

Bacterial community shifts in taxa and diversity in response to localized organic loading in the deep sea

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Summary

The deep sea is a unique and extreme environment characterized by low concentrations of highly recalcitrant carbon. As a consequence, large organic inputs have potential to cause significant perturbation. To assess the impact of organic enrichment on deep sea microbial communities, we investigated bacterial diversity in sediments underlying two whale falls at 1820 and 2893 m depth in Monterey Canyon, as compared with surrounding reference sediment 10–20 m away. *Bacteroidetes*, *Epsilonproteobacteria* and *Firmicutes* were recovered primarily from whale fall-associated sediments, while *Gammaproteobacteria* and *Planctomycetes* were found primarily within reference sediments. Abundant *Deltaproteobacteria* were recovered from both sediment types, but the *Desulfobacteraceae* and *Desulfobulbaceae* families were observed primarily beneath the whale falls. UniFrac analysis revealed that bacterial communities from the two whale falls (~30 km apart) clustered to the exclusion of corresponding reference sediment communities, suggesting that deposition of whale fall biomass is more influential on deep sea microbial communities than specific seafloor location. The bacterial population at whale-1820 at 7 months post deposition was less diverse than reference sediments, with *Delta*- and *Epsilonproteobacteria* and *Bacteroidetes* making up 89% of the community. At 70 months, bacterial diversity in reference sediments near whale-2893 had decreased as well. Over this time, there was a convergence of each community's membership at the phyla level, although lower-taxonomic-level composition remained distinct. Long-term impact of organic carbon loading from the whale falls was also evident by elevated total organic carbon and enhanced proteolytic activity for at least 17–70 months. The response of the sedimentary

microbial community to large pulses of organic carbon is complex, likely affected by increased animal bioturbation, and may be sustained over time periods that span years to perhaps even decades.

Introduction

Organic material in the oceans, originating from primary production in surface waters, is recycled within the water column, leaving only small amounts to aggregate, sink and eventually become buried. This sedimentation of particulate organic matter to the seafloor results in low concentrations (< 0.1% by weight) of highly recalcitrant carbon in deep sea sediments (Mackin and Swider, 1989; Paull *et al.*, 2006). The deep sea is also unusual in that it is characterized by high pressure, low temperature and variable oxygen levels. Despite these physical and biochemical constraints, deep sea sediments have been shown to support abundant and diverse populations of microorganisms (Li *et al.*, 1999; Vetriani *et al.*, 1999; Dhillon *et al.*, 2003; Knittel *et al.*, 2005; Inagaki *et al.*, 2006). Traditionally, however, the metabolic activity of these microbes has been thought to be slower than shallow-living relatives (e.g. Jannasch and Wirsen, 1973), and the juxtaposition of extreme physical conditions and decreased rate of carbon delivery to the seafloor make the determination of what specifically controls their metabolic activity difficult to measure. Many deep sea bacterial isolates possess a high number of ribosomal operon copies relative to their genome size, suggestive of opportunistic lifestyles (*r*-strategy and a high degree of gene regulation) and possibly fast response to environmental change (Klappenbach *et al.*, 2000; Lauro and Bartlett, 2008). In the laboratory, certain deep sea isolates have demonstrated extreme resistance to starvation, and the retained ability, after decades in some cases, for exponential growth upon exposure to nutrients (Lauro and Bartlett, 2008). How and to what extent the structure of deep sea bacterial assemblages varies in response to large organic accumulations *in situ*, however, has yet to be determined.

In localized areas, stimulated microbial biomass and activity do occur in the permanently cold depths of the deep sea (see Jørgensen and Boetius, 2007). For example, whale falls form persistent, regionally abundant, organic-rich habitat islands on the deep seafloor (Smith

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et al., 1989; Naganuma *et al.*, 1996; Baco and Smith, 2003; Goffredi *et al.*, 2004; Braby *et al.*, 2007). Whale carcasses deliver large amounts of organic material to the seafloor at a rate much greater than the supply of planktonic marine snow (Van Dover, 2000; Baco and Smith, 2003), thus resulting in a carbon shunt to sedimentary communities. The microbial ecology within these organic-rich deep sea habitats has the potential to be unique, particularly with regard to interactions among microbial populations and the facilitation of specific pathways for nutrient cycling. Previous studies on whale falls suggest that elevated concentrations of bioavailable carbon allow for the unusual coexistence of methanogenic archaea and sulfate-reducing bacteria, possibly supported by enhanced rates of fermentation and associated hydrogen production (Goffredi *et al.*, 2008; Treude *et al.*, 2009). Decomposition of complex organic carbon involves an assembly line of various microbial metabolisms, initiated by the extracellular hydrolysis of particulate organic matter to high-molecular-weight dissolved organic compounds, which subsequently supplies lower-molecular-weight compounds (organic acids, H₂) for terminal metabolism (Capone and Kiene, 1988). The specific groups of indigenous microorganisms responsible for this initial breakdown of organic carbon have not been well characterized, nor is it known how the diversity of microorganisms is influenced by large organic carbon inputs to the deep sea.

In Monterey Canyon, two distinct whale falls within ~50 km of shore offered a unique opportunity to better understand the impact of nutrient loading on deep sea microbial community structure and adaptability among various microbial groups. Events such as a whale fall are expected to result in an ecologically distinct bacterial community, as compared with surrounding seafloor sediments. Following an earlier study that documented temporal changes in processes involving archaea (i.e. methanogenesis; Goffredi *et al.*, 2008), we assessed differences in the distribution and activity of bacteria involved in the hydrolytic and fermentative breakdown of organic carbon, as well as terminal metabolism (i.e. bacterial sulfate, and possible hydrogen, respiration). Sampling of two deep sea whale falls in Monterey Canyon over a collective period of ~6 years allowed for the investigation of factors that control the spatial and temporal distribution of microbial assemblages involved in the diagenesis of organic matter, a task that is often difficult in the deep sea.

Results and discussion

Local organic enrichment has been linked to changes in microbial community composition in a variety of natural and perturbed marine environments (Holmer and

Kristensen, 1992; Moezelaar *et al.*, 1996; Weston and Joye, 2005). It is not known, however, how microbial communities in the permanently cold depths of the ocean respond to large organic inputs. Whale carcasses that fall to the seafloor ('whale falls') represent localized areas of extreme organic enrichment in the otherwise nutrient-poor deep sea and, thus, provide an opportunity to determine the responsiveness and adaptability of deep sea sedimentary bacteria.

As discrete resource patches, whale falls are thought to contribute significantly to habitat heterogeneity, and thus may contribute to the proliferation of unique microbial assemblages (Grassle and Morse-Porteous, 1987; Baco and Smith, 2003). At present there are only a handful of studies investigating microbial assemblages associated with whale falls. Previous investigations include surveys of whale bone surfaces (Deming *et al.*, 1997; Tringe *et al.*, 2005) and characterizations of whale fall sediments, including measurements of community methane production and sulfate consumption (Treude *et al.*, 2009), biomarker profiling of specific functional groups of methanogens and sulfate-reducing bacteria (Naganuma *et al.*, 1996), and molecular and biochemical surveys of archaea and genes related to methane cycling (Goffredi *et al.*, 2008). Collectively, these studies revealed an unrecognized niche for methanogenic and methanotrophic archaea, and reported the presence of sulfur-based microbial communities associated with whale falls. Little attention, however, has been paid to the microbial assemblages involved in early stages of carbon degradation.

Sediment geochemistry and carbon loading

All sediments collected under whale falls in Monterey Canyon (including three additional whale falls not included in this study) showed significant levels of total organic carbon (1–3.5% TOC, Fig. 1; Table 1; Goffredi *et al.*, 2008), relative to typical deep sea conditions (~0.1% TOC at a depth of 1200 m, Paull *et al.*, 2006). Similarly, whale fall-impacted sediments were depleted in sulfate, relative to reference sediments (Table 1), likely a result of carbon degradation coupled to microbial sulfate reduction. Elevated levels of methane and sulfide have also previously been observed for sediments underlying whale-2893, suggesting that despite low temperatures and overlapping distribution with active sulfate-reducing microorganisms, methanogenesis, a terminal step in the diagenesis of organic matter, also occurs in these near seafloor habitats (Naganuma *et al.*, 1996; Goffredi *et al.*, 2008). Organic carbon concentration and net carbon mineralization rates, in particular, have been identified as a primary influence on sedimentary bacterial communities and may serve as good predictors of the abundance of

Table 1. Summary of samples used in the construction of 16S rRNA libraries, and selected chemical conditions, including TOC and sulfate.

Depth (m) ^a	Date (dive #)	Months	Description (PushCore #)	Sediment depth (cm)	Library name ^b	No. of clones screened	TOC (%)	SO ₄ (mM)
1820	October 2006 (T1048)	7	0 m (PC50)	0–3	7mos_0s	60	1.5 ^c	13.7
				12–15	7mos_0d	42	1.5 ^c	nm
			10 m (PC53)	0–3	7mos_10s	46	nm	28.0
				12–15	7mos_10d	43	nm	27.1
2893	November 2004 (T769)	33	0 m (PC18)	0–2.5	33mos_0s	81	1.5	11.0
				12–15	–	nm	2.2	nm
			20 m (PC55)	0–2.5	33mos_20s	68	1.9	nm
				12–15	–	nm	1.6	nm
	December 2007 (T1162)	70	0 m (PC49)	0–3	70mos_0s	66	1.5	28.8
				6–9	70mos_0d	67	0.9	26.7
			20 m (PC71)	0–3	70mos_20s	67	1.2	29.0
				9–12	–	nm	1.2	29.4

a. The 1820 m whale fall was intentionally implanted in March 2006, while the 2893 m whale fall was a natural whale fall discovered in February 2002.

b. Library names represent (# months since implantation or discovery)_(distance from whale as 0 or 10 m)(s or d to indicate shallow 0–3 cm or deep 6–15 cm core horizon).

c. Values for samples taken at 10 months after implantation on the seafloor (January 2007, dive T1071). nm = not measured. – = no library available.

some bacterial groups (Jørgensen, 1982; Bissett *et al.*, 2006; Hansel *et al.*, 2008 and references therein).

