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Comparative Genomic Evidence for a Close Relationship between the Dimorphic Prosthecate Bacteria *Hyphomonas neptunium* and *Caulobacter crescentus*

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The dimorphic prosthecate bacteria (DPB) are α-proteobacteria that reproduce in an asymmetric manner rather than by binary fission and are of interest as simple models of development. Prior to this work, the only member of this group for which genome sequence was available was the model freshwater organism *Caulobacter crescentus*. Here we describe the genome sequence of *Hyphomonas neptunium*, a marine member of the DPB that differs from *C. crescentus* in that *H. neptunium* uses its stalk as a reproductive structure. Genome analysis indicates that this organism shares more genes with *C. crescentus* than it does with *Silicibacter pomeroyi* (a closer relative according to 16S rRNA phylogeny), that it relies upon a heterotrophic strategy utilizing a wide range of substrates, that its cell cycle is likely to be regulated in a similar manner to that of *C. crescentus*, and that the outer membrane complements of *H. neptunium* and *C. crescentus* are remarkably similar. *H. neptunium* swarmer cells are highly motile via a single polar flagellum. With the exception of *cheY* and *cheR*, genes required for chemotaxis were absent in the *H. neptunium* genome. Consistent with this observation, *H. neptunium* swarmer cells did not respond to any chemotactic stimuli that were tested, which suggests that *H. neptunium* motility is a random dispersal mechanism for swarmer cells rather than a stimulus-controlled navigation system for locating specific environments. In addition to providing insights into bacterial development, the *H. neptunium* genome will provide an important resource for the study of other interesting biological processes including chromosome segregation, polar growth, and cell aging.

Unlike most bacteria, which reproduce by symmetric binary fission, dimorphic prosthecate bacteria (DPB) reproduce by asymmetric binary fission (e.g., *Caulobacter crescentus*) or budding (e.g., *Hyphomonas* and *Hyphomicrobium* species) to produce a motile swarmer cell from a nonmotile mother cell (68). The mother cell is distinguished by a presence of an appendage termed a prostheca or stalk (84), as well as generally having a holdfast that allows the cell to adhere to a surface (36). The swarmer cells, which are unable to reproduce, undergo a developmental process that results in their conversion to mother cells.

The life cycle of DPB is analogous in many ways to that of dimorphic invertebrates, and this analogy is further supported by studies that have shown that the motile offspring of DPB, as in the multicellular case, are in a "juvenile" condition in which most energy is expended on motility and little on growth (26, 67). These facts suggest that DPB are good model systems for understanding the evolution and biology of dimorphic life in general.

DPB are ubiquitous in both freshwater and marine environments but are found also to a lesser degree in soil (68). They are of considerable environmental interest since many *Hyphomicrobium* species can mineralize pollutants such as aromatic hydrocarbons (54), methyl chloride (34), and various alcohols, including methanol (40). In addition, various *Hyphomonas* species are primary colonizers of marine surfaces (6) and form biofilms necessary for the recruitment of invertebrate larvae such as those of oysters (15). DPB are members of the α-proteobacteria but currently are not considered a coherent taxonomic unit (68), and *Hyphomonas* and *Caulobacter* are even classified as members of different orders.

One member of the DPB, *C. crescentus* CB15, has been the subject of a genome sequencing study (62). The genus *Hyphomonas* was selected for sequencing as a second member of the DPB, not only for comparative purposes but also because, unlike *C. crescentus*, the stalk in *Hyphomonas* is a reproductive

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structure. The developing bud receives proteins, DNA, and other cellular components from the mother cell through the stalk (36). Electron micrographs suggest that these components are transported through the stalk within membranous swellings termed pseudovesicles (98). Another significant distinction in reproduction between *C. crescentus* and budding, prosthecate bacteria is that the reproductive ability of the latter is quite limited, with typically only about eight offspring formed during the life of the mother cell (55), while *C. crescentus* mother cells typically create 100 or more offspring over their lifetimes (1). This suggests that *Hyphomonas* is a good model system for the study of senescence.

The species of *Hyphomonas*, *Hyphomonas neptunium*, was selected for a variety of reasons. It has a faster swarmer cycle (time for new swarmer cells to mature into reproductive cells and initiate bud formation) than other *Hyphomonas* species (55), and its prostheca are easily distinguishable by light microscopy (12). In addition, the use of the stalk as a conduit for transfer of macromolecules from the mother cell to the developing bud in *Hyphomonas* in general (98) was originally discovered in *H. neptunium* (92). This is the first report of a whole genome sequence of a member of the family *Hyphomonadaceae* (47), a group of bacteria that is believed to include primary colonizers of surfaces in the ocean (6).

MATERIALS AND METHODS

Genome sequencing and assembly. The genome of *H. neptunium* ATCC 15444^T (the type strain of the species) was sequenced by means of the whole genome shotgun method as previously described (25). A total of 41,941 usable sequence reads were generated, of which 41,461 were incorporated into the initial assembly, yielding an average of 8.1-fold coverage across the genome. Further details of sequencing and closure are available in document 1 posted at http://www.hyphomonas.com/hnep_supp.html.

Sequence annotation. The prediction of putative-protein coding genes and functional annotation were performed as previously described (88). The program GLIMMER (20) was used to identify the initial set of putative coding regions; from this initial list candidates consisting of fewer than 30 codons and those containing overlaps were eliminated. Frameshifts and point mutations were detected and corrected where appropriate. Additional phylogenetic analyses were performed using APIS (for automated phylogenetic inference system) (unpublished). APIS automates the process of sequence similarity, alignment, and phylogenetic inference for each protein in a genome (see below).

