signal transduction mechanisms (Category T)*

Two-component signal transduction is a stimuli-response mechanism important for microorganisms to cope with changing environmental conditions. Positive correlations in genome size and enrichment of signal transduction functions have been reported, as well as poor representation of these functions in the dominant surface marine bacterial genomes [3,51]. The PRT nonredundant protein set was highly enriched in signal transduction functions, particularly FOG: PAS/PAC domain proteins (COG2202), FOG: CheY-like receiver protein (COG0784), signal transduction histidine kinases (COG0642), and FOG: GGDEF, EAL, and GAF domain proteins (COG2199, COG5001, COG2200, and COG2203) (Fig. S2). The PAS/PAC domain proteins (COG2202) were particularly enriched in the PRT. PAS domain-containing proteins are located in the cytosol where they function as internal sensors of redox potential and oxygen [52]. These findings support the proposed hypothesis that deep ocean microbial assemblages possess functions to cope with resource scarcity and a high diversity of molecular substrates. While the HOT4000 metagenome was enriched in the sensory domain-containing proteins listed above compared to the Sargasso Sea, the PRT metagenome had a more prominent enrichment in all cases, which could suggest a requirement for increasing numbers of signal transduction pathways with increasing depth.

*Transcription (Category K)*

The deep ocean nonredundant proteins, and the PRT in particular, displayed an enrichment of transcriptional regulators (COG0583, transcriptional regulator; COG2207,

AraC-type DNA binding domain-containing protein; COG1595, DNA-directed RNA polymerase specialized sigma subunit sigma24 homolog; COG0640, predicted transcriptional regulators; and others) (Fig. S3). The enrichment of COG1595, the specialized sigma24 homolog, or *rpoE*-like, is intriguing since this alternative RNA polymerase sigma factor plays a role in outer membrane protein synthesis and growth at low-temperature, high-pressure conditions in the piezophile *Photobacterium profundum* SS9 [53]. In general, these are trends for transcriptional regulation functionalities representative of a copiotrophic lifestyle strategy [54] found within the PRT metagenome and the two other deep ocean metagenomes. Expanded gene families for transcription and in particular, transcriptional regulation, are important features in the piezophile and piezotolerant genomes *P. profundum* SS9 and *Shewanella piezotolerans* WP3, respectively [11,13].

*Inorganic ion transport and metabolism (Category P)*

One striking example within category P is the overrepresentation of arylsulfatase A and related enzymes (COG3119) in the deep nonredundant proteins, particularly for the PRT metagenome (Fig. S4). Sulfatases catalyze the hydrolysis of sulfate esters for the degradation of sulfated polysaccharides, and are highly abundant in the phyla Planctomycetes and Lentisphaerae [55,56]. Notably, the piezotolerant bacterium *S. piezotolerans* WP3 contains eight putative sulfatase genes [11]. While the role of these numerous sulfatases is not clear, one hypothesis based on studies of *Rhodopirellula baltica* [57] suggests that in addition to polymer degradation, sulfatases are involved in structural remodeling during morphological differentiation. Interestingly, the overrepresentation of sulfatases displayed one of the greatest phylogenetically-driven

abundances (mainly within the phylum Lentisphaerae) when the orthologous groups were further divided into phylum-level groupings based on the APIS classifications (Fig. S5).

**Transporters.** Transport mechanisms are one of the major cellular processes differentially influenced by hydrostatic pressure changes in transcriptomic analyses in the piezophilic bacterium *P. profundum* strain SS9 [58]. Additionally, the most pressure-regulated proteins produced by this bacterium are outer membrane porin proteins [59,60]. Since high hydrostatic pressure acts to reduce the system volume, and consequently modifies the cellular membrane, the structural diversity, specificity, and variety of transporters would presumably be distinct in deep ocean microbial assemblages compared to surface seawater counterparts. To address this question, the nonredundant protein sets were classified using the Transporter Classification Database (TCDB) [30]. The overall diversity of transporter families identified was comparable across the metagenomes, generally with an even distribution and representation (Fig. 5). However, comparisons of the deep and surface seawater metagenomes revealed that 281 transporter classifications (TC IDs) within 116 transporter families (out of a total of 610 transporter families) were significantly different ($p < 0.05$), and 155 of the 281 TC IDs were enriched in the deep-sea compared to the shallow-water datasets. These included the general secretory and outer membrane protein secreting pathways, many outer membrane proteins (most are members of the outer membrane receptor family, the outer membrane porin family, the OmpA-OmpF porin family, and the FadL outer membrane family), diverse cation transporters (sodium symporters, monovalent cation antiporters, cation diffusion facilitators, ferrous iron and magnesium transporters), including many

associated with heavy metals (chromate, arsenical resistance family, resistance-nodulation-cell division, arsenite-antimonite efflux, iron lead transporters, mercuric ion permeases, P-type ATPases). Also enriched were peptide transporters, including those linked with carbon starvation, mono, di- and tri-carboxylate transporters, mechanosensitive ion channels, members of the major facilitator superfamily, tripartite ATP-independent periplasmic transporters and ATP-binding cassette superfamily transporters.

The largest, significantly different group of ABC transporter families overrepresented in the deep ocean metagenomes were of the Peptide/Opine/Nickel Uptake Transporter (PepT) Family (TC3.A.1.5), including non-specific oligopeptide transport, glutathione porter, and probable rhamnose and xylose porters (Fig. 5B). The deep metagenomes, and the PRT in particular, were also enriched in nitrogen uptake transporters of the families Nitrate/Nitrite/Cyanate (NitT; TC3.A.1.16), Quaternary Amine (QAT; TC3.A.1.12), Taurine (TauT; TC3.A.1.17), and the nitrate/nitrite porter (NNP; TC2.A.1.8.2). The total organic nitrogen (TON) concentration in the PRT was significantly higher compared to the overlying surface seawater (~29 μM compared to 5.7 μM), as was the inorganic nitrate concentration [16]. The enrichment in nitrogen uptake systems is therefore congruent with the greater concentration of nitrogen and indicates the ability of the PRT microbial assemblage to utilize this nitrogen source.

All transporters classified within the Heavy Metal Efflux (HME) Family (TC2.A.6.1), within the Resistance-Nodulation-Cell Division (RND) Superfamily, were overrepresented in the PRT compared to the Sargasso Sea (Fig. 5A). This is an intriguing finding considering that the majority of heavy metal efflux systems currently

characterized are found within contaminated environments [61]. The overrepresentation of heavy metal efflux pumps (COG3696) in the PRT metagenome compared to surface seawater datasets is observed in the two other deep ocean datasets, yet is more pronounced in the PRT metagenome comparisons (Fig. S4). These include efflux systems such as the CzcCBA and CusCFBA $H^+$-antiport systems for $Ni^{2+}$, $Co^{2+}$, $Zn^{2+}$, $Cd^{2+}$, $Cu^+$, and $Ag^+$ efflux [62]. Additionally, nine families within the P-type ATPase Superfamily (TC3.A.3) were significantly overrepresented, all more abundant in the deep and almost all associated with heavy metal translocation for $Cu^+$, $Ag^+$, $Zn^{2+}$, and $Cd^{2+}$. The enrichment of both $H^+$ and ATP-driven efflux systems indicates diverse mechanisms to deal with elevated concentrations of trace metals in the hadopelagic.

Dramatic changes in the elemental composition of sinking particulates with depth have been documented in the Sargasso Sea, where sinking material (such as marine snow) can become rapidly depleted in organic matter, while becoming enriched in lithogenic, authigenic minerals, and redox sensitive elements that are scavenged on particles [63,64]. The chemical speciation of trace metals (free hydrated ions, inorganic complexes, and organic complexes) in the deep ocean is poorly understood, which has dramatic consequences for the different biogeochemical interactions of the microbial assemblage. Total and free (bioavailable) copper concentrations are highly elevated in the deep ocean relative to the surface ocean, which could necessitate the particular efflux pumps identified from the PRT metagenome [65,66].

The elemental composition of not only sinking particulates, but also neutrally-buoyant macroscopic particles might also call for a diverse array of heavy metal efflux systems in the deep. Bochdansky and colleagues found pronounced peaks of macroscopic

particles (> 500 um) within the deep Antarctic Bottom water (AABW) and two branches of the North Atlantic Deep Water (NADW), suggesting these macroscopic particles can act as microbial 'hot-spots' [67]. In addition to the proposed impact of laterally-advected macroscopic particles entrained within the AABW, which flows into the PRT from the South Atlantic, the unique topography of the PRT lends itself to turbidity flows and inputs of terrigenous detritus from the nearby continental shelf [15]. These particular oceanographic considerations undoubtedly contribute to the types of functional features of this unique hadal microbial assemblage. Further work is clearly needed to investigate the association between the enrichment of heavy metal efflux systems in deep ocean microbial assemblages and the specific concentrations (and speciation) of trace metals in hadal environments.

**Multiple Displacement Amplification (MDA) of four uncultivated single cells from the PRT.** Single-cell genomics was pursed as a route to obtain further insight into the PRT community. Single cells were isolated by fluorescence activated cell sorting (FACS) and their DNA amplified using Multiple Displacement Amplification (MDA) [18,19]. Four unique uncultivated bacterial cells were selected for sequencing (Table 3). Although genome recovery was minimal, for reasons likely related to DNA damage associated with sample handling and cell lysis for MDA, the data obtained exceeded the single-cell sequence information available in fosmid clones derived from environmental DNA (See Supplementary Methods, Table S1). The phylogenetic affiliations of the four single cells are detailed in Table 4.

***Alphaproteobacterium.*** The Rhodospirillales bacterium JCVI-SC AAA001 single cell sequence data was the most complete of the four single cells studied, consisting of 310 kbp of assembled sequence data (Table 3). A complete methionine biosynthetic pathway was identified with an initial succinylation step catalyzed by homoserine trans-succinylase (HTS – EC 2.3.1.46). The sulfur inclusion second step could proceed through direction incorporation of sulfide (sulfhydrylation) to form homocysteine either via a putative sulfhydrolase (contig00083 - Cystathionine beta-lyase/cystathionine gamma-synthase) or an intermediate associated with the cysteine synthase A (contig00025). Some organisms have active copies of the CGS (cystathionine gamma-synthase), CBL (Cystathionine beta-lyase), and HS (homocysteine synthase) and are capable of carrying out both direct incorporation and transsulfuration capacities [68]. Lastly, methylation of the homocysteine via an identified cobalamin-dependent methionine synthase completed the pathway. Additionally, a SAM (S-adenosylmethionine) riboswitch (RF00521) was identified on contig00002, which could be associated with the methionine biosynthetic genes identified on various other contigs. The riboswitch identified is a SAM-II aptamer with close identity and structure to other riboswitches found predominantly in Alphaproteobacteria [69].

A two component system for regulating transport and catabolism of phosphorous-containing compounds (PhoBR) and an ABC transporter for $P_i$ compounds was identified (contig00193). The phosphorous concentrations measured in the PRT were two-orders of magnitude greater than the overlying surface seawater [16], so presumably the ability to utilize phosphorous efficiently is an important metabolic feature in the deep. There were also three components of the ABC-$Fe^{3+}$ transport system (AfuBCA) (contig00162), a

high-affinity TRAP C4-dicarboxylate transport (Dct) system utilizing an electrochemical gradient instead of ATP (contig00059), and assimilatory nitrogen metabolic components, such as nitrate and nitrite reductases in addition to an ABC-type transport system for nitrate/sulfonate/bicarbonate (contig00165). Interestingly, the Pho genes were flanked by integrase sequences, and transposase IS sequences flanked the nitrate and nitrite transporters as well, suggesting that mobile genetic elements may influence the transfer of these and other genes between members of the deep ocean microbial community.

***Gammaproteobacterium*.** The genomic information recovered from the Oceanospirillales bacterium JCVI-SC AAA002 single cell consisted of 190 kbp of assembled sequence data (Table 3) and had the closest reference genome match to *Kangiella koreensis* DSM 16069, isolated from a tidal flat using dilution-to-extinction culturing in a rich marine medium [70]. A complete biotin biosynthetic cluster was identified (*bioBFHCD*, contig00007) along with multiple ribosomal proteins localized on two main contigs (contig00048 and contig00049). Interestingly, a system for copper homeostatis (*copAB* copper resistance proteins) and efflux (*cusCBA*) were identified (contig00022), providing support for the overrepresentation of $H^+$ and P-type ATPase heavy metal efflux systems in the PRT metagenomic data.

***Bacteroidetes.*** The Flavobacteriales bacterium JCVI-SC AAA003 single cell sequence data had the longest contig of the four single cells (114 kbp of a total 209 kbp recovered) and encoded 182 putative orfs (Table 3). A complete ribosomal operon (5S-23S-Ile.tRNA-Ala.tRNA-16S) was present on the largest assembled contig (contig00082),

with two additional tRNAs identified (Arg and Val). The closest completed reference genomes are *Flavobacterium johnsoniae* UW101 (6.1 Mbp) and *Flavobacterium psychrophilum* JIP01/86, (2.86 Mbp).

A complete assimilatory nitrogen metabolic pathway was recovered (contig00082) for the conversion of nitrate to L-glutamate, with the identified enzymatic components including nitrate and nitrite reductases, glutamine synthetase (EC6.3.1.2), and glutamate synthase (EC1.4.7.1). As with the Alphaproteobacterium single cell, the identification of nitrogen uptake components gives genomic context to the functional enrichment of nitrogen uptake systems identified within the PRT metagenome and is congruent with the greater concentration of nitrogen in the hadopelagic. Also of interest were multiple putative sulfatases and sulfatase precursors, including a putative arylsulfatase with closest sequence similarity to a *Robiginitalea biformata* HTCC2501 sulfatase ([71]; NCBI locus: YP_003196467), a putative secreted sulfatase *ydeN* precursor with closest similarity to *Lentisphaerae araneosa* HTCC2155 ([56]; NCBI locus: ZP_01872651), and two truncated iduronate-2- sulfatase precursors also with closest similarity to *Lentisphaerae araneosa* HTCC2155 ([56]; NCBI locus: ZP_01873063). The representation of multiple sulfatases from the Bacteroidetes single cell and the overrepresentation of sulfatases in PRT metagenome further supports the hypothesis that there is a potentially multifaceted role for these enzymes in the hadopelagic, including polymer degradation and structural remodeling of the cell wall.

***Planctomycetes.*** The recovered Planctomycetes bacterium JCVI-SC AAA004 single cell sequence data was the most fragmentary, with 48 putative proteins (32 with annotations)

encoded on thirteen contigs. The Planctomycetes phylum in general is underrepresented in the sequence databanks, currently with 13 genome projects (GOLD [72], October 2010; of which only four are closed and finished) and a handful of fosmids [73,74]. Consistent with the genome architecture and genomic repertoire of the marine Planctomycetes, the PRT Planctomycetes bacterium contains numerous hypothetical genes and a lack of apparent operon structure for essential pathways. Eleven hypothetical proteins were identified, one of which contained DUF1570 (PF07607), a family of hypothetical proteins in *Rhodopirellula baltica* $SH1^T$ [55]. The sequenced Planctomycetes contain unlinked *rrn* operons, for example, the *Rhodopirellula baltica* $SH1^T$ genome contains a 460 kbp region separating the 16S from the 23S-5S [55]. We were unable to assess whether the single cell rRNA operon was linked or not, since the 16S rRNA and 23S-5S rRNA genes were not located on the same contig. The flagellar biosynthesis gene *flhF* and a putative flagellar RNA polymerase sigma factor (RNA polymerase sigma factor *whiG/fliA*) were present, suggestive of a motile lifestyle.

**High recruitment of the PRT metagenome to the PRT single cells.** Fragment recruitment of the PRT raw metagenomic reads to the four single cells yielded extremely high recruitment compared to recruitment to 172 sequenced marine microbial genomes (Fig. 6). The majority of PRT reads which recruited to the single-cell genomes were matches to portions of the ribosomal operons, with percent identity ranging from 84.5 to 88.0%, as well as transfer-RNA sequences. The Planctomycetes bacterium JCVI-SC AAA004 was the only single cell to recruit fragments of the PRT metagenome to all

contigs of the dataset, in addition to recruiting the most reads relative to the size of the genome for any of the genomes compared (45,373 hits/Mbp).

The number of reads recruited from the PRT metagenome to any given reference marine genome was low compared to the Sargasso Sea metagenome (Fig. 6). Thus, despite the poor recovery of genome sequence data from the four single cells, the high level of PRT metagenome recruitment demonstrates both the low representation of deep-ocean microbial genomes currently available and the power of single-cell genomics to complement metagenomic coverage of an environment. A similar analysis was performed for the HOT4000 dataset and demonstrated relatively high recruitment to the four PRT single cells, although not as heavily as the PRT metagenome recruitment. These data indicate the distinct composition of deep-ocean microbial genomes, which are not well represented in currently available marine microbial genome sequences.

**Summary.**

This study has provided the first large-scale molecular sequence dataset from a hadopelagic environment. The data demonstrate that the PRT microbial community possesses larger genomes that are enriched in signal transduction, particularly PAS domain-containing proteins that function as internal sensors of redox potential and oxygen, transcriptional regulators and alternative sigma factors like RpoE that have been shown to play a role in growth at low-temperature and high-pressure, and transposable elements. A distinctive collection of transporter mechanisms was identified, including numerous transporters associated with heavy metal resistance. An overabundance of metabolic pathways associated with aerobic carbon monoxide (CO) oxidation and

oxidative carbohydrate metabolism was present in the PRT dataset, along with sulfated polysaccharide degradation, which was particularly prevalent within the PRT members of the phylum Lentisphaerae. Partial single-cell genomes from members of the PRT Alphaproteobacteria, Gammaproteobacteria, Bacteroidetes and Planctomycetes were investigated and found to highly recruit the PRT metagenome gene sequences, as well as providing further genomic context to some of the trends observed in the PRT metagenome. Future work to delineate the metabolic potential from other deep-ocean environments and single-cells, as well as cultivation approaches to obtain a more phylogenetically-diverse sets of reference piezophiles, will shed further light on the diversity, evolution and adaptations of microbial life in the dark ocean.

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**AUTHOR CONTRIBUTIONS**

Conceived and designed the experiments: EE RL SW DB. Performed the wet-lab experiments: EE DF MN ML JY-G. Performed the bioinformatic analyses: EE LZA MK SY. Analyzed and interpreted the data: EE DW MN LZA MK ML JY-G SY EA RL SW DB. Wrote the paper: EE DB.

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76. Letunic I, Bork P (2007) Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation. Bioinformatics 23: 127-128.

**Table 1.** General features of the metagenomic dataset from the Puerto Rico Trench.

| **Feature** | |
|---|---|
| Total Unique sequence (Mbp) | 145.4 |
| Total # Non-redundant contigs | 25,776 |
| Total # Non-redundant singleton reads | 331,384 |
| | |
| Largest contig (bp) | 16,963 |
| Average contig size (bp) | 596 |
| | |
| Total # SSU rRNA | 496 |
| Bacteria | 463 |
| Archaea | 23 |
| Eukarya | 10 |
| | |
| Total # LSU rRNA | 1,216 |
| Bacteria | 1,196 |
| Archaea | 2 |
| Eukarya | 18 |
| | |
| Total # tRNAs | 1,884 |
| | |
| Average G+C content (%) | 52.2 |

**Table 2.** Functional annotations for nonredundant proteins from the PRT metagenome and comparison metagenomes.

| | Sample | | | | |
|---|---|---|---|---|---|
| **Characteristic** | **PRT** | **HOT4000** | **DeepMed** | **GS00c** | **GS00d** |
| Total proteins annotated | 379,908 | 111,746 | 12,635 | 599,097 | 541,789 |
| Unique protein clusters | 351,799 | 103,569 | 11,304 | 345,380 | 340,046 |
| Total matches against Pfam | 146,797 | 87,332 | 7,515 | 234,669 | 226,740 |
| Clusters with Pfam matches (PfamA) | 127,721 (36.3%) | 60,201 (58.1%) | 5,759 (50.9%) | 172,735 (50.0%) | 168,214 (49.5%) |
| Clusters assignable to extended OGs | 172,071 (48.9%) | 73,025 (70.5%) | 6,934 (61.3%) | 203,709 (59.0%) | 201,185 (59.2%) |
| Clusters assignable to KOs | 140,571 (40.0%) | 59,186 (57.1%) | 5,882 (52.0%) | 174,796 (50.6%) | 171,227 (50.4%) |
| Cluster matches to PRT | | 76,002 (73.4%) | 7,732 (68.4%) | 199,620 (57.8%) | 188,926 (55.6%) |
| Avg aa identity against PRT (%) | | 64.9 | 60.3 | 51.3 | 51.2 |
| Estimated genome size (complete sample) | 3.56 | 2.92 | 3.56 | 1.85 | 1.75 |
| Estimated genome size (bacteria/archaea only) | 3.07 | 2.52 | 3.07 | 1.60 | 1.51 |

**Table 3.** Single cell sequencing, assembly, and annotation statistics.

| | Alphaproteobacterium Rhodospirillales bacterium JCVI-SC AAA001 | Gammaproteobacterium Oceanospirillales bacterium JCVI-SC AAA002 | Bacteroidetes Flavobacteriales bacterium JCVI-SC AAA003 | Planctomycetes bacterium JCVI-SC AAA004 |
|---|---|---|---|---|
| # Reads Input | 113,421 | 189,378 | 130,439 | 42,704 |
| # Bases Input | 24,465,204 | 37,000,539 | 27,133,706 | 11,443,900 |
| Total # Contigs | 249 | 66 | 84 | 144 |
| % Chimeric Reads | 15.42 | 9.9 | 16.05 | 50.21 |
| Total # Non-contaminant contigs | 42 | 9 | 14 | 13 |
| Total sequence after filters (kbp) | 310 | 190 | 209 | 58 |
| Largest contig (kbp) | 51 | 71 | 114 | 24 |
| Total # Non-contaminant orfs | 276 | 159 | 182 | 48 |

**Table 4.** Comparison of single cell phylogeny to closest sequenced isolate genome.

| Single cell phylogeny | Closest sequenced relative (Finished genome) | 16S rRNA % ID | Genome size (Mbp) |
|---|---|---|---|
| Gammaproteobacterium Oceanospirillales bacterium JCVI-SC AAA002 | *Kangiella koreensis* DSM 16069 | 90 | 2.85 |
| Alphaproteobacterium Rhodospirillales bacterium JCVI-SC AAA001 | *Magnetospirillum magneticum* AMB-1 | 88 | 4.97 |
| | *Rhodospirillum centenum* SW | 86 | 4.36 |
| | *Rhodospirillum rubrum* ATCC 11170 | 86 | 4.35 (53.7 kbp plasmid) |
| Bacteroidetes Flavobacteriales bacterium JCVI-SC AAA003 | *Flavobacterium johnsoniae* UW101 | 88 | 6.1 |
| | *Flavobacterium psychrophilum* JIP01/86 | 87 | 2.86 |
| Planctomycetes bacterium JCVI-SC AAA004 | *Rhodopirellula baltica* SH 1 | 81 | 7.15 |

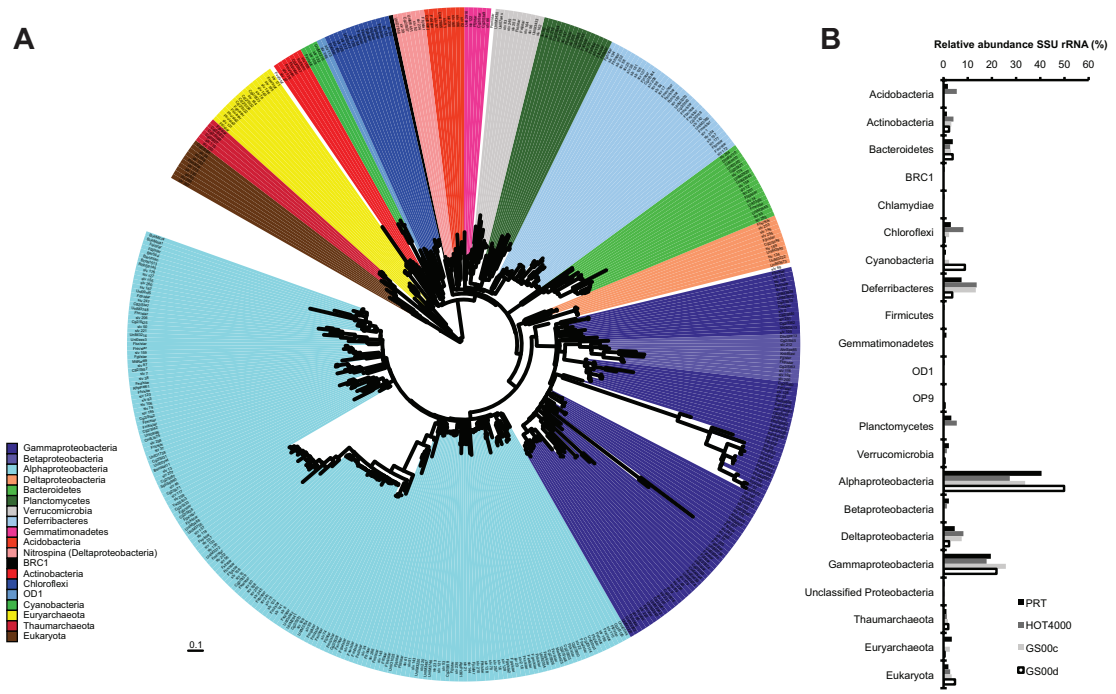



**Figure 1. Phylogenetic distribution of partial SSU ribosomal genes.** SSU rRNA genes identified from the (A) PRT metagenome, and (B) comparison to the SSU ribosomal gene distribution from the HOT4000, GS00c, and GS00d metagenomes. The sequences were aligned using the SINA Webaligner [23], uploaded into the ARB program [75] and manually checked with the ARB_EDIT4 tool. Phylogenetic placement was additionally verified against best blastn hits to the Silva SSU reference dataset (release 102). Tree topology was exported directly from the ARB database and imported into iToL for visualization [76]. Singleton sequences not colored in the tree include representatives from the phyla Chlamydiae, Firmicutes, and an unclassified Proteobacterial sequence. Values in parentheses are the number of SSU rRNA genes identified in the respective metagenome.