Proteolytic enzyme activity

Hydrolytic and fermentative bacteria are key players in the early stages of organic carbon breakdown and are expected to respond quickly to increased carbon loading. To determine the potential for organic carbon breakdown, relative proteolytic enzyme activity, a measure of protein hydrolysis, was determined in sediments immediately underlying whale-1820 at 7 months post deposition, and at 3 and 10 m distance from the whale fall (Fig. 2). Proteolytic activity, in particular, can be quite high in organic-rich sediments (Wakeham and Canuel, 2006). In whale fall sediments, the highest protease activity was measured directly under the whale carcass within the top 3 cm of sediment (78.7 mU), while activity in reference sediments at 3 and 10 m distance was 2–6× lower (16.8 and 33.2 mU respectively, Fig. 2). Elevated protease activity under the whale fall was still detected at 17 months (53.8 mU, data not shown), suggesting that enzymatic breakdown of proteinaceous material continues for years following initial deposition of a whale carcass on the seafloor. In comparison, protease activity under whale-2893 after 54 months was comparable with reference sediments (4.8 mU). While this assay provided assessment of proteolytic potential only, it is likely that heterotrophic microorganisms found under whale falls are also able to breakdown and utilize other classes of macromolecules (Arnosti *et al.*, 1995). Many heterotrophic species observed in abundance under the whale falls in this study (e.g. the *Bacteroidetes*) produce proteases used in the breakdown of exogenous insoluble proteins.

General trends in bacterial community composition

Our analysis of whale fall and reference sediments (pooled across core depths, including 0–3 and 6–15 cm, Table 2) revealed distinct differences in bacterial community composition. *Deltaproteobacteria* dominated both sediment types (ranging from 22% to 39% of the recovered ribotypes; Fig. 3B), but the abundant families within this subdivision were differentially distributed, with

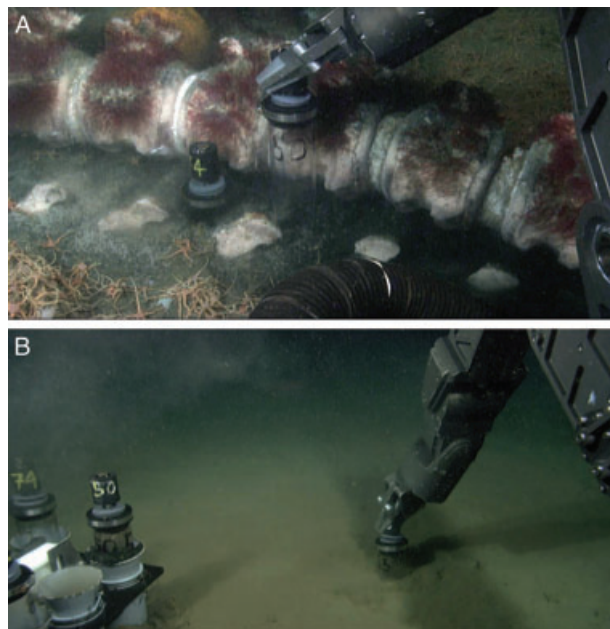


Fig. 1. Collection of sediment samples in Monterey Canyon either in (A) direct association with a whale fall, collected at 7 months post deposition (whale-1820) or (B) at 10 m away, considered to be 'reference' sediment, initially outside of the influence of the whale fall.

Table 2. Comparison of bacterial communities associated with whale fall and reference sediments.

Bacterial group	Number of clones in the following sediments ^a					
	Whale 1820	Whale 2893		Reference 1820	Reference 2893	
	7 mos ^b	33 mos	70 mos ^b	7 mos ^b	33 mos	70 mos
<i>Bacteroidetes</i>	24 (15/9)	17	39 (13/26)	6 (4/2)	5	12
<i>Deltaproteobacteria</i>	38 (19/19)	21	34 (15/19)	35 (17/18)	15	18
<i>Epsilonproteobacteria</i>	29 (20/9)	12	16 (16/0)	2 (2/0)	–	1
<i>Gammaproteobacteria</i>	1 (1/0)	6	15 (15/0)	17 (8/9)	17	16
<i>Alphaproteobacteria</i>	2 (1/1)	1	2 (2/0)	6 (5/1)	1	3
<i>Firmicutes</i>	4 (2/2)	6	16 (3/13)	3 (2/1)	1	–
<i>Planctomycetes</i>	–	1	–	5 (3/2)	11	5
<i>Verrucomicrobia</i>	–	4	–	2 (0/2)	4	2
<i>Acidobacteria</i>	2 (2/0)	–	1 (0/1)	5 (2/3)	3	3
<i>Spirochaetes</i>	–	–	4	3 (0/3)	–	–
Unknown	2	9	7	8	11	7
Total	102	81	133	89	68	67

Phyla representation (based on 16S rRNA) is shown for samples underlying two whale falls (at 1820 and 2893 m depth) and at nearby reference sites (10–20 m distant), at three sampling times, in both shallow and deep sediment horizons.

a. Whale fall-associated sediments came from an artificially deposited whale carcass at 1820 m, sampled 7 months post deposition, and a naturally deposited carcass at 2893 m, sampled at 33 and 70 months post discovery.

b. Number of clones of each phylum in each sediment core, followed by when measured, the distribution between two depth horizons (0–3/6–15 cm). – = not detected.

Desulfobacteraceae and *Desulfobulbaceae* recovered primarily from whale fall sediments. Whale fall sediments also had a comparatively higher proportion of *Bacteroidetes*, *Epsilonproteobacteria* and *Firmicutes* (collectively averaging 51% of the recovered bacterial diversity versus 14% in reference sediments), and uniquely contained low numbers of *Spirochaetes* (~4% of recovered ribotypes, Fig. 3B and Table 2). By contrast, reference assemblages had comparatively more *Gammaproteobacteria* and *Planctomycetes* (together averaging 32% of the diversity versus ~7% under the whale falls), as well as ribotypes associated with *Acidobacteria* and *Verrucomicrobia* (3–6% of recovered ribotypes). The ribotypes recovered from whale-associated and reference sediments spanned a wide phylogenetic range within each phylum and are shown in Figs 4–8. While the majority of sequences could be assigned unambiguously to known phyla based on comparison with previously described 16S rRNA data sets (GenBank), 2–13% of the

total ribotypes in each library were not definitively affiliated with any known group ('unknown' in Table 2).

Diversity indices

Parametric, non-parametric and phylogenetic approaches were used to assess the microbial community diversity within and between environments (Bohannan and Hughes, 2003). The Shannon–Wiener diversity index suggested that at 7 months, the bacterial population under whale-1820 was less diverse ($H' = 3.99$), compared with reference sediments ($H' = 4.32$, Table 3). Lower microbial diversity has been similarly observed for chemically extreme, perturbed and organically enriched shallow marine habitats, such as sediments impacted by fish farming and pollution (Benlloch *et al.*, 1995; Torsvik *et al.*, 1998; Sievert *et al.*, 1999; Bissett *et al.*, 2006; Zhang *et al.*, 2008; Kawahara *et al.*, 2009). At 33 months, there was no difference in diversity

Table 3. Biological diversity indices for bacterial sequences recovered from both whale fall and reference sediment types over three separate collection times, including the Shannon–Wiener diversity index (H') and the Chao1 non-parametric estimator (S_{Chao1}).

Sediment type	Months ^a	Clones screened	OTUs ^b	Singletons	H'	S_{Chao1}
Whale-1820	7	102	71	46	3.99	152
Whale-2893	33	81	66	54	4.11	212
Whale-2893	70	133	114	102	4.59	857
Reference-1820	7	89	85	78	4.32	1099
Reference-2893	33	68	66	63	4.17	728
Reference-2893	70	67	55	46	3.93	231

a. After discovery (whale-2893) or intentional implantation (whale-1820).

b. Defined by a minimum threshold of 97% similarity.

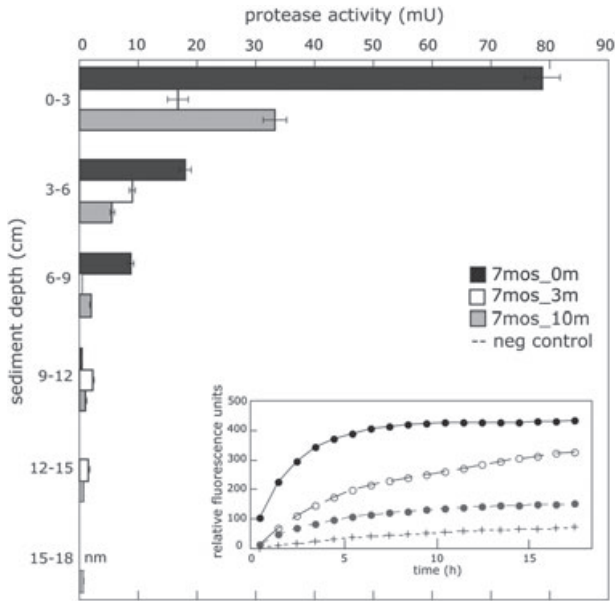


Fig. 2. Proteolytic enzyme activity in sediments immediately underlying whale-1820 at 7 months post deposition, and at 3 and 10 m distance from the whale fall. Protease activity is expressed in mU g^{-1} wet weight, with 1 U equal to the amount of enzyme necessary to liberate $1 \mu\text{mol casein min}^{-1}$. Inset: activity, shown here over a period of 0–17 h, usually plateaued within ~3 h, thus most measurements were made during this time frame. Slopes from the linear portion of the curve were used to calculate protease activity. nm = not measured.

between sediment under whale-2893 and the reference ($H' = 4.11\text{--}4.17$), but at 70 months the bacterial community diversity in surrounding reference sediments had fallen behind that of whale fall sediments ($H' = 3.9$ versus 4.6 respectively; Table 3). This eventual decrease in diversity in surrounding reference sediments may be explained by succession of the microbial communities and the movement of a ‘dispersing front’ of whale-associated nutrients, which could result in a steep drop in diversity at its leading edge as it passes through microbial communities at successively greater distance from the whale fall.