APIS. APIS is a system for automatic creation and summarizing of phylogenetic trees for each protein encoded by a genome. It is implemented as a series of Ruby scripts, and the results are viewable on an internal web server which allows the user to explore the data and results in an interactive manner. The homologs used by APIS for each phylogenetic tree are obtained by comparing each query protein against a curated database of proteins from complete genomes using WU-BLAST (30). The full-length sequences of these homologs are then retrieved from the database and aligned using MUSCLE (24), and bootstrapped neighbor-joining trees are produced using QuickTree (37). As QuickTree (unlike most programs) produces bootstrapped trees with meaningful branch lengths, the inferred tree is then midpoint rooted prior to analysis, allowing automatic determination of the taxonomic classification of the organisms with proteins in the same clade as the query protein. APIS was created to address some of the weaknesses of existing automated phylogenetic systems such as PyPhy (79), in which a general-purpose protein database such as Swiss Prot (78) is used, weakening the interpretation of clades because the absence of proteins from organisms which have not had their genomes completely sequenced cannot be taken as biological evidence of the nonexistence of such proteins.

Assertions of orthology. Putative orthologs between genomes were established by the following method. First, two BLASTP (30) analyses were run; then all proteins encoded by the first genome were compared against a database of proteins encoded by the second, and vice versa. The threshold used in these comparisons was 10^{-9}. Orthology was asserted if two proteins were each other's best BLASTP hit (best reciprocal match).

TABLE 1. General features of the genome of *H. neptunium* ATCC 15444^T

Parameter[a]	Value
Size (bp)	3,705,021
% G+C content	62
Predicted no. of protein CDS	3,521
Avg CDS size (bp)	953
No. of unconserved hypothetical proteins	400 (11%)
No. of conserved hypothetical proteins	506 (14%)
% Coding of genome	91
No. of rRNA operons	1
No. of tRNAs	43

[a] CDS, coding sequences.

Identification of outer membrane proteins. A set of curated outer membrane proteins with experimental evidence were retrieved from the membrane transport protein classification database (http://www.tcdb.org) (77). The representative hidden Markov models (HMMs) for each individual family were retrieved from the Pfam protein families database (7).

The complete predicted protein sequences of *H. neptunium* were first searched against this outer membrane protein database (75) for similarity to known outer membrane proteins using BLASTP and for matches to the HMMs using the program hmmsearch. All of the query proteins with significant hits (cutoff of 10^{-3}) were collected and searched against the NCBI nonredundant protein database. A web-based interface that incorporates the number of hits to the outer membrane protein database, BLASTP and HMM search E-value and score, and the description of top hits to the nonredundant protein database was implemented to facilitate the annotation processes. Up-to-date results can be viewed at the TransportDB website (http://www.membranetransport.org/) (75, 76), and the initial analysis used in the annotation of the genome is available in Table 1 posted at http://www.hyphomonas.com/hnep_supp.html.

Chemotaxis assays. Chemotaxis assays were performed using motile cells from an overnight culture of *H. neptunium* in a modified TY (2.5 g of Bacto-tryptone plus, 1.5 g of yeast extract per liter [pH 6.9], supplemented with 8 mM calcium chloride) medium washed three times in chemotaxis buffer (10 mM phosphate buffer, pH 7.0, 8 mM calcium chloride). About 50% of the cells were motile under these conditions. The swarm plate assay was performed on cells inoculated in the center of modified TY medium, LB medium, or marine broth (Gibco) solidified with 0.3% agar, an agar concentration that was found to be optimal for bacterial growth and motility under the conditions of the experiments. The chemical-in-plug assays and the temporal gradient assays were performed essentially as described previously (2, 3, 89). The following chemicals were tested in a chemical-in-plug assay and a temporal gradient assay at concentrations of 0.1 mM, 1 mM, and 10 mM: glutamate, aspartate, oxaloacetate, succinate, pyruvate, and malate. The substituted quinone, 1,4-benzoquinone, was tested as a potential repellent at concentrations of 0.1 mM, 1 mM, and 10 mM. Oxygen was tested in a temporal gradient assay as described previously (99).

Nucleotide sequence accession number. The annotated genome sequence of *H. neptunium* ATCC 15444^T has been deposited in the GenBank database as accession number CP000158.

RESULTS AND DISCUSSION

General characteristics. The genome of *H. neptunium* ATCC 15444^T is composed of a single circular chromosome. General features of the genome can be found in Table 1.

Repetitive and mobile DNA. The *H. neptunium* genome was analyzed for the existence of repetitive elements. The results are summarized in Table 2. Besides repeats due to the highly conserved duplicated genes EF-Tu and DUF227, the *H. neptunium* genome contains transposons related to two families found in *C. crescentus*, ISCc2 and ISCc3. In addition, the genome contains six copies of a 370-bp intergenic repeat that may have some regulatory function. It is perhaps significant that four of these copies flank the presumed origin of replication at an approximate distance of ±100 and ±200 kbp.

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TABLE 2. Repetitive elements in the *H. neptunium* genome[a]

Class	Avg size (bp)	No. of copies	Function[b]
1	1,265	4	ISCc3 transposase
2	1,019	8	ISCc2 transposase
3	608	2	EF-Tu C terminus
4	542	2	EF-Tu N terminus
5	370	6	Conserved intergenic region
6	647	2	DUF227 C terminus
7	320	2	DUF227 N terminus

[a] Repeats listed are those over 200 bp in length as found by REPuter (42).
[b] DUF227 is a family of proteins containing a conserved domain of unknown function.

Metabolism. Cultured *Hyphomonas* species preferentially use amino acids as their carbon and energy sources (56). Consistent with this, *H. neptunium* ATCC 15444^T did not use glucose in semisynthetic growth medium (93, 91) but was capable of using pyruvate, α-ketoglutarate, fumarate, or malate as carbon sources (22).