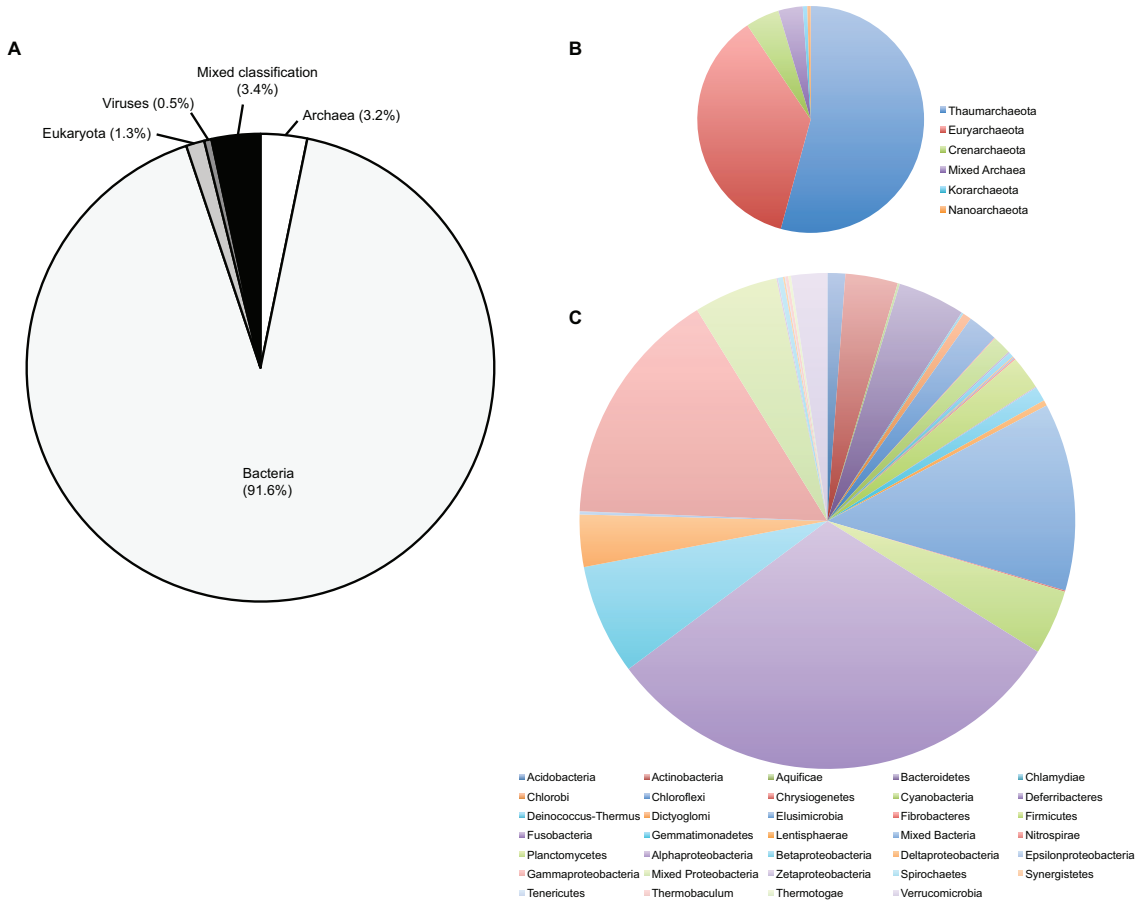



**Figure 2. Taxonomic affiliation of protein sequences using the Automated Phylogenetic Inference System (APIS).** (A) Division-level distribution. (B) Archaeal phyla. (C) Bacterial phyla with further division of Proteobacterial classes.

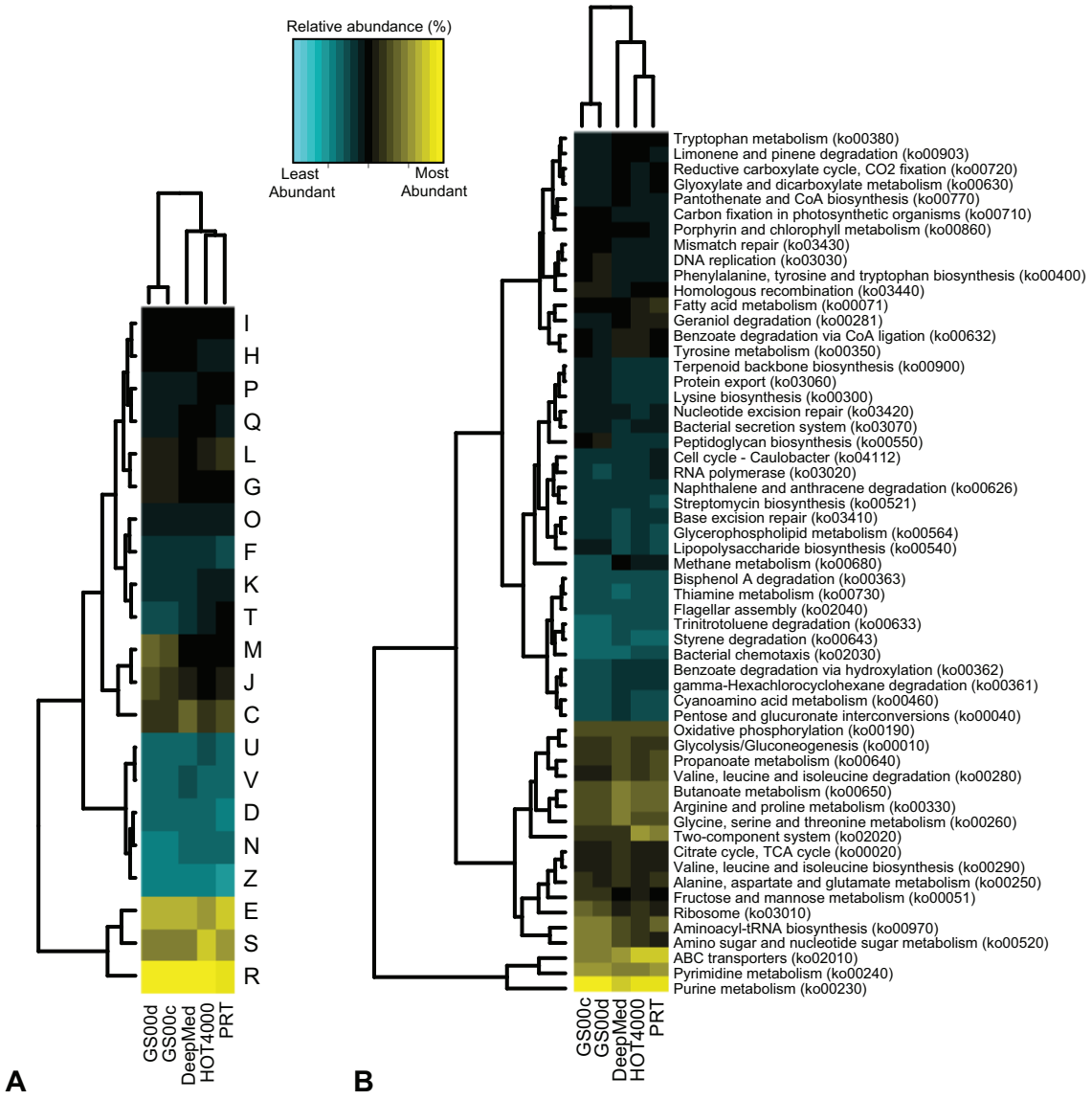



**Figure 3. Cluster analysis of (A) COG categories and (B) KEGG pathways.** Analysis was based on the relative abundances of the nonredundant protein dataset within each metagenome. Only COG categories and KEGG pathways that were represented by ≥ 0.2% of the total are shown. COG categories are as follows: C, energy production and conversion; D, cell division, chromosome partitioning; E, amino acid transport and metabolism; F, nucleotide transport and metabolism; G, carbohydrate transport and metabolism; H, coenzyme transport and metabolism; I, lipid transport and metabolism; J, translation and biogenesis; K, transcription; L, replication, recombination, and repair; M, cell wall/membrane/envelope; N, cell motility; O, protein turnover, chaperones; P, inorganic ion transport and metabolism; Q, secondary metabolism; R, general function prediction only; S, function unknown; T, signal transduction mechanisms; U, intracellular trafficking and secretion; V, defense mechanisms; and Z, cytoskeleton.

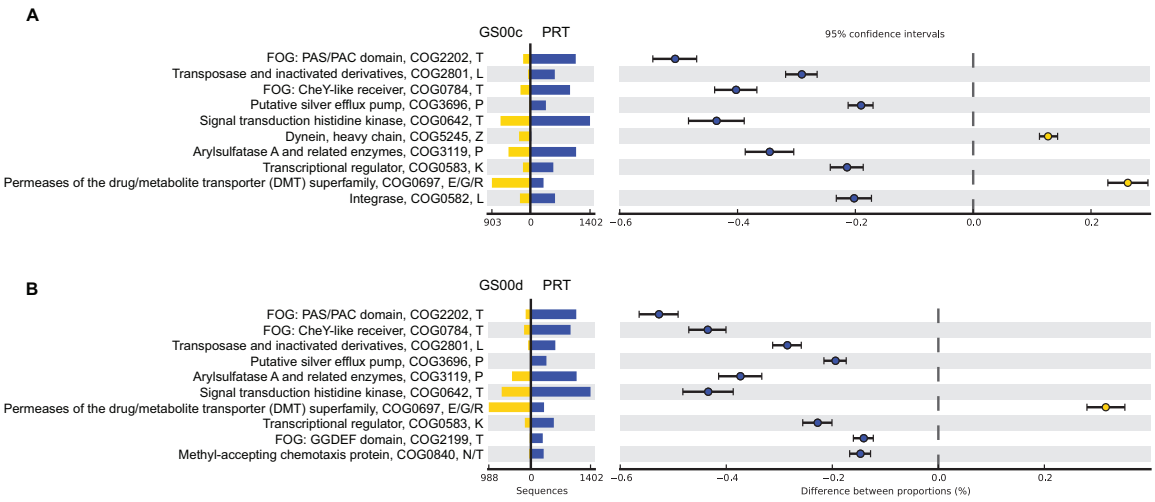



**Figure 4. Metagenomic profile comparisons of COG families for the PRT and Sargasso Sea nonredundant proteins.** Extended error bar plot for the top ten COGs, ordered according to significance, identified in the PRT compared to (A) GS00c and (B) GS00d, indicating the effect size and associated confidence intervals for each significantly different COG family. COG descriptions are listed along with the COG category letters, E, amino acid transport and metabolism; G, carbohydrate transport and metabolism; K, transcription; L, replication, recombination, and repair; N, cell motility; P, inorganic ion transport and metabolism; R, general function prediction only; T, signal transduction mechanisms; and Z, cytoskeleton.

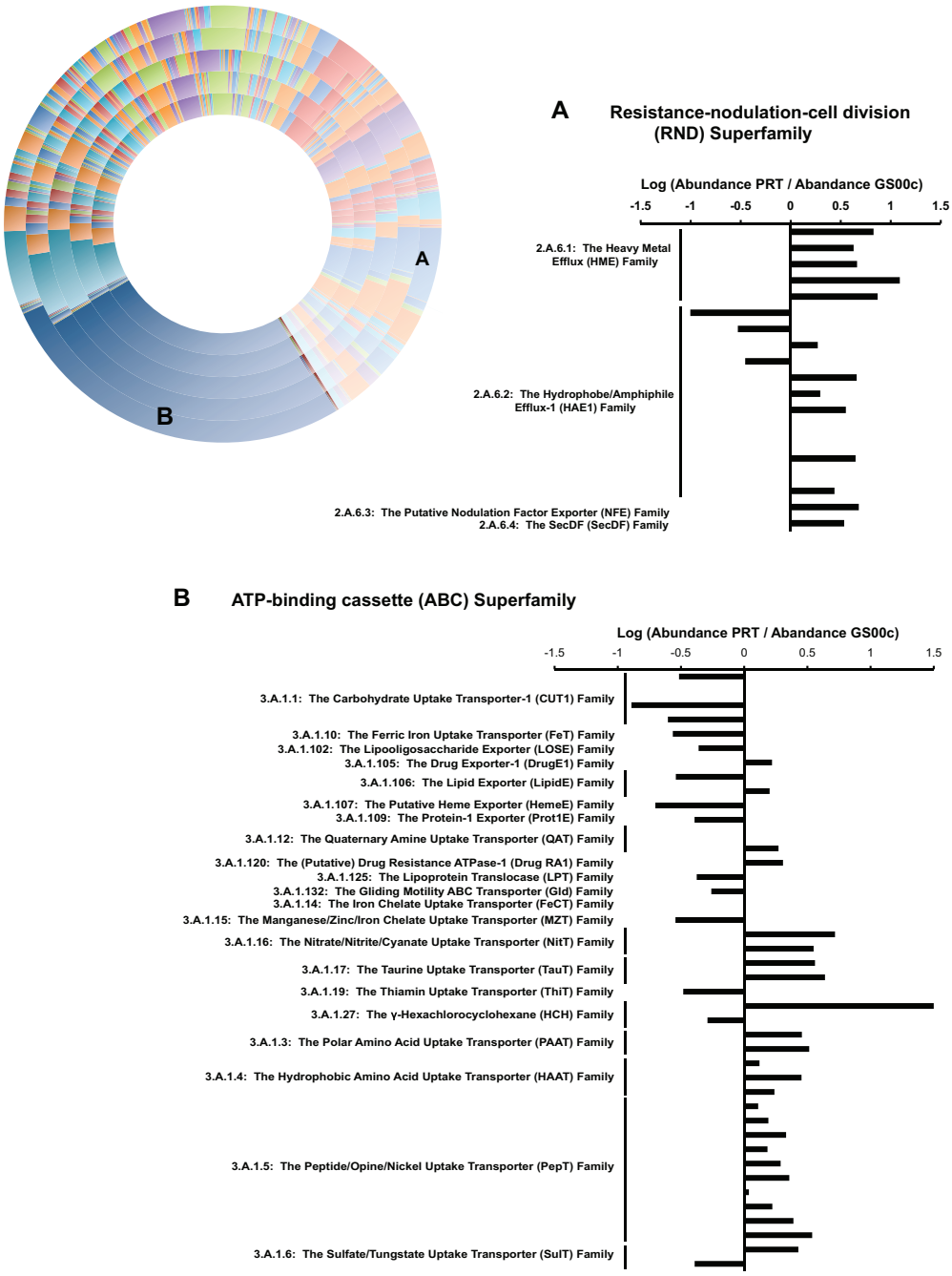



**Figure 5. Transporter family distribution.** Outer circle moving inwards: PRT, HOT4000, DeepMed, GS00d, GS00c. Log abundance profiles for (A) the Resistance-nodulation-cell division (RND) superfamily and (B) the ATP-binding cassette (ABC) superfamily are shown for the PRT compared to the GS00c (similar profiles were obtained for comparisons to GS00d). Positive values in the bar chart denote greater abundances in the PRT, while negative values are greater abundances in the GS00c for the given transporter family member. Similar results were observed for PRT and GS00d metagenome comparisons.

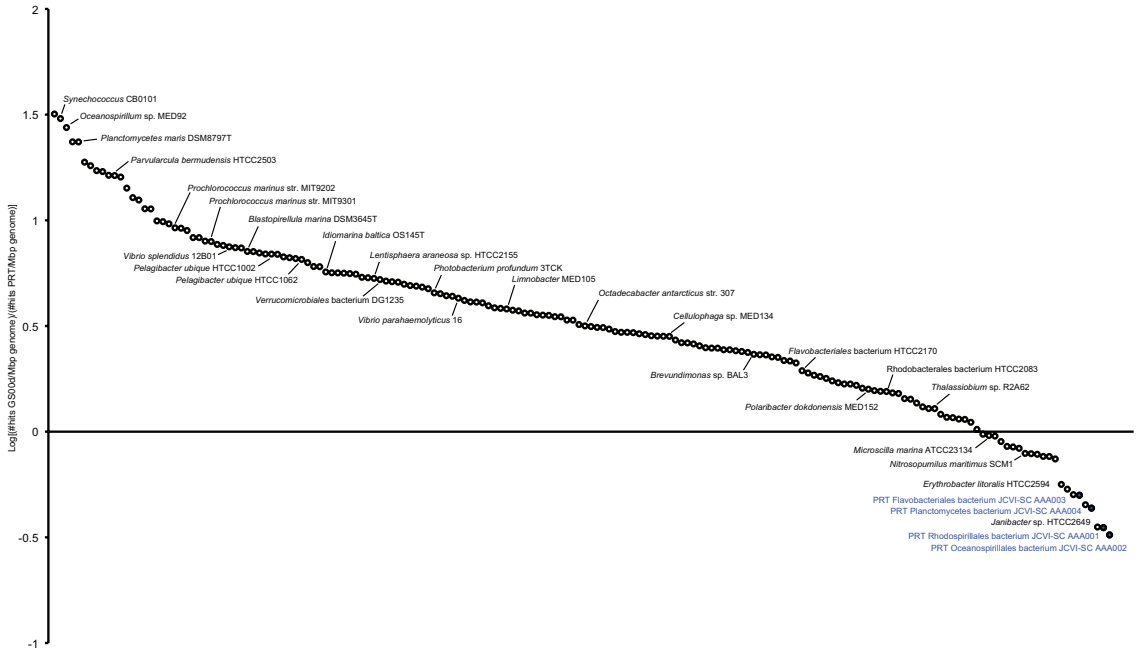



**Figure 6. Comparative fragment recruitment for the PRT and GS00d metagenomes.** Recruitment of the PRT metagenome compared to the GS00d metagenome to the Marine Microbial Genome Sequencing Project (MMGSP) genomes, *Nitrosopumilus maritimus* SCM1 [39], Candidatus *Pelagibacter ubique* HTCC1062 [40], and the four PRT single-cell genomes. The number of reads recruited from each metagenome was normalized to the size (in Mbp) of the reference genome as well as to the number of total reads in the metagenome. Each circle represents the position of a reference genome in relation to recruitment to either the GS00d or PRT metagenomes. References genomes with a greater normalized relative recruitment to the PRT are below the zero line, while genomes above the zero are better recruiters for the GS00d dataset. Similar results were observed for PRT and GS00c metagenome comparisons.

**Supplementary Material – Figures.**

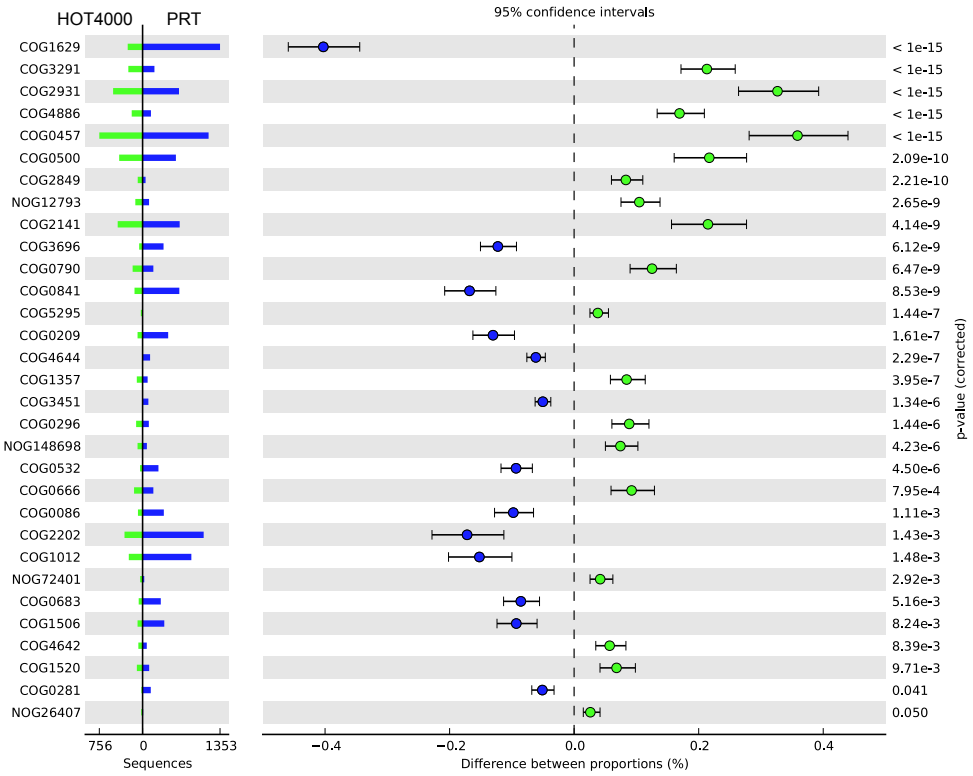
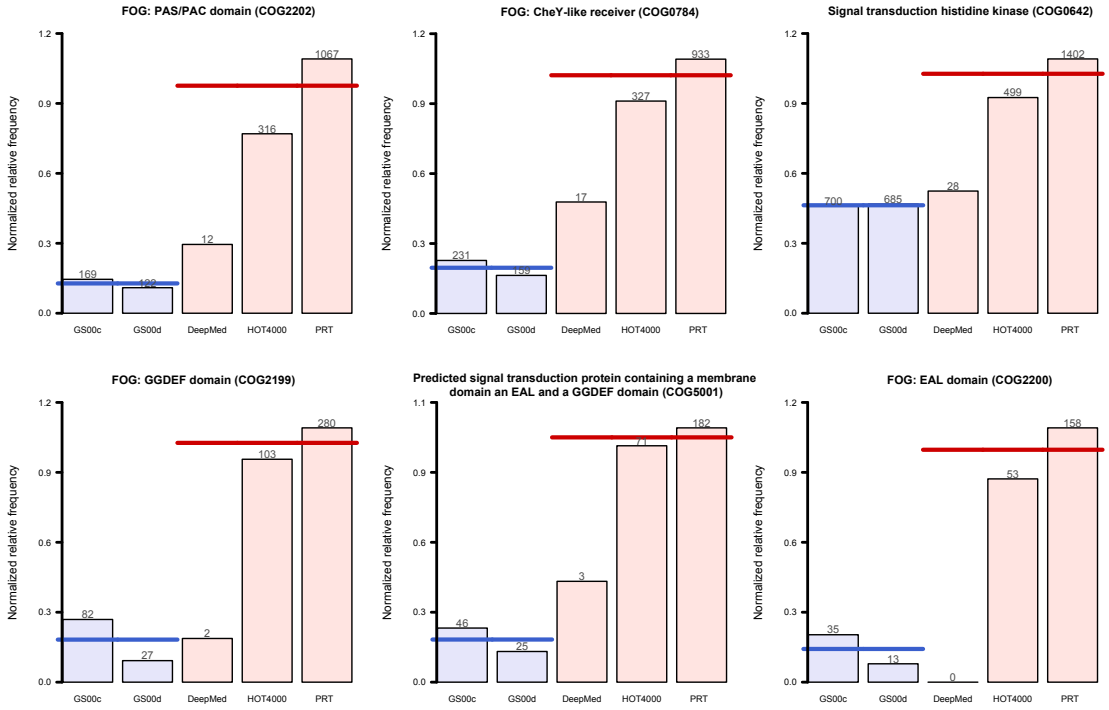
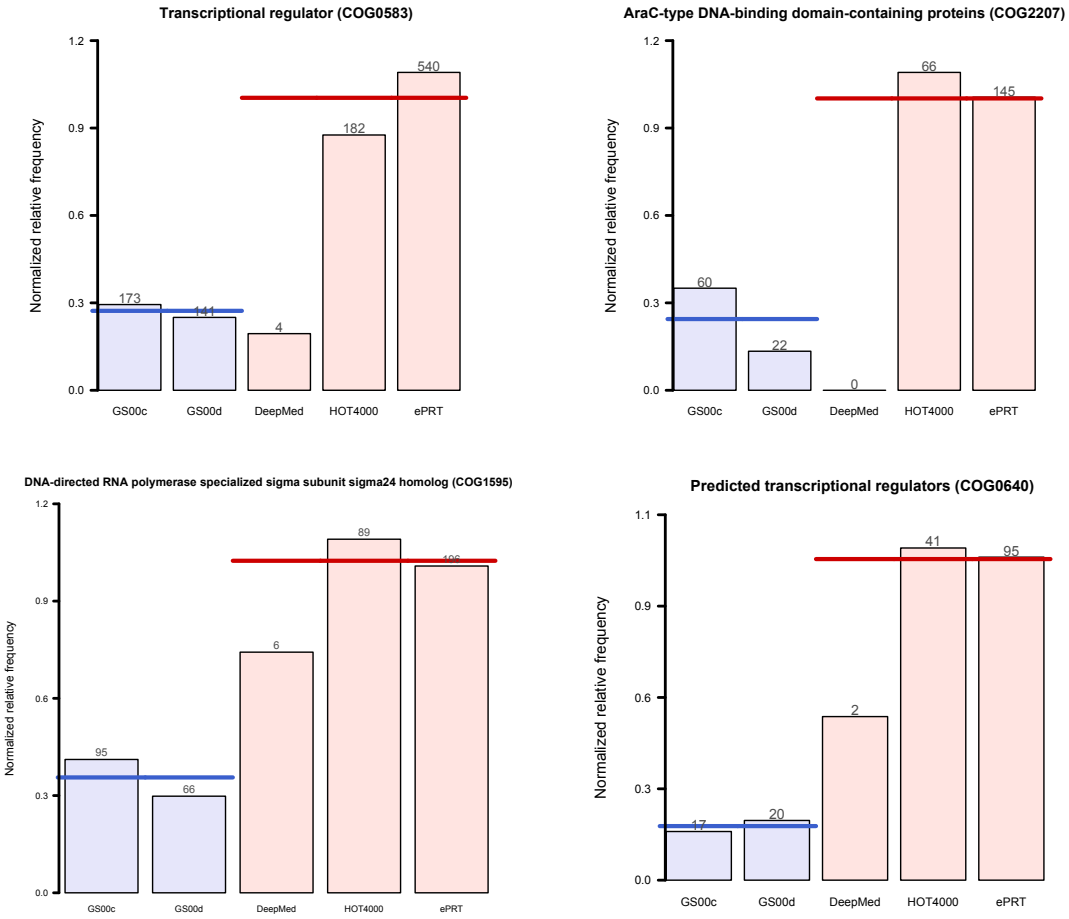



**Figure S1.** Statistical hypothesis testing implemented in the program STAMP [1] for differentially abundant orthologous groups (OGs) between the PRT metagenome and the HOT4000 metagenome. Results are shown for the Fisher's exact test using the Newcombe-Wilson method for calculating confidence intervals (CIs) at the 95% nominal coverage and a Bonferroni multiple test correction. Only 31 OGs were identified having significant differences ( $p < 0.05$). Subsequent filtering of these significant differences taking into account effect size, with the difference between proportions set to a value of 0.5% and the ratio of proportions set to 2.0, resulted in no significant difference between the two metagenomes.