The non-parametric S_{Chao1} estimator allowed a focus on the richness component of diversity, and like the diversity index showed opposite temporal trends, with increasing richness below the whale fall and decreasing richness at the reference sites over time. The total number of estimated OTUs ranged from ~150 to 1100 (Table 3), suggesting that actual diversity in some samples was much greater than detected, with only ~10% of the total diversity sampled. Such undersampling of richness is commonly observed in bacterial surveys in a range of environments (Bohannan and Hughes, 2003; Kemp and Aller, 2004; Hansel *et al.*, 2008). An increasing number of habitats appear to contain a ‘rare biosphere’ with an extraordinarily large number of diverse, low-abundance ribotypes (Sogin *et al.*, 2006; Huber *et al.*, 2007; Fuhrman, 2009). Both the frequency and significance of this rare biosphere remain unclear. One suggested description of these low-

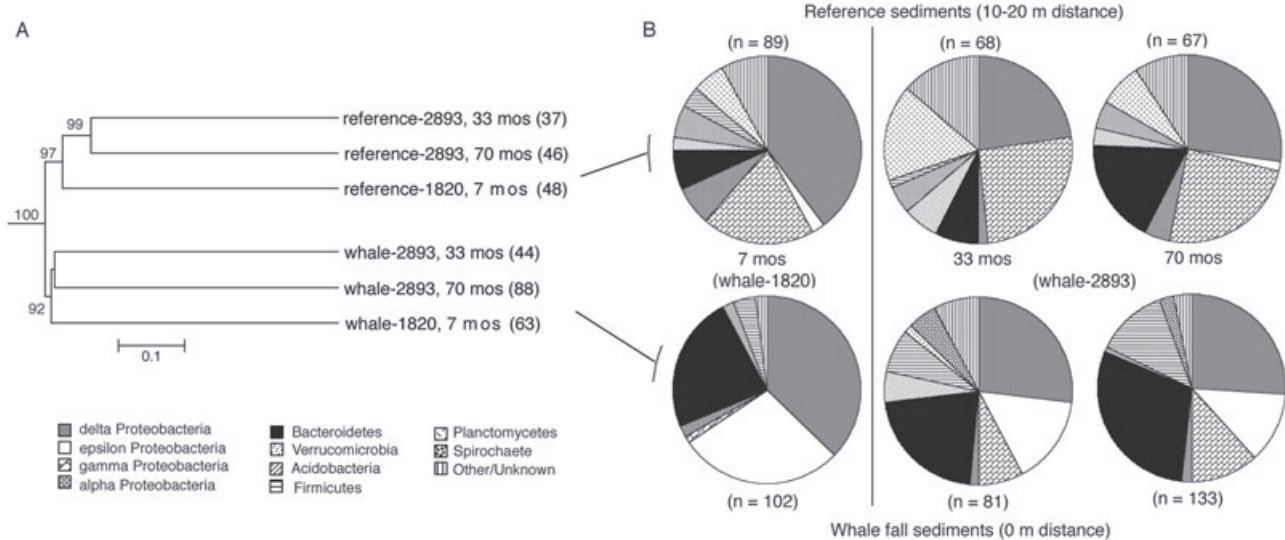


Fig. 3. Bacterial groups associated with whale fall and reference sediment in Monterey Canyon. A. Jackknife environment cluster tree (unweighted UniFrac metric, based on an ARB neighbor-joining tree of 16S rRNA gene sequences) showing the collective phylogenetic relationships among specific bacterial lineages (Gamma- and *Deltaproteobacteria* and *Bacteroidetes*) recovered from whale fall and reference sediments in this study. One hundred jackknife replicates were calculated, and each node is labelled with the jackknife value (all nodes are robust with values > 92%). The number of sequences that represent each environment is indicated next to the sample name. Scale bar: distance between the environment in UniFrac units. B. Proportions of bacterial groups within clone libraries obtained from whale fall and reference sediment within Monterey Canyon. Sampling occurred at 7 months following implantation of whale-1820 and at 33 and 70 months following the discovery of whale-2893.

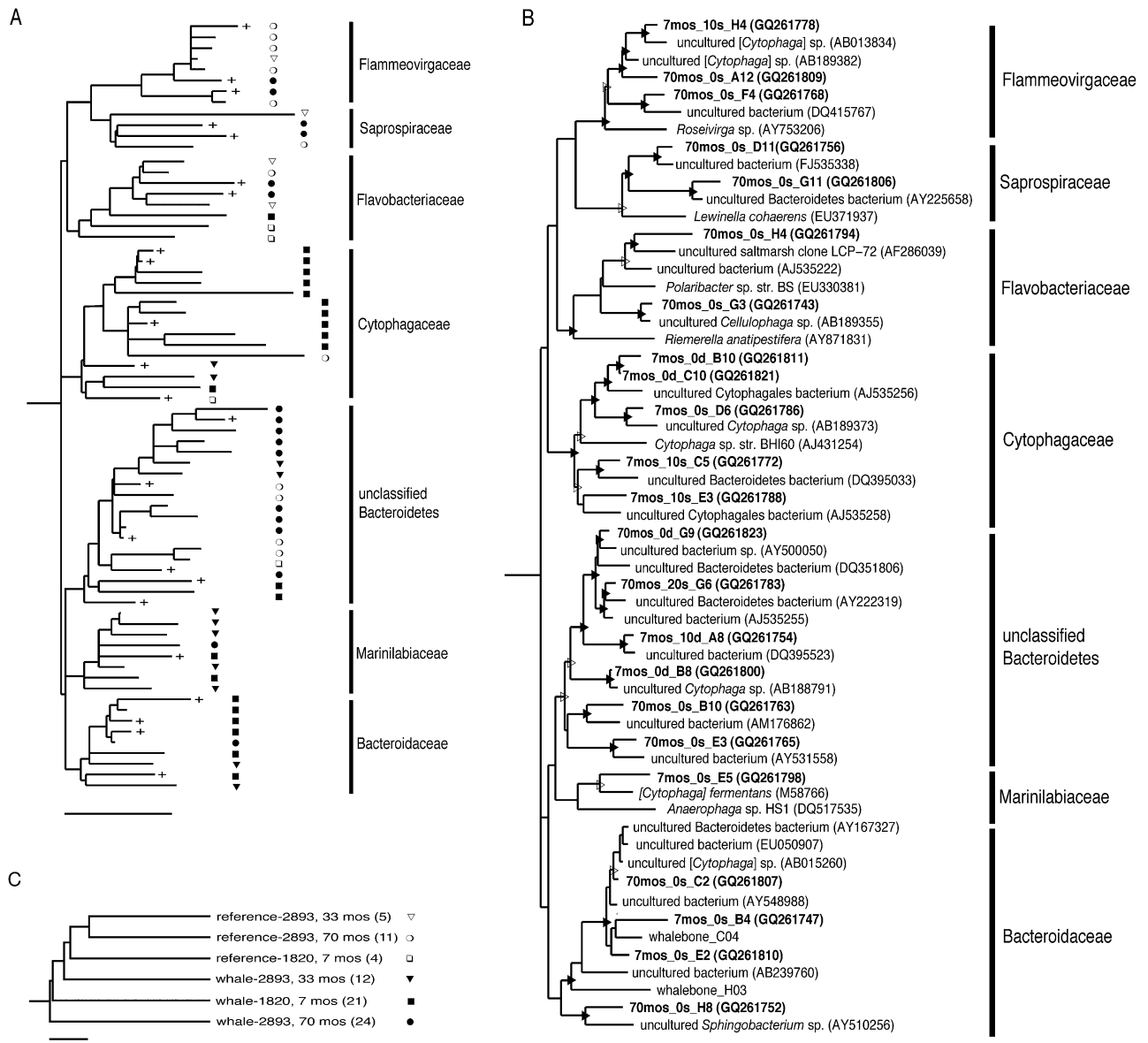


Fig. 4. Phylogenetic relationships of *Bacteroidetes* associated with whale falls in Monterey Canyon (CA), based on sequence divergence within the 16S rRNA gene.

A. Neighbor-joining tree (Olsen correction) of 16S rRNA genes recovered in this study only, from both whale fall and reference sediments, made by parsimony insertion of partial sequences into a backbone tree of a subset of near full-length sequences. The plus (+) sign denotes ribotypes that were selected for near full-length sequencing and are included in the tree in (B). The symbol with which the sample is represented is shown in (C).

B. Phylogenetic position of 16S rRNA genes recovered in this study relative to selected cultured and environmental sequences in public databases. *Thermotoga maritima* (AJ401021) was used as an outgroup (not shown). Symbols next to nodes correspond to bootstrap values based on neighbor-joining distance and 5000 replicates (open symbol = 60–90%, closed symbol = 90+% bootstrap support). For library designations, refer to Table 2. Taxon designations correspond to those listed in Table 4.

C. Jackknife environment cluster tree (unweighted UniFrac metric, based on an ARB neighbor-joining tree of 16S rRNA gene sequences). The number of sequences that represent each environment is indicated next to the sample name, as well as the symbol with which the sample is represented. All scale bars represent 10% divergence in either rRNA sequence (A/B) or distance between each environment in UniFrac units (C).

abundance members are those adapted to different nutrient conditions from commonly found in a given habitat, but poised for rapid growth should conditions change. The response of the deep sea oligotrophic sediment environ-

ment to the massive nutrient input of a whale carcass presents an ideal opportunity to test this hypothesis in the future. Deeper sequencing (e.g. ‘Tag sequencing’, Sogin *et al.*, 2006) from each sediment type may help differen-

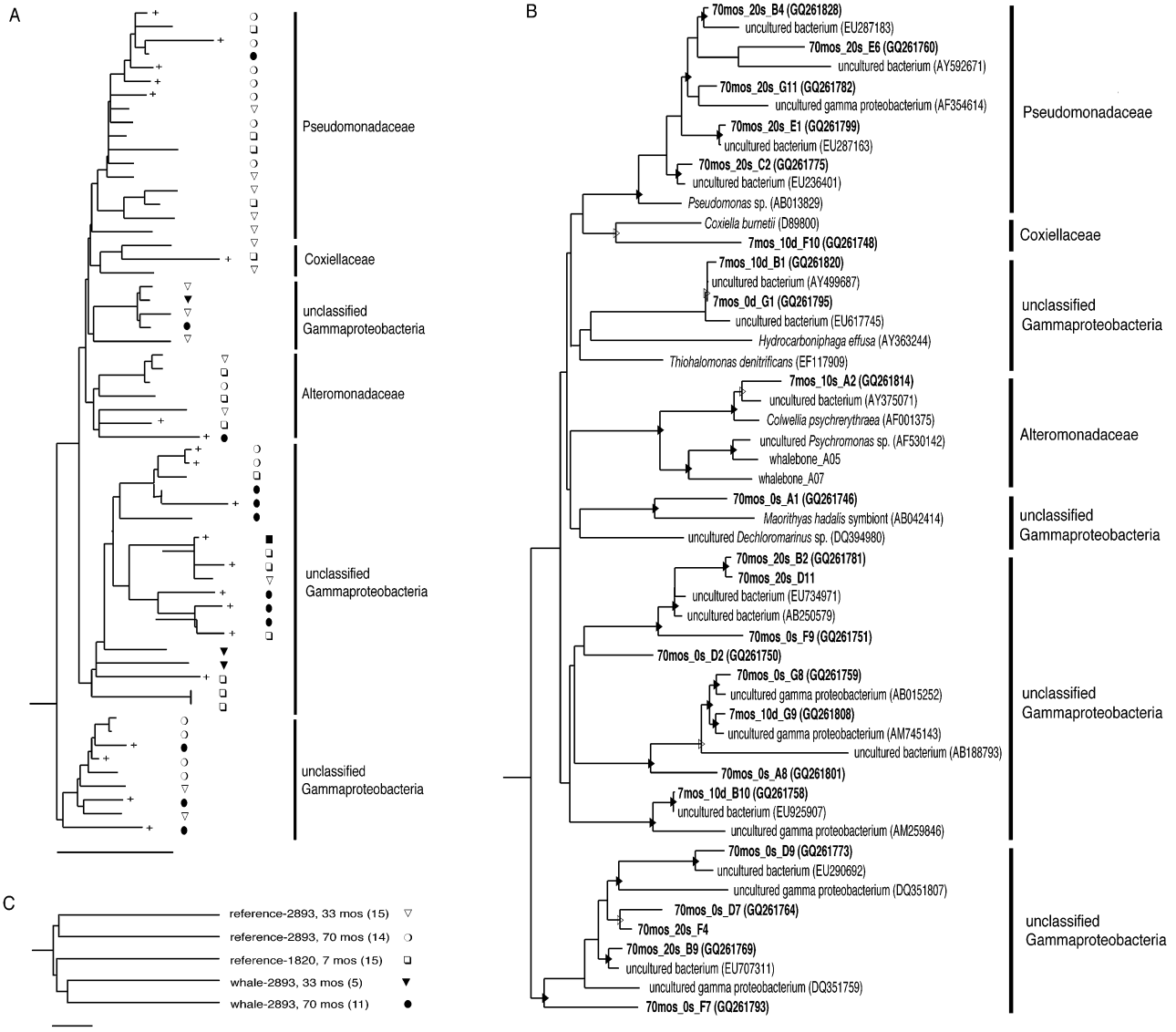


Fig. 5. Phylogenetic relationships of *Gammaproteobacteria* associated with whale falls in Monterey Canyon (CA), based on sequence divergence within the 16S rRNA gene.