Despite these experimental results, it appears that the *H. neptunium* genome possesses all of the genes needed for both the glycolytic conversion of glucose to pyruvate and pentose phosphate biosynthetic pathways. This suggests that, while *H. neptunium* may not have used glucose under the conditions tested, it does have the ability to utilize glucose under some conditions. Since *H. neptunium* possesses genes encoding enzymes for a complete tricarboxylic acid cycle along with enzymes for the glyoxylate shunt, the ability of *H. neptunium* to utilize organic acids (22) is supported by genome analysis. Additionally, the genome analysis suggests that glycerol can serve as a carbon source for *H. neptunium* by feeding the end product of glycerol dehydrogenase into the glycolytic pathway. As expected, genes for the complete degradative pathways of the 20 standard amino acids are also present.

Aromatic and halogenated compounds also appear to be metabolized by *H. neptunium* since the genome contains genes that are predicted to encode enzymes involved in the degradation of various such substrates. While identifying specific substrates will require experimental evidence, it is clear that proteins encoded by certain genes (e.g., HNE_0817, HNE_0958, HNE_0987, HNE_1322, HNE_1435, HNE_1602, HNE_1663, HNE_2413, HNE_2751, and HNE_3259) are involved. It is understandable that the ability to utilize aromatic and halogenated compounds would confer adaptive advantage upon *H. neptunium* as it was isolated from Barcelona harbor (51), which is contaminated with such compounds (60).

The results from the Genome Properties system of The Institute for Genomic Research (33) are posted at http://www.hyphomonas.com/hnep_supp.html as Table 2. The Genome Properties system consists of a series of properties (including evidence for many metabolic pathways) that can be expressed in numerical or controlled vocabulary terms, thus easing the comparison of properties across different genomes.

Comparison with *C. crescentus* and *Silicibacter pomeroyi*. The predicted proteins of the *H. neptunium* genome were compared against those of *C. crescentus* as well as those of the only other completely sequenced member of the *Rhodobacterales*, *S. pomeroyi* (57), which is not a DPB. A Venn diagram summarizing the comparison is presented as Fig. 1. As shown, while each of the three genomes encodes numerous proteins without apparent orthologs in the other two, a core of 1,241 proteins is shared by all. As expected, this core consists primarily of proteins involved in essential processes such as transcription, translation, and basic metabolism. *H. neptunium* and *C. crescentus* share 594 proteins to the exclusion of *S. pomeroyi*, and many of these proteins may be related to the lifestyle of DPB (discussed in detail in subsequent sections). *S. pomeroyi* and *H. neptunium* share only 307 proteins to the exclusion of *C. crescentus*, among which are many flagellar proteins, transporters, and permeases.

There is a striking similarity between the outer membrane protein complements of *C. crescentus* and *H. neptunium* that is not shared by *S. pomeroyi*, namely the proliferation of predicted TonB-dependent receptors, which catalyze energy-dependent transport across the outer membrane. The *C. crescentus* genome was originally predicted to encode 65 TonB-dependent receptors, the highest number yet found in sequenced bacterial genomes. Subsequent studies have revealed equally high, or greater, numbers (25 to 115) in the genomes of *Bacteroides* species, pseudomonads, and plant-pathogenic xanthomonads. The genome of *H. neptunium* is predicted to encode 43 TonB-dependent receptors, in marked contrast to that of *S. pomeroyi*, which lacks them completely. Similarly, the genomes of both *H. neptunium* and *C. crescentus* encode predicted vitamin B12 receptors that feed directly into an ABC-type iron compound importer, while *S. pomeroyi* appears to lack such a construct. Proliferation of TonB-dependent receptors suggests that both *C. crescentus* and *H. neptunium* take up macromolecules (e.g., iron-siderophore complexes and vitamin B12) that are too large to be obtained via passive diffusion through the outer membrane porins. Nine of the 43 *H. neptunium* receptors were predicted to interact with siderophores on the basis of HMM analysis, while three appeared to bind vitamin B12; the substrate of the remainder could not be predicted with a reasonable degree of confidence. One *C. crescentus*

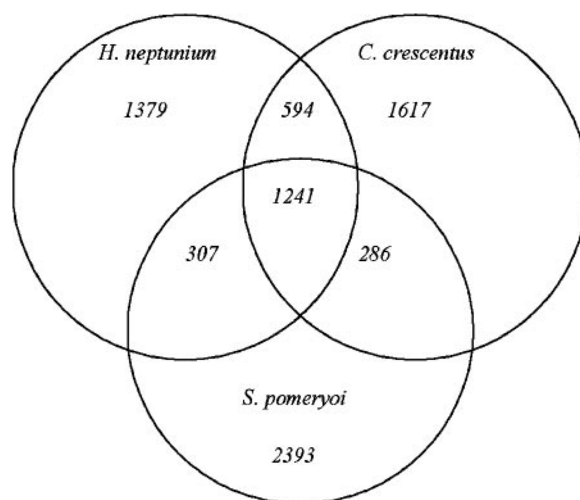

FIG. 1. Venn diagram showing the shared gene content between *H. neptunium*, *C. crescentus*, and *S. pomeroyi*. Orthology was assumed using the best reciprocal BLASTP matches, with a *P* value cutoff of 10^{-9}.

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TonB-dependent receptor has recently been shown to be involved in the uptake of maltodextrins (61), suggesting that these receptors in *H. neptunium* may similarly, actively take up nutrients in a nutrient-poor environment.