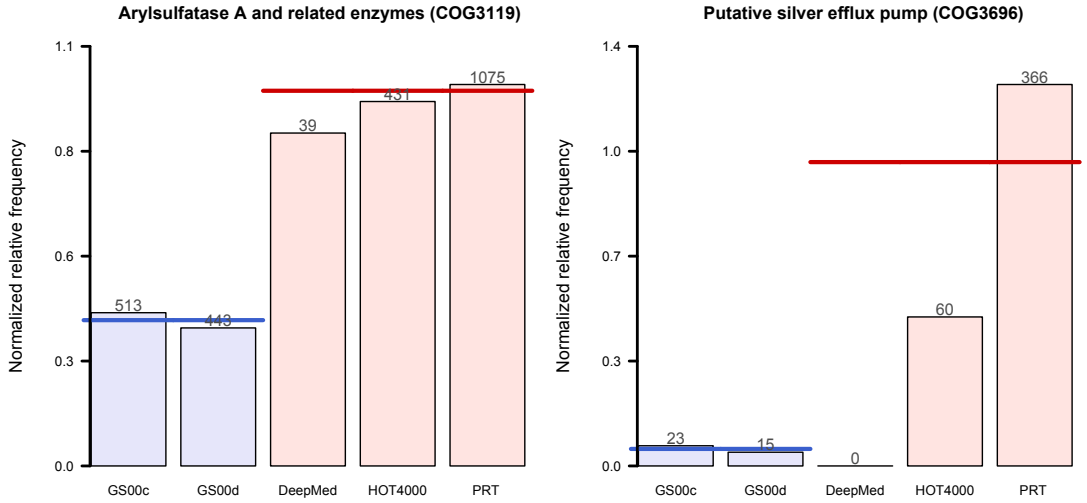

| GeneFamily | Coefficient | AIC | P-value (BH) | Annotation | Class |
|---|---|---|---|---|---|
| COG2202 | -2.03 | 102.81 | 6.41E-299 | FOG: PAS/PAC domain | [T] |
| COG0784 | -1.65 | 70.90 | 2.99E-214 | FOG: CheY-like receiver | [T] |
| COG0642 | -0.80 | 69.91 | 1.27E-110 | Signal transduction histidine kinase | [T] |
| COG2199 | -1.73 | 71.40 | 5.07E-67 | FOG: GGDEF domain | [T] |
| COG5001 | -1.75 | 39.92 | 9.37E-45 | Predicted signal transduction protein containing a membrane domain an EAL and a GGDEF domain | [T] |
| COG2200 | -1.95 | 50.33 | 1.11E-41 | FOG: EAL domain | [T] |
| COG2203 | -1.28 | 49.95 | 4.64E-40 | FOG: GAF domain | [T] |
| COG2197 | -1.92 | 44.04 | 4.28E-37 | Response regulator containing a CheY-like receiver domain and an HTH DNA-binding domain | [TK] |
| COG3920 | -2.43 | 33.44 | 3.23E-24 | Signal transduction histidine kinase | [T] |
| COG2206 | -1.72 | 35.94 | 2.48E-23 | HD-GYP domain | [T] |
| COG4585 | -2.24 | 24.82 | 2.88E-14 | Signal transduction histidine kinase | [T] |
| COG3706 | -1.27 | 44.41 | 1.16E-13 | Response regulator containing a CheY-like receiver domain and a GGDEF domain | [T] |
| COG5278 | -2.14 | 31.38 | 2.36E-13 | Predicted periplasmic ligand-binding sensor domain | [T] |
| COG2766 | -2.93 | 24.14 | 6.01E-13 | Putative Ser protein kinase | [T] |
| COG1716 | -2.13 | 25.90 | 5.89E-12 | FOG: FHA domain | [T] |
| COG3300 | -3.03 | 25.28 | 6.52E-11 | MHYT domain (predicted integral membrane sensor domain) | [T] |
| COG4251 | -2.48 | 28.36 | 1.03E-10 | Bacteriophytochrome (light-regulated signal transduction histidine kinase) | [T] |
| COG0664 | -0.74 | 37.00 | 1.44E-10 | cAMP-binding proteins - catabolite gene activator and regulatory subunit of cAMP-dependent protein kinases | [T] |
| COG1639 | -2.05 | 27.53 | 1.72E-07 | Predicted signal transduction protein | [T] |
| COG4566 | -3.07 | 21.11 | 2.07E-07 | Response regulator | [T] |
| COG3614 | -2.23 | 21.73 | 2.55E-07 | Predicted periplasmic ligand-binding sensor domain | [T] |
| COG1217 | -0.65 | 36.36 | 6.88E-07 | Predicted membrane GTPase involved in stress response | [T] |
| COG2198 | -1.64 | 25.18 | 4.25E-06 | FOG: HPt domain | [T] |
| COG3605 | -1.13 | 28.38 | 6.54E-06 | Signal transduction protein containing GAF and PtsI domains | [T] |
| COG3322 | -21.71 | 16.80 | 7.01E-05 | Predicted periplasmic ligand-binding sensor domain | [T] |
| COG1966 | -1.36 | 28.53 | 7.01E-05 | Carbon starvation protein predicted membrane protein | [T] |
| COG2114 | -0.32 | 38.11 | 7.53E-05 | Adenylate cyclase family 3 (some proteins contain HAMP domain) | [T] |
| COG4191 | -2.07 | 20.72 | 1.24E-04 | Signal transduction histidine kinase regulating C4-dicarboxylate transport system | [T] |
| COG2204 | -0.34 | 47.57 | 2.12E-04 | Response regulator containing CheY-like receiver AAA-type ATPase and DNA-binding domains | [T] |
| COG2337 | -2.66 | 18.55 | 3.51E-04 | Growth inhibitor | [T] |
| COG2905 | -2.66 | 18.46 | 3.51E-04 | Predicted signal-transduction protein containing cAMP-binding and CBS domains | [T] |

**Figure S2.** Abundance of the functional OG category Signal Transduction (T) for deep ocean metagenomes compared to the Sargasso Sea metagenomes.




| GeneFamily | Coefficient | AIC | P-value (BH) | Annotation | Class |
|---|---|---|---|---|---|
| COG0583 | -1.30 | 65.62 | 7.64E-88 | Transcriptional regulator | [K] |
| COG2207 | -1.41 | 56.93 | 1.83E-27 | AraC-type DNA-binding domain-containing proteins | [K] |
| COG1595 | -1.06 | 38.72 | 6.19E-25 | DNA-directed RNA polymerase specialized sigma subunit sigma24 homolog | [K] |
| COG0640 | -1.78 | 29.68 | 4.70E-24 | Predicted transcriptional regulators | [K] |
| COG1309 | -0.94 | 49.31 | 5.21E-23 | Transcriptional regulator | [K] |
| COG2771 | -1.94 | 30.18 | 6.01E-15 | DNA-binding HTH domain-containing proteins | [K] |
| COG1396 | -0.85 | 42.21 | 4.86E-11 | Predicted transcriptional regulators | [K] |
| COG3437 | -1.69 | 25.79 | 4.51E-09 | Response regulator containing a CheY-like receiver domain and an HD-GYP domain | [KT] |
| COG4977 | -1.49 | 25.47 | 5.32E-08 | Transcriptional regulator containing an amidase domain and an AraC-type DNA-binding HTH domain | [K] |
| COG2378 | -1.83 | 24.02 | 5.36E-08 | Predicted transcriptional regulator | [K] |
| COG2909 | -2.73 | 22.51 | 2.71E-07 | ATP-dependent transcriptional regulator | [K] |
| COG1522 | -0.67 | 40.03 | 4.70E-07 | Transcriptional regulators | [K] |
| COG1733 | -1.54 | 24.81 | 8.22E-07 | Predicted transcriptional regulators | [K] |
| COG1802 | -0.88 | 32.83 | 1.63E-06 | Transcriptional regulators | [K] |
| COG2186 | -1.31 | 25.15 | 4.63E-06 | Transcriptional regulators | [K] |
| COG1695 | -1.61 | 24.55 | 8.17E-06 | Predicted transcriptional regulators | [K] |
| COG4941 | -3.46 | 20.47 | 8.66E-06 | Predicted RNA polymerase sigma factor containing a TPR repeat domain | [K] |
| COG2808 | -2.54 | 19.60 | 1.55E-05 | Transcriptional regulator | [K] |
| COG1349 | -1.85 | 28.08 | 2.60E-05 | Transcriptional regulators of sugar metabolism | [KG] |
| COG4957 | -2.50 | 21.08 | 3.43E-05 | Predicted transcriptional regulator | [K] |
| COG3829 | -3.30 | 17.17 | 1.14E-04 | Transcriptional regulator containing PAS AAA-type ATPase and DNA-binding domains | [KT] |
| COG2183 | -1.35 | 29.49 | 1.92E-04 | Transcriptional accessory protein | [K] |
| COG3604 | -1.44 | 24.56 | 2.23E-04 | Transcriptional regulator containing GAF AAA-type ATPase and DNA binding domains | [KT] |
| COG4646 | -2.66 | 19.80 | 3.51E-04 | DNA methylase | [KL] |

**Figure S3.** Abundance of the functional OG category Transcription (K) for deep ocean metagenomes compared to the Sargasso Sea metagenomes.




| GeneFamily | Coefficient | AIC | P-value (BH) | Annotation | Class |
|---|---|---|---|---|---|
| COG3696 | -2.88 | 109.60 | 1.25E-124 | Putative silver efflux pump | [P] |
| COG3119 | -0.95 | 46.09 | 1.46E-116 | Arylsulfatase A and related enzymes | [P] |
| COG2217 | -1.43 | 53.54 | 2.38E-50 | Cation transport ATPase | [P] |
| COG1230 | -1.93 | 37.86 | 3.77E-24 | Co/Zn/Cd efflux system component | [P] |
| COG0155 | -1.38 | 31.38 | 6.33E-17 | Sulfite reductase beta subunit (hemoprotein) | [P] |
| COG0715 | -1.09 | 35.41 | 2.59E-16 | ABC-type nitrate/sulfonate/bicarbonate transport systems periplasmic components | [P] |
| COG0753 | -3.10 | 28.47 | 3.13E-16 | Catalase | [P] |
| COG3158 | -21.81 | 27.95 | 5.54E-16 | K+ transporter | [P] |
| COG3667 | -2.84 | 22.07 | 1.70E-11 | Uncharacterized protein involved in copper resistance | [P] |
| COG1629 | -0.25 | 221.37 | 5.47E-11 | Outer membrane receptor proteins mostly Fe transport | [P] |
| COG4773 | -2.39 | 32.78 | 2.44E-09 | Outer membrane receptor for ferric coprogen and ferric-rhodotorulic acid | [P] |
| COG0370 | -1.56 | 29.81 | 6.47E-09 | Fe2+ transport system protein B | [P] |
| COG0659 | -0.91 | 35.65 | 1.24E-08 | Sulfate permease and related transporters (MFS superfamily) | [P] |
| COG4774 | -2.80 | 26.09 | 5.26E-08 | Outer membrane receptor for monomeric catechols | [P] |
| COG0475 | -0.91 | 35.23 | 1.82E-07 | Kef-type K+ transport systems membrane components | [P] |
| COG0053 | -1.23 | 29.24 | 2.51E-07 | Predicted Co/Zn/Cd cation transporters | [P] |
| COG0598 | -2.23 | 25.24 | 2.55E-07 | Mg2+ and Co2+ transporters | [P] |
| COG2060 | -22.91 | 13.74 | 4.53E-06 | K+-transporting ATPase A chain | [P] |
| COG3712 | -1.70 | 26.79 | 9.11E-06 | Fe2+-dicitrate sensor membrane component | [PT] |
| COG0025 | -0.96 | 43.81 | 9.98E-06 | NhaP-type Na+/H+ and K+/H+ antiporters | [P] |
| COG3131 | -3.41 | 16.55 | 2.05E-05 | Periplasmic glucans biosynthesis protein | [P] |
| COG1116 | -0.77 | 30.68 | 5.57E-05 | ABC-type nitrate/sulfonate/bicarbonate transport system ATPase component | [P] |
| COG0306 | -0.82 | 30.19 | 7.62E-05 | Phosphate/sulphate permeases | [P] |
| COG2216 | -21.64 | 14.88 | 1.70E-04 | High-affinity K+ transport system ATPase chain B | [P] |
| COG3746 | -1.63 | 23.88 | 1.83E-04 | Phosphate-selective porin | [P] |
| COG2223 | -1.63 | 22.11 | 1.83E-04 | Nitrate/nitrite transporter | [P] |
| COG4638 | -0.32 | 41.92 | 2.16E-04 | Phenylpropionate dioxygenase and related ring-hydroxylating dioxygenases large terminal subunit | [PR] |
| COG0600 | -0.62 | 44.97 | 4.78E-04 | ABC-type nitrate/sulfonate/bicarbonate transport system permease component | [P] |

**Figure S4.** Abundance of the functional OG category Inorganic ion transport and metabolism (P) for deep ocean metagenomes compared to the Sargasso Sea metagenomes.

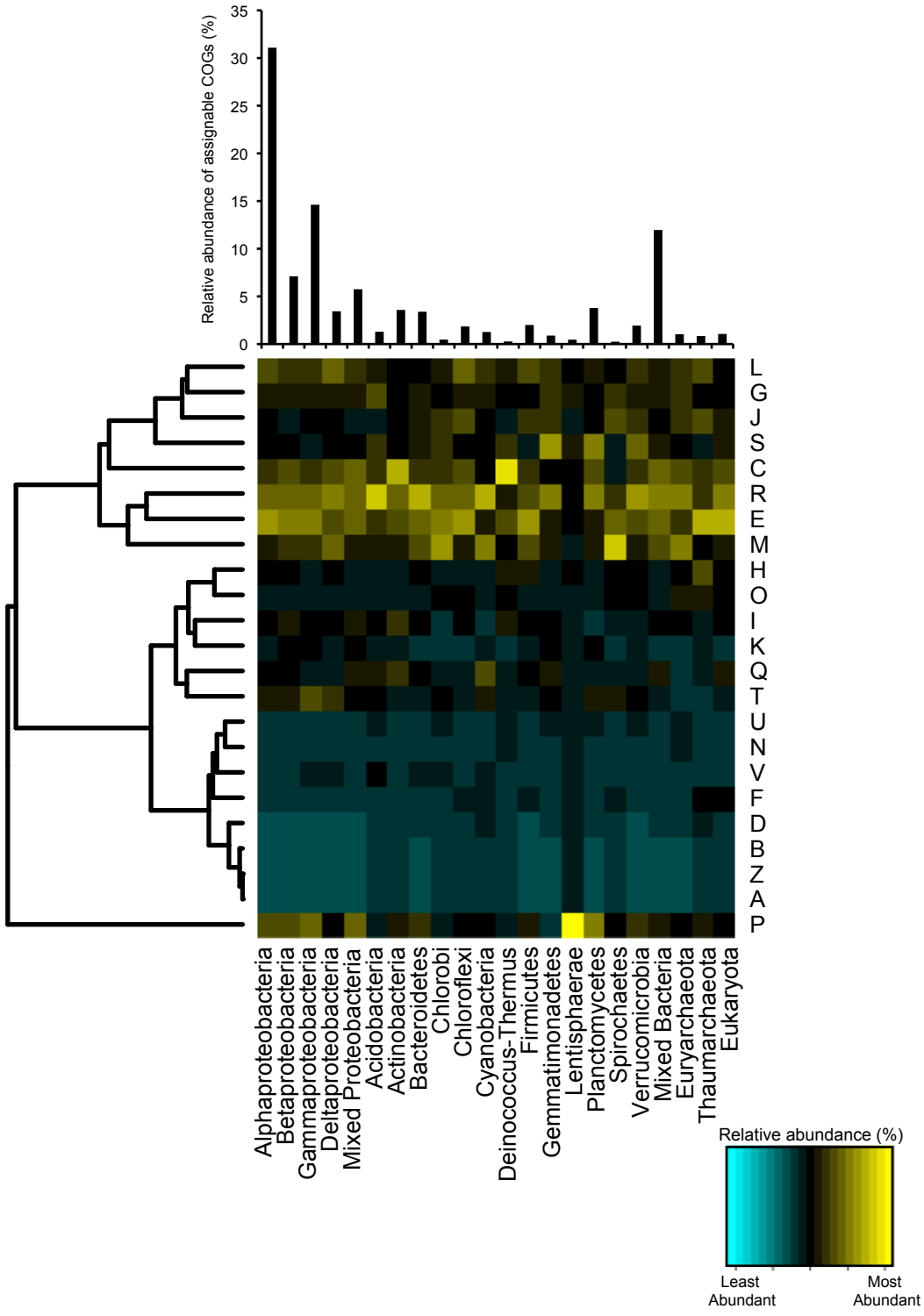



**Figure S5.** Relative abundance of assignable COG categories and distribution within phylum-level (and class-level for the Proteobacteria) groupings based on APIS. Only phyla contributing ≥ 0.2% of the total proteins classified are shown.

**Supplementary Material – Tables.**

**Table S1.** Detailed assembly and putative contaminant statistics for single-cell genomes. Clean datasets consisted of removal of all contigs less than 1 kb in length, as well as contigs greater than 1 kb with predicted proteins that had a phylogenetic affiliation different from the 16S rRNA phylogeny as determined using APIS.

| | Alphaproteobacterium Rhodospirillales bacterium JCVI-SC AAA001 | Bacteroidetes Flavobacteriales bacterium JCVI-SC AAA003 | Gammaproteobacterium Oceanospirillales bacterium JCVI-SC AAA002 | Planctomycetes bacterium JCVI-SC AAA004 |
|---|---|---|---|---|
| Total # reads used for assembly | 113,421 | 130,439 | 189,378 | 42,704 |
| Total bases used for assembly (bp) | 24,465,204 | 27,133,706 | 37,000,539 | 11,443,900 |
| Total assembled contigs (bp) | 471,029 | 241,120 | 282,158 | 159,263 |
| Total # contigs | 249 | 84 | 66 | 144 |
| # contigs < 1kb | 188 | 70 | 44 | 114 |
| Putative contaminant sequences > 1kbp (bp) | 60,520 | 0 | 69,190 | 38,950 |
| Clean assembled contigs (bp) | 310,294 | 209,062 | 190,170 | 58,361 |

**Supplementary Material – Experimental Procedures.**

**Preparation of DNA for 454 pyrosequencing.** DNA was randomly sheared, end polished with Bal31 nuclease/T4 DNA Polymerase (NE Biolabs), and carefully size-selected on a 1% low-melting-point agarose gel. Adaptors were appended to the DNA fragments followed by amplification with Phusion DNA Polymerase (Finnzymes, Espoo, Finland) for 15 cycles. Amplified DNA fragments were purified through three rounds of gel purification using 1% low-melting-point agarose (Invitrogen). DNA was fragmented further and 500 – 800bp fragments were size-selected. Adaptors, serving as priming sites for amplification and sequencing, were ligated onto the fragments. After library quantification, amplification was carried out in an emulsion-based format and subsequently purified using AMPure beads (Beckman Coulter), selectively binding targeted DNA fragments while allowing for the removal of primers and other small DNA fragments. The library was processed through the breaking and enriching steps, followed by sequencing on the Genome Sequencer FLX System (454 Life Sciences) at the Joint Technology Center of the J. Craig Venter Institute (Rockville, MD).

**Functional annotations.** All nonredundant proteins were searched using blastp against the STRING database (v8.3) [2] to assign top hits to orthologous groups (OGs) in the eggNOG database, as well as queried against the KEGG orthologs (KOs) and Pfams with an e-value $\leq 1 \times 10^{-5}$. For OGs and Pfam searches, top hits were allowed to map to multiple OG categories and multiple Pfam motifs per query. The nonredundant proteins were additionally queried using blastp as described above against the Transporter

Classification Database to assign transporter classifications (TC IDs) for membrane transport proteins [3].

**Metagenome comparisons.** Pair-wise direct comparisons were carried out in the program ShotgunFunctionalizeR [4] using a binomial method with the Benjamini-Hochberg False Discovery Rate correction to adjust q-values for multiple testing. Additionally, direct comparisons between the groups "shallow" (referring to surface seawater derived datasets, GS00c and GS00d) versus "deep" (referring to the three datasets derived from depth: DeepMed, HOT4000, and PRT) enabled testing for gene families which were significantly represented in deep versus shallow metagenomes using the Poisson model implemented in ShotgunFunctionalizeR. A coefficient from the Poisson model, which is the estimated difference between the two groups, as well as the Akaike information criterion (AIC) as a measure of the model fit, are reported for all of the OG families tested (Figs. S2-4).

A short discussion is warranted here for these differential comparisons with regard to genome size differences estimated from the surface- and deep-ocean metagenomes. Considering the average EGS for the PRT metagenome compared to the two Sargasso Sea metagenomes is approximately 2-fold greater, the calculated differences in the functional gene abundance profiles is in part a reflection of this increase in genome size [5]. However, considering the estimate uncertainty associated with incorporating this biasing factor is at this time unclear, we have refrained from including the EGS in our quantitative comparative analyses to avoid unwanted propagation of this uncertainty.

**Assembly of 454-pyrosequencing MDA data.** MDA samples present challenges during assembly of the sequencing data, which include both over- and under-representation of regions of the genome, chimera formation, and sometimes the presence of contaminating DNAs [6,7]. In the analysis of the four deep trench samples, we addressed these issues in the following manner. Reads and contigs from initial *de novo* Newbler assembly were processed through the JCVI metagenomic 16S rRNA pipeline and searched against the PANDA database to provide taxonomic classification of ribosomal and non-ribosomal sequences [8]. Because over amplification of random regions creates uneven distribution of coverage, the contigs from the initial *de novo* assembly were screened for short contigs with high coverage. The reads from these tagged contigs were reduced in coverage and the filtered dataset was reassembled. The Newbler read type classification was used from a reference mapping assembly to screen for chimeric reads. The initial *de novo* consensus was used as a reference in a mapping assembly of the raw reads. Potential chimeric reads were removed from the readset and the filtered dataset was reassembled. This final iteration of assembly resulted in the non-chimeric assembly from which orf calling and functional annotation was performed.

Assemblies were manually curated using a conservative approach as follows. All contigs less than 1,000 bp in length were removed. Taxonomic affiliations of the predicted protein sequences assigned using APIS [9], and contigs which contained a majority of proteins with taxonomic affiliations other than the phylum-level classification associated with the 16S rRNA gene were removed. While this curation removed a sizeable fraction of the single cell sequence data downstream analysis (Supplementary

Table 1), it rendered confidence in the final datasets since taxonomic affiliation of the peptides was in accordance with the 16S rRNA phylogeny.

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# Chapter IV

## The elusive art of microbial cultivation

**PREFACE**

One of the most challenging issues facing environmental microbiologists is the fact that the majority of the microbial members identified in a given community using molecular techniques appear recalcitrant to cultivation efforts. What is the nature and extent of this paradigm, and will researchers be able to overcome the cultivation 'bottleneck'? In this Perspective, we discuss the barriers to traditional and non-traditional cultivation efforts and propose a shift in 'culture' to encompass a continuum of cultivation systems.

## INTRODUCTION

The elusive nature of microbial cultivation has prompted thoughtful consideration from numerous researchers[1-13], and remains one of the most challenging issues facing microbiologists. Estimates of microbial diversity and abundances vastly exceed our previous conceptions by orders of magnitude[14] and for complex environments, the majority of the microbial community identified using molecular techniques has proven recalcitrant to cultivation efforts[8]. This problem reflects the staggering number of nutrient and energy-substrate types and quantities to which microorganisms are fine-tuned, as well as physiochemical adaptations, extracellular cue requirements, and growth modes. The task of phylogenetically identifying a microorganism and linking metabolic capabilities to individuals, and the ability to realize the full potential of the microbial community in a given biome is daunting.