A. Neighbor-joining tree (Olsen correction) of 16S rRNA genes recovered in this study only, from both whale fall and reference sediments, made by parsimony insertion of partial sequences into a backbone tree of a subset of near full-length sequences. The plus (+) sign denotes ribotypes that were selected for near full-length sequencing and included in the tree in (B). The symbol with which the sample is represented is shown in (C).

B. Phylogenetic position of 16S rRNA genes recovered in this study relative to selected cultured and environmental sequences in public databases. *Desulfocapsa sulfexigens* (Y13672) was used as an outgroup (not shown). Symbols next to nodes correspond to bootstrap values based on neighbor-joining distance and 5000 replicates (open symbol = 60–90%, closed symbol = 90+% bootstrap support). For library designations, refer to Table 2. Taxon designations correspond to those listed in Table 4.

C. Jackknife environment cluster tree (unweighted UniFrac metric, based on an ARB neighbor-joining tree of 16S rRNA gene sequences). Scale bar: distance between the environment in UniFrac units. The number of sequences that represent each environment is indicated next to the sample name, as well as the symbol with which the sample is represented. Only one ribotype was recovered from sediment immediately underlying whale-1820, at 7 months post deposition, thus this category was removed from the UniFrac analysis. All scale bars represent 10% divergence in either rRNA sequence (A/B) or distance between each environment in UniFrac units (C).

tiate whether the community response to nutrient loading is driven by shifting abundances of lineages already present in the sediment at appreciable numbers, or by the rapid response of organisms normally at extremely low abundance.

In addition to the within-community measures of diversity, phylogenetic divergence-based comparison of the diversity among the communities was also made. UniFrac analysis indicated a well-supported clustering of the total bacterial diversity in sediments associated with two inde-

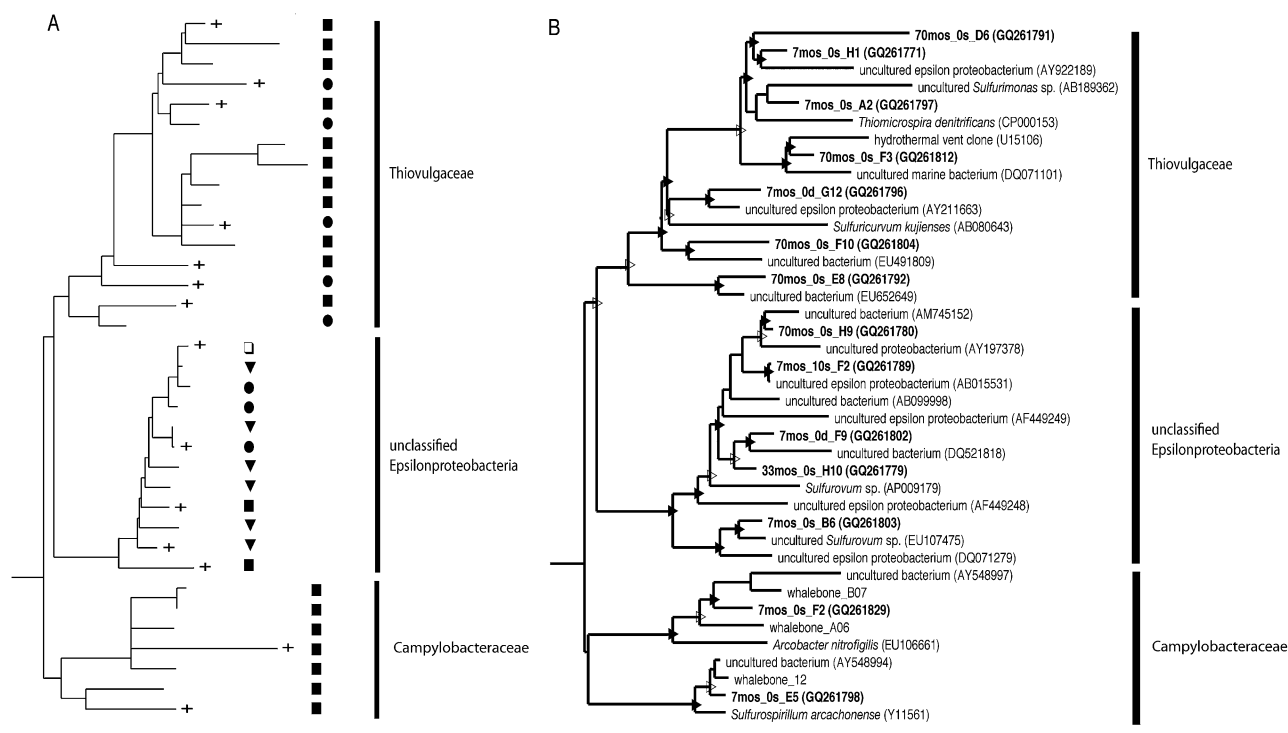


Fig. 6. Phylogenetic relationships of *Epsilonproteobacteria* associated with whale falls in Monterey Canyon (CA), based on sequence divergence within the 16S rRNA gene.

A. Neighbor-joining tree (Olsen correction) of 16S rRNA genes recovered in this study only, from both whale fall and reference sediments, made by parsimony insertion of partial sequences into a backbone tree of a subset of near full-length sequences. The plus (+) sign denotes ribotypes that were selected for near full-length sequencing and included in the tree in (B). The symbol with which the sample is represented is shown in (C).

B. Phylogenetic position of 16S rRNA genes recovered in this study relative to selected cultured and environmental sequences in public databases. *Geobacter* sp. (DQ394958) was used as an outgroup (not shown). Symbols next to nodes correspond to bootstrap values based on neighbor-joining distance and 5000 replicates (open symbol = 60–90%, closed symbol = 90+% bootstrap support). For library designations, refer to Table 2. Taxon designations correspond to those listed in Table 4. Both scale bars represent 10% divergence in rRNA sequence.

pendent whale falls (~30 km apart at 1820 m and 2893 m depth), to the exclusion of corresponding reference sediment communities (jackknife values > 96, data not shown). This suggests that the deposition of whale fall biomass on the seafloor is more influential on the bacterial community than specific location. To that end, UniFrac was also used to compare the shared, dominant bacterial phyla (*Gamma*- and *Deltaproteobacteria* and *Bacteroidetes*) within the different habitats versus time of collection (Fig. 3A). These three bacterial groups were observed in both whale fall and reference sediments and collectively comprised 54–69% of the recovered ribotypes from each sediment sample at any given collection time (Fig. 3B). Likewise, results indicated that the bacterial assemblages within whale fall sediments clustered together (regardless of collection time – 7, 33, 70 mos), by the unweighted-pair group method using average linkages (jackknife values > 92, Fig. 3A). Thus, although higher-taxonomic groups were broadly shared between whale fall and reference sediments, at finer taxonomic resolution the specific bacterial assemblages within each

habitat were phylogenetically unique. This pattern was repeated when each dominant bacterial group was compared separately (Figs 4C, 5C, and 7C).

Variation in bacteria between whale fall and reference sediment

The *Bacteroidetes* are responsible for the breakdown of a major fraction of complex organic matter (Kirchman, 2002; Reichenbach, 2006). They are abundant in many oceanic habitats, are known for macromolecule hydrolysis and fermentative capabilities, and have been shown previously to respond quickly to organic carbon enrichment of marine sediments (Rossello-Mora *et al.*, 1999; Kirchman *et al.*, 2003). Members of the *Bacteroidetes* in our study were abundant within whale fall-associated sediments (21–29% of the recovered ribotypes) and clades existed that were generally unique to whale fall sediment (Fig. 4). For example, at least four groups, including the families *Cytophagaceae*, *Marinilabiaceae* and *Bacteroidaceae* were observed almost exclusively underlying both whale