Approximately the same fraction of TonB-dependent receptors was predicted to bind siderophores in both *H. neptunium* and *C. crescentus*. Neither organism appears to encode proteins for siderophore production, suggesting that both organisms may exploit exogenous siderophores, as has been suggested for *Nitrosomas europaea*. Like *N. europaea*, both *H. neptunium* and *C. crescentus* (but not *S. pomeroyi*) also seem to possess a tandem pair of *fecIR*, genes involved in ferric citrate transport mediated by transmembrane signaling. Thus, *H. neptunium* and *C. crescentus* (and *N. europaea*) appear to employ similar iron-scavenging strategies in marine and freshwater environments, respectively. The lack of such strategies in *S. pomeroyi* may indicate that this organism is less likely to encounter iron limitation in its native environment. Coastal salt marshes, such as the marsh from which *S. pomeroyi* was isolated (32), have been shown to have significantly higher iron concentrations in vegetated soils than nearby unvegetated areas (23). It is possible that *S. pomeroyi* encounters available iron from such an iron-rich source.

To explore further the genes shared between *C. crescentus*, *H. neptunium*, and *S. pomeroyi*, a phylogenetic profiling (66) analysis was performed. Phylogenetic profiling is a method in which the presence or absence of presumed orthologs to a protein is examined across many complete genomes. Proteins that are only present in the genomes of organisms sharing a particular characteristic are good candidates for being involved in that feature. Consistent with the orthology study above, *H. neptunium* shared the highest number of proteins, 62, with *C. crescentus* to the exclusion of other organisms. Also consistent with the orthology study was that the second-highest number, 10, was between *H. neptunium* and *S. pomeroyi*. As expected, functions have not been ascribed to most of the 62 proteins uniquely shared between *H. neptunium* and *C. crescentus* (Table 3). This list of proteins, however, includes numerous transcriptional regulators that may be involved in the cell cycle or stalk biogenesis and several putative lipoproteins that may be involved in stalk formation or oligotrophy. Of the 10 proteins shared with *S. pomeroyi* (Table 4), four are flagellar proteins. Functional homologs of these flagellar proteins occur in many other bacteria, but the sequences of the *S. pomeroyi* and *H. neptunium* proteins are sufficiently divergent that they scored below the cutoff for orthology with flagellar proteins from other bacteria, suggesting that the *S. pomeroyi* and *H. neptunium* flagellar genes were derived from a common ancestor.

Selenoproteins represent a unique feature of *H. neptunium* in comparison to *C. crescentus* and *S. pomeroyi*. *H. neptunium* encodes an L-seryl-tRNA selenium transferase (HNE_2489), a selenocysteine-specific translation elongation factor (HNE_2488), and an apparent selenoprotein of unknown function (HNE_2485).

Surface colonization. Swarmer cells of both *Caulobacter* and *Hyphomonas* are dedicated to finding a suitable surface for colonization prior to their morphogenesis into reproductive cells. Having oligotrophic capability, these prosthecates may serve important roles as primary colonizers by initiating biofilm development (15). *Caulobacter* and some species of *Hyphomonas* synthesize a combination of retractable pili and polysaccharide holdfasts (normally polar capsules), which function in surface attachment and colonization (13), while other species of *Hyphomonas* use two polysaccharide structures for these functions (45). For example *Hyphomonas adhaerens* synthesizes both an adhesive holdfast (71) and pili (70), whereas *Hyphomonas rosenbergii* synthesizes a polysaccharide holdfast and a capsule that surrounds the cell (44). Interestingly, the holdfasts of 15/16 marine species and 6/10 freshwater species of *Caulobacter* contain GlucNac (*N*-acetylglucosamine) (53), as do all *Hyphomonas* species that have been examined to date (71). The integrity of the GlcNac is critical for the elastic properties of the holdfast (50). Thus, the presence of GlucNac is suggested to be characteristic of adhesive polysaccharides (44). It is not clear yet whether this is an example of converging or diverging evolution.

Genomic analysis suggests that *H. neptunium* synthesizes pili and polysaccharides. There are at least 13 open reading frames (ORFs) coding for pili biosynthesis (aside from those annotated as being involved in type IV fimbrae syntheses) and a number of ORFs potentially involved in surface polysaccharide biosynthesis. Interestingly, one of these ORFs, (HNE_3005), codes for a putative O-linked GlcNac transferase that may be involved in biosynthesis of the GlucNac residues in the holdfast. However, despite the fact that *H. neptunium* mother cells have holdfasts, many of the known proteins for holdfast synthesis and attachment in *C. crescentus* appear not to have orthologs encoded by the *H. neptunium* genome. For example, none of the holdfast attachment proteins encoded by the *C. crescentus hfaABD* gene cluster (17, 41) have apparent orthologs encoded in the *H. neptunium* genome, and of the proteins encoded by the *hfsDAB* gene cluster for holdfast synthesis in *C. crescentus* (82), only *HfsB* seems to have an ortholog in *H. neptunium* (HNE_2240). Therefore, it appears that holdfast synthesis and attachment in *H. neptunium* must be performed differently than in *C. crescentus*.

Motility. The *H. neptunium* swarmer cell has a single, polar flagellum, which it sheds prior to differentiation into a mature mother cell (90, 92). The developing bud can elaborate a flagellum while still attached to the mother cell as cells undergoing budding are often motile, suggesting that expression of the *H. neptunium* flagellar genes is temporally and spatially regulated as it is in *C. crescentus*. Most of the genes encoding structural proteins of the flagellum and components required for flagellar assembly and function are found in a region consisting of 35 contiguous ORFs (HNE_0241 to HNE_0275) that are arranged in at least 8 operons (see Table 3 and Fig. 1 posted at http://www.hyphomonas.com/hnep_supp.html). This island of motility is similar to that in the *S. pomeroyi* DSS-3^{T} genome but is in contrast to the organization of flagellar genes in *C. crescentus*, where most of these genes are distributed in 13 major clusters that are scattered throughout the genome to r-scan statistics (21, 39) (see Fig. 2 posted at http://www.hyphomonas.com/hnep_supp.html). Interestingly, for predicted products of most of the flagellar genes within the *H. neptunium* island of motility, the closest BLAST matches are with *S. pomeroyi* DSS-3^{T} homologs (24 of 31) (see Fig. and Table 3 posted at http://www.hyphomonas.com/hnep_supp.html), suggesting a common ancestry for these flagellar genes. Genes outside the major *H. neptunium* flagellar gene cluster