The "Great Plate Count Anomaly" was formally detailed by Staley and Konopka[15] in 1985 (although observations were documented as early as 1898[13]), and describes the observation that viable plate counts recover only a small fraction of bacteria in a natural sample compared to that which could be counted by direct microscopic enumeration. Soon thereafter, the use of small-subunit ribosomal gene surveys to explore the diversity of microbes in natural environments further enlightened researchers to the vast uncultivated microbial majority[16, 17]. The surge of environmental genomics and high-throughput cultivation-independent techniques have grossly added to the inadequacies of what our current culture collections hold in terms of the diversity of microbes worldwide[18-21]. The underlying cause of the "Great Plate Count Anomaly[15]" and systematic exploration of the nature of this issue remains obscured from researchers,

leaving a severe gap in our knowledge of how to establish microbial cultivation studies and models. In this Perspective, we provide a synthesis of the historical barriers to both traditional and non-traditional cultivation efforts, and emphasize the need to redefine the conceptual basis of cultivation. This proposed 'culture' shift places cultivation studies along a continuum, from isolated monocultures to highly tractable model systems, to underscore the different ways in which researchers can access the 'uncultivated microbial majority' in a culture-based setting.

## THE CULTIVATION 'BOTTLENECK'

The cultivation 'bottleneck' describes the limitations of culture-based approaches to identify the extant microbial majority. The advent of environmental genomics and high-throughput cultivation-independent techniques has yielded remarkable insight into the complex diversity of bacteria and archaea whilst circumventing the arduous task of cultivation[18-21]. New (meta)genomic, (meta)transcriptomic and (meta)proteomic approaches (collectively referred to as '-omics' approaches hereafter) have all but eclipsed traditional microbiological techniques. It is increasingly apparent that the time and energy necessary to isolate and characterize a new microorganism substantially outweighs the effort required to obtain massively parallel molecular datasets. DeLong[22] describes this conundrum as an "impedance mismatch," in that it is currently not feasible to obtain corresponding massively parallel physiological data. We posit that without the ability to experimentally interrogate a microbial member within a culture-based setting, researchers will be left with only '-omics'-enabled inferences and an overwhelming amount of sequence data. The physiological, biochemical, and genetic data are crucial to

giving sequence data biological context. While '-omics' techniques can provide potential insights into microbial physiology, we would like to stress that these are computational inferences that *must* be verified through experimentation. In this respect, there is no substitution for cultivation efforts. Cultivation studies enable hypothesis-driven science in that a system can be experimentally manipulated to elicit a specific response in an organism.

## LIMITATIONS OF '-OMICS'

Three considerations are provided that highlight the importance of studying cultivated microorganisms and reasons why we *still* need to justify cultivation efforts.

## QUALIFY FUNCTIONAL AND METABOLIC NOVELTY

Functional novelty is inherently linked with phylogenetic novelty. The primary limitation of '-omics' techniques is that the biological inferences gained rely exclusively on the quality and content of the reference database derived from isolated microorganisms. An excellent example of how expansion of the genomic database based on phylogenetic novelty of cultivated isolates can benefit biological inferences through '-omics' data is the Genomic Encyclopedia of Bacteria and Archaea (GEBA) project, where the number of novel protein families was extended by orders of magnitude[23].

The cultivation of representatives from novel phyla, previously represented only by environmental 16S rRNA sequence data, can illustrate how functional and metabolic novelty can be experimentally qualified. For example, the newly cultured OP5 isolate *Caldisericum exile* sp. nov.[24] and the enrichment culture of *Candidatus* Methylomirabilis

oxyfera[25, 26] harbor the unexpected metabolic properties of anaerobic sulfur respiration and anaerobic methane oxidation coupled to nitrite reduction, respectively (Box 1). Importantly, metagenomic surveys in search of novelty are limited by comparisons to the functional properties and metabolic repertoires that currently populate sequence databases.

## ESTIMATES OF BIOGEOCHEMICAL TRANSFORMATIONS

In an effort to assimilate microorganisms into ecosystem-based models of biogeochemical processes, understanding which phylogenetic groups and metabolic activities are responsible for relevant chemical transformations is essential[27]. Only within the past few decades have cultivated representatives of the numerically dominant and functionally important marine microbial lineages emerged, including the unicellular photoautotrophs *Synechococcus*[28] and *Prochlorococcus*[29] (major producers of global atmospheric oxygen), the ubiquitous heterotroph SAR11[30] (accounting for one quarter of marine microbial plankton), and coastal sulfur-cycling Roseobacters[31]. These groups of microorganisms are responsible for major global chemical transformations of oxygen, carbon, nitrogen, phosphorous, and sulfur[32]. For example, members of the Roseobacter clade are involved in the degradation of dimethylsulfoniopropionate (DMSP), which has consequences for the release of climate-relevant dimethylsulfide (DMS) and the global marine sulfur cycle[33].

The detailed physiological study of the first ammonia-oxidizing marine group I crenarchaeote *Nitrosopumilus maritimus* SCM1[34] is a prominent example of how probing the physiology of a cultured isolate can advance our understanding of how microbes

contribute to major biogeochemical cycles. Martens-Habbena and colleagues recently[35] elucidated the kinetics of oligotrophic ammonia oxidation by *N. maritimus* SCM1, which correlated strikingly well with *in situ* oceanic nitrification measurements and demonstrated the ability of *Nitrosopumilus*-like ammonia-oxidizing archaea (AOA) to efficiently out-compete ammonia-oxidizing bacteria (AOB).

**ANNOTATION OF 'HYPOTHETICALS'**

Our molecular databases are overwhelmed with 'hypotheticals.' This fact is a serious issue that demands further physiological, biochemical, and genetic characterization of microorganisms. Alternative methods attempting to elucidate the functions of 'hypotheticals' in the absence of a cultivated isolate primarily rely on structural analyses of uncharacterized protein domains[36]. While this is a step in the right direction, the ability to functionally annotate these uncharacterized gene products through meaningful experimental data rests in the physiology, biochemistry, and genetics of cultivated microorganisms. An eloquent example of how detailed study of a cultivated isolate can lead to the annotation of previously uncharacterized genes is from the elucidation of the circadian clock gene complement in cyanobacteria[37]. The biochemical and physiological nature of the endogenous clock for the model cyanobacterium *Synechococcus elongatus* provides a mechanistic basis for the unique synchronous behavior of the circadian oscillator[38].

Similarly, the detailed characterization of 'well-characterized' genes and pathways in diverse cultivated isolates can lead to profound insights and further expand functional roles. For example, the transcription factor SoxR, a well-characterized

response regulator in enteric bacteria controlling oxidative stress, has an alternative role in the pathogen *Pseudomonas aeruginosa*, where the primary function is to mediate response to the redox-active phenazine pyocyanin[39]. The findings of Dietrich and colleagues[39] demonstrate how primary functional annotations in one bacterial lineage can be an evolutionary exception, and it is therefore imperative to experimentally characterize the physiological, biochemical, and genetic aspects of diverse microbial isolates in an effort to further annotate novel genes and pathways.

## THE NATURE OF THE 'ANOMALY'

Is it necessary to have a "green thumb" to coax a microbe into isolation? Why have researchers fallen short of isolating a given microbe in pure culture? Four major challenges and proposed solutions pertaining to the nature of the 'Great Plate Count Anomaly[15]' are examined and highlight the historical reasons for cultivation difficulties.

## IDENTIFYING SUBSTRATES, SIGNALS, AND METABOLIC REQUISITES

Epstein relates that "the finger was always pointing to media deficiencies," a sentiment dating back to Robert Koch and Petri in the 1880s[13]. Manipulation of culture conditions, such as modifications of medium constituents or physical parameters (temperature, light regime, or anaerobic conditions), have and will continue to the primary tool for microbial cultivation. The two most advantageous modifications resulting in successful isolation include mimicking natural concentrations of substrates and extending incubation times[10, 11, 30, 40-52]. The first modification exploits the concept of 'substrate-accelerated death,' where high nutrient concentrations used in typical

microbiological media inhibit cultivation of oligotrophic microbes[12]. For example, the usable (or labile) dissolved organic carbon (DOC) in seawater can range from < 1% to 45% of the total measurable DOC, yet the standard ZoBell Marine Medium used for culturing heterotrophic bacteria contains concentrations two thousand times greater than that of the measureable DOC in seawater[53]. Continuous-flow chemostat systems are excellent means of mimicking natural concentrations of substrates and environmental parameters to establish a tractable microbial community and enriching for a particular microorganism [54-56]. Similarly, long-term incubations (on the order of months) can increase recovery of rarely isolated groups from soil samples by an order of magnitude[41]. Additionally, signaling molecules, such as *N*-acyl homoserine lactones and cyclic AMP[57, 58], signaling peptide [59], "helper" bacteria[60], siderophores[61], heterogeneous cell exudates[62], and co-culture approaches[63] have all resulted in cultivation successes and describe intrinsic microbial properties that warrant further mechanistic experimentation.

With genomic reconstruction as the template to informing cultivation strategies, the use of "-omics"-informed cultivation methods holds remarkable promise to obtain isolated cultures of microorganisms of interest. Two recent successes of "-omics"-enabled cultivation efforts include the isolation of the obligate intracellular pathogen *Coxiella burnetii*, the causative agent of Q fever[64], and the autotrophic ferrous iron oxidizing bacterium *Leptospirillum ferrodiazotrophum*, the key nitrogen-fixing member of an Acid Mine Drainage (AMD) consortium[7, 65]. In both instances, systematic '-omics' techniques led to the identification of key conditions necessary to selectively target and enrich each microorganism.

## THE PHYSIOLOGICAL STATE OF THE CELL

A major facet of cultivation rests in the physiological state of the cells. In response to various environmental stresses, like nutrient starvation, microorganisms can enter a reversible state of reduced metabolic activity, or dormancy. The explicit mechanisms and quantified extent of this dormancy remains unknown in microbes from a diverse array of environments. The concept of the bet-hedging strategy of microbial dormancy has recently received attention and is the topic of a recent review by Lennon and Jones[66]. Microbial dormancy is presumably more prevalent than previously thought, describing both spore forming and non-spore forming microbes[66-68], and undoubtedly plays a critical role in cultivation ability. The work of Shah and colleagues eloquently demonstrates how muropeptide fragments of the cell wall from growing bacterial cells are potent germinants of dormant *Bacillus subtilis* spores[69, 70]. As described previously, the ability of signaling molecules to mediate physiological state and induce favorable growth conditions are excellent avenues for further exploration of cultivation strategies.

A controversial, and lesser understood physiological state of microbes is the "viable-but-non-culturable" (VBNC) semi-dormant state, which decreases the ability to culture bacterial populations by several orders of magnitude[71]. This phenomenon is largely associated with cellular damage caused by intracellular reactive oxygen species (ROS). To counter the effect of ROS, the expression of superoxide dismutase and catalase, or the addition of ROS scavenger pyruvate, has been successfully used to re-initiate growth[72, 73]. Models that describe this phenomenon include (1) stochastic cellular deterioration similar to senescence in ageing organisms, (2) genetic modules mediating programmed cell death, and (3) adaptive pathways to generate dormant forms for survival

under stressful environmental conditions[74]. How cells have evolved mechanisms for survival, persistence, and distribution in the environment in the VBNC state, and how to resuscitate them for cultivation efforts, are still unknown[75].

## NEW TOOLS AND NEW TECHNIQUES

The emergence of single-cell and high-throughput cultivation techniques have surged in recent years, enabling increasingly refined methods for targeting and enriching a diversity of microbes. The lack of tools to access microbial members of interest in a given environment, therefore, is becoming less of a hindrance to cultivation studies. Particularly, single-cell cultivation technologies, including micromanipulation, gel microdroplet encapsulation, flow cytometry, and the application of micro-engineered mechanical systems (MEMS), have provided some of the most promising platforms for cultivation studies (Table 1). Micromanipulation and flow cytometry in combination with phylogenetic identification tools (for taxon-specific isolation) or fluorogenic indicators of a particular metabolic process are the most precise methods available[76-79]. The application of MEMS[80], the micro-Petri dish for example[81], have recently provided extreme high-throughput cultivation methods and screening capabilities on par with the high-throughput nature of molecular sequence retrieval.

## LACK OF EFFORT

From a pragmatic standpoint, cultivation studies are time consuming, laborious, and wrought with low success rates. These aspects have very real consequences for funding resources. For example, efforts to expand the number of representative cultivated

microorganisms from the deep ocean (piezophiles), the largest biome on Earth harboring the greatest numbers and diversity of aquatic microorganisms[14], have proven elusive. Many researchers have turned to molecular surveys to catalogue the phylogenetic diversity and metabolic potential from this vast habitat[82-85]. Our own work to cultivate novel microbes from the deep ocean has been met with challenges, yet by employing extended incubation times in combination with a seawater-based medium, a representative from the dominant lineage (the Alphaproteobacteria) identified in deep ocean environments worldwide has been successfully isolated. The lack of a sustained cultivation effort, therefore, is another reason contributing to the 'Anomaly.' The establishment of cultivation centers, similar to microbial observatories, would enable a more dedicated effort to explore cultivation strategies, with the primary goal of expending effort and resources to cultivate microbes from diverse environments.

**A 'CULTURE' SHIFT**

So where does this leave us in efforts to resolve the 'Anomaly'? We believe the first task in resolving the 'Great Plate Count Anomaly' involves redefining the conceptual basis of cultivation, and in particular, to expand the terminology of 'culture' to include highly tractable model systems in addition to isolated monocultures.

Model microbial communities, such as the acid mine drainage (AMD) biofilm consortia[86], are exemplary examples of how population heterogeneity, organismal interactions, evolutionary constraints, and functional and metabolic attributes can be experimentally followed in a natural system. In more complex environments, experimental perturbation in microcosms and '-omics'-based tracking of community

changes[87, 88] can lead to robust observations of microbial functionality. A 'culture' shift therefore, refers to what we term the 'cultivation landscape,' a continuum of cultivation systems ranging from experimentally controlled microcosm studies to the highly-defined laboratory-based studies of isolated 'model organisms' (Fig.1). Through this proposed shift, we hope to reverse the perception that the uncultivated microbial majority is beyond the reach of cultivation techniques and place emphasis on using this continuum of cultivation systems to access diverse aspects of 'as-yet-uncultivated' microbes. We ask microbial ecologists to consider this shift in 'culture' as an effort to better integrate cultivation approaches with large-scale, high-throughput molecular surveys of microbial assemblages.

Practical issues aimed at resolving the 'Great Plate Count Anomaly' involve further detailed study of model organisms to better comprehend the vast majority of underlying mechanisms of microbial physiological states, such as dormancy and the semi-dormant VBNC state, as proxies to test in uncultivated organisms. Also, the ability to utilize new technologies, particularly single-cell technologies using inocula from diverse samples, will enable avenues for targeting and enriching a diversity of microbes. This is not the "end of the pure culture era," rather it is the beginning of the development of new culturing platforms.

## FUTURE PERSPECTIVES

We are at a stage in the study of microorganisms, where '-omics' techniques are revolutionizing the field in such an unprecedented way that the immensity of data is overwhelming. The way in which we do science has transformed into an ever-more

complex and computational, sequence-based arena. While it is well recognized that '-omics' studies by themselves are not a panacea, the synergy between '-omics' and microbial cultivation continues to favor high-throughput molecular techniques at the expense of cultivation-dependent studies. The ability to keep pace in this high-throughput molecular world will depend heavily on the integration of interdisciplinary studies combining genomics, metagenomics, microbial physiology, biochemistry, ecology and ecosystem response[22]. To aid in accomplishing this necessary integration, we propose a framework for cultivation efforts in the '-omics' era through the 'cultivation landscape,' which can act as a scaffold for how researchers can use a cultivation continuum to inform '-omics' studies at the appropriate spatial and temporal scales.

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**Box 1 Phylogenetic novelty: The case for '*Candidate'* divisions.**

Since the recognition of the 'uncultured microbial majority[8],' there are still major recognized microbial divisions (or candidate divisions[89]) known only through environmentally derived molecular surveys. The *'Candidate'* status is employed for classification above Class rank as well as for as-yet-uncultivated microorganisms, and is a mechanism to record putative taxa based on sequence identity, experimental *in situ* detection of cells, and all available data regarding morphology, metabolism, physiology, or other phenotypic properties[90]. To put the need for culture-based research of these novel *'Candidate'* lineages into perspective, Rappé and Giovannoni[8] delineated 26 candidate phyla without cultured representatives in 2003. Currently, only four of the original 26 candidate phyla have cultured representatives and the number of candidate phyla has grown exponentially[91]. Pictured below is a phylogenetic tree of the 8,029 type strains currently documented as part of the All-Species Living Tree Project[92, 93], along with the growth in SSU sequences available through the Silva database[94] compared to the number of validated bacterial and archaeal species. While comparing the number of SSU sequences to cultivated isolates is clearly not a valid comparison considering a 16S phylotype does not represent a 'species,' this serves as a benchmark for the discrepancies between the number of validated species and the putative diversity that has been sampled from natural environments. Images are modified, with permission, from[92, 94], and data derived for validated type strains is presented with permission from J.P. Euzéby (http://www.bacterio.cict.fr/index.html).

**Box 2 Targeting a single cell at a time.**

Technological advances go hand-in-hand with advances in our understanding of the microbial world, dating back to the development of some of the first microscopes by Hooke and van Leeuwenhoek[95]. The ability to not only identify cells of a particular phylogenetic lineage using fluorescence *in situ* hybridization (FISH), but to specifically identify the activities and chemical cycling in a single cell has yielding captivating insights (see Amman and Fuchs[96]). Stable- and radioactive-isotope techniques have allowed for the direct probing of individual cells from an environmental sample to link physiological activities to as-yet-uncultivated microorganisms. The first example of coupling FISH with secondary ion mass spectrometry (SIMS) demonstrated phenomenal ability to identify a specific archaeal group associated with methane consumption in anoxic marine sediments[97]. Since the development of high-resolution SIMS (nanoSIMS), the ability to track substrate use in single cells has led to extraordinary findings, such as identification of cyanophycin granules as transient storage bodies for carbon and nitrogen fixation in *Trichodesmium*[98] and the metabolic variability of individual purple and green sulfur bacterial cells[99].

Single-cell genomics is another avenue to gain insight into microbial lifestyles of uncultivated groups, using the ϕ-29 polymerase for whole-genome amplification[100]. Remarkable work at the single-cell level has yielded insight into the uncultured candidate phylum TM7[101, 102] and novel marine Bacteriodetes[103, 104]. Innovative research combining stable-isotope probing and genome amplification from either a subset of the microbial population or from a single cell are enabling fine-scale assessment of microbial physiology and community dynamics in fascinating ways[105, 106]. The world of single-cell

technologies is greatly expanding and incorporating multiple technologies to gain detailed physiological, biochemical, and genomic information.

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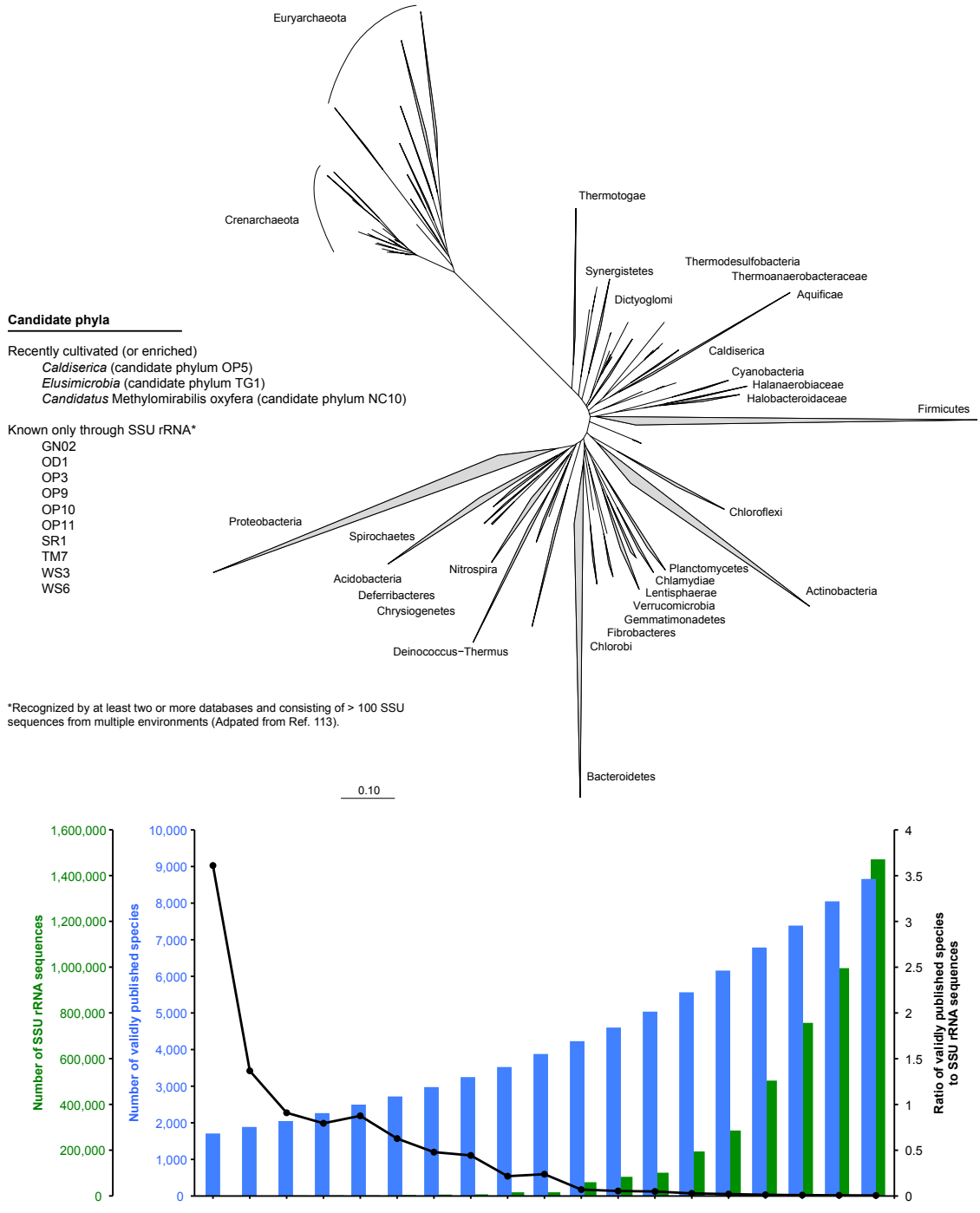


**Figure 1. Phylogenetic novelty: The case for '*Candidate*' divisions.**

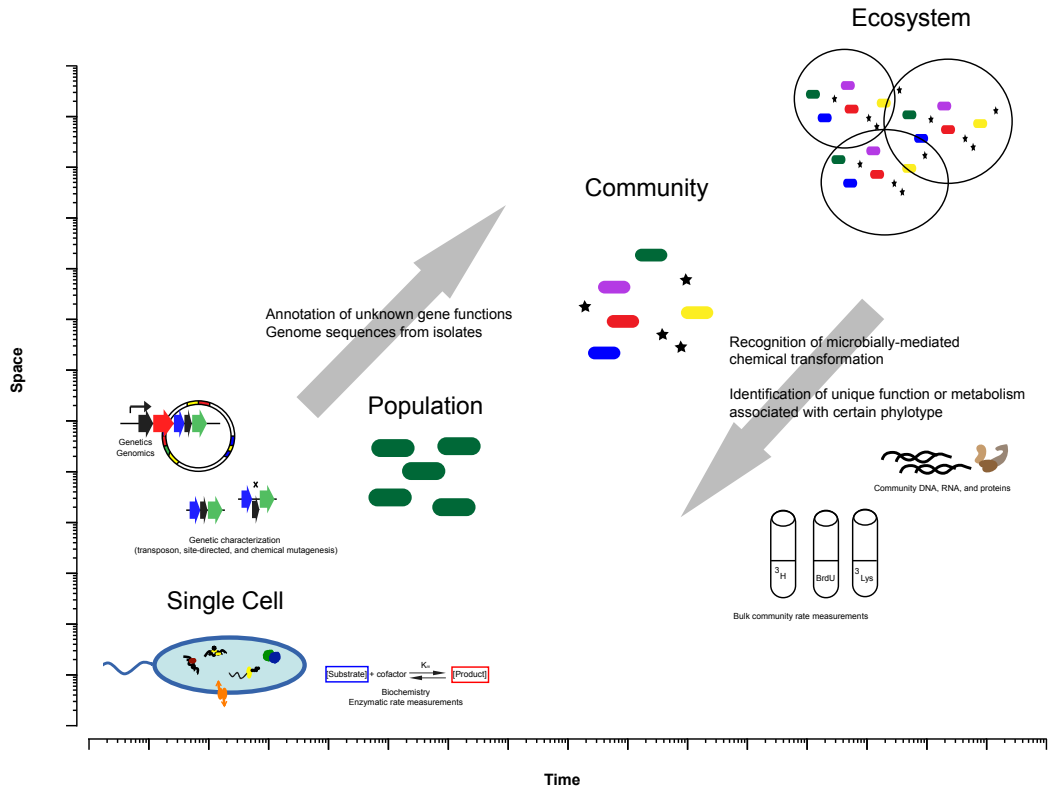


**Figure 2. The 'cultivation landscape.'** This proposed framework relates the continuum of cultivation studies at the relative spatial and temporal scales. At the finest scale, single microbial cells can be systematically probed to investigate enzymatic activity or phylogenetic affiliation (see Box 2). These microbial cells can further be selectively targeted for cultivation efforts mimicking natural conditions, extended incubation times, and addition of signaling molecules or activator constituents. Enrichments leading to isolated monocultures will serve as the templates for genome sequencing, in addition to physiological, biochemical, and genetic studies. Co-cultivation systems, chemostats, and microcosms can similarly lead to informed linkages of biological corollaries to observed phenomena. In this framework, the continuum of cultivation efforts, ranging from the single-cell to model community systems, will allow researchers to gain access to uncultivated microbes and test hypotheses generated by '-omics' techniques.