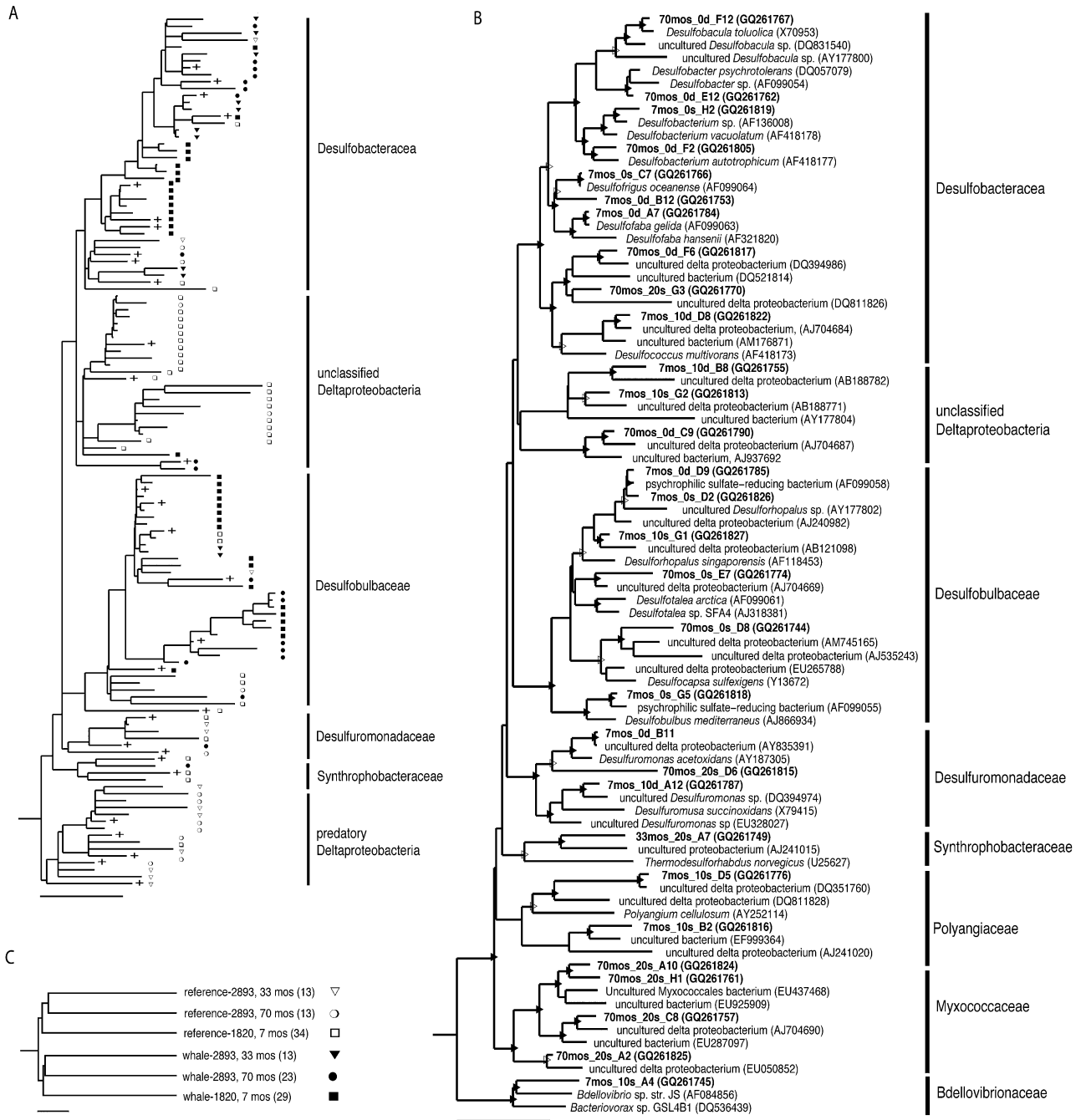


Fig. 7. Phylogenetic relationships of *Deltaproteobacteria* associated with whale falls in Monterey Canyon (CA), based on sequence divergence within the 16S rRNA gene.

A. Neighbor-joining tree (Olsen correction) of 16S rRNA genes recovered in this study only, from both whale fall and reference sediments, made by parsimony insertion of partial sequences into a backbone tree of a subset of near full-length sequences. The plus (+) sign denotes ribotypes that were selected for near full-length sequencing and included in the tree in (B). The symbol with which the sample is represented is shown in (C).

B. Phylogenetic position of 16S rRNA genes recovered in this study relative to selected cultured and environmental sequences in public databases. *Vibrio cholerae* (X74695) was used as an outgroup (not shown). Symbols next to nodes correspond to bootstrap values based on neighbor-joining distance and 5000 replicates (open symbol = 60–90%, closed symbol = 90+% bootstrap support). For library designations, refer to Table 2. Taxon designations correspond to those listed in Table 4.

C. Jackknife environment cluster tree (unweighted UniFrac metric, based on an ARB neighbor-joining tree of 16S rRNA gene sequences). The number of sequences that represent each environment is indicated next to the sample name, as well as the symbol with which the sample is represented. All scale bars represent 10% divergence in either rRNA sequence (A/B) or distance between each environment in UniFrac units (C).

falls (Fig. 4A and B). While functional similarity cannot be inferred from low (< 97%) 16S rRNA similarity, it is interesting that many of the *Bacteroidetes* sequences recovered here grouped with other organisms from organic-rich environments. For example, ribotypes related to an organism recovered from organically enriched fish farm sediments comprised ~9% of the deep horizon library under the whale fall at 70 months (70mos_0d_G9, 97% similar, Table 4; Bissett *et al.*, 2006). Other ribotypes exclusively associated with whale fall sediments also clustered adjacent to groups shown to respond to elevated levels of organic carbon, including *Lewinella* (family *Saprospiraceae*), [*Cytophaga*] *fermentans* and the strictly anaerobic *Anaerophaga* sp. (both within the family *Marinilabiaceae*), and the aerobic genus *Cellulophaga* (family *Flavobacteriaceae*, Fig. 4B). All of these chemoorganotrophs have been observed in carbon-rich marine habitats, such as activated sludge, sewage, estuaries and fish hatcheries (Bachmann, 1955; Johansen *et al.*, 1999; Bowman, 2000; Denger *et al.*, 2002; Lee and Reichenbach, 2006). Surprisingly, *Bacteroidetes* in general were not reported as being dominant on the bones of a whale carcass from Santa Cruz Basin (Tringe *et al.*, 2005), suggesting that their preferred habitat is within marine sediments, rather than on bone surfaces.

Consistent with previous studies on deep sea or permanently cold sediments, recovery of *Bacteroidetes* from reference sediments was generally lower (~7% of recovered ribotypes; Ravenschlag *et al.*, 1999). Their presence, however, appeared to increase over time, ultimately comprising 18% of clones recovered from reference sediment at 70 months (Fig. 3B). Many of these ribotypes were related to the *Flammeovirgaceae* and *Flavobacteriaceae*, a large, predominantly chemoheterotrophic, obligate group of aerobes, common in many aquatic ecosystems (Bernardet and Nakagawa, 2006; Bowman, 2006). This temporal increase in *Bacteroidetes* within reference sediments, some of which were also observed in whale fall sediments, may be attributed to the lateral dispersion of whale fall-associated nutrients to the surrounding seafloor.

Organic carbon does appear to be translocated laterally through the sediment away from the whale fall and exceeds enrichment observed in deeper horizons immediately under the whale fall (Goffredi *et al.*, 2008; see also 7 months 1–1.5%, Table 1). We observed a significant increase in fauna surrounding both whale falls, including polychaetes, molluscs, crustaceans and echinoderms (Goffredi *et al.*, 2004; Braby *et al.*, 2007) and, thus, attribute this lateral expansion of organic carbon beyond the initial radius of the whale fall to be the result of increased animal activity and related bioturbation. Residence times for organic matter in marine sediments is known to vary as a result of bioturbation, which can effec-

tively mix the labile and refractory organic components, resulting in enhanced degradation of the latter (Canfield, 1994; Wakeham and Canuel, 2006). Enhanced bioturbation observed at both whale falls may, thus, contribute to the observed changes in bacterial assemblages and diversity over time, especially in sediments initially thought to be beyond the influence of the whale fall biomass (e.g. 10–20 m distance).

Firmicutes were another relatively abundant group found primarily in whale fall-associated sediments (4–12% of recovered ribotypes, Fig. 3B). Although members of this group are metabolically diverse and reported from a range of environments, ribotypes recovered in our study were similar to those reported from carbon-rich marine environments, such as hydrocarbon contaminated salt marsh sediments (Pearson *et al.*, 2008), the surfaces of corals (Table 4, Fig. 8, Sekar *et al.*, 2008) and deep sea methane-seep sediments (Li *et al.*, 1999). Most of the whale fall-associated ribotypes were distinct but broadly related to members of the anaerobic family *Clostridiaceae*, including the genera *Clostridia* and *Alkaliphilus* (Fig. 8). Cultured members of these fermentative bacteria have broad hydrolytic properties and are known to evolve hydrogen in the course of metabolizing fatty acids and proteinaceous substrates, frequently in close association with terminal metabolizers (Smith, 1970; Cao *et al.*, 2003; Wiegel *et al.*, 2006). Molecular hydrogen, in turn, is an important energy source and intermediate substrate for the further anaerobic degradation of organic materials and its *in situ* concentration can serve as a predictor of the dominant metabolic processes (Lovley and Goodwin, 1988; Hoehler *et al.*, 1998; Finke, 2003). A surplus of hydrogen has been measured in sediments under whale-2893 (up to 300 nM H₂, W. Ziebis, pers. comm.) and these elevated concentrations, presumably linked to enhanced fermentation, are suspected to contribute to overlapping niches for both hydrogenotrophic methanogens and sulfate-reducing bacteria, previously observed in sediments under the whale fall (Goffredi *et al.*, 2008).

As observed with the *Bacteroidetes*, *Gammaproteobacteria* also showed significant differences in diversity and ribotype abundance between whale fall and reference sediments. *Gammaproteobacteria* were the most commonly recovered group from reference sediments, comprising 19–25% of the total bacterial community (Fig. 3B), with most ribotypes related to the family *Pseudomonadaceae*, a common and diverse group of nutritional generalists (Moore *et al.*, 2006), and a minor proportion of sequences related to those found in deep sea and Arctic Ocean sediments (Fig. 5; Ravenschlag *et al.*, 1999; Bowman and McCuaig, 2003). In contrast, *Gammaproteobacteria* were in low abundance in whale

Table 4. Summary of representative 16S rRNA bacterial ribotypes recovered from sediments associated with whale falls and reference sites within Monterey Canyon, along with closest relatives from GenBank.