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TABLE 3. The 62 predicted proteins in *H. neptunium* sharing a putative ortholog only with *C. crescentus*, according to phylogenetic profiling, together with their putative orthologs

H. neptunium protein		*C. crescentus* protein	
ORF	Description	ORF	Description
HNE_0127	Conserved hypothetical protein	CC0136	Hypothetical protein
HNE_0203	Conserved hypothetical protein	CC3602	Hypothetical protein
HNE_0204	OstA family protein	CC3601	Hypothetical protein
HNE_0317	Putative chorismate mutase, type II	CC1116	Chorismate mutase, putative
HNE_0320	Hypothetical protein	CC1485	Hypothetical protein
HNE_0394	Conserved hypothetical protein	CC0224	Hypothetical protein
HNE_0430	Ribosomal protein L35	CC1046	Ribosomal protein L35
HNE_0439	Phosphocarrier protein HPr	CC0241	phosphocarrier protein HPr
HNE_0477	Conserved hypothetical protein	CC2977	Transcriptional regulator, TetR family
HNE_0479	Putative membrane protein	CC1840	RNase BN, putative
HNE_0570	Transcriptional regulator, TetR family	CC2493	Transcriptional regulator, TetR family
HNE_0573	Thioesterase family protein	CC3309	Hypothetical protein
HNE_0634	Conserved hypothetical protein	CC3551	Hypothetical protein
HNE_0654	Conserved hypothetical protein	CC0356	Hypothetical protein
HNE_0679	Putative lipoprotein	CC1285	Hypothetical protein
HNE_0697	Conserved domain protein	CC1987	Hypothetical protein
HNE_0728	Conserved hypothetical protein	CC0948	Hypothetical protein
HNE_0830	Conserved hypothetical protein	CC0922	Medium-chain fatty acid, CoA ligase
HNE_0851	Putative lipoprotein	CC0699	Hypothetical protein
HNE_0983	Putative membrane protein	CC0636	Hypothetical protein
HNE_1015	Conserved hypothetical protein	CC2228	Hypothetical protein
HNE_1037	Tetratricopeptide repeat protein	CC2031	TPR domain protein
HNE_1131	Conserved domain protein	CC1589	Hypothetical protein
HNE_1193	Conserved hypothetical protein	CC0163	Hypothetical protein
HNE_1388	Conserved hypothetical protein	CC0495	Transcriptional regulator, TetR family, putative
HNE_1417	Putative lipoprotein	CC2104	Hypothetical protein
HNE_1569	Transcriptional regulator, TetR family	CC2493	Transcriptional regulator, TetR family
HNE_1578	Conserved hypothetical protein	CC1167	Hypothetical protein
HNE_1665	Conserved hypothetical protein	CC1177	Hypothetical protein
HNE_1732	Hypothetical protein	CC2341	Hypothetical protein
HNE_1746	Conserved hypothetical protein	CC1951	Hypothetical protein
HNE_1840	Exodeoxyribonuclease VII, small subunit	CC2070	Exodeoxyribonuclease small subunit
HNE_1984	Conserved hypothetical protein	CC1842	Response regulator, putative
HNE_2049	Sporulation related repeat protein	CC2007	Hypothetical protein
HNE_2081	Phosphoglycerate mutase family protein	CC1966	Hypothetical protein
HNE_2096	Conserved hypothetical protein	CC1308	Hypothetical protein
HNE_2106	Toluene tolerance protein, Ttg2 family	CC3693	Hypothetical protein
HNE_2138	Toluene tolerance protein, Ttg2 family	CC3693	Hypothetical protein
HNE_2240	Conserved hypothetical protein	CC2430	Hypothetical protein
HNE_2241	Putative chain length determinant protein	CC2431	Hypothetical protein
HNE_2337	Conserved domain protein	CC3555	Hypothetical protein
HNE_2339	Sensor histidine kinase	CC3474	Sensor histidine kinase, putative
HNE_2440	Transcriptional regulator, TetR family	CC1345	Transcriptional regulator, TetR family
HNE_2607	Transcriptional regulator, TetR family	CC1855	Transcriptional regulator, TetR family
HNE_2647	Antibiotic biosynthesis monooxygenase domain protein	CC2132	Hypothetical protein
HNE_2661	Peptidase, S54 (rhomboid) family	CC2627	Rhomboid family protein
HNE_2737	Conserved hypothetical protein	CC1453	Hypothetical protein
HNE_2875	Conserved hypothetical protein	CC2822	Hypothetical protein
HNE_2914	Conserved hypothetical protein	CC2480	Hypothetical protein
HNE_2952	RusA family protein	CC2107	Hypothetical protein
HNE_2965	Transcriptional regulator, TetR family	CC1664	Transcriptional regulator, TetR family
HNE_2977	Transcriptional regulator, TetR family	CC0772	Transcriptional regulator, TetR family
HNE_3033	Putative MraZ protein	CC2563	Hypothetical protein
HNE_3111	Putative lipoprotein	CC0125	Hypothetical protein
HNE_3125	Conserved hypothetical protein	CC3104	Hypothetical protein
HNE_3206	Conserved hypothetical protein	CC3441	Arylesterase-related protein
HNE_3320	Conserved hypothetical protein	CC0155	Hypothetical protein
HNE_3401	Conserved hypothetical protein	CC3115	Hypothetical protein
HNE_3416	S4 domain protein	CC0656	S4 domain protein
HNE_3520	Transcriptional regulator, MarR family	CC3677	Transcriptional regulator, MarR family
HNE_3553	Thiamine monophosphate synthase family protein	CC3746	Hypothetical protein
HNE_3556	Putative lipoprotein	CC3748	Hypothetical protein