**Table 1.** Single-cell technologies implemented in cultivation strategies.

| Cultivation strategy | Advantage | Disadvantage | Study (Ref) |
|---|---|---|---|
| Micromanipulation | • Specifically identify cells of interest based on:<br>(1) Morphology<br>(2) Phylogenetic identity with fluorescent probes<br><br>• Visualize and manipulate viable cell *in situ* | • Low throughput<br><br>• Requires extensive practice and skill | Isolation of the hyperthermophilic archaeon *Thermosphaera aggregans* M11TL$^{T}$ [107, 108]<br><br>Symbiotic *Frankia* cells associated with root nodules[109] |
| Fluorescence-activated cell sorting (FACS) | • Specifically identify cells of interest based on:<br>(1) Morphology<br>(2) Phylogenetic identity with fluorescent probes<br><br>• High throughput | • Difficulties in working with non-aqueous samples<br><br>• Requires skill in handling machinery | Actively oxidizing methylotrophs in lake sediment[79]<br><br>LNA-containing *Polynucleobacter* strains[110] |
| Gel microdroplet encapsulation (GMDs) | • Incubation step in growth column, allowing natural chemical exchange<br><br>• Secondary FACS step to identify microcolonies in GMDs<br><br>• High throughput | • Heating sample during encapsulation process with agar (typically 40ºC)<br><br>• Limited ability to target specific phylogenetic groups | Isolation of thousands of microcolonies from seawater, alkaline lake sediment, and soil[42, 111] |
| Micro-engineered mechanical systems (MEMS) | • Microfabricate cultivation devices based on the needs of the researcher<br><br>• Ability to use microscale imaging for high sensitivity detection of growth<br><br>• Extreme high throughput | • Requires equipment for microfabrication and screening<br><br>• Limited ability to target specific phylogenetic groups | micro-Petri dish[81]<br><br>Microfluidic devices[112] |

**GLOSSARY TERMS**

1. Biogeochemical cycle – Abiotic and biologically mediated chemical transformations.

2. Candidate division – Unaffiliated lineage having < 85% identity to reported sequences determined using multiple analyses of data sets with varying types and number of taxa (defined in ref. 89).

3. Dimethylsulfoniopropionate (DMSP) – Sulfur-containing organic metabolite produced by marine algae; the volatile cleavage product DMS is a climate-important compound in the global sulfur cycle.

4. Dissolved organic carbon (DOC) – Carbon-containing organic matter operationally-defined based on filtration. This size-continuum includes a range of macromolecules, colloidal nano- and micro-gels, viruses, and some bacteria.

5. Fluorescence-activated cell sorting (FACS) – Specialized type of flow cytometry able to separate individual cells in a sample based on size and intensity of fluorescent signal.

6. Fluorescence *in situ* hybridization (FISH) – Technique to identify and enumerate microorganisms *in situ* using fluorescently labeled oligonucleotide probes that hybridize to a specific complementary target sequence (generally 16S rRNA) and can be visualized with a fluorescence microscope.

7. Low-nucleic acid (LNA) containing cells –Method-defined population of cells based on low fluorescent signal (indicating low nucleic-acid content) and sideward scatter (indicating cell size) detected using flow cytometry. LNA containing cells generally have low fluorescent signal, when assayed with fluorescent nucleic acid staining dyes, and are small in size.

8. Piezophile – Organism with a maximal growth rate at a pressure in excess of atmospheric pressure (0.1 MPa).

Chapter 4 is an invited Perspective article that is in preparation for submission under the title “The elusive art of microbial cultivation”, with permission from coauthor D. H. Bartlett.

# Chapter V

## Isolation and characterization of the first psychropiezophilic Alphaproteobacterium

**ABSTRACT**

Cultivated psychropiezophilic ('low-temperature and high-pressure adapted') bacteria are currently restricted to a phylogenetically-narrow grouping capable of growth under nutrient-replete conditions, limiting current knowledge of the extant functional attributes and evolutionary constraints from diverse microorganisms inhabiting the cold, deep ocean. This study documents the first isolation of a deep-sea bacterium following dilution to extinction cultivation using a natural seawater medium at high-hydrostatic pressure and low temperature. This isolate, designated PRT1, is the slowest growing (minimal doubling time 36 h) and lowest cell density producing (maximal densities 5.0 x $10^6$ cells $ml^{-1}$) piezophile yet obtained. Optimal growth was at 80 MPa, correlating with the depth of capture (8,350 m) and 10°C, with average cell sizes of 1.46 μm length and 0.59 μm width. Through detailed growth studies, we provide further evidence for the temperature-pressure dependence of the growth rate for deep-ocean bacteria. PRT1 was phylogenetically placed within the Roseobacter clade, a bacterial lineage known for widespread geographic distributions and assorted lifestyle strategies in the marine environment. Additionally, the gene transfer agent (GTA) *g5* capsid protein gene was amplified from PRT1, indicating a potential mechanism for increased genetic diversification through horizontal gene transfer within the hadopelagic environment. This study provides a phylogenetically-novel isolate for future investigations of high-pressure adaptation, expands the known physiological traits of cultivated members of the Roseobacter lineage, and demonstrates the feasibility of cultivating novel microbial members from the deep ocean using natural seawater.

## INTRODUCTION

High hydrostatic pressure is one of the most unique physical parameters in deep ocean environments and plays a significant, albeit under-appreciated, role in the distribution of life in the biosphere. The effects of high hydrostatic pressure on microbial physiology are pervasive, with influences ranging from macromolecular structures to diverse cellular processes such as cell division and motility (8).

Study of microbial assemblages from the abyssal- (4,000 m – 6,000 m) and hadopelagic (> 6,000 m) environments are riddled with methodological issues including, but not limited to (1) difficulty and expense of sample collection, (2) bulk seawater collection that mechanically disrupts delicate colloidal and particulate microenvironments, and (3) specialized high-pressure equipment to maintain samples at *in situ* pressure and temperature once shipboard (6, 50, 88). Intrinsic biological factors similarly compound sampling from depth, the most challenging of which is the mixing of allochthonous microbial members derived from the vertical transport of aggregated material from the overlying surface seawater. These 'hitchhiking' microorganisms, which are not adapted to the prevailing conditions of the deep, render taxonomic cataloguing and diversity estimates of authentic microbial members difficult. Allochthonous members can readily be recovered in molecular surveys, cultivation efforts, and influence activity measurements from deep ocean environments (21, 29, 40, 50, 60). Thus, it is imperative to corroborate the putative high-diversity of microorganisms from deep ocean environments identified using molecular techniques (72) with detailed experiments of phylogenetically-diverse psychropiezophilic ('low-temperature and high-pressure adapted') isolates.

Over thirty years ago, Yayanos and colleagues reported the isolation of the first piezophilic bacterium from the cold, deep ocean (90). Since then, cultivation efforts have yielded psychropiezophilic isolates from a phylogenetically narrow grouping within the Gammaproteobacteria and two gram-positive *Carnobacteriaceae* strains (15, 17, 29, 33-35, 39, 53-57, 84-87, 89, 90). These isolates have generation times ranging from 6 to 35 h under copiotrophic, or nutrient-replete, conditions at deep-sea temperatures and pressures. Cultivation of meso- and hyperthermophilic isolates has yielded a more diverse grouping, including members of the phyla Actinobacteria, Thermotogae, and Euryarchaeota in addition to the Proteobacteria (76) (Table 1). Biochemical, physiological, genetic and genomic studies of these isolates have provided valuable information about the mechanisms of high-pressure adaptation (4, 15, 20, 38, 79, 81, 87, 88). However, the high phylogenetic and metabolic diversity identified from metagenomic (16, 36, 46) and small-subunit ribosomal gene surveys (21, 28, 62, 72) beg the question whether the same adaptive mechanisms identified in the isolated piezophiles are representative of phylogenetically-diverse piezophiles.

Dilution to extinction cultivation methods have resulted in the isolation of numerous members of the bacterioplankton community, including representatives from the ubiquitous SAR11 clade and phylogenetically-novel microorganisms from previously uncultivated phyla (14, 26, 64, 69, 70). While natural seawater as a growth medium, either unamended or supplemented with additional nutrients and vitamins, has been widely successful in obtaining novel microbial isolates from surface seawater assemblages, this methodology has not been extended to cultivation attempts with deep ocean samples. An exploration of dilution to extinction cultivation attempts at high

hydrostatic pressure would be a distinct method to isolate microbes from the cold, deep ocean.

In this study, the isolation and characterization of the first psychropiezophilic member of the Alphaproteobacteria, designated PRT1, is presented. This is the first dilution to extinction cultivation effort using a natural seawater medium at high-hydrostatic pressure and low temperature.

## MATERIALS AND METHODS

**Sample collection and high-pressure cultivation conditions.** Hadal seawater was collected from 8,350 m (19.763°N, 66.379°W) within the Puerto Rico Trench (PRT) aboard the R/V *Pez Mar* in November 2006 using the Deep Ocean Vehicle (DOV) "Bobby Ray," an untethered free-fall/free-ascent vehicle designed and constructed by Scripps engineer Kevin Hardy. Upon recovery of DOV "Bobby Ray," hadal seawater was returned to *in situ* temperature and pressure conditions using stainless steel pressure vessels (91). Seawater samples were maintained in 15 ml polyethylene transfer pipet bulbs (Samco) and heat-sealed with a handheld heat-sealing clamp (Nalgene) until further processing at Scripps Institution of Oceanography.

Dilution to extinction cultivation methods were carried out at high hydrostatic pressure using a natural seawater medium supplemented with 1.0 μM $NH_4Cl$ and 0.1 μM $KH_2PO_4$ as described by Rappé *et. al.* (64). Natural seawater was collected from the Scripps pier (La Jolla, CA) and subsequently filtered through a 47 mm diameter 0.22 μm pore-size Supor-200 membrane (Pall Corporation), sparged with $CO_2$ (3 minutes per 1 liter of seawater), and autoclaved for thirty minutes. Sterilized seawater was stored in the

dark at 4ºC to age for at least 3 months. Prior to culture transfers, the medium was again filtered though a sterile 0.22 μm pore-size Supor-200 membrane (Pall Corporation) membrane and supplemented either with phosphate and ammonium, or phosphate, ammonium, Thauer vitamin mixture (83) diluted $10^{-4}$ and a defined mixture of organic carbon compounds (0.001% (w/v) d-glucose, d-ribose, succinic acid, pyruvic acid, glycerol, *N*-acetyl glucosamine, 0.002% (v/v) ethanol (64)). For serial dilution transfers, samples were briefly decompressed and immediately inoculated into chilled seawater medium on ice, which was then transferred into 15 ml polyethylene transfer pipet bulbs (Samco), heat-sealed and pressurized to 80 MPa in pressure vessels (88, 91). All transfers and culture manipulations were exposed to atmospheric pressure for less than 30 min and were performed in the dark under a yellow fluorescent lamp (General Electric) to minimize exposure to light. Enrichments were carried out at 80 MPa (corresponding approximately to the *in situ* pressure condition) and at 8ºC. Cell abundances were monitored microscopically using the nucleic acid stain DAPI (H-1200, Vector laboratories Inc.) for evidence of bacterial growth.

**16S rRNA sequencing and phylogenetic analysis.** Genomic DNA was extracted from 150 ml of culture filtered onto a 0.22 μm pore-size Supor-200 membrane (Pall Corporation). The filter was submerged in 3 ml lysis buffer (50 mM Tris-HCl, 40 mM EDTA, 0.75 M sucrose, 1 mg/ml lysozyme) and incubated at 37ºC for 1 h with gentle mixing. Proteinase K (final concentration 0.5 mg/ml) and 1% SDS were added, mixed by inversion, subjected to two freeze/thaw cycles, and incubated at 55ºC for 2 h. Phenol:chloroform:isoamyl alcohol (25:24:1; Invitrogen) was used to purify DNA, which

was subsequently precipitated with 100% ethanol and resuspended in TE (10 mM Tris, 1 mM EDTA) buffer. The 16S ribosomal gene was amplified by PCR using bacterial 16S-specific primers 27F and 1492R (25). The PCR product was purified with a QIAquick PCR purification kit (Qiagen) and directly sequenced using the general eubacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3'), 518R (5'-GTATTACCGCGGCTGCTG-3'), 530F (5'-GTGCCAGCAGCCGCGG-3'), 907R (5'-CCGTCAATTCATTTGAGT-3'), 926F (5'-ACTCAAAGGAATTGACGG-3'), and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The sequences were assembled using the CLC Sequence Viewer (CLC bio), aligned using the SINA Webaligner (63), uploaded into the ARB program (42) and manually checked with the ARB_EDIT4 tool. A filter was applied to aligned sequences to exclude positions < 50% conservation and *E. coli* positions 59 to 1,406 were exported for bootstrap analysis using the maximum likelihood methods implemented in RaxML (73) with the JTT model for evolutionary distances through the CIPRES portal (48). Neighbor-joining and parsimony methods implemented in PHYLIP (23) were additionally utilized to compare phylogenies and tree topology. Bootstrap support for nodes are indicated for values > 50%. The outgroup used to calculate phylogeny was *Sinorhizobium meliloti* (GenBank accession D14509). Sequence similarity analysis was performed within the ARB program (42).

**Identification of putative gene transfer agent (GTA) using PCR and microscopy.** Degenerate primers developed by Zhao and colleagues (93) were used to screen for the presence of the GTA *g5* gene (encoding the major capsid protein) from PRT1 genomic DNA extracted as described above. PCR amplification was carried out as described

previously (93), the product was purified with a QIAquick PCR purification kit (Qiagen), and sequenced directly. The PRT1 *g5* DNA sequence was translated and aligned with 156 putative GTA g5 proteins (Supplementary Table 1) using MUSCLE (19). Phylogenetic trees were constructed from the aligned amino acid sequences consisting of 241 unambiguous positions using the maximum likelihood methods implemented in RaxML (73) with the JTT model for evolutionary distances through the CIPRES portal (48).

GTA particles were observed using epifluorescence microscopy with SYBR Green I (Invitrogen) using the staining method described by Patel and colleagues (58).

**Growth characterization at varying temperature and hydrostatic pressure conditions.** For growth-rate determinations as a function of pressure and temperature, mid-log cultures were inoculated 1:100 into 100 ml sterilized Kapak bags (Kapak Corporation) with an attached 0.045 inch-diameter polyethylene tube to allow sub-sampling from the same culture. All cultures were incubated with sterile glass beads in a temperature-controlled rocking water bath to ensure mixing within the pressure vessels. The same collected batch of natural seawater supplemented with phosphate, ammonium, Thauer vitamins and the mixture of organic carbon compounds was used for all growth characterization experiments. At 24 hr time intervals, pressure vessels were decompressed and 2 ml of culture was removed, fixed with formaldehyde solution (3% final concentration), and frozen at -80ºC until analysis.

Growth was monitored with a Becton-Dickson FACsort flow cytometer. Samples were thawed on ice, diluted 10-fold, and stained for 15 minutes in the dark with SYBR Green I (Invitrogen) (44). Quantification of cells was based on green fluorescence and

side scatter, and each sample was run with a known concentration of 0.9-µm standard beads. CytoWin (78) was used to process cytometric plots and the resultant growth curves were fit to a parametric logistic growth model using the program grofit implemented in R (31). The exponential growth rate constants ($k$) were derived for seventeen growth conditions at varying temperatures and pressures from a total of thirty discrete growth experiments (experiments resulting in no growth were assigned $k = 0$). The values for $k$ were used to construct the contour plot for the pressure-temperature dependency of the growth rate constant ($k$) with the DIVA (Data-Interpolating Variational Analysis) gridding software implemented in Ocean Data View (ODV) (68).

**Microscopy.** *Epifluorescence microscopy for cell sizing.* Formaldehyde-fixed mid-log cultures were filtered onto 0.2 µm Isopore polycarbonate membranes (Millipore) and stored at -80ºC until further analysis. Two different staining methods were utilized to obtain cell size measurements as described by Malfatti *et. al.* (43), the nucleic acid stain DAPI (H-1200, Vector laboratories Inc.) and the protein stain NanoOrange (N-6666, Invitrogen). The stained samples were viewed at 1,000 X on an Olympus BX51 microscope (Olympus) and images were processed using ImageJ (1, 65). Length and width for 100 cells were measured for both DAPI and NanoOrange stained samples.

*Atomic force microscopy.* A sample was fixed and filtered onto 0.2 µm polycarbonate membranes as described above. Atomic force microscopy (AFM) imaging was performed with a MFP-3D BIO (Asylum Research, Santa Barbara, USA) as described by Malfatti *et. al.* (43). Briefly, images were acquired in AC mode in air with a silicon nitride cantilever (AC160TS; Olympus), with a spring constant of 42 N/m and

scan rates of 1 Hz. The height and phase channels were recorded since the height channel gives quantitative data on the topography of the sample, while the phase channel can suggest qualitative data on the visco-elastic properties of the sample. Topography images were processed with Planfit and Flatten functions.

*cryo-Transmission electron microscopy.* 3.5 μL mid-log culture samples were applied to holey carbon grids (Quantifoil) that had been glow-discharged in an Emitech K350 evaporation unit. Grids were then vitrified using a Vitrobot robot (FEI) and transferred into a pre-cooled, FEI Polara, multi-specimen holder, which maintained the grids at liquid nitrogen temperature. Micrographs were recorded on a Gatan 4K2 CCD camera at 300 keV in an FEI Polara microscope under low-dose conditions (3 e-/$Å^2$) at nominal magnifications of 12,000× and 23,000× (CCD, 1.88 Å per pixel) and an objective lens defocus setting of -15 μM.

**Nucleotide sequence accession numbers.** The 16S rRNA and GTA sequences were deposited in GenBank under accession numbers JF303756 and JF303757, respectively.

## RESULTS AND DISCUSSION

### Isolation of PRT1 using natural seawater medium at high hydrostatic pressure.

Dilution to extinction cultivation methods using a natural seawater medium supplemented with ammonium and phosphate as described by Rappé *et. al.* (64) were carried out at 80 MPa and 8°C. Eleven end-point serial dilution transfers over the course of approximately two years resulted in the isolation of strain PRT1, which was verified using 16S rRNA gene sequencing.

Representative growth plots are presented in Figure 1. PRT1 was originally isolated and propagated with natural seawater amended only with 1.0 μM ammonium ($NH_4Cl$) and 0.1 μM phosphate ($KH_2PO_4$). Under these conditions, growth rates ranged from 0.3 – 0.4 $d^{-1}$. Subsequent growth experiments included supplement with a dilute vitamin solution and a defined mixture of organic carbon compounds, resulting in marginal growth rate increases (0.4 – 0.43 $d^{-1}$) and higher cell abundances (maximal cell densities 5.0 x $10^6$ cells $ml^{-1}$). These growth rate values are comparable to surface seawater oligotrophic isolates (0.40 – 0.58 $d^{-1}$ for *Pelagibacter ubique* HTCC1062 (64); 0.40 – 0.73 $d^{-1}$ for four oligotrophic isolates (70)), yet are substantially lower compared to all cultured psychropiezophilic isolates (Table 1). However, a compilation of 375 growth rate measurements from microbes in the bathypelagic realm (1,000 – 4,000 m) yielded an average growth rate of 0.061 $d^{-1}$ (± 0.008 SE, median = 0.019)(6). Therefore, the growth rate of PRT1 might be more representative of natural rates of reproduction in the deep ocean than the growth rates of previously described psychropiezophilic isolates grown under nutrient-replete conditions, and might actually be considered one of the relatively faster-growing representatives from the deep ocean.

The growth yield of PRT1 was similarly distinct from all cultured psychropiezophilic isolates, reaching cell densities (~ $10^6$ cells $ml^{-1}$) at least an order of magnitude less than even the slowest growing, most piezophilic isolate reported under optimal growth conditions (*Colwellia* sp. MT41, ~ $10^7$ cells $ml^{-1}$)(89). While most psychropiezophilic growth can be tracked using optical density (OD) measurements since cultures reach $10^8$ – $10^9$ cells $ml^{-1}$, the growth of PRT1 was monitored using flow cytometric techniques in the absence of visible turbidity. The maximal cell densities

varied between different batches of seawater collected, as well as the amount of time allowed for the seawater to age. This variation has similarly been observed for the growth properties of SAR11 (64) and suggests that intrinsic factors in the seawater contribute to the growth characteristics of PRT1. Efforts to propagate PRT1 in a variety of synthetic seawater media were unsuccessful. Additionally, increasing the concentration of organic carbon compounds by one and two orders of magnitude (0.01% and 0.1%, respectively) substantially decreased growth yields (maximal cell densities $2.6 \times 10^4$ cells $ml^{-1}$).

Cell shapes ranged from bi-lobes to rods, with an average length of 1.46 μm (SE ± 0.039, n=100) and an average width of 0.59 μm (SE ± 0.0064, n=100). Size estimates were obtained using NanoOrange staining cell-sizing methods (the ratio of DAPI to NanoOrange sizing was 0.87 and 0.78 for length and diameter, respectively) (43). PRT1 cells were further visualized using more refined techniques, including cryo-TEM as well as atomic force microscopy (AFM) to observe morphological features (Fig. 2). Interestingly, AFM imaging yielded identification of small (~20 nm in diameter) pilus-like appendages that were consistently found associated or detached from the cell surfaces and were distinct from flagellar filaments due to their small size (Fig. 2C).

**PRT1 is an obligate psychropiezophile.**

PRT1 exponential growth rate constants (*k*) were derived from a total of thirty discrete growth experiments (using seawater collected from the same batch for consistency purposes) and used to construct the contour plot for the pressure-temperature dependency of the growth rate constant (Fig. 4). These detailed growth experiments at varying temperature and pressure conditions demonstrate the prominence of the

'piezophilic trait' and provide further evidence for the temperature-pressure dependence of the growth rate for deep-ocean bacteria as previously described by Yayanos (87). PRT1 was piezophilic under all temperatures tested (4 – 12ºC) and found to be an obligate piezophile, or hyperpiezophile ($P_{kmax}$ > 60 MPa). No growth was observed at atmospheric pressure (Fig. 4). As has been observed previously (87), the growth rate varied with temperature and pressure indicating a correlation with the habitat pressure (or capture depth) of the isolate. The growth rate of PRT1 near the pressure of the depth of capture (8,350 m) increased with increasing temperature over the interval of 6 – 10ºC, with the maximal growth rate among all temperatures ≈ 8 – 10ºC, consistent with the work of Yayanos and colleagues (87, 88). This isolate further supports the assertion that the 'piezophilic trait' is invariant with nutritional state and that, in the absence of a high-nutrient medium (such as 2216 Marine broth), bacterial isolates can be recovered that demonstrate adaptations to the prevailing low temperature and high hydrostatic pressure conditions found in the deep ocean.

**PRT1 is a member of the *Roseobacter* clade within the Alphaproteobacteria.**

Sequence analysis of the 16S small-subunit ribosomal gene placed PRT1 within the Roseobacter clade, a diverse Alphaproteobacterial lineage found ubiquitously in the marine environment (Fig. 3). The within-taxon phylogeny of the Roseobacter lineage is poorly resolved using 16S rRNA phylogenies (12, 13, 52), rendering reliable phylogenetic reconstruction for PRT1 difficult. A more robust phylogenetic analysis of over 6,000 Rhodobacterales sequences indicated PRT1 was affiliated with the abundant marine cluster NAC11-7 (13), a group represented by numerous environmental clone

sequences yet only two cultivated members (13, 70). The two cultivated members were both obtained using dilution to extinction cultivation methods, with a draft genome available for one of the isolates (Rhodobacterales bacterium HTCC2255; https://moore.jcvi.org/moore/), yet little additional physiological data is available since the initial description of the isolation. The NAC11-7 clade has typically been associated with phytoplankton blooms (82), yet has also been identified in diverse environments including oxygen minimum zones (74, 80), associated with deep-sea corals (61) and the hydrothermal vent worm *Riftia pachyptila* (41), hydrothermal chimney biofilms (11), deep ocean sediments (67), and numerous coastal environments worldwide (66, 75). PRT1 16S rRNA sequence similarity to the 32 Roseobacter isolates with available genome sequences ranged from 90.8 – 94% (Fig. 3B), which is consistent with the large sequence variation (~ 11%) documented for the greater than 45 described genera of the Roseobacter lineage (52).

The identification of a gene component, the *g5* capsid protein gene, for the positive detection of a gene transfer agent (GTA) from PRT1 further supports the phylogenetic placement within the Rhodobacterales Order, since almost all members contain this gene (10). GTAs are novel virus-like elements that function in the transfer of random fragments of genomic DNA (4.5-kb for the *R. capsulatus* RcGTA), while conferring no documented negative effects associated with transfer (i.e. cell lysis) (10, 37). While the g5 phylogeny has previously been found to be congruent with the 16S rRNA phylogeny (93), a more recent study of Rhodobacterales GTAs from the subarctic North Atlantic demonstrated that using a subset of the gene sequence information does not necessarily reflect the expected phylogenetic relationships (24). Analysis of the PRT1

g5 capsid protein with 156 available Rhodobacterales g5 capsid protein sequences (Fig. S1) did not help resolve phylogenetic placement of PRT1, although since HTCC2083 and HTCC2255 appear to lack the genetic complements for GTA production, this finding is not surprising. The sequence similarity for the PRT1 g5 capsid protein sequence ranged from 81.1 – 67.4% compared to the g5 capsid protein sequences from the 32 Roseobacter isolates with available genome sequences (Fig. 3B). The identification of the *g5* capsid protein gene from PRT1 provides insight into a potential mechanism of increased genetic diversification through horizontal gene transfer from GTAs within the hadopelagic environment.