Group ribotype ID ^a	Representative closest relative (Acc. no.)	% Similarity	Group ribotype ID ^a	Representative closest relative (Acc. no.)	% Similarity
Bacteroidetes					
7mos_10s_H4	Unc <i>Cytophaga</i> , Japan Trench (AB013834)	97	Proteobacteria		
7mos_0s_A12	Unc <i>Cytophaga</i> , Japan Trench (AB189382)	94	Delta group		
7mos_0s_F4	Unc, cave biofilm (DQ415767)	95	70mos_0d_F12	<i>Desulfohalobium toluolica</i> (X70953)	98
7mos_0s_D11	Unc, HTV Kermadec Arc (FJ535338)	95	70mos_0d_E12	<i>Desulfobacter</i> sp., Arctic sediments (AF099054)	97
7mos_0s_G11	Unc, Atlantic HT vent (AY225658)	96	7mos_0s_H2	<i>Desulfobacterium</i> sp., Arctic sediments (AF136008)	97
7mos_0s_H4	Unc, salt marsh sediments (AF286039)	94	70mos_0d_F2	<i>Desulfobacterium autotrophicum</i> (AF418177)	95
7mos_0s_G3	Unc <i>Cellulophaga</i> , Japan Trench (AB189355)	97	7mos_0s_C7	<i>Desulfofrigus oceanense</i> (AF099064)	99
7mos_0d_B10	Unc, Cascadia Margin (AJ535256)	93	7mos_0d_B12	<i>Desulfofrigus oceanense</i> (AF099064)	94
7mos_0d_C10	Unc, Cascadia Margin (AJ535256)	95	7mos_0d_A7	<i>Desulfohalobium toluolica</i> (X70953)	99
7mos_0s_D6	Unc, Cascadia Margin (AJ535256)	95	70mos_0d_F6	Unc, polluted harbour (DQ394986)	96
7mos_10s_C5	Unc, Cascadia Margin (AJ535256)	96	70mos_0d_G3	Unc, mangrove soil (DQ811826)	93
7mos_10s_E3	Unc, polluted harbour sediments (DQ395063)	93	70mos_10d_D8	Unc, Haakon Mosby mud volcano (AJ704684)	98
7mos_0d_G9	Unc, Cascadia Margin (AJ535256)	91	7mos_10d_B8	Unc, Sagami Bay (AB188782)	97
7mos_20s_G6	Unc, fish farm sediments (AY500050)	97	7mos_10s_G2	Unc, Sagami Bay (AB188771)	95
7mos_10d_A8	Unc, echinoid gut (AY222319)	98	70mos_0d_C9	Unc, Haakon mud volcano (AJ704687)	99
7mos_0d_B8	Unc, deep sea coral (DQ395523)	98	7mos_0d_D9	Unc, Arctic sediments (AF099058)	98
7mos_0s_E3	Unc <i>Cytophaga</i> , Sagami Bay (AB188791)	98	7mos_0s_D2	Unc <i>Desulfohalobium</i> , Antarctic (AY177802)	98
7mos_10s_E5	Unc, mangrove sediments (AM176862)	92	7mos_10s_G1	Unc, Japan Sea, deep (AB121098)	98
7mos_0s_C2	Unc, snail epibiont (AY531558)	95	70mos_0s_E7	Unc, Haakon mud volcano (AJ704669)	95
7mos_0s_B4	<i>Anaerophaga</i> sp. HS1 (DQ517535)	87	70mos_0s_D8	Unc, Cascadia margin sediments (AJ535243)	94
7mos_0s_E2	Unc <i>Cytophaga</i> , Japan Trench (AB015260)	98	7mos_0d_B11	Unc, Arctic sediments (AF099055)	99
7mos_0s_H8	Unc, polychaete epibiont (AY548988)	93	70mos_20s_D6	<i>Desulphuromonas svalbardensis</i> (AY835391)	99
Firmicutes	Unc, Arctic sediments (EU050907)	96	7mos_10d_A12	<i>Desulphuromonas</i> , oily soil (EU328027)	87
33mos_0s_F7	Unc <i>Sphingobacterium</i> , cave (AY510256)	90	33mos_20s_A7	Unc <i>Desulphuromonas</i> , polluted harbour (DQ394974)	96
33mos_0s_A2	Unc, coral surfaces (EF123524)	88	7mos_10s_D5	Unc, deep sea sediments (AJ241015)	91
33mos_0s_F3	Unc, deep sea sediments (AY588963)	92	7mos_10s_B2	Unc, North Sea sediments (DQ351760)	98
33mos_0s_A4	Unc, anaerobic digester (EU888001)	93	70mos_20s_A10	Unc, estuary (EF999364)	94
33mos_0s_A3	Unc, human gut (DQ800999)	90	70mos_20s_H1	Unc, marine sediments (EU437468)	93
7mos_10s_E4	Unc, bovine rumen fluid (AF463535)	89	70mos_20s_C8	Unc, Bering Sea (EU925909)	93
7mos_10s_D1	Unc, grassland soils (Y07574)	96	70mos_20s_A2	Unc, Haakon Mosby mud volcano (AJ704690)	96
	Unc, marine sediments (EU374022)	99	7mos_10s_A4	Unc, Arctic sediments (EU050852)	99
				<i>Bdellovibrio</i> sp. JS2 (AF084856)	92

Table 4. cont.

Group ribotype ID ^a	Representative closest relative (Acc. no.)	% Similarity	Group ribotype ID ^a	Representative closest relative (Acc. no.)	% Similarity
<i>Proteobacteria</i>					
Gamma group					
70mos_20s_B4	Unc, Arctic Ocean sediments (EU287183)	99	Proteobacteria		
70mos_20s_E6	Unc, Bering Sea (EU734942)	97	Epsilon group		
70mos_20s_G11	Unc, Arctic Ocean sediments (EU287226)	97	70mos_0s_D6	Unc, whale bones (AY922189)	94
70mos_20s_E1	Unc, Arctic Ocean sediments (EU287163)	99	70mos_0s_A2	Unc <i>Sulphurimonas</i> , Japan Trench (AB189362)	95
70mos_20s_C2	Unc, Arctic Ocean sediments (EU287159)	98	70mos_0s_H1	Unc, whale bones (AY922189)	98
70mos_10d_F10	Unc, ocean crust (EU491259)	92	70mos_0s_F3	Unc, Atlantic Ocean sediment (DQ071101)	97
70mos_10d_B1	Unc, fish farm sediments (AY499687)	99	70mos_0d_G12	Unc, Nankai Trough sediments (AB013263)	98
70mos_0d_G1	Unc, Yellow Sea (EU617745)	95	70mos_0s_F10	Unc, ocean crust (EU491809)	94
70mos_10s_A2	Unc, Pacific Ocean sediments (AY375071)	96	70mos_0s_E8	Unc, Yellow Sea (EU652649)	96
70mos_0s_A1	<i>Maorithyes hadalis</i> symbiont (AB042414)	92	70mos_0s_H9	Unc, Gulf of Mexico sediments (AM745152)	98
70mos_20s_B2	Unc, Bering Sea sediment (EU734971)	97	70mos_10s_F2	Unc, deep sea (AB015531)	99
70mos_0s_F9	Unc, tidal flat sediments (EU623015)	99	70mos_0d_F9	Unc, Gulf of Mexico sediments (DQ521818)	98
70mos_0s_D2	Unc, Kazan Mud Volcano (FJ712524)	97	33mos_0s_H10	Unc, deep sea sediments (AB015582)	98
70mos_0s_G8	Unc, Japan Trench (AB015252)	97	70mos_0s_B6	Unc <i>Sulphurovum</i> , epibiont (EU107475)	97
70mos_10d_G9	Unc, Gulf of Mexico (AM745143)	98	70mos_0s_F2	Unc, Japan Trench (AB189374)	96
70mos_0s_A8	Unc, cold seep sediments (FJ264671)	96	70mos_0s_E5	<i>Sulphurospirillum arcachonense</i> (Y11561)	99
70mos_10d_B10	Unc, Bering Sea (EU925907)	99	<i>Planctomycetes</i>		
70mos_0s_D9	Unc, marine sediments (EU290692)	97	70mos_10s_H3	<i>Rhodopirellula</i> sp. (FJ624356)	95
70mos_0s_D7	Unc, Bering Sea (EU925834)	95	33mos_20s_E1	Unc, deep Indian Ocean (FM214461)	92
70mos_20s_B9	Unc, Wadden Sea (EU707311)	98	33mos_20s_D5	Unc, Antarctic sediments (AF424500)	94
70mos_0s_F7	Unc, contaminated sediments (AM882579)	94	33mos_20s_F2	Unc, Bering Sea sediments (EU735024)	95
<i>Verrucomicrobia</i>			70mos_20s_E8	Unc, Antarctic sediments (AF424482)	93
33mos_20s_F7	Unc, polluted marine sediments (DQ351738)	98	33mos_20s_H1	Unc, deep sea (AB015544)	95
33mos_20s_H7	Unc, Antarctic sediments (AF424509)	94	33mos_20s_C3	Unc, Japan Trench (AB189369)	95
70mos_10d_A7	Unc, fish farm sediments (AY500062)	96	33mos_0s_B10	Unc Lentsphaerales, Nankai Trough (AB240714)	92
33mos_0s_F2	Unc, vent polychaete mucous (AJ441222)	97	70mos_10d_G7	Unc, hydrothermal sediment (EU983151)	93
33mos_20s_E3	Unc, Arctic Ocean sediments (AY028222)	97	70mos_20s_D4	Unc, Antarctic sediments (AF424475)	98
<i>Spirochaetes</i>			33mos_20s_D7	Unc, biofilm reactor (FJ710625)	94
70mos_0d_C1	Unc, sulfidic mud (AF211321)	91	<i>Acidobacteria</i>		
70mos_0d_E9	Unc, anoxic fjord sediments (FJ615155)	93	70mos_20s_A4	Unc, marine sediments (EU373925)	96
33mos_0s_E11	Unc, whalebones (AY922204)	95	33mos_20s_E10	Unc, estuary (DQ395012)	97
70mos_0d_F3	Unc, <i>Spirochaeta litoralis</i> (M88723)	96	70mos_20s_D7	Unc, Arctic Ocean sediments (AJ241004)	95
33mos_0s_F11	Unc, Antarctic sediments (M87055)	96	70mos_10d_C7	Unc, pacific warm pool (AY375095)	90
			70mos_20s_G4	Unc, river sediments (DQ017927)	97
			70mos_10d_G8	Unc, <i>Holophaga</i> Japan Trench (AB189335)	98

a. Representative ribotype IDs correspond to those shown in Figs 4–8. Unc. = uncultured.

fall sediments (ex. only 1% of recovered ribotypes under whale-1820), indicating that this subdivision may be selected against during the initial conditions of high organic carbon loading. This phenomenon is consistent with patterns in diversity reported for marine sediments impacted by fish farming and other aquaculture (Asami *et al.*, 2005; Kawahara *et al.*, 2009). Although this group comprised a relatively minor percentage of the bacterial community initially, the abundance of *Gammaproteobacteria* within and surrounding the whale fall habitat appeared to be dynamic over time. Members of the *Gammaproteobacteria* were distinctly higher under whale-2893 at 33 and 70 months than under whale-1820 at 7 months (7–11% versus 1%, Fig. 3B). These were predominately affiliated with uncultured relatives reported from deep sea and permanently cold sediments, and relatives of free-living and endosymbiotic sulfur oxidizers (Fig. 5, Table 4). While white bacterial mats resembling the large sulfide oxidizing genus *Beeggiatoa* were occasionally observed in small patches adjacent to the whale fall, no sequences belonging to this *Gammaproteobacterial* group were recovered in sediments directly underlying the whale carcass. In general, the diversity of *Gammaproteobacteria* in the sediments underlying the whale fall appeared to be distinct from the microflora colonizing bone surfaces (e.g. *Psychromonas*; see ribotypes listed as 'whalebone' in Fig. 5. S. Goffredi, unpubl. obs.).