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TABLE 4. The 10 predicted proteins in *H. neptunium* sharing a putative ortholog only with *S. pomeroyi*, according to phylogenetic profiling, together with their putative orthologs

H. neptunium protein		*S. pomeroyi* protein	
ORF	Description	ORF	Description
HNE_1542	Conserved hypothetical protein	SPO0172	Hypothetical protein
HNE_1330	Conserved hypothetical protein	SPO3675	Hypothetical protein
HNE_0593	Radical SAM domain protein	SPO2049	Radical SAM domain protein
HNE_0272	Flagellar biosynthetic protein fliQ	SPO0179	Flagellar biosynthetic protein FliQ
HNE_0269	Putative flagellar protein	SPO0182	Flagellar basal-body rod protein; FlgB
HNE_0266	Putative flagellum biosynthesis repressor protein FlbT	SPO2036	Sensory box sensor histidine kinase/ response regulator
HNE_0259	Hypothetical protein	SPO0174	Hypothetical protein
HNE_0248	Putative flagellar hook-associated protein FlgL	SPO0194	Flagellar hook-associated protein FlgL family protein
HNE_3425	Glyoxalase family protein	SPO0313	Glyoxalase family protein
HNE_3078	Conserved hypothetical protein	SPO3859	Hypothetical protein

that have predicted roles in motility include a *cheY* homolog (HNE_0639), a *cheR* homolog (HNE_0640), and two potential flagellar regulatory genes, *flhF* (HNE_0942) and *motR* (HNE_0943; also referred to as *flhG* or *fleN*). The *flhF* and *motR* genes are not found in enteric bacteria, but they do occur in a number of flagellated bacteria, including *Pseudomonas* species, where they have been implicated in the polar placement of flagella or the regulation of flagellar number (18, 65).

Where it has been examined in other bacteria, a transcriptional hierarchy controls the expression of flagellar genes (52, 97). Initiation of the flagellar gene hierarchy in *C. crescentus* requires the response regulator CtrA (72, 74). *H. neptunium ctrA* (HNE_0944) appears to be in an operon with *flhF* and *motR* (see Fig. 1 posted at http://www.hyphomonas.com/hnep_supp.html), suggesting a role for *ctrA* in flagellar gene regulation. The activity of CtrA in *C. crescentus* is controlled in response to cell cycle and developmental cues by a multicomponent signal transduction network consisting of the sensor kinases CckA, PleC, DivJ, and DivL and the response regulator DivK (5). *H. neptunium* possesses homologs of *cckA* (HNE_0507), *divJ* (HNE_0746), and *divL* (HNE_0399), indicating that the earliest events in the regulation of flagellar biogenesis in *H. neptunium* may be similar to those in *C. crescentus*. Transcription of many of the flagellar genes in *C. crescentus* requires σ^{54}-RNA polymerase holoenzyme in conjunction with the σ^{54}-dependent activator FlbD and FliX, a protein that functions to both inhibit and stimulate FlbD activity (8, 14, 58, 59, 73, 95, 96). *H. neptunium* possesses an *rpoN* homolog (encodes σ^{54}; HNE_0206) but not homologs of *flbD* or *fliX*. Inspection of sequences upstream of the flagellar genes failed to identify any good matches for potential σ^{54}-dependent promoters [the consensus sequence is TGGCAC-N_4-TTTGC(A/T)]. Taken together, these observations suggest that σ^{54} is not involved in *H. neptunium* flagellar biogenesis. Proteobacteria often employ σ^{28} for the expression of specific flagellar genes (43, 49, 69, 85), but *H. neptunium* lacks this alternative σ factor. Thus, the transcriptional control of *H. neptunium* flagellar genes needed in later stages of flagellar biogenesis appears to involve a mechanism that has not been described in other bacteria.

The ability of flagellated motile bacteria to seek optimum positions in gradients by chemotaxis is normally dependent on the presence of methyl-accepting chemotaxis proteins (MCPs) and a conserved set of chemotaxis genes. Bacterial chemotaxis occurs by modulation of the probability of locomotor-directional changes, brought about by reversals of the direction of flagellar rotation or modulation of flagellar rotary speed (9). CheY, which is a response regulator of a conventional two-component system, controls the probability of directional changes, and the activity of CheY is modulated by the MCPs and conserved cytoplasmic chemosensory proteins (4). An unexpected finding from the *H. neptunium* genome was the absence of homologs of MCPs and most of the other chemotactic proteins, including CheA, CheW, and CheB. The absence of conserved chemosensory proteins together with the presence of genes required for the biogenesis of functional flagella was also reported for *Aquifex aeolicus* (19), *S. pomeroyi* (57), and *Pirellula* sp. strain 1 (31), now reclassified as *Rhodopirellula baltica*, a member of the *Planctomycetes* that, like *H. neptunium* and *C. crescentus*, has a dimorphic cell cycle. In contrast to *H. neptunium*, *C. crescentus* possesses ORFs for 18 MCPs and two complete sets of chemotaxis signal transduction proteins (62). *H. neptunium* possesses CheY and CheR homologs, which appear to be encoded within the same operon. CheR catalyzes the methylation of specific glutamate residues within the cytoplasmic domains of MCPs (83). Since MCPs are the only known protein substrates for CheR, it is not clear which proteins, if any, *H. neptunium* CheR methylates or if CheR has a role in motility in *H. neptunium*.