A detailed analysis of available marine Roseobacter genomes indicated the gene repertoire for a particular trophic strategy (heterotrophy, photoheterotrophy, or autotrophy) was the best predictor of clustering relationships (52). The findings that trophic strategy, and not necessarily phylogeny, is the best framework for predicting genome content is in line with the hypothesis that horizontal gene transfer (potentially mediated by GTAs) may be the dominant evolutionary forces for this generalist lineage. Importantly, the results from the eloquent analysis of available Roseobacter genomes demonstrate the uniqueness of each genome (52), therefore rendering inferences about physiological traits based on phylogenetic relatedness impossible. PRT1 is the third documented psychrophile within the clade after *Octadecabacter arcticus* 238 and *Octadecabacter antarcticus* 307 (27). These psychrophilic Roseobacter genomes contained the greatest number of unique genes, the majority of which were annotated as phage or transposase-related, consistent with the proposed hypothesis that transposases play an important role in psychrophilic genome evolution (5). One could hypothesize that

the genome of PRT1 is similarly unique compared to the two psychrophilic Roseobacter genomes, and sufficiently divergent from other mesophilic Roseobacters, since it must cope with not only cold temperatures, but also adaptational constraints imposed by high-hydrostatic pressure. Indeed, molecular analysis of piezophilic Gammaproteobacterial isolates suggests high-pressure adaptation is preceded by pre-existing adaptation to low temperature (39).

The majority of published representatives from the *Roseobacter* lineage form colonies on nutrient agar media, yet more recent cultivation strategies using dilution to extinction methods have resulted in novel isolates unable to form colonies, such as Rhodobacterales bacterium HTCC2150 (32). While methods to detect isolated colonies at high hydrostatic pressure are available (18, 51), PRT1 was unable to form colonies under the conditions tested using either gelatin or agar as the solidifying agent. Although PRT1 was not found to form colonies, cells were observed to clump together and form weakly associated aggregates during growth, which might be indicative of the general hypothesis suggested by Moran and colleagues that members of the Roseobacter clade live in nutrient-replete aggregates (e.g. marine snow) found in the oligotrophic ocean (49). The identification of small pilus-like appendages associated with PRT1 mentioned previously might contribute to the clumping growth characteristics observed and additional work is warranted to explore potential colonization and colony formation abilities beyond this initial observation. It is possible these appendages are pili and would be consistent with recent genomic analyses indicating all Roseobacter genomes harbor the genetic complement for assembly of adhesive Flp (fimbrial low-molecular-weight protein) pili (71).

**Implications for isolating new piezophiles from novel phylogenetic lineages.**

Autochthonous microorganisms of the deep ocean are inherently adapted to the ambient conditions of their environment, thus to the high-pressure, low-temperature conditions found throughout the deep ocean. The utility of extinction to dilution cultivation techniques has further been demonstrated as a unique and distinctive strategy to isolate new psychropiezophiles from diverse phylogenetic groups. Isolation of PRT1, the first piezophilic Alphaproteobacterial representative, demonstrates (1) the ability to cultivate a phylogenetically-novel piezophile outside of the previously cultivated Gammaproteobacterial families *Colwelliaceae*, *Moritellaceae*, *Psychromonadaceae*, *Shewanellaceae*, and *Vibrionaceae* from the cold, deep ocean; (2) the ability to cultivate a low-nutrient-adapted piezophilic isolate using a natural seawater medium, thus providing the fist evidence for a piezophilic phenotype under oligotrophic conditions; and (3) enables future biochemical and physiological characterization to further document the unique adaptations of high-pressure-adapted bacteria to the psychropiezosphere. A cautionary note, however, regarding PRT1 is that the slow growth and minimal cell yields preclude many additional biochemical characterizations that require substantial biomass, such as membrane lipid composition or cytochrome analyses.

This study has demonstrated the effectiveness of dilution to extinction cultivation efforts using a natural seawater medium at high-hydrostatic pressure and low temperature. The isolation of PRT1 and subsequent growth characterization has provided a more comprehensive view of psychropiezophile growth and physiology.

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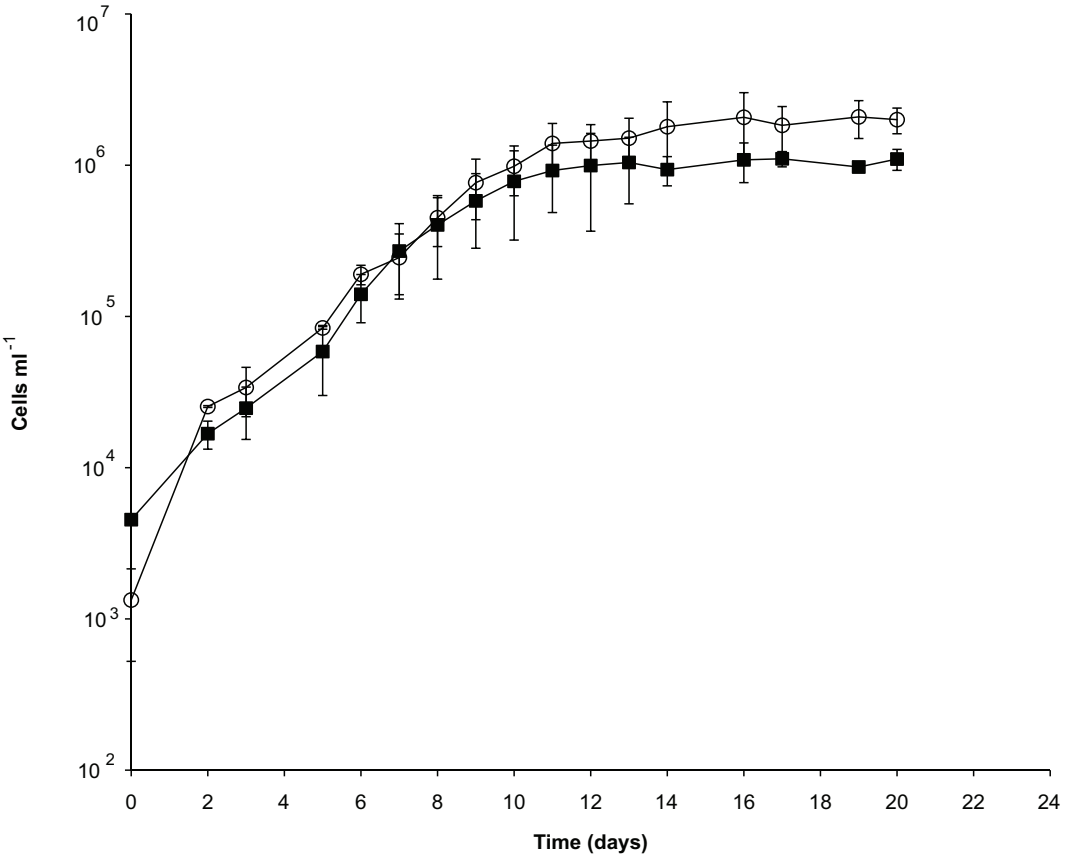


**Figure 1.** Growth of PRT in natural seawater medium amended with ammonium and phosphate (■) and ammonium, phosphate, Thauer vitamins, and a mixed carbon solution (○) measured using flow cytometry. Error bars represent standard deviations of duplicate incubations at 80 MPa and 8°C.

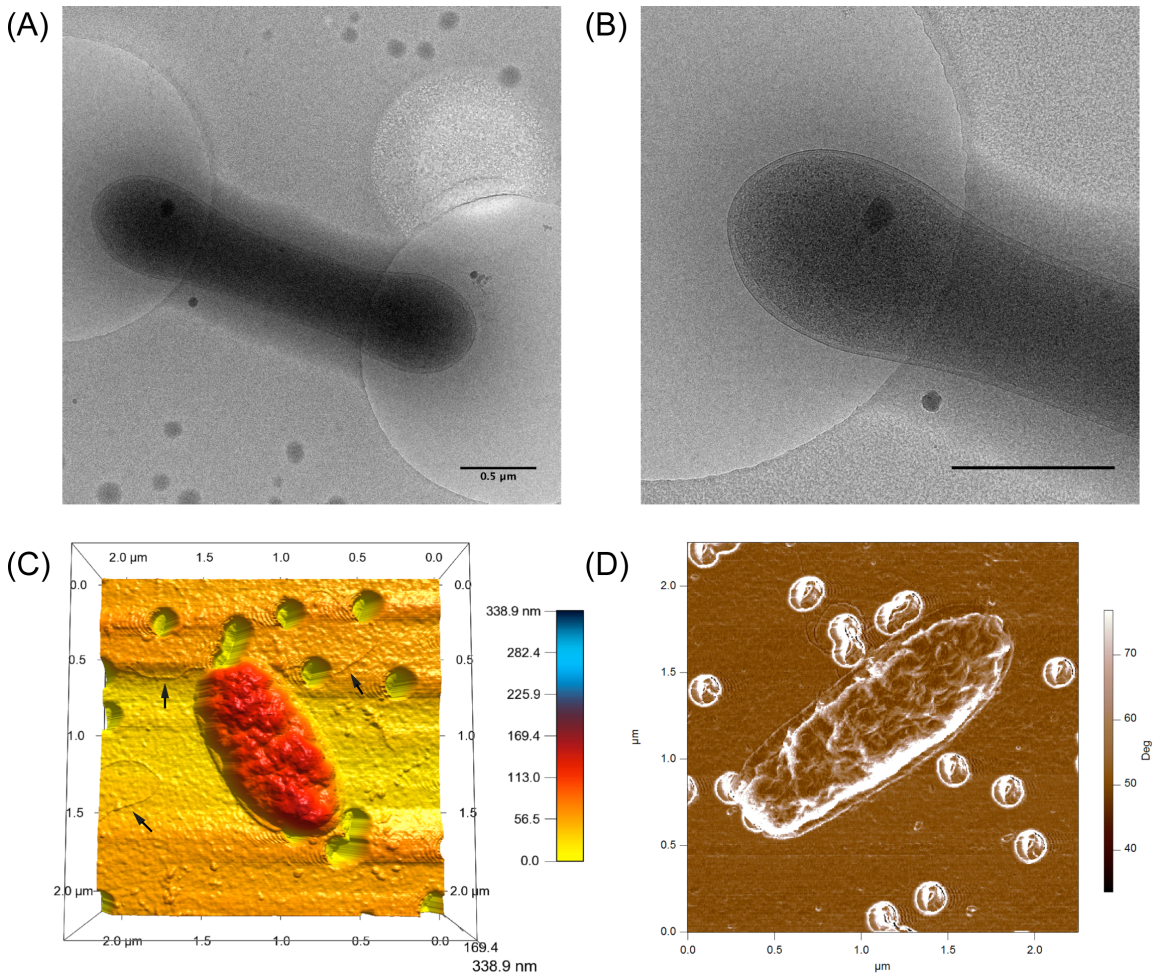


**Figure 2**. Morphological features of PRT1 visualized using cryo-transmission electron microscopic (cryo-TEM) and atomic force microscopic (AFM) techniques. Cryo-TEM images of PRT1 at (A) 12,000× magnification and (B) 23,000× magnification. Scale bar, 0.5 µm. (C, D) AFM images of individual PRT1 cells. Color bars indicate the cell height (*Z*) range in nm (C) and degrees (height contrast from the filter base) (D). Black arrows indicate pilus-like appendages (C).

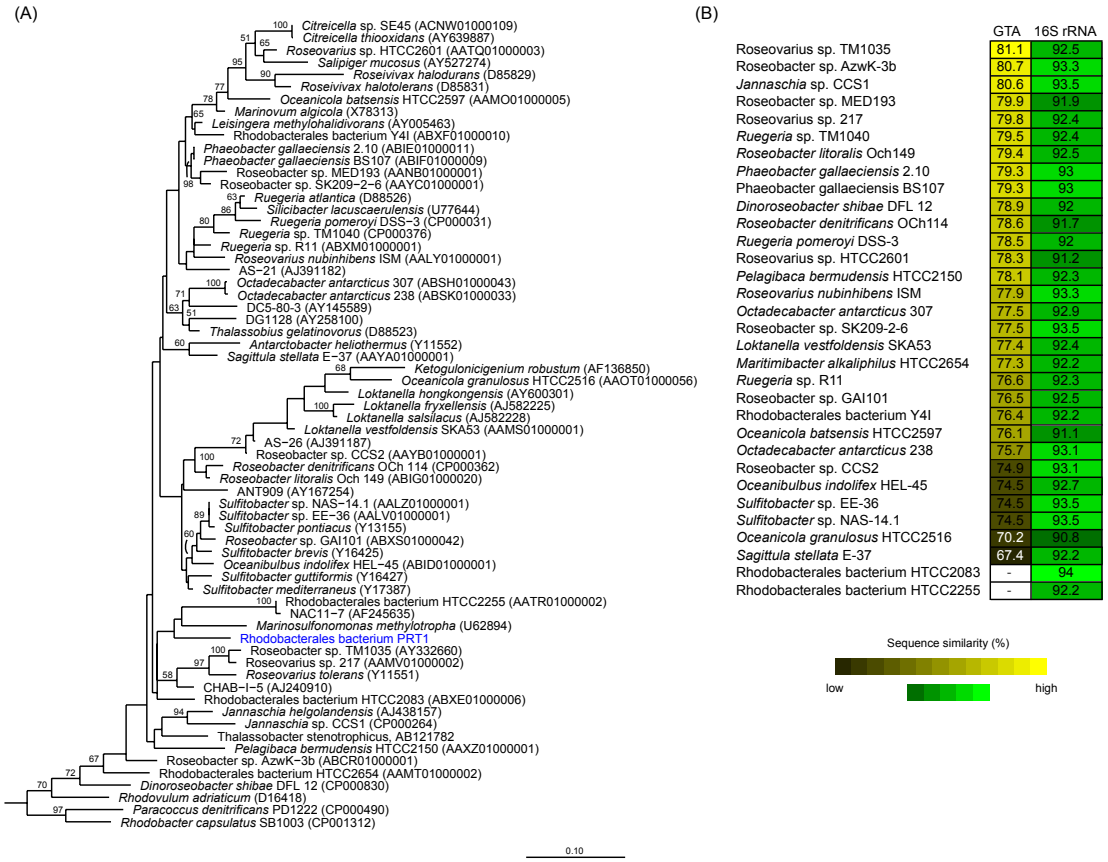


**Figure 3.** Phylogenetic placement of PRT1 within the Roseobacter clade. (A) The PRT1 16S rRNA gene sequence was aligned, compared to reference Roseobacter sequences, and phylogeny inferred using the maximum likelihood method implemented in RaxML (73) with the JTT model for evolutionary distances through the CIPRES portal (48). Bootstrap support (100 bootstrap replicates) for nodes are indicated for values > 50%. GenBank accession numbers are indicated in parentheses. (B) Sequence similarity analysis for the PRT1 16S rRNA and gene transfer agent (GTA) g5 capsid protein compared to the 32 cultivated members of the Roseobacter lineage with available genome sequence information.

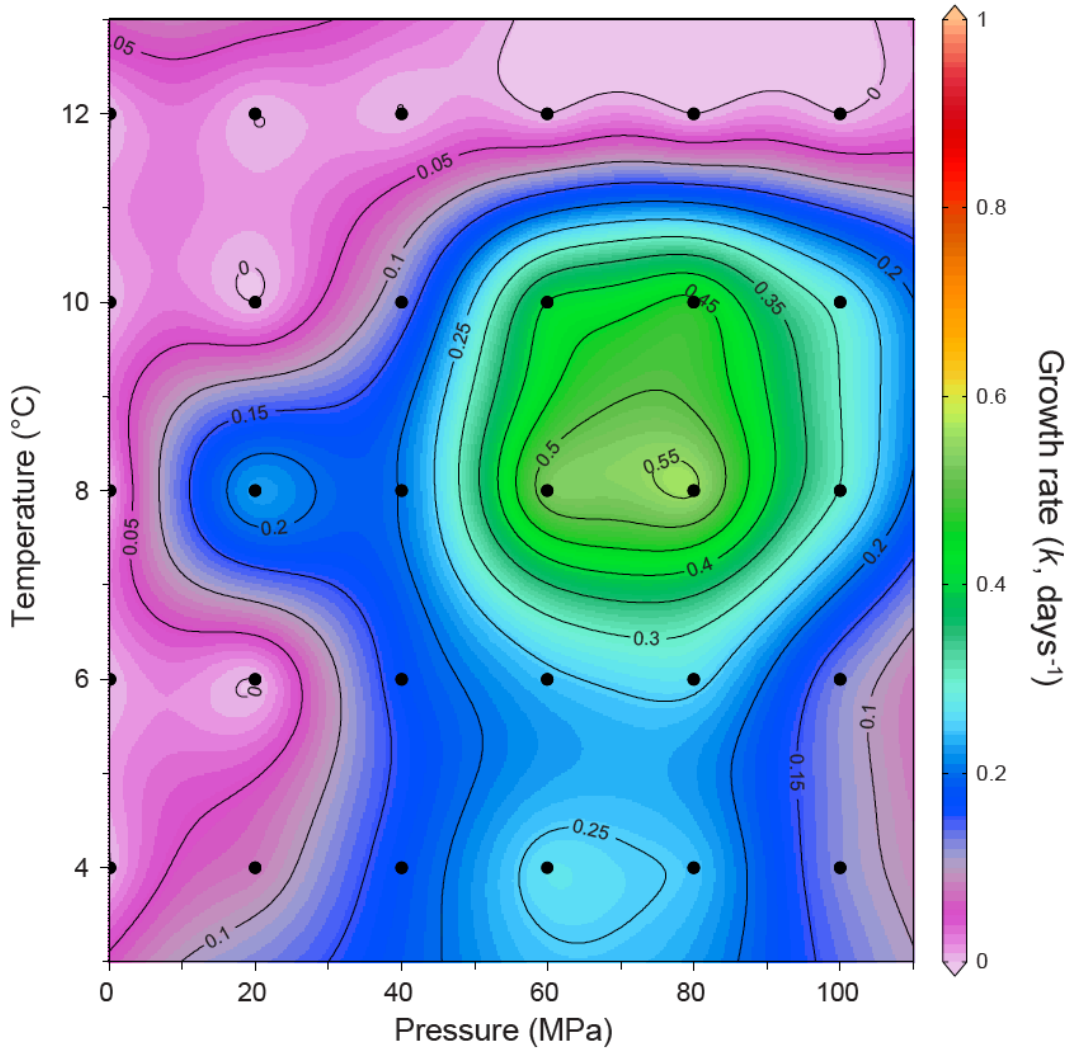


**Figure 4.** Interpolated contour diagram of the temperature-pressure dependence of the exponential growth rate constant ($k$) of PRT1. Black dots represent individual growth experiments at the given temperatures and pressures, with color shading and contour lines integrated using the DIVA (Data-Interpolating Variational Analysis) gridding software. The growth rate constant ($k$) is expressed in days$^{-1}$.

**Table 1.** Growth properties of selected cultivated piezotolerant and piezophilic isolates.

| Isolate | $T_{opt}$ (°C) | $P_{opt}$ (MPa) | Maximal growth rate ($h^{-1}$) | Isolation source (depth) | Ref |
|---|---|---|---|---|---|
| ***Colwelliaceae*** | | | | | |
| *Colwellia piezophila* Y223G$^T$ | 10 | 60 | 0.14 | Japan Trench, sediment (6,278 m) | (53) |
| *Colwellia hadaliensis* BNL-1$^T$ | 10 | 90 | 0.12 | Puerto Rico Trench (7,410 m) | (17) |
| *Colwellia* sp. MT41 | 8 | 103 | ~ 0.07 | Mariana Trench, decaying amphipod (10,476 m) | (87, 89) |
| ***Psychromonadaceae*** | | | | | |
| *Psychromonas profunda* 2825$^T$ | 10 | 25 | ~ 0.15 | Atlantic Ocean sediment (2,770 m) | (86) |
| *Psychromonas kaikoae* JT7304$^T$ | 10 | 50 | ~ 0.15 | Japan Trench, cold-seep sediment (7,434 m) | (56) |
| *Psychromonas* sp. CNPT3 | 12 | 52 | ~ 0.19 | Central North Pacific, decaying amphipod (5,800 m) | (87, 90) |
| *Psychromonas hadalis* K41G$^T$ | 6 | 60 | ~ 0.14 | Japan Trench, sediment (7,542 m) | (54) |
| ***Moritellaceae*** | | | | | |
| *Moritella profunda* 2674$^T$ | 6 | 30 | ~ 0.17 | Atlantic Ocean, sediment (2,815 m) | (85) |
| *Moritella abyssi* 2693$^T$ | 10 | 30 | ~ 0.20 | Atlantic Ocean, sediment (2,815 m) | (85) |
| *Moritella* sp. PE36 | 10 | 41 | ~ 0.28 | Pacific Ocean, amphipod trap water (3,584 m) | (87) |
| *Moritella japonica* DSK1 | 15 | 50 | ~ 0.4 | Japan Trench, sediment (6,356 m) | (35) |
| *Moritella yayanosi* DB21MT-5 | 10 | 80 | ~ 0.2 | Mariana Trench, sediment (10,898 m) | (33, 55) |
| ***Vibrionaceae*** | | | | | |
| *Photobacterium profundum* DSJ4 | 10 | 10 | ~ 0.45 | Ryukyu Trench, sediment (5,110 m) | (57) |
| *Photobacterium profundum* SS9 | 15 | 28 | ~ 0.5 | Sulu Trough, amphipod homogenate (2,551 m) | (15) |
| ***Shewanellaceae*** | | | | | |
| *Shewanella violacea* DSS12 | 10 | 30 | ~ 0.28 | Ryukyu Trench, sediment (5,110 m) | (35) |

**Table 1.** Continued.

| Isolate | $T_{opt}$ (°C) | $P_{opt}$ (MPa) | Maximal growth rate ($h^{-1}$) | Isolation source (depth) | Ref |
|---|---|---|---|---|---|
| *Shewanella benthica* F1A | 8 | 30 | ~ 0.15 (at 3°C, 30 MPa) | Atlantic Ocean, water column (4,900 m) | (29, 84) |
| *Shewanella benthica* DB6101 | 10 | 50 | ~ 0.35 | Ryukyu Trench sediment (5,110 m) | (35) |
| *Shewanella benthica* DB5501 | 15 | 60 | ~ 0.35 | Suruga Bay, sediment (2,485 m) | (35) |
| *Shewanella benthica* DB6705 | 15 | 60 | ~ 0.4 | Japan Trench, sediment (6,356 m) | (35) |
| *Shewanella benthica* DB6906 | 15 | 60 | ~ 0.35 | Japan Trench, sediment (6,269 m) | (35) |
| *Shewanella benthica* DB172R | 10 | 60 | ~ 0.45 | Izu-Bonin Trench, sediment (6,499 m) | (34) |
| *Shewanella benthica* DB172F | 10 | 70 | ~ 0.41 | Izu-Bonin Trench, sediment (6,499 m) | (34) |
| *Shewanella benthica* DB21MT-2 | 10 | 70 | ~ 0.17 | Mariana Trench sediment (10,898 m) | (33, 55) |
| *Shewanella* sp. KT99 | ~ 2 | ~ 98 | | Kermadec Trench, amphipod homogenate (9,856 m) | (39) |
| **Non-Gammaproteobacteria** | | | | | |
| *Thioprofundum lithotrophica* 106 | 50 | 15 | 0.3 | Mid Atlantic Ridge, black smoker chimney (3,626 m) | (76) |
| *Desulfovibrio profundus* 500-1$^{T}$ | 25 | 15 | -* | Japan Sea, sediment core 518 mbsf** (900 m) | (7) |
| *Carnobacterium* sp. AT7 | 20 | 20 | | Aleutian Trench, water column (2,500 m) | (39) |
| *Methanopyrus kandleri* 116 | 105 | 20 | ~ 0.75 | Central Indian Ridge, black smoker fluid (2,415–2,460 m) | (77) |
| *Desulfovibrio hydrothermalis* AM13$^{T}$ | 35 | 26 | ~ 0.05 | East Pacific Rise, hydrothermal vent chimney (2,600 m) | (3) |
| *Piezobacter thermophilus* 108 | 50 | 35 | 0.46 | Mid Atlantic Ridge, Black smoker chimney (3,626 m) | (76) |
| *Marinitoga piezophila* KA3$^{T}$ | 65 | 40 | ~ 1.9 | East Pacific Rise, hydrothermal vent (2,630 m) | (2) |
| *Dermacoccus abyssi* MT1.1$^{T}$ | 28 | 40 | -$^{\#}$ | Mariana Trench, sediment (10,898 m) | (59) |

**Table 1.** Continued.

| Isolate | $T_{opt}$ (°C) | $P_{opt}$ (MPa) | Maximal growth rate ($h^{-1}$) | Isolation source (depth) | Ref |
|---|---|---|---|---|---|
| *Pyrococcus abyssi* GE5 | 100 | 40 | 0.98 | Fiji Basin, hydrothermal vent (2,000 m) | (22) |
| *Thermococcus barophilus* $MP^T$ | 85 | 40 | ~ 1.5 | Mid Atlantic Ridge, hydrothermal vent chimney (3,550 m) | (45) |
| *Methanococcus thermolithotrophicus* | 65 | 50 | ~ 0.58 | Geothermal heated sediments, Italy (0.5 m) | (9) |
| *Pyrococcus* sp. CH1 | 98 | 52 | -## | Mid Atlantic Ridge, hydrothermal vent (4,100 m) | (92) |
| *Methanococcus jannaschii* | 86 | 75 | 2.36 | East Pacific Rise, hydrothermal vent (2,610 m) | (30, 47) |
| Rhodobacterales bacterium PRT1 | 10 | 80 | 0.019 | Puerto Rico Trench, seawater (8,350 m) | This study |

* Growth tracked by sulfide production (7).
**mbsf, meters below the seafloor.
#Rate not reported, viable count $3.6 \pm 0.4 \times 10^6$ c.f.u. $ml^{-1}$ (60% increase over growth at atmospheric pressure) (59).
##Rate not reported, maximal growth yields $5 \times 10^8$ cells $ml^{-1}$ under optimal growth conditions (92).