Epsilonproteobacteria were found overwhelmingly in association with whale fall sediments, representing 12–28% of recovered ribotypes, as compared with the reference site (< 2%, Figs 3B and 5). Members of the *Epsilonproteobacteria* are rarely recovered from deep sea or permanently cold sediments (Bowman and McCuaig, 2003), but have been previously noted on the bone surfaces of whale falls (~22% of the bacterial community primarily associated with the *Campylobacteraceae*; Tringe *et al.*, 2005). Among many unclassified members of this subdivision, whale fall-associated ribotypes recovered in this study were most closely related to uncultured *Sulphurimonas* sequences from the Japan Trench (> 95% similarity; family *Thiovulgaceae*, Campbell *et al.*, 2006), as well as *Arcobacter* and *Sulphurospirillum* (93% and 99% similarity respectively, family *Campylobacteraceae*, Fig. 6, Table 4). Ribotypes related to this group are abundant in marine sediments impacted by fish farming (Bissett *et al.*, 2006; Kawahara *et al.*, 2009) and are common in methane seep sediments (Inagaki *et al.*, 2002; Teske *et al.*, 2002; Harrison *et al.*, 2009). Many of the cultured chemoorganotrophic *Epsilonproteobacteria* can oxidize sulfide and other intermediate sulfur compounds, or alternatively gain energy from hydrogenotrophic growth. Specifically, heterotrophic *Sulphurospirillum* species are capable of hydrogen use and have been

observed in diverse environments including freshwater and marine sediments, oil fields and deep sea hydrothermal vents (Finster *et al.*, 1997; Schwartz and Freidrich, 2006). In general, *Epsilonproteobacteria* 16S rRNA phylogeny appears to be well correlated with ecotype and metabolic capability (Campbell *et al.*, 2006), and it is likely that the ribotypes recovered in our study are also capable of sulfur-based metabolism and hydrogen utilization, which may be favoured in the organic-rich whale fall environment.

Sulfate-reduction is one of the dominant pathways for the mineralization of organic matter, particularly in organic-rich marine environments (Jørgensen, 1982; Canfield, 1994). A significant proportion of the organic molecules (e.g. fatty acids and alcohols) and hydrogen generated during fermentation and polymer hydrolysis are ultimately oxidized through sulfate respiration. In sediments underlying both whale falls, a large percentage of the bacterial clone libraries were associated with sulfate-reducing *Deltaproteobacteria* (25–37% of recovered ribotypes), supporting the importance of this guild in the terminal remineralization processes within these carbon-rich seafloor habitats (Treude *et al.*, 2009). Members of the *Deltaproteobacteria* also comprised a large fraction of the bacterial assemblage recovered from reference sediments (22–39%); however, there was a clear difference in the specific community composition between the two environments (Fig. 7). Clades were generally unique to either whale fall or reference sediment, with very little overlap. In reference sediments, *Deltaproteobacteria* within the *Desulphuromonadaceae*, *Syntrophobacteraceae*, various predatory families predominated (Fig. 7A and B). Under the whale fall, members of the sulfate-reducing *Desulfobacteraceae* and *Desulfobulbaceae* were abundant, primarily affiliated with cultured representatives, including *Desulphuromonas*, *Desulfobacterium*, *Desulfofrigus* and *Desulfobacula* (all > 96% similarity; Fig. 7, Table 4). Some of these *Deltaproteobacterial* groups have been found in other permanently cold environments (Sahm *et al.*, 1999) suggesting that the groups recovered in this study may be adapted to the low and stable temperatures of the deep sea. Cultured representatives of many of these sulfate-reducing genera are collectively capable of metabolizing a range of carbon sources, with some (e.g. *Desulforhopalus* and *Desulfobulbus* sp.) also capable of consuming hydrogen during heterotrophic growth (Isaksen and Teske, 1996; Schwartz and Freidrich, 2006). Relatives of both genera were recovered in whale fall sediments and may be important consumers of hydrogen within this environment.

In addition to their role as terminal mineralizers of organic carbon, some of these *Deltaproteobacterial* representatives may be involved in the cycling of intermediate sulfur compounds, in addition to sulfate, within the

whale fall habitat. An exceptional trait of the family *Desulfobulbaceae*, one of the dominant *Deltaproteobacterial* groups recovered from both whale falls, is their capability for sulfur disproportionation, a process that converts sulfur species of intermediate oxidation states to sulfate and sulfide (Finster *et al.*, 1998; Frederiksen and Finster, 2004). Specifically, ribotypes related to the obligate sulfur disproportionator *Desulfocapsa sulfexigens* comprised ~9% of the bacterial assemblage under whale-2893 (70mos_0s_D8, 93% similar; Finster *et al.*, 1998). Sulfur disproportionation is more energetically favourable when oxidized metals are available as sulfide scavengers, maintaining environmental sulfide concentrations below 1 mM (Thamdrup *et al.*, 1993; Böttcher and Thamdrup, 2001). Initial assessment of the geochemical conditions at whale-2893 harbouring *Desulfocapsa*-affiliated ribotypes suggest that disproportionation may be favoured in this environment. While confirmation of this process in whale fall sediments requires further investigation, the high levels of total iron and zinc (~20 000 and 150 p.p.m. respectively, S.K. Goffredi, unpubl. obs.) and unexpectedly low sediment pore fluid sulfide levels (< 60 µM, Goffredi *et al.*, 2008), likely derived from metal sulfide precipitation and/or biological sulfide oxidation (e.g. *Epsilonproteobacteria* discussed previously), are consistent with marine environments previously shown to support sulfur disproportionation (Jørgensen, 1990; Canfield and Thamdrup, 1996).

Conclusion

The majority of deep sea microbial communities experience very low organic carbon availability; however, within localized whale fall habitats, microorganisms are exposed to organic carbon levels significantly higher than that sourced by primary production in surface waters. As such, whale falls provide a unique ecological niche and have a dynamic impact on microbial diversity and activity in associated sediments over time. Whale fall-associated microorganisms encompass a myriad of microbial taxa that, despite the deep and permanently cold environment, appear capable of rapidly responding to and degrading large inputs of organic carbon, both from the whale fall as well as from the abundant macrofauna that colonize and enhance bioturbation within and surrounding the site. Bacteria that were commonly enriched in the two whale fall habitats included members of the *Bacteroidetes*, *Firmicutes*, *Epsilonproteobacteria* and the *Deltaproteobacteria* families *Desulfobacteraceae* and *Desulfobulbaceae*, suggesting that eutrophication in the deep sea can result in significant shifts in bacterial phyla and lower level taxa. A variety of carbon compounds and available hydrogen are thought to support these specialized microbial groups and allow for the

simultaneous occurrence of fermentation, sulfate reduction and methanogenesis in the shallow near seafloor sediments. Initially, the whale fall caused large perturbations in the natural environmental conditions, and resulted in a decrease in overall diversity in comparison with the surrounding seafloor sedimentary communities. Over time, the sphere of influence of whale fall-derived nutrients expanded laterally, with changes in bacterial diversity and increases in TOC observed up to 20 m away. At 33–70 months, variations in the adjacent reference community included a greater proportion of *Bacteroidetes* and a general decrease in total diversity, suggesting that temporal effects of large organic inputs to the deep sea occur within a few years and likely extend beyond the time scales of this study.

Experimental procedures

Site description and sampling

Sediment samples were obtained from two whale falls at 30 km distance from each other in Monterey Canyon, CA. One whale fall was originally discovered in February 2002 ('whale-2893', 36.613°N/122.434°W, 2893 m depth; Goffredi *et al.*, 2004) and the other was intentionally implanted on the seafloor in March 2006 ('whale-1820', 36.708°N/122.105°W, 1820 m depth; Braby *et al.*, 2007). At the time of discovery, it was estimated that whale-2893 was within a year of deposition on the seafloor (Goffredi *et al.*, 2004). Sediment samples were collected by means of the remotely operated vehicle *Tiburon* (owned and operated by the Monterey Bay Aquarium Research Institute, Fig. 1). Sampling occurred at 7 months following implantation of whale-1820 (October 2006, Tiburon dive #1048) and at 33 and 70 months (November 2004 – Tiburon dive #769 and December 2007 – Tiburon dive #1162 respectively) following discovery of whale-2893. Sediments were sampled directly beneath each whale fall ('0 m' distance) and at a reference site 10–20 m distance from the centre of the carcass, beyond visible signs of influence of each whale fall (Fig. 1). Sediment samples were collected via push core and stored at 4°C until processed (usually shipboard within 0–5 h). From the push core sleeve, samples were extruded upwards in 3-cm-thick sections and subsampled using cut-off 10cc syringes. Shallow (0–3 cm) and deep (6–9, 9–12 or 12–15 cm, depending on the core) horizons were sampled to allow depth comparisons (Table 1). Sediment samples were subdivided for nucleic acid and chemical analyses (~15 g of sediment frozen at –80°C), enzyme assays (~1 g flash frozen in liquid nitrogen) and for immediate collection of pore fluids (described below).

Nucleic acid extraction

For 16S rRNA analysis, total nucleic acids were extracted from ~3-cm-depth sediment intervals. Cell lysis and DNA extraction from ~0.5 g (wet weight) of sediment was conducted using the Ultra-Clean Soil DNA kit (MoBio Labo-