We investigated motility and chemotaxis in *H. neptunium* using a set of standard techniques and found that the bacteria are capable of swimming motility with directional changes, resulting in a three-dimensional random walk. The bacteria swam at speeds ranging from 10 to 90 μm per s by modulating the rotary speed of a single unidirectional flagellum. Swimming cells, however, did not respond to chemotactic stimuli, including oxygen, which triggers a tactic response in all bacteria studied to date (87). In addition, in either spatial or temporal gradients of potential attractants (oxaloacetate, succinate, pyruvate, malate, aspartate, glutamate, and oxygen) and repellents (1,4-benzoquinone and oxygen), no chemotactic rings or bands were formed, and the probability of directional changes and swimming speed remained unchanged. These results are consistent with the absence of a complete set of the genes that

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TABLE 5. Comparison of two-component signal transduction genes shared by *C. crescentus* and *H. neptunium*

C. crescentus ORF	Gene name	Type[a]	Essential?	*H. neptunium* ORF	*S. pomeroyi* ORF	Phenotypic characteristic[b]							
						Cell cycle	Doubling time	Swarm size	Swarm density	Motility	Stalk	Pili	Cell length
CC0138	*shkA*	HK		HNE_0294		Y	−	−	+	−	−		−
CC0237		RR		HNE_1408	SPO0710			+	−	−			
CC0238		HK		HNE_1404	SPO0711			+	−				−
CC0289		HK		HNE_3058	SPO1947			+	−				−
CC0294	*phoB*	RR		HNE_2028	SPO1953		−	+	−	−	−		
CC0530	*cenK*	HK	+	HNE_0407	SPO0541								
CC0744		RR		HNE_0229		Y	−	−	+	−	−		−
CC1063	*divJ*	HK		HNE_2910	SPO0094	Y	−	−	+	−	−		−
CC1078	*cckA*	HK	+	HNE_0507	SPO2036								
CC1114	*shpA*	HPT		HNE_0318		Y	−	−	−	−	−		−
CC1740		HK		HNE_2012	SPO2088		−		−				
CC1741		RR		HNE_2011	SPO2087		−		−				
CC1743		RR	+	HNE_2009	SPO2085								
CC2462	*pleD*	RR		HNE_2284	SPO2753	Y		−	+	−	−		
CC2463	*divK*	RR	+	HNE_2285									
CC2482	*pleC*	HK		HNE_2910		Y		−	+	−	−	−	
CC2755		HK		HNE_1311	SPO1623			−					
CC2931		RR	+	HNE_3093	SPO0251								
CC2932		HK	+	HNE_3094	SPO2173								
CC3035	*ctrA*	RR	+	HNE_0944	SPO1679								
CC3315	*tacA*	RR		HNE_3039		Y	−	−	+	−	−		−
CC3471		RR		HNE_0639				−	+	−	−		−
CC3474		HK		HNE_2339		Y		+	−	−			
CC3477		RR		HNE_2344		Y		+	−	−			
CC3484	*divL*	HK	+	HNE_0399	SPO3868								
CC3743	*cenR*	RR	+	HNE_3550	SPO3426								

[a] HK, histidine kinase; RR, response regulator; HPT, histidine phosphotransferase.
[b] Phenotypic characterization of deletions is from reference 81. Genes whose corresponding deletion strain was identified as having a cell cycle, growth, motility, or morphogenesis defect are marked with a plus sign (+). Cell cycle-regulated genes according to reference 46 are indicated by Y. For all other categories, a plus sign indicates that the phenotypic characteristic is larger than wild type and a minus (−) sign indicates that a characteristic is smaller than wild type.

are normally required for efficient navigation in chemical gradients. Motility that is not coupled to temporal sensing machinery may serve as a mechanism of population dispersion rather than stimulus-controlled navigation toward specific environments. A recent model for the bacterial chemotactic response (16) suggested that bacterial chemotaxis provides a competitive advantage to motile bacteria only under conditions where the cells can detect and adapt to stimuli within short distances (fewer than a few millimeters) or a short timescale (shorter than the cell division time). An *H. neptunium* swarmer cell, which does not replicate, may travel a relatively great distance from the attached reproductive cell that spawned it before it finds a suitable niche for attachment, differentiation, and growth. We hypothesize that *H. neptunium* swarmer cells are well adapted to survival in oligotrophic environments because their motility provides an efficient means for dispersion.

Signal transduction. *H. neptunium* contains several genes encoding two-component signal transduction proteins: 17 sensor histidine kinase genes, 23 response regulator genes, and 3 combined sensor histidine kinase/response regulator genes were identified. Four of the histidine kinase genes were located adjacent on the chromosome to a response regulator, indicating possible functional units. Although two-component signal transduction systems were originally characterized for their role in adaptive responses to environmental changes, work in *C. crescentus* has revealed a major role for these signaling systems in controlling core physiological processes, including cell cycle progression and programs of morphogenesis inherent to the *Caulobacter* life cycle (38, 64, 80). A recent study reported the systematic deletion of each of the 106 two-component signal transduction genes encoded in the *C. crescentus* genome. Phenotypic analysis of these deletion strains identified 39 signaling genes required for proper cell cycle progression, cell growth, or morphogenesis, 9 of which are essential for viability (81). Since these 39 signal transduction genes are important for normal growth and cell cycle progression, we expected that many would be conserved in the closely related *H. neptunium* genome. In fact, *H. neptunium* encodes orthologs for 25 of the 39 (64%) genes required for normal growth or morphology, which includes 9 genes that are essential for viability (Table 5). In contrast, only 18 (27%) of the 67 other two-component genes in *Caulobacter* for which no clear phenotype was identified had orthologs in *H. neptunium*. Similarly, *S. pomeroyi* contains orthologs for 17 of the 39 (44%) *Caulobacter* genes required for normal growth or morphology (including 8 of the 9 essential genes), but only 9 of the 67 genes (13%) without clear phenotypes. Interestingly, 17 of the 39 *Caulobacter* two-component signaling genes with growth or morphology phenotypes have orthologs in both *H. neptunium* and *S. pomeroyi* (Table 5). This set of genes includes the major cell cycle regulators *ctrA*, *cckA*, *divL*, and *divJ* and includes the recently identified two-component pair *cenK*-*cenR*, which is essential for growth of *Caulobacter* owing to a role in regulating the cell envelope. These comparisons demonstrate that two-component signaling genes critical to core physiological processes are more highly conserved than other classes of