**Supplementary Material – Figures.**

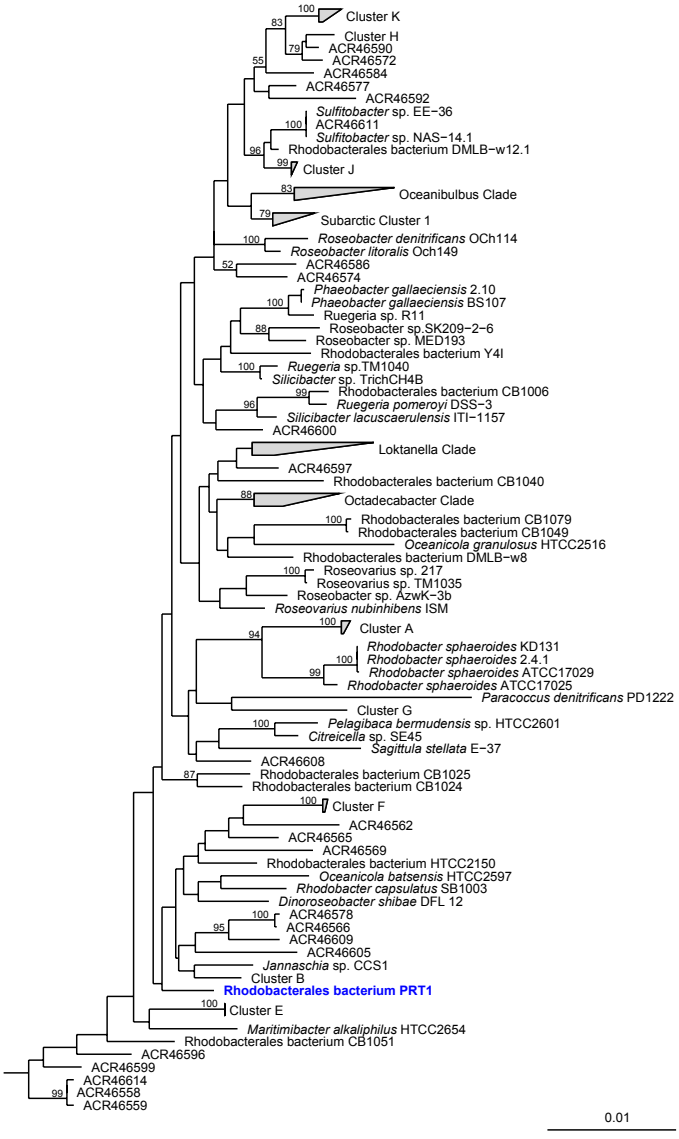


**Figure S1.** Phylogenetic placement of the PRT1 g5 GTA capsid protein with 156 putative Rhodobacterales GTA g5 proteins. Alignments were made using MUSCLE (19) and phylogenetic trees were constructed from the aligned amino acid sequences consisting of 241 unambiguous positions using the maximum likelihood methods implemented in RaxML (73) with the JTT model for evolutionary distances through the CIPRES portal (48). Bootstrap support (100 replicates) for nodes are indicated for values > 50%. The outgroup used to calculate phylogeny was *Brucella abortus* (GenBank accession YP221349). Clade designations are denoted for clusters previously identified by Zhao *et. al.* (93) and Fu *et. al.* (24).

Chapter 5 is a full-length manuscript in preparation for submission under the title 'Isolation and characterization of the first psychropiezophilic Alphaproteobacterium,' with permission from coauthor D. H. Bartlett.

# Chapter VI

## The deep-sea bacterium *Photobacterium profundum* SS9 utilizes separate flagellar systems for swimming and swarming under high-pressure conditions

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# The Deep-Sea Bacterium *Photobacterium profundum* SS9 Utilizes Separate Flagellar Systems for Swimming and Swarming under High-Pressure Conditions▽†

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**Motility is a critical function needed for nutrient acquisition, biofilm formation, and the avoidance of harmful chemicals and predators. Flagellar motility is one of the most pressure-sensitive cellular processes in mesophilic bacteria; therefore, it is ecologically relevant to determine how deep-sea microbes have adapted their motility systems for functionality at depth. In this study, the motility of the deep-sea piezophilic bacterium *Photobacterium profundum* SS9 was investigated and compared with that of the related shallow-water piezosensitive strain *Photobacterium profundum* 3TCK, as well as that of the well-studied piezosensitive bacterium *Escherichia coli*. The SS9 genome contains two flagellar gene clusters: a polar flagellum gene cluster (PF) and a putative lateral flagellum gene cluster (LF). In-frame deletions were constructed in the two flagellin genes located within the PF cluster (*flaA* and *flaC*), the one flagellin gene located within the LF cluster (*flaB*), a component of a putative sodium-driven flagellar motor (*motA2*), and a component of a putative proton-driven flagellar motor (*motA1*). SS9 PF *flaA*, *flaC*, and *motA2* mutants were defective in motility under all conditions tested. In contrast, the *flaB* and *motA1* mutants were defective only under conditions of high pressure and high viscosity. *flaB* and *motA1* gene expression was strongly induced by elevated pressure plus increased viscosity. Direct swimming velocity measurements were obtained using a high-pressure microscopic chamber, where increases in pressure resulted in a striking decrease in swimming velocity for *E. coli* and a gradual reduction for 3TCK which proceeded up to 120 MPa, while SS9 increased swimming velocity at 30 MPa and maintained motility up to a maximum pressure of 150 MPa. Our results indicate that *P. profundum* SS9 possesses two distinct flagellar systems, both of which have acquired dramatic adaptations for optimal functionality under high-pressure conditions.**

The deep sea constitutes the largest habitat in the biosphere, where physiochemical parameters such as low temperature, refractory organic carbon, and high hydrostatic pressure structure diverse communities. It has been hypothesized that the evolutionary modalities of autochthonous microbial residents are significantly affected by hydrostatic pressure at depths greater than 2,000 m (45). Hydrostatic pressure alters the free energy of equilibria, macromolecular packing, and hydration via influences on system volume changes (39). Piezophilic ("pressure-loving") microorganisms harbor unique adaptations to cope with deep-sea high-pressure conditions (reviewed in reference 4). A bacterial strain with a $P_{kmax}$ (pressure at which the growth rate is maximal) of >0.1 MPa and <60 MPa is termed a piezophile (46); a strain with a $P_{kmax}$ of <30 MPa might be termed a "moderate" piezophile and one with a $P_{kmax}$ of >60 MPa a "hyperpiezophile."

Motility is considered one of the most pressure-sensitive cellular processes in nonpiezophilic microorganisms (4, 31). In *Escherichia coli*, increased hydrostatic pressure acts as an inhibitor of the formation of new flagella and of the functioning of previously assembled filaments (31). The bacterial flagellum is a helical filament of approximately 15 μm consisting of ~30,000 flagellin monomers that polymerize to form a functional filament (24). High-resolution structures obtained from X-ray crystallography and electron cryomicroscopy of the *Salmonella enterica* serovar Typhimurium flagellin F41 fragment reveal four linearly connected domains (D0, D1, D2, and D3) with a 3-dimensional structure in the shape of a uppercase Greek gamma (Γ) (36, 48). The flagellar filament is composed of 11 protofilaments that form two conformations based on supercoiling: a left-handed (L type, or "normal") and a right-handed (R type, or "curly") symmetry. The N-terminal and C-terminal regions form an α-helical coiled-coil (D0–D1 domains) that constitutes a densely packed filament core and are highly conserved among eubacterial flagellins (6). Domains D2 and D3, on the other hand, are hypervariable regions projecting out from the filament core. These two regions vary both in sequence and in length and are thought to be important for folded flagellin conformation stability (26). In vitro studies of *Salmonella* serovar Typhimurium flagellin show an increase in partial molar volume of 340 $cm^3$/mol upon polymerization, where filaments irreversibly depolymerize at approximately

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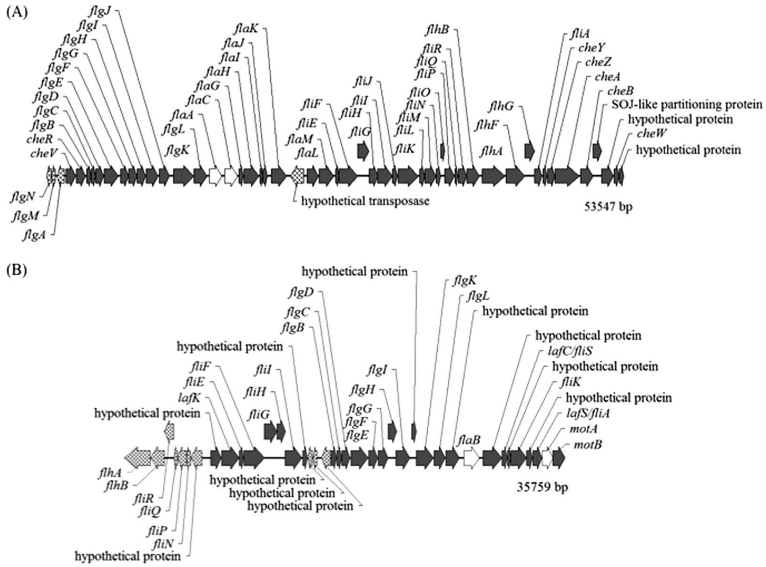


FIG. 1. Polar (A) and lateral (B) flagellar gene clusters in *P. profundum* SS9. Additional genes not found in the main clusters include the putative sodium motor genes *motAB* (PBPRA0808 and PBPRA0809, chromosome 1, positions 895239 to 896941), *motX* (PBPRA3344, chromosome 1, positions 3792907 to 3793536), and *motY* (PBPRA2571, chromosome 1, positions 2976252 to 2977142). Four of the five genes deleted via in-frame mutagenesis are represented by open arrows.

340 MPa (40). It would therefore appear that the assembly and functioning of the flagellar apparatus under high hydrostatic pressure in deep-sea bacteria would necessitate adaptation.

The psychrotolerant, moderately piezophilic organism *Photobacterium profundum* SS9 has been extensively studied using genetic, genomic, and functional genomic approaches (12, 23, 41). Two flagellar motility gene clusters have been identified: a polar flagellum (PF) gene cluster and a potential lateral flagellum (LF) gene cluster (12, 41). Microarray hybridization experiments indicate that one of the most notable differences between SS9 and its shallow-water, pressure-sensitive relative *Photobacterium profundum* strain 3TCK is that the latter lacks the putative LF cluster found in SS9 (12). Based on its higher GC content, the LF cluster could have been horizontally transferred to SS9 (12).

The organization of the *P. profundum* SS9 PF and LF clusters (Fig. 1) is almost identical to that of the major flagellar loci in *Vibrio parahaemolyticus* BB22 (28). The genes found in the PF cluster appear to be organized into nine operons based on close or overlapping sequences and the absence of transcriptional terminators. Only three flagellin genes are present in SS9, compared to the six polar flagellin genes of *Vibrio parahaemolyticus* and *Vibrio fischeri* and the five polar flagellin genes of *Vibrio cholerae* and *Vibrio anguillarum* (21, 29, 30, 32). *flaA* and *flaC* reside within the PF gene cluster, while *flaB* is the single flagellin gene present within the LF gene cluster. Interestingly, *P. profundum* SS9 *flaA* is differentially upregulated at atmospheric pressure compared to 28 MPa (12). Furthermore, *flaC* is expressed at a higher level than *flaA* based on microarray mean fluorescent values and is presumably incorporated into the filament more extensively.

The LF cluster in *P. profundum* SS9 contains a number of sequences related to genes present in the *V. parahaemolyticus* LF system, with the notable exception that the lateral cluster in *P. profundum* SS9 is present on chromosome 1 instead of chromosome 2 and is not similarly organized into two regions (28). The first 15 genes from *V. parahaemolyticus* region 2 are inverted and reside upstream of the region 1 genes in the *P. profundum* SS9 LF system. Many of the genes in the SS9 LF cluster are annotated as hypothetical proteins due to low amino acid identity and sequence similarity to known flagellar components. *P. profundum* SS9 *flaB* is highly divergent and only weakly resembles a lateral flagellin. Instead, it is most closely related at the amino acid level to a *Pseudomonas fluorescens* flagellin (corresponding to GenBank accession number AAC63947; 41% identity).

Consistent with the *V. parahaemolyticus* motility system, *P. profundum* SS9 appears to possess two kinds of rotary motors for propulsion: a sodium-driven complex associated with PF rotation and components for a proton-driven motor found in the LF cluster. Four genes are linked to the sodium type polar motor, with *motAB2* residing upstream of the PF cluster: *motA* (GenBank locus tag PBPRA0808), *motB* (PBPRA0809), *motX* (PBPRA3344), and *motY* (PBPRA2571). The putative proton-driven components *motAB1* (PBPRA0048, PBPRA0049) reside in the LF cluster. Chemotaxis genes and numerous

TABLE 1. Bacterial strains and plasmids used in this study

| Strain or plasmid | Genotype or description[a] | Reference or source |
|---|---|---|
| Strains | | |
| *P. profundum* | | |
| SS9R | Rif$^r$ SS9 derivative | 13 |
| EAE1 | Δ*flaA* SS9R; Rif$^r$ | This study |
| EAE2 | Δ*flaB* SS9R; Rif$^r$ | This study |
| EAE3 | Δ*flaC* SS9R; Rif$^r$ | This study |
| EAE4 | Δ*motA1* SS9R; Rif$^r$ ($H^+$ driven) | This study |
| EAE5 | Δ*motA2* SS9R; Rif$^r$ ($Na^+$ driven) | This study |
| *E. coli* | | |
| ED8654 | pRK2073 maintenance | 33 |
| DH5α | RecA$^-$; cloning | 18 |
| XL1-Blue | RecA$^-$; cloning | Stratagene, La Jolla, CA |
| TOP10 | RecA$^-$; cloning | Invitrogen, Carlsbad, CA |
| Plasmids | | |
| pRK2073 | *tra* genes for conjugal transfer | 9 |
| pRL271 | *sacB*-containing suicide plasmid; Cm$^r$ | 10 |
| pMB2190 | pBR327 derivative; Kn$^r$ | 2 |
| pEAE1k | *flaA* deletion construct::pRL271; Kn$^r$ | This study |
| pEAE2k | *flaB* deletion construct::pRL271; Kn$^r$ | This study |
| pEAE3k | *flaC* deletion construct::pRL271; Kn$^r$ | This study |
| pEAE4k | *motA1* deletion construct::pRL271; Kn$^r$ | This study |
| pEAE5k | *motA2* deletion construct::pRL271; Kn$^r$ | This study |

[a] Kn$^r$, kanamycin resistance; Cm$^r$, chloramphenicol resistance; Rif$^r$, rifampin resistance.

methyl-accepting chemotaxis protein genes are distributed throughout the genome.

In this study, the first examination of motility as a function of pressure in a deep-sea microbial species is reported. The results indicate that the SS9 PF and LF systems are fully functional and are adapted for swimming and swarming, respectively, at depth.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The strains and plasmids used in this work are described in Table 1. *P. profundum* SS9 strains were cultured aerobically in 2216 medium (28 g/liter; Difco Laboratories) at 16°C. *E. coli* strains were grown aerobically in Luria-Bertani (LB) medium at 37°C (37). High-pressure growth of SS9 strains was performed anaerobically at 16°C in 2216 medium supplemented with 20 mM glucose and 100 mM HEPES buffer (pH 7.5) (Sigma). Late-exponential-phase cultures were diluted 500-fold into fresh medium and used to fill 4.5-ml polyethylene transfer pipettes (Samco). Transfer pipettes were heat sealed with a handheld heat-sealing clamp (Nalgene) and incubated at 0.1 MPa and 30 MPa in stainless-steel pressure vessels (47). Antibiotics (Sigma) were used at the following concentrations: chloramphenicol, 30 μg ml$^{-1}$; kanamycin, 75 μg ml$^{-1}$ for *E. coli* and 200 μg ml$^{-1}$ for SS9; rifampin, 100 μg ml$^{-1}$.

**Measurement of swimming speed under high hydrostatic pressure.** The HPDS (Hartmann, Pfeifer, Dornheim, Sommer) high-pressure cell (Technische Universität München, Freising-Weihenstephan, Germany) was used to examine and analyze the swimming behavior of *P. profundum* strain 3TCK, *Escherichia coli* strain W3110, the parental strain SS9R, and its motility mutants under high hydrostatic pressure (16, 19). The HPDS high-pressure cell was fixed to an inverted microscope (DM-IRB; Leica, Germany) and examined using a 40× phase-contrast objective giving 0.5-μm resolution. The temperature and pressure were measured through a transducer and sent, via a DAQ (data acquisition hardware) device custom developed for use with the HPDS cell, to a computer for monitoring with a custom-developed program made in LabView, version 5.1 (National Instruments) (for details, see reference 19). Mid-exponential-phase (optical density at 600 nm [$OD_{600}$], 0.3) cultures were diluted 1:10, and 3.5 μl (~$3.5 \times 10^3$ cells) was loaded into the HPDS cell for viewing. Samples were maintained at atmospheric pressure (0.1 MPa) for 2 min before the pressure was increased. Pressure treatment involved a stepwise increase by 10 MPa (up to 150 MPa) every 30 s and a final decompression. A 2/3-in charge-coupled device digital camera (Basler, Germany) (19) displayed live output feed, which was directly recorded to iMovie format and subsequently converted into MPEG image sequence files for analysis. Image analysis software (NIH ImageJ, version 1.36 for Mac; http://rsb.info.nih.gov/ij/) (1) was used to measure the swimming speeds of individual moving bacteria manually in 25 successive frames corresponding to 1 s. Between 50 and 100 individual swimming tracks were measured for each condition with approximately 20 to 30 cells/frame.

**Generation of flagellin and motor protein mutants.** Marker exchange-eviction mutagenesis was performed using the *sacB*-containing suicide vector pRL271 (10, 35). Plasmids were constructed as described by Welch and Bartlett (43). First, a 2,642-bp region upstream (primers, FlaAUPF [5′-AGTCTCGAGTGATCGGCAGTGGGCATACC-3′] and FlaAUPR [5′-GATGCGGCCGCTTTGCTCTCCTTTGACTTTTCAC-3′]) and a 2,564-bp region downstream (primers, FlaADNF [5′-GATGCGGCCGCACACAGCTACAGTAAATATTG-3′] and FlaADNR [5′-Phos-GCGAGCGCCATTATCATCTTTG-3′]) of *flaA* were PCR amplified from wild-type SS9 using the Expand Long-Template PCR system (Roche Applied Science). The amplified flanking regions were digested with NotI, ligated using Quick-stick ligase (Bioline), and reamplified using primers FlaAUPF and FlaADNR. The resulting deletion construct was digested with XhoI and cloned into the XhoI-NaeI sites of the suicide plasmid pRL271, creating pEAE1. A SalI-digested fragment containing the kanamycin resistance gene from pMB2190 was cloned into pEAE1, creating plasmid pEAE1k. pEAE1k was then conjugated into SS9R, a rifampin-resistant derivative of wild-type SS9, by triparental conjugations using helper plasmid pRK2073 as described by Chi and Bartlett (13), with kanamycin selection to identify exconjugants with pEAE1k integrated into the *flaA* gene. pEAE1k contains the *sacB* gene, the product of which is lethal in gram-negative bacteria in the presence of sucrose (35). When exconjugants were plated onto 2216 medium with 5% sucrose, only clones that had undergone a second recombination event that excises *sacB* grew. A *flaA* deletion mutant was identified from the two possible recombination events by PCR with primers FlaACTRLF (5′-CCAAAGTGACGGTAACCCCAAAAA-3′) and FlaACTRLR (5′-TGTTTTGCTCACCGGTTTTATCTG-3′) and was designated EAE1. In-frame deletions of the *flaB*, *flaC*, *motA1*, and *motA2* genes were similarly constructed and verified by PCR. Primer sequences used for the construction of pEAE1k to pEAE5k are listed in Table S1 in the supplemental material.

**Microscopy.** A 0.5-μl aliquot of a stock solution of the fluorescent protein stain NanoOrange (Molecular Probes, Invitrogen) was added to 10-μl mid-exponential-phase ($OD_{600}$, 0.3) liquid cultures on a microscope slide (17). Slides were incubated in the dark for 10 min to allow staining and were then examined on an inverted epifluorescence microscope (model IX71; Olympus) at a magnification of ×60 with a blue filter (excitation wavelength, 490 nm; emission wavelength, 520 nm). Images were captured with a MicroFire 2/3-in charge-coupled device digital camera and examined with the PictureFrame imaging application system (Optronics).

**Motility assay.** Motility phenotypes for the flagellin and motor protein mutants were investigated qualitatively using 2216 medium–0.3% agar polyethylene transfer pipette bulbs. Late-exponential-phase cultures were inoculated into the bulb in a straight line by using a thin inoculation rod, and the bulb was subsequently heat sealed. The parental strain, SS9R, and its mutants were incubated for 48 h at 16°C and 0.1 MPa or 30 MPa in stainless-steel pressure vessels. Polyvinylpyrrolidone, with an average molecular weight of 360,000 (PVP-360; Sigma), was used as a viscosity-increasing agent in the 2216 medium–0.3% agar bulbs at increments of 1.25%, 2.5%, 5%, and 10% to assay for lateral motility (7). pFL185 (a plasmid expressing β-galactosidase) was conjugated into SS9R and motility mutants by using helper plasmid pRK2073. Plasmid-containing strains were inoculated into 2216 medium–0.3% agar bulbs with added S-Gal (3,4-cyclohexenoesculetin-β-D-galactopyranoside; Sigma) and ferric ammonium citrate (20).

**RT-PCR expression analysis.** Reverse transcription-PCR (RT-PCR) was used to assess differential expression of flagellin and motor-protein genes from the parental strain, SS9R, under conditions of varying viscosity and high pressure. Mid-exponential-phase ($OD_{600}$, 0.3) 30-ml cultures grown with or without 5%

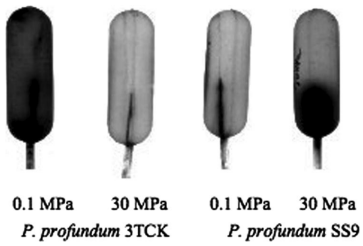


FIG. 2. Growth-based 0.3% agar bulb assay to qualitatively assess motility for *P. profundum* strains 3TCK and SS9 at atmospheric pressure (0.1 MPa) and high pressure (30 MPa).

PVP-360 (Sigma) in 2216 medium (20 mM glucose and 100 mM HEPES buffer) at 30 MPa and 16°C were harvested for RNA extraction. Cell pellets were resuspended in 4 ml Trizol reagent (Invitrogen), and 800 μl chloroform was added. The solution was thoroughly mixed, incubated on ice for 5 min, and centrifuged at 9,000 × *g* for 15 min. Two milliliters of the aqueous phase was transferred to a new tube, and 2 ml isopropanol was added and then incubated at room temperature for 10 min. Tubes were centrifuged at 12,000 × *g* for 5 min, washed with 4 ml 75% ethanol, and centrifuged again at 9,000 × *g* for 5 min. RNeasy columns (Qiagen) were used for the second RNA cleanup with additional DNase treatment according to the manufacturer's instructions. RT-PCR was carried out using a Qiagen One-Step RT-PCR kit according to instructions with an added RNase inhibitor (Ambion). An internal fragment of *flaB* was amplified using primers flaBRNAF (5′-TGGCGGTTCAGTCTAAAAAT-3′) and flaBRNAR (5′-AATACCAGTACCGGCATCCTCAGT-3′). Uridine phosphorylase (PBPRA1431) (primers, udpF [5′-GTGCACCGTCAGCCATTATCG-3′] and udpR [5′-CGCCCAGCACGCCTTTCT-3′]) was used as a control due to low expression as determined from microarray data (12).

## RESULTS

**Agar matrix motility assays at high hydrostatic pressure.** Qualitative analyses of microbial motility are typically performed by inoculating cell cultures into plates with a low percentage of agar and visually inspecting the diameter of the growing cell population and the associated chemotaxis patterns (32). This method, however, is impractical at high hydrostatic pressure. Instead, pressurizable plastic pipette bulbs containing a low-percentage agar medium and a β-galactosidase indicator dye, and lacking significant air space, were utilized (see Materials and Methods). Inoculation into the bulbs of bacteria genetically modified to produce high levels of β-galactosidase activity, and their subsequent incubation at appropriate temperatures and pressures, provided a convenient 3-dimensional view of cell growth and movement over time.