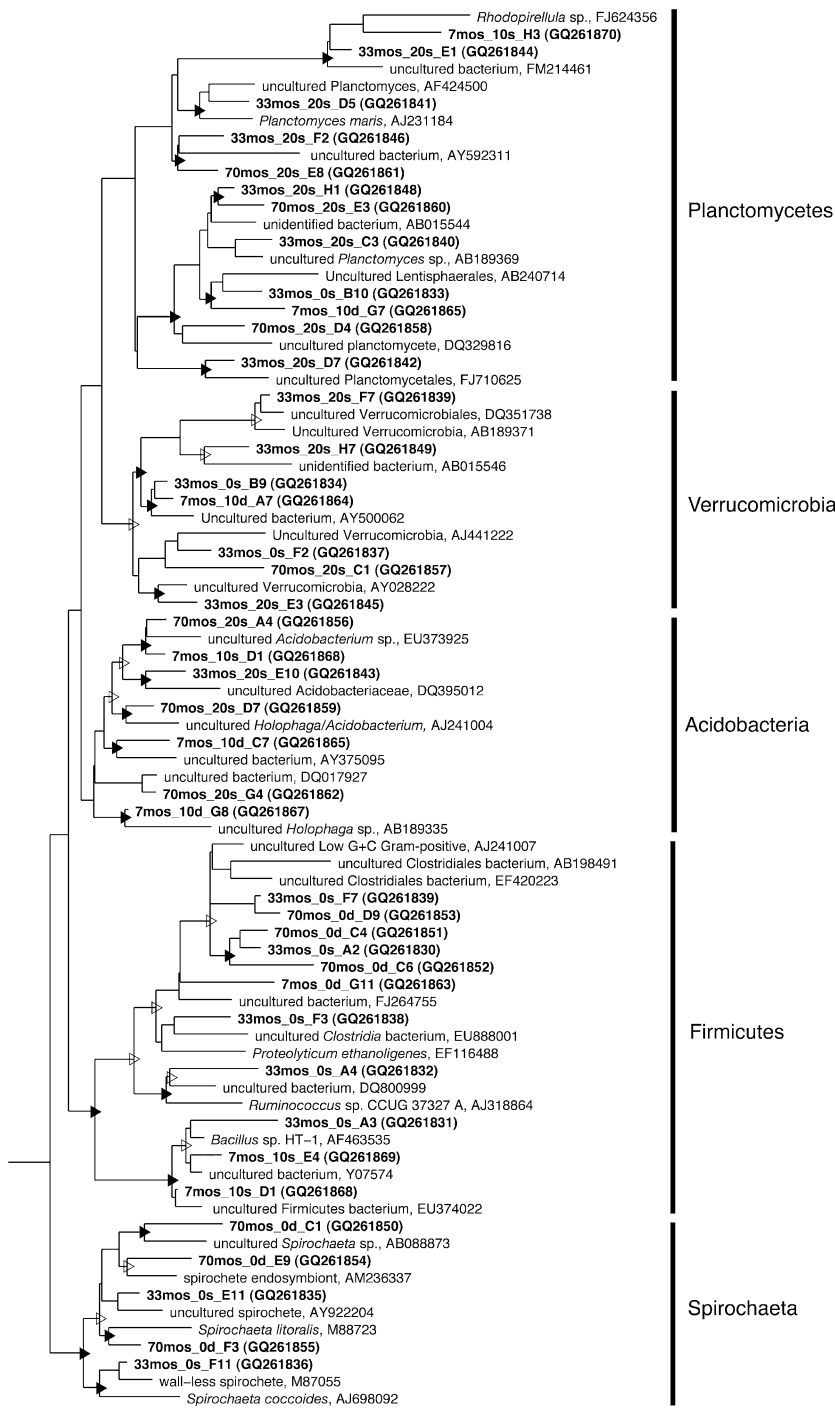


Fig. 8. Phylogenetic relationships of *Planctomycetes*, *Verrucomicrobia*, *Acidobacteria*, *Firmicutes* and *Spirochaetes* associated with whale falls in Monterey Canyon (CA), based on sequence divergence within the 16S rRNA gene, relative to selected cultured and environmental sequences in public databases. *Thermotoga maritima* (AJ401021) was used as an outgroup (not shown). Symbols next to nodes correspond to bootstrap values based on neighbor-joining distance and 5000 replicates (open symbol = 60–90%, closed symbol = 90+% bootstrap support). For library designations, refer to Table 2. Taxon designations correspond to those listed in Table 4.

ratories, Carlsbad, CA). The protocol was modified by two initial 5 min incubations at 70°C followed by bead beating (using a Bio101 Fastprep machine, speed 5.5 for 45 s, Thermo Electron Corporation, Waltham, MA). The remainder of the extraction procedure was carried out according to the manufacturer's instructions, with the exception of a 4°C incubation in IRS solution between solutions S2 and S3.

Polymerase chain reaction, clone library construction and sequencing

SSU rRNA (16S) was amplified by polymerase chain reaction (PCR) from extracted DNA. The PCR mixtures (25 µl) contained 0.4 µM 16S rRNA primers, 27F and 1492R (Lane, 1991), and 2.5 µl of 10 X PCR buffer (containing 2 mM

MgCl₂), 0.2 mM each deoxynucleotide triphosphates and 0.025 U of Eppendorf HotMaster Taq (Westbury, NY). Thermal cycling conditions included 45 s each of denaturation at 94°C, annealing at 54°C, elongation at 72°C (25–30 cycles) and a final 6 min of elongation at 72°C. The PCR products were pooled and cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Clone libraries of PCR amplified bacterial 16S rRNA genes were constructed from one to two depth horizons from each core, with 42–81 clones analysed for each library (Table 1). Library designations are 'collection time (in months)_ distance from whale (0, 10, or 20 m) followed by depth horizon ['s' for shallow (0–3 cm) or 'd' for deep (6–15 cm)]_clone library position'. For example, '7mos_0 s_E4' indicates a clone ('E4') recovered from sediments immediately underlying the whale fall at the shallow (0–3 cm) depth horizon ('0s') at 7 months time ('7mos') following deposition on the seafloor. Inserts from clones were amplified with M13F/R primers, Taq polymerase from Promega (Madison, WI) and thermal cycling conditions of an 8 min initial denaturation, followed by 30 s each of denaturation at 94°C, annealing at 54°C, elongation at 72°C (30 cycles), and a final 6 min of elongation at 72°C. M13 amplicons were cleaned prior to sequencing with MultiScreen HTS plates (Millipore Corporation, Bedford, MA). Sequencing reactions were performed using the Genome Laboratory DTCS Quick Start Kit and run on a CEQ 8800 Genetic Analysis System (Beckman Coulter, Fullerton, CA). Representative ribotypes (based on 97% sequence similarity) were selected for nearly full-length sequencing, using an additional 519F internal primer, for complete overlap within the 16S rRNA region. Representative sequences acquired during this study were deposited in GenBank (Table 4, accession numbers GQ261743–GQ261870).

Sequence homology searches within GenBank were performed using blastn (NCBI website). The bacterial nomenclature found in the second edition of Bergey's Manual of Systematic Bacteriology (<http://www.bergeys.org>) was used. Sequences were compiled and aligned using ARB Fast Aligner (Ludwig *et al.*, 2004). Initial neighbor-joining trees of partial sequences recovered in our study were made via parsimony insertion of partial sequences (500–600 bp) within a tree of 'near full length' sequences (~1465 bp, for examples, see Figs. 4A and 6A). Additional sequences were obtained from GenBank and Greengenes and compiled and aligned with our 16S rRNA sequences using the ARB automated alignment tool with subsequent manual refinements (Ludwig *et al.*, 2004). Greengenes was also used to check for potential chimeras (DeSantis *et al.*, 2006), of which 15 were identified and removed from our data set. For near full-length representatives and closest relatives, neighbor-joining analysis was conducted with Olsen distance correction, using 5000 bootstrap replicates to assign confidence levels to nodes (shown if > 60% confidence, Figs 4B–7B and 8).

Statistical analysis of molecular data

The Shannon–Wiener diversity index (H') of species number and evenness was calculated according to the equation $H' = -\sum(p_i)(\ln p_i)$, where p_i was the number of ribotypes in each OTU group, as defined by 97% similarity, divided by the total number of ribotypes in each library. The non-parametric

Chao1 estimator of species richness was calculated using the equation $S_{\text{chao1}} = S_{\text{obs}} + (Q_1^2/2Q_2)$, where S_{obs} was the total number of ribotypes, Q_1 was the number of singleton ribotypes observed only once and Q_2 was the number of ribotypes observed twice (Magurran, 2004). To test for differences between the bacterial communities from each sample, we used the UniFrac computational tool (Lozupone and Knight, 2005). The UniFrac measure of phylogenetic relatedness between two communities was performed using the hierarchical clustering UPGMA (unweighted pair-group method with arithmetic mean) algorithm and a jackknife analysis with 100 permutations to access confidence in nodes of the resulting tree.

Enzyme activity

Protease activity was measured using either the EnzChek Ultra Protease Assay Kit (E33757) or the Protease Assay Kit (E6638, Molecular Probes, Eugene, OR). Reactions included 50 µl BODIPY FL casein substrate (10 µg ml⁻¹) and 50 µl of either 1× digestion buffer (containing 10 mM Tris-HCl, 0.1 mM sodium azide, pH 7.8), as a negative control, or 50 µl prepared sediment sample. Frozen (at -80°C) sediment samples were thawed (only once) and mixed, by vortexing, with 1:1 (w/v) digestion buffer. This slurry was allowed to sit on ice for 15 min before centrifugation at 6500 g for 2 min. Quadruplicate aliquots of the resulting supernatant were measured for protease activity. No loss of activity resulted from centrifugation (data not shown). Sonication did not result in higher measures of activity, suggesting that membrane bound enzymes, if present, were liberated during the cold digestion. Negative controls included samples boiled for 20 min. A commercially available non-specific protease isolated from *Streptomyces griseus* (Sigma, P8811) was used to generate standard curves (0.05–0.2 U ml⁻¹). Enzyme activity was measured in U g⁻¹ wet weight, with 1 U equal to the amount of enzyme necessary to liberate 1 µmol casein min⁻¹. Reactions were incubated in darkness at room temperature and fluorescence intensity was measured using a CytoFluor4000 microplate reader (PerSeptive Biosystems) set for excitation at 485 ± 20 nm and emission detection at 530 ± 25 nm, over a period of 0–17 h (Fig. 3, inset). Activity plateaued within ~3 h, thus most measurements were made during this time frame of observed linear increase in activity. Fluorescent reference standards, including 1 mM BODIPY FL propionic acid and a fluorescein component dissolved in 0.1 M NaOH (Molecular Probes Reference Dye Sampler Kit, R14782) were used to check for day-to-day variation in the plate reader and reagents, as well as to calculate BODIPY FL dye equivalents.

Sediment chemistry

Sediments were collected as described in Goffredi and colleagues (2008). Briefly, for sediment pore fluid collection, sediment samples were centrifuged (1380 g for 15 min) in cut-off, stoppered 10-cc syringes. Pore fluid samples were transferred to gas-tight syringes via a needle through the side-wall of the 10-cc syringe, minimizing oxygen exposure and sulfide oxidation (for accurate sulfate measures), and

preserved immediately in 0.5 M barium chloride (1:1). Sulfate in the pore fluids was determined by turbidometry using a spectrophotometer (Gieskes *et al.*, 1991). The turbidity of a given reaction was measured at 420 nm and expressed in units derived from a standard curve prepared by use of barium sulfate suspensions (0–28 mM). Total organic carbon was measured in bulk sediments as described in Goffredi and colleagues (2008). Sediment total metal concentrations were determined via inductively coupled plasma mass spectrometry. Approximately 0.5 g of frozen sediment was transferred to a digitube (SCP Science, Champlain, NY) and extracted for metals using hot (85°C) nitric acid digestion. Metal concentrations were surveyed using the semiquantitative analysis mode with an HP 4500 series ICP-MS. Nebulization was effected with a flow of 1.3 lpm argon using a Babbington type nebulizer in a pyrex Scott-type spray chamber. The argon plasma power was 1200 W with a flow of 15 l min⁻¹ and an auxiliary flow of 1.1 lpm. A solution containing lithium, yttrium, cerium and thallium at 10 µg l⁻¹ served as an external calibration. Chemstation software was used to estimate concentrations for other elements by accounting for differences in ionization energy and isotopic abundance of the reference elements. We estimate achieving accuracy within a factor of two via this method. Samples were matrix matched to the reference solution (2% nitric acid). Samples found to have elements at high concentration were diluted quantitatively in 2% nitric acid and re-measured to minimize matrix effects.

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