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genes. This analysis further suggests that the two-component genes with growth and morphology phenotypes in *Caulobacter* that are conserved in all three bacteria are crucial players in regulating cell cycle progression. A detailed investigation into the role of these genes should be productive. Similar comparisons of other classes of genes may help to identify important cell cycle regulatory genes in these organisms. Our analysis further suggests that two-component signaling genes unique to each organism or only conserved in two of the three may mediate responses or events specific to each organism. For example, in *Caulobacter* a phosphorelay, comprised of the kinase *shkA* (CC0138), the histidine phosphotransferase *shpA* (CC1114), and the response regulator *tacA* (CC3315), regulates stalk biogenesis during cell cycle progression (11). Orthologs of each gene are found in *H. neptunium*, which also has stalks, but are not found in *S. pomeroyi*, which lacks stalks. This example suggests that further comparative analysis of these genomes may unveil other genes and pathways responsible for the lifestyle of a prosthecate bacterium.

Cell aging and death. Senescence is the decrease in survival or reproduction with age and has been well documented in unicellular microorganisms with asymmetric division. For example, stalked cells of *C. crescentus* exhibit decreased reproductive output (number of progeny produced per cell per hour) with age (1). In this study, some *C. crescentus* stalked cells produced up to 130 progeny over the 300-h time course of the experiment, but many divided more slowly or stopped dividing completely with increased age. Recent studies with *Escherichia coli* showed that daughter cells that inherit the older poles exhibit slower growth rates, decreased reproductive output, and an increased likelihood of death, suggesting that bacteria that undergo morphologically symmetric division also exhibit senescence (86). Prosthecate, budding bacteria display an extreme form of senescence in which the mother cell gives rise to a very limited number of progeny (55, 94). *H. neptunium* mother cells produce up to nine progeny (R. M. Weiner, unpublished data). This pronounced senescence may be related to polar growth and the use of the stalk as a reproductive structure since the developing bud likely receives newly synthesized macromolecules (e.g., components of the cell envelope) that are not renewed in the reproductive cell. Thus, senescence of the *H. neptunium* mother cell may result from the accumulation of damaged cellular components or the loss of components required for reproduction following a limited number of budding events.

Alternatively, the cessation in reproductive output by *H. neptunium* may be mediated by a specific genetic program. Programmed cell death occurs in some bacteria with distinct developmental cycles, such as during *Bacillus subtilis* sporulation and fruiting body formation in *Myxococcus xanthus* (48). If the *H. neptunium* cell cycle involves a programmed cell death, candidate genes for such a program include toxin-antitoxin (TA) loci and homologs of eukaryotic apoptotic proteins. These genes are found in a variety of bacteria, but this does not preclude their potential involvement in *H. neptunium* senescence. TA loci have been suggested to be involved in programmed cell death in *E. coli*, but this idea remains controversial (27, 28, 29, 35, 63). *H. neptunium* has two sets of TA loci (HNE_2664/HNE_2665 and HNE_0347/HNE_0348). TA loci consist of two genes organized in an operon, one encoding a stable toxin and the other an unstable antitoxin. They were described first in plasmid and phage genomes, where they contribute to episome stability by selectively killing segregants cured of the episome, but were later found in the chromosomes of many bacteria (28, 29, 35, 63). Homologs of eukaryotic apoptotic proteins found in bacteria include caspase-like proteases, HtrA-like serine proteases, apoptotic ATPases, and NACHT family NTPases. The marine cyanobacterium *Trichodesmium* sp. apparently undergoes a programmed cell death in aging cultures and also in response to certain environmental and physiological stresses, and this process is correlated with an increased level and activity of caspase-like proteases (10). *H. neptunium* possesses three HtrA-like serine proteases (HNE_0472, HNE_1313, and HNE_2644) which could have roles in a potential cell death program.

Conclusion. The analysis of the genome sequence of *H. neptunium* ATCC 15444^T and its comparison to that of *C. crescentus* CB15 has revealed some unexpected results. For example, although the analysis is congruent with phenotypic similarities between the two organisms, the fact that *H. neptunium* shares twice as many proteins with *C. crescentus* than it does with its fellow Rhodobacter *S. pomeroyi* conflicts with the currently accepted 16S rRNA phylogeny. This suggests that *Hyphomonas* can be reclassified.

Genomic and experimental evidence indicate that *H. neptunium* cells are motile but not chemotactic. Furthermore, while some of the flagellar genes such as *flgL* and *fliQ* appear to be much closer to their *S. pomeroyi* homologs than to their homologs in *C. crescentus*, *H. neptunium* contains the potential flagellar regulatory genes *flhF* and *flhG*, which are not encoded by either of the other two genomes. The presence of additional regulatory flagellar genes that are not found in closely related organisms is consistent with the notion that motility plays an important role in the lifestyle of this organism. We speculate that motility among *Hyphomonas* swarmer cells is a random dispersal method. The lack of evidence for chemotaxis further supports this hypothesis.

Finally, comparative genomics has yielded valuable insights into prokaryotic diversity and evolution. The availability of the complete *H. neptunium* genome will be an important tool for examining unique aspects of the biology of DBP, including cell development, stalk biogenesis, and strategies for oligotrophic lifestyles. Moreover, the *H. neptunium* genome will provide an important resource for the study of processes for which prosthecate, budding bacteria may serve as model systems, including chromosome segregation, polar growth, and cell aging.

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