Motility bulb assay characteristics were obtained for derivatives of the *P. profundum* piezosensitive strain 3TCK and the *P. profundum* piezophilic strain SS9, along with its isogenic *fla* and *mot* mutants (Fig. 2 and 3). Flagellin and motor component deletion mutants were checked for growth defects to ensure that there were no pressure effects on the growth rate that would affect the interpretation of the growth-based motility agar assays. All five flagellin and motor component mutants were found to have growth rates comparable to that of the parental strain, SS9R (data not shown). 3TCK displayed vigorous swimming ability at 0.1 MPa, with cells moving out to the sides of the 140-mm bulb after 24 h at 16°C, while little swimming ability was evident at 30 MPa. The opposite was true for SS9, which filled the entire bulb after 48 h at 16°C and 30 MPa. The PF *flaA*, *flaC*, and *motA2* mutants were nonmotile

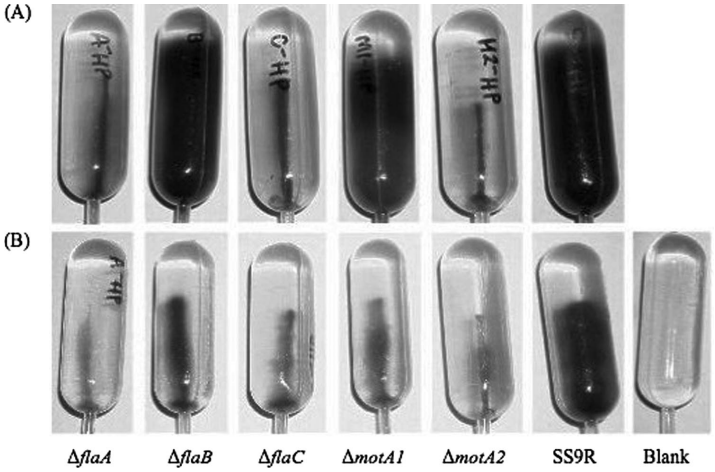


FIG. 3. Growth-based agar bulb assay to qualitatively assess motility under high-hydrostatic-pressure and high-viscosity conditions. (A) Swimming motility bulbs under high-pressure conditions (30 MPa) with 0.3% agar. (B) Swarming motility bulbs under high-pressure (30 MPa) and increased-viscosity (0.3% agar with 2.5% PVP-360) conditions. The strains examined were SS9R (the parental strain) and the Δ*flaA*, Δ*flaB*, Δ*flaC*, Δ*motA1*, and Δ*motA2* mutants.

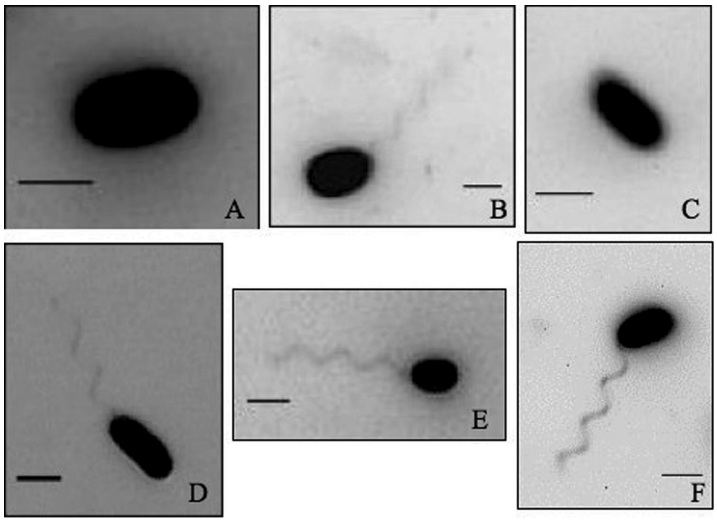


FIG. 4. Flagella visualized with NanoOrange staining for *P. profundum* SS9 and deletion mutants. The Δ*flaA* (A) and Δ*flaC* (C) mutants do not produce a filament. The Δ*flaB* (B) and proton-motor (Δ*motA1*) (D) mutants were seen to produce a polar flagellum comparable to that of the parental strain, SS9R (F). The sodium motor (Δ*motA2*) (E) mutant was found to produce an intact flagellum yet was nonmotile. Scale bars, 2 μm.

both at 0.1 MPa and at 30 MPa, whereas the LF *flaB* mutant and the *motA1* mutant appeared to possess wild-type motility at 30 MPa (Fig. 3A). NanoOrange fluorescent microscopic visualization of these cells indicated that they all are monotrichously flagellated except for the *flaA* and *flaC* mutants (Fig. 4). Taken together, the results indicate that *P. profundum* possesses a single polar flagellum, which in the case of 3TCK functions optimally at atmospheric pressure and in the case of SS9 functions best at elevated pressure. SS9 FlaA and FlaC are required components of the polar flagellum, which is most likely driven by sodium motive force based on the sequence similarity of the *motAB* gene products to confirmed sodium-driven motors in *V. parahaemolyticus* (3).

**SS9 swarming motility.** Lateral flagellar motility has been studied on 0.6 to 0.8% agar plates containing growth media with reduced iron levels and in similar liquid media containing viscosity-increasing agents (7). Viscosity-increasing agents such as polyvinylpyrrolidone, polyethylene glycol, and Ficoll effectively induce swarmer cell differentiation in *Vibrio parahaemolyticus* (7). The possible presence of a lateral flagellar motility system in SS9 was examined by modifying the bulb motility assays described above to include PVP-360 (Sigma) in increments of 1.25%, 2.5%, 5%, and 10%. Under these conditions, all of the SS9R-derived strains were nonmotile, except for the parental strain, SS9R. While the *flaB* and *motA1* mutants were motile at high pressure under standard bulb assay conditions, they displayed nonmotile phenotypes when grown under conditions of high pressure and increased viscosity (Fig. 3B). These results provide the first phenotypic evidence for a functional lateral flagellar motility apparatus in SS9, a feature that is evident only under conditions of both high pressure and high viscosity. However, both NanoOrange fluorescence microscopy and scanning electron microscopy failed to reveal lateral flagella on SS9 cells, although these structures were clearly visible in control cultures of *V. parahaemolyticus* (data not shown).

The results from the motility assays suggested induction of a lateral flagellar system under high-pressure and high-viscosity conditions, and therefore the possibility of upregulation of the transcription of the components of the lateral flagella and their associated motor proteins. The transcript abundances of two genes associated with putative lateral flagellar function, *flaB* and *motA1*, were assessed under high-pressure and high-viscosity conditions by semiquantitative RT-PCR, with uridine phosphorylase (*udp*; PBPRA1431) as a control (Fig. 5). Uridine phosphorylase was used as a reference due to its low-level constitutive expression, based on transcriptome data (13), which was comparable to the low expression levels of *flaB*. The results indicated that mid-log-phase cultures of SS9R grown under high-pressure conditions induce *flaB* at least fivefold under increased-viscosity conditions (5% PVP-360), based on the ratio of gene amplification under high-pressure conditions to that under high-pressure plus high-viscosity conditions relative to *udp* control expression. *flaB* was constitutively expressed at the same level in *flaA*, *flaC*, and *motA2* mutants under conditions of high pressure with or without increased viscosity, suggesting that the upregulation of *flaB* and the production of lateral flagella are dependent on a functional polar flagellum.

**Direct visualization of motility as a function of pressure.** The HPDS high-pressure cell was used to examine and analyze the swimming behavior of *Escherichia coli* strain W3110, *Photobacterium profundum* strains 3TCK and SS9R, and the SS9R

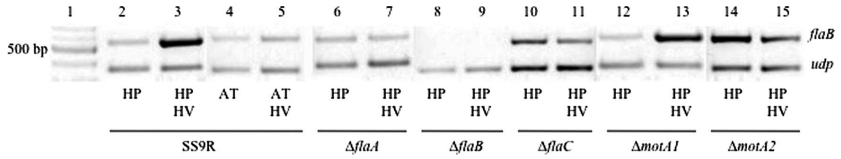


FIG. 5. Flagellin B levels increase under conditions of high pressure and increased viscosity. Shown are results of semiquantitative RT-PCR analysis of *flaB* from cells grown under conditions of high pressure (HP) or atmospheric pressure (AT), with or without increased viscosity (HV) (5% PVP-360). Lane 1, 2-log ladder (New England Biolabs); all other lanes are as marked. The upper band is an internal fragment of *flaB*; the lower band is a constitutively expressed control gene, uridine phosphorylase (*udp*; PBPRA1431).

motility mutants under high hydrostatic pressure (16, 19). The temperature used for these experiments was 20°C. This system has been used previously to examine *Spirogyra* algae and human B cells, but this was the first application to measure swimming speeds under high hydrostatic pressure. *E. coli* was used as a reference because the swimming behavior of this species as a function of pressure has been investigated previously by capillary tube assays (31). Consistent with these prior results, *E. coli* strain W3110 in the HPDS high-pressure cell displayed its highest average swimming speed at atmospheric pressure (12.8 $\mu m\ s^{-1}$), and its motility was completely abolished at 50 MPa (Fig. 6; see also Table S2 in the supplemental material). In contrast to *E. coli*, both 3TCK and SS9R displayed an extremely broad pressure range for swimming motility. 3TCK and SS9R displayed the highest average swimming speeds at their corresponding optimal growth pressures (21.7 $\mu m\ s^{-1}$ for 3TCK at 0.1 MPa; 28.2 $\mu m\ s^{-1}$ for SS9R at 30 MPa) (Fig. 6). Stepwise increases in hydrostatic pressure resulted in a striking decrease in swimming velocity for *E. coli* and a much more gradual reduction for 3TCK, which proceeded up to 120 MPa, while SS9R showed increased swimming velocity at 30 MPa and maintained motility up to a maximum pressure of 150 MPa (Fig. 6).

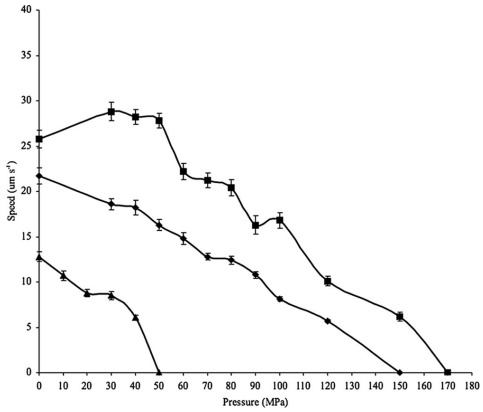


FIG. 6. Swimming velocity as a function of increasing hydrostatic pressure for the piezophile *P. profundum* SS9 (squares), the pressure-sensitive strain *P. profundum* 3TCK (diamonds), and *E. coli* W3110 (triangles). For pressures up to 100 MPa, data are mean velocity measurements from 50 individual cells; beyond 100 MPa, velocity measurements for 3TCK and SS9 were taken for 10 to 20 individual cells. Error bars, standard errors.

## DISCUSSION

In this study the PF and LF systems of a deep-sea bacterium have been explored using genetics in concert with novel phenotypic screening as a function of hydrostatic pressure. The results suggest that while motility is one of the most pressure-sensitive cellular processes in mesophilic microorganisms, piezophilic bacteria possess uniquely adapted motility systems to maintain movement under the high pressures found in the deep ocean. The combination of culture-based agar bulb assays and culture-independent short-term microscopic visualizations made it possible to examine motility under conditions requiring the assembly of new flagella (the bulb assay) or only the function of preexisting flagella (high-pressure microscopy). The former assay could have been addressed by using a variation of the Dorayaki plate method, in which colonial growth under high-pressure conditions is achieved by sandwiching cells between slabs of agar growth medium (34). However, while this method is useful for screening the growth of many strains on a solid medium under high-pressure conditions, bacterial growth tends to spread out in the layer between the two agar sections, which is especially problematic in media with a low percentage of agar. In our hands the bulb assay provided more-consistent results.

Bacterial motility in a liquid environment under high-pressure conditions has been investigated previously. Meganathan and Marquis utilized a capillary assay to examine *E. coli* swimming behavior under high-pressure conditions over periods as long as 48 h (31). Although they utilized a different method and a different *E. coli* strain, their results were very similar to those reported here using the high-pressure microscope. High-pressure microscopy provides a window into the behavior of individual cells as well as cell populations. In the future it would be useful to use the HPDS system to further examine motility as a function of pressure, temperature, viscosity, and time.

The results of motility assays for the *P. profundum* strains were particularly revealing. Not surprisingly, the swimming of SS9, like that of other marine bacteria (22, 25, 38), appears to be sodium powered, and its swarming motility, like that of *V. parahaemolyticus* (3), appears to be proton powered, based on the phenotypic characterization of the two *motA* deletion mutants and the deduced MotA primary structures. However, in

contrast to the observation that because of redundancy none of the six polar flagellin genes are required for swimming motility in *V. parahaemolyticus*, *flaA* and *flaC* in SS9 are essential for swimming. Another major surprise is that both 3TCK and SS9 are capable of short-term swimming under pressures well above the known upper pressure limit for microbial life (45). Their pressure optima for swimming matched those for growth, suggesting that swimming analyses are a good proxy for the overall pressure adaptation of the microbe under examination. The swimming speeds measured for *P. profundum* in this study are comparable to those of *Vibrio* species (20 to 65 $\mu m\ s^{-1}$) and reflect the high-velocity swimming characteristics of marine bacteria compared to the lower velocities of *E. coli* (8, 22, 25, 38).

While we were unable to visualize lateral flagella, phenotypic characterizations of *flaB* and *motA1* mutants, as well as *flaB* expression analysis, indicate a functional lateral motility system under conditions of high pressure and increased viscosity. *V. parahaemolyticus* lateral flagella have been visualized using transmission electron microscopy under careful handling conditions with phosphotungstic acid staining (as opposed to uranyl acetate staining, since uranyl acetate destroys lateral flagella) (29). Lateral flagella are extremely fragile structures, so one explanation for our inability to visualize lateral flagella might be filament loss or damage following decompression and subsequent sample processing.

The functionality of the SS9 LF system is particularly noteworthy when SS9 is compared to its deep-sea relative *P. profundum* DSJ4, which also possesses an LF gene cluster, and the recently characterized dual motility system of *Shewanella piezotolerans* WP3 (12, 42). In the case of *S. piezotolerans* WP3, the two sets of flagellar systems were found to be inversely regulated: the LF system is upregulated at low temperatures, and the PF system is upregulated under high-pressure conditions (42). Our results suggest that *P. profundum* SS9 regulates its LF genes differently from *S. piezotolerans* WP3, since the lateral system is expressed only under conditions of high pressure and increased viscosity.

The SS9 lateral flagellum is a complex organelle encoded by almost 40 genes, which were potentially acquired via lateral gene transfer (12). Although it is not known how the LF block of 35 kbp was obtained, recently, within the family *Vibrionaceae*, *Vibrio cholerae* was demonstrated to be capable of chitin-mediated transformation of as much as 42 kbp of DNA, resulting in lipopolysaccharide serogroup conversion (11). Presumably, SS9 LF development requires a functional PF, since induction occurs only under conditions of high pressure and increased viscosity, and the regulation of LF flagellin (*flaB*) by viscosity is dependent on the expression of *flaA*, *flaC*, and *motA2*. In *V. parahaemolyticus*, the functionality of the polar flagellum is coupled to the transcription of *laf* genes, where physical or genetic disruption of the polar flagellum results in induction of lateral flagella (27). More work will be needed to sort out the details of the SS9 PF signal transduction process under high-pressure and increased-viscosity conditions, including more quantitative studies of *laf* gene expression and gene product abundance.

These results provide the first phenotypic evidence for a functional LF motility apparatus in SS9, a feature that is evident only under conditions of high pressure and increased viscosity. The ecological significance of having a dual motility system in the deep sea could stem from the particular lifestyle of *P. profundum* SS9, which was isolated from a deep-sea scavenging amphipod (5, 14). While the deep sea is generally described as an oligotrophic environment with limited utilizable carbon (44), the ability to attach to and colonize particles or animals would enable deep-sea bacteria to access a more dependable source of organic matter. Indeed, surface-adapted motility systems could assume greater significance at depth. Additional deep-sea bacteria have been found to contain LF gene components (42; also our unpublished results), and environmental genomic surveys suggest a potentially greater role for a surface-attached lifestyle in deeper-water microbial communities (15). It will be intriguing in the future to compare the motility systems of other piezophilic bacteria in order to further substantiate the ecological relevance and mechanisms of motility in the deep sea.

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**Table S1.** Primer sequences used in this study for *fla* and *mot* deletions.

| Primer name | Sequence |
|---|---|
| Deletion Constructs | |
| FlaA UP F | 5'-AGTCTCGAGTGATCGGCAGTGGGCATACC-3' |
| FlaA UP R | 5'-GATGCGGCCGCTTTGCTCTCCTTTGACTTTTCAC-3' |
| FlaA DN F | 5'-GATGCGGCCGCACACAGCTACAGTAAATATTG-3' |
| FlaA DN R | 5'-Phos-GCGAGCGCCATTATCATCTTTG-3' |
| FlaB UP F | 5'-AGTCTCGAGGGCAACCGGTAAGTATGT-3' |
| FlaB UP R | 5'-GATGCGGCCGCGATAAGTCTTCCTTTAATTCGA-3' |
| FlaB DN F | 5'-GATGCGGCCGCATTGTTGTAGTCAGCCAGTGGT-3' |
| FlaB DN R | 5'-Phos-CTGCCCATAATGCCCTGAAGT-3' |
| FlaC UP F | 5'-AGTCTCGAGCCGCGATGAATCTGGCAACTATG-3' |
| FlaC UP R | 5'-GATGCGGCCGCAATTGTATCTCCTTAATTATTCT-3' |
| FlaC DN F | 5'-GATGCGGCCGCTCCAGCTAAGAACGGCAAGGGT-3' |
| FlaC DN R | 5'-Phos-CTGCGCAAACTAATAATAATGTCT-3' |
| MotA1 UP F | 5'-CCGAGATCTATATACGTTTAGACCCGCCTGAAT-3' |
| MotA1 UP R | 5'-GATGCGGCCGCGTGACAACTCCGCTAAACTGAC-3' |
| MotA1 DN F | 5'-GATGCGGCCGCTCAATCATGAGTAAAGAACCC-3' |
| MotA1 DN R | 5'-CCGAGATCTCGTTGCATCGTAAGTGTATT-3' |
| MotA2 UP F | 5'-AGTCGTGCATGCCCCGCTTGACTGGCTGACTGAG-3' |
| MotA2 UP R | 5'-GATGCGGCCGCGAAAAGCTCCCCTTTTTGTGACG-3' |
| MotA2 DN F | 5'-GATGCGGCCGCGAGGTCACAATGGAAGAAGATG-3' |
| MotA2 DN R | 5'-AGTCGTGCATGCACGTAAAATCATGCTGTCCA-3' |
| | |
| Control verification | |
| FlaA CTRL UP | 5'-CCAAAGTGACGGTAACCCCAAAAA-3' |
| FlaA CTRL DN | 5'-TGTTTTGCTCACCGGTTTTATCTG-3' |
| FlaB CTRL UP | 5'-TGCGGGCACCAAGACAGATAC-3' |
| FlaB CTRL DN | 5'-TTGCGCTGTGATAGTGGCTGAAAT-3' |
| FlaC CTRL UP | 5'-ATGAATCGCCAGCAAATAAAAGT-3' |
| FlaC CTRL DN | 5'-TCTTCCATCGATAAACAACTG-3' |
| MotA1 CTRL UP | 5'-AGCAGCTGCAGGCGAGTGTAGA-3' |
| MotA1 CTRL DN | 5'-GCACAGCAAGCAGATTCAG-3' |
| MotA2 CTRL UP | 5'-TCAAATGATCAAAAGGCGTGTTA-3' |
| MotA2 CTRL DN | 5'-TGCGTACTGTTATATCTGGGTTCT-3' |

**Table S2.** Swimming velocity as a function of increasing hydrostatic pressure for the piezophile *P. profundum* SS9, the pressure-sensitive *P. profundum* 3TCK, and *E. coli* W3110. Mean velocity measurements from 50 individual cells, ± standard error; nm: not measured.

| | **Mean velocity (μm $s^{-1}$); Maximum velocity (μm $s^{-1}$)** | | |
|---|---|---|---|
| Pressure (MPa) | *E. coli* W3110 | *P. profundum* 3TCK | *P. profundum* SS9 |
| 0.1 | 12.8 ± 0.51; 23.1 | 21.7 ± 0.91; 35.8 | 25.8 ± 0.98; 40.0 |
| 10 | 10.7 ± 0.51; 20.9 | nm | nm |
| 20 | 8.8 ± 0.42; 17.7 | nm | nm |
| 30 | 8.5 ± 0.47; 17.1 | 18.6 ± 0.59; 28.8 | 28.8 ± 1.0; 49.4 |
| 40 | 6.1 ± 0.23; 11.3 | 18.2 ± 0.79; 29.4 | 28.2 ± 0.81; 42.2 |
| 50 | 0 | 16.3 ± 0.59; 26.0 | 27.8 ± 0.85; 41.1 |
| 60 | 0 | 14.8 ± 0.62; 25.4 | 22.2 ± 0.89; 33.5 |
| 70 | 0 | 12.8 ± 0.40; 18.7 | 21.2 ± 0.82; 33.5 |
| 80 | 0 | 12.4 ± 0.45; 19.5 | 20.4 ± 0.88; 30.6 |
| 90 | 0 | 10.8 ± 0.37; 16.2 | 16.3 ± 1.0; 22.6 |
| 100 | 0 | 8.1 ± 0.24; 12.5 | 16.8 ± 0.87; 23.7 |

Chapter 6 is a full reprint of the publication: Eloe E. A., F. M. Lauro, R. F. Vogel, and D. H. Bartlett. 2008. The deep-sea bacterium *Photobacterium profundum* SS9 utilizes separate flagellar systems for swimming and swarming under high-pressure conditions. *Applied and Environmental Microbiology*. 74(20): 6298-6305, with permission from all coauthors.

# Chapter VII

# Deep perspectives: Concluding remarks

The research contained within this dissertation has broadened our current understanding of the taxonomic, functional and metabolic properties of indigenous deep ocean microorganisms. Molecular techniques were utilized to elucidate community structure and metabolic potential, novel cultivation techniques at high pressure were optimized, and a detailed genetic investigation using the model deep-sea bacterium *P. profundum* SS9 was successfully completed. Through the use of small-subunit ribosomal gene sequencing described in this work, a greatly expanded catalogue of bacteria, archaea, and eukarya diversity from deep ocean environments exists. The results, additionally, provide insight into a size-fractionated phylogenetic composition not previously appreciated that will help inform future sampling practices and diversity estimates from deep ocean habitats.

Data presented in this dissertation highlights the putative functional and metabolic properties identified from deep ocean microbial assemblages by providing the first analysis of the unique attributes from a hadopelagic community. The distinctive collection of transporters associated with heavy metal resistance was a particularly intriguing finding. The concentrations and distributions of trace metals from trench environments are unknown, further highlighting the importance of coupling physical, chemical, and biological measurements to support molecular sequence data. Interestingly, *cusCBA* cassettes are present in all the piezophilic Gammaproteobacterial genomes (*P. profundum* SS9, *Shewanella* sp. KT99, *Moritella* sp. PE36, *Psychromonas* sp. CNPT3, *S. piezotolerans* WP3, and *Colwellia* sp. MT41), with multiple copies in the two *Shewanella* genomes. Thus, future lines of investigation to characterize these systems, particularly in the genetically-tractable SS9 or WP3, are needed to shed light on the curious

representation of heavy metal resistance systems in deep-ocean microbial genomes and their functionality at high hydrostatic pressure.

While the use of single-cell genomic techniques holds tremendous potential for accessing a wide diversity of microbes from the deep-sea, research is needed to optimize the recovery of more complete genomes from individual cells. The challenges encountered in the research described here include low recovery of positive MDA reactions for the initial screening coupled with bioinformatic hardships in assembly and contaminant sequence removal. Refined single-cell technology using microfluidic cells together with digitized MDA reactions will substantially aid in contaminant identification and optimization of genome recovery (1). Thus, with continuing advances in single-cell technologies, this line of research will provide valuable data from uncultivated deep-sea bacteria and archaea. As demonstrated in this dissertation, even partial genome data from single-celled members of the dominant PRT phylogenetic groups provided a genomic context for some of the trends observed in the PRT metagenome by highly recruiting the metagenome gene sequences.

The cultivation of phylogenetically novel microbes from deep ocean environments will undoubtedly be an important avenue for furthering our understanding of the physiology and biochemistry of piezophilic microbes. The utility of extinction to dilution cultivation techniques has been demonstrated as a unique and distinctive strategy to isolate new psychropiezophiles, such as PRT1, the first piezophilic Alphaproteobacterial representative. The growth characterization of PRT1 provided the first evidence for a piezophilic phenotype under oligotrophic conditions, suggested previously by Yayanos and colleagues (6). However, the difficulties involving future

cultivation attempts are not to be taken lightly. The slow growth rates and low cell yields rendered characterization of PRT1 challenging, where further characterization of membrane lipid composition and cytochrome components were not feasible due to the relatively large biomass necessary for these analyses. Therefore, the detailed genetic tools and subsequence phenotype characterization available for the model deep-sea bacterium *P. profundum* SS9 provide attractive methods to test hypotheses and will continue to provide invaluable data regarding the influences of pressure (2, 5). The genetic characterization of the motility system in SS9 described in this dissertation highlights a cellular process that has acquired dramatic adaptations for optimal functionality at high pressure.

The future study of microbial assemblages at depth will be heavily dependent on advances in both sampling strategies and methodologies in order to access the remote depths of the oceans and maintain the prevailing *in situ* pressure and temperature conditions in downstream experimentation. Untethered vehicles, like the DOV 'Bobby Ray' used to collect samples from the Puerto Rico Trench, will be invaluable technologies to obtain both pelagic and benthic samples. Establishment of dedicated microbial observatories to monitor variations in chemical, physical, and biological features over long temporal scales will enable researchers to establish a robust understanding of the processes and interactions of deep ocean ecosystems.

Much remains unanswered concerning microbial distribution and function within the dark pelagic realm. Are the high taxonomic diversity and expanded genomic repertoires we observed indicative of the autochthonous, metabolically active members of the community? Analyses of 16S rRNA libraries constructed from both RNA and DNA

in an effort to discriminate the active members of the community have indicated that there is a great disparity between the active and inactive fractions at depth (4). Who are the key players in the microbial loop in the dark ocean and how do they structure the community over the appropriate spatial and temporal scales? Highly localized and patchy diversity patterns in bacterioplankton composition have been observed (3), yet what are the predictors (eg. water mass characteristics) that inform these patterns? Will more concerted cultivation efforts under low temperature and high-pressure conditions result in piezophilic representative from phyla outside of those groups previously characterized? Considering the progress this field has experienced over the last few decades, researchers are well poised to address these challenges in a highly interdisciplinary and informed fashion.

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