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Evolutionary innovation: a bone-eating marine symbiosis

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Summary

Symbiotic associations between microbes and invertebrates have resulted in some of the most unusual physiological and morphological adaptations that have evolved in the animal world. We document a new symbiosis between marine polychaetes of the genus *Osedax* and members of the bacterial group Oceanospirillales, known for heterotrophic degradation of complex organic compounds. These organisms were discovered living on the carcass of a grey whale at 2891 m depth in Monterey Canyon, off the coast of California. The mouthless and gutless worms are unique in their morphological specializations used to obtain nutrition from decomposing mammalian bones. Adult worms possess elaborate posterior root-like extensions that invade whale bone and contain bacteriocytes that house intracellular symbionts. Stable isotopes and fatty acid analyses suggest that these unusual endosymbionts are likely responsible for the nutrition of this locally abundant and reproductively prolific deep-sea worm.

Introduction

The evolution of a number of animal groups has been influenced significantly by the symbiotic condition, including the evolution of novel structures, organs and even biochemical pathways, resulting in both a departure from free-living existence and sometimes very unique ecological strategies. Endosymbioses involving invertebrates and bacteria play critical roles in sustaining the high productivity of many marine environments, including deep-sea hydrothermal vents, cold seeps, anoxic basins and other sites of organic enrichment (reviewed in Van Dover, 2000; Smith and Baco, 2003). Exploitation of energy in these atypical deep-sea environments often requires metabolic and physiological capabilities that cannot be accomplished by most metazoans alone. Symbiotic microbes typically provide a nutritional bridge between high-energy compounds and the animals that exploit them. Here we examined an unusual association between newly discovered polychaete annelids and bacterial endosymbionts that allows for the exploitation of decaying whale bones.

In February 2002, a dense assemblage of deep-sea invertebrates was discovered in association with the carcass of a juvenile grey whale (*Eschrichtius robustus*) in the axis of Monterey Canyon, California (Goffredi *et al.*, 2004). The carcass, found at 2891 m depth (36°36.8′N; 122°26.0′W), is one of the deepest large whalefalls discovered to date. Such massive pulses of organic material serve as habitat islands for unique deep-sea benthic communities that can persist from several decades to hundreds of years (Brunn, 1957; Smith and Baco, 2003). The Monterey whalefall community was particularly noteworthy for its abundance of unusual polychaetes, including a new polychaete genus *Osedax*, the dominant animal occupying the surfaces of exposed bones (Rouse *et al.*, 2004). During visits to the carcass in February and October 2002, one species, *Osedax rubiplumus* (Rouse *et al.*, 2004), occurred in large numbers on highly calcified bones, including ribs and skull (Fig. 1A). Their density was high, rivalling previous estimates of single-species densities for other taxa on whalebones (Van Dover, 2000; Smith and Baco, 2003). During subsequent visits to this carcass in August 2003 and January 2004, we collected a second species, *Osedax frankpressi* (Fig. 1B; Rouse *et al.*, 2004), that had replaced *O. rubiplumus* as the dominant occupant of the whale bones.

Morphological and molecular evidence reveal that the genus *Osedax* is part of the polychaete family Siboglinidae (Rouse *et al.*, 2004). Like other siboglinid worms, including vestimentiferans and pogonophorans, *Osedax* lack a mouth and functional gut. Unlike vestimentiferans and pogonophorans, however, *Osedax* also lack an obvi-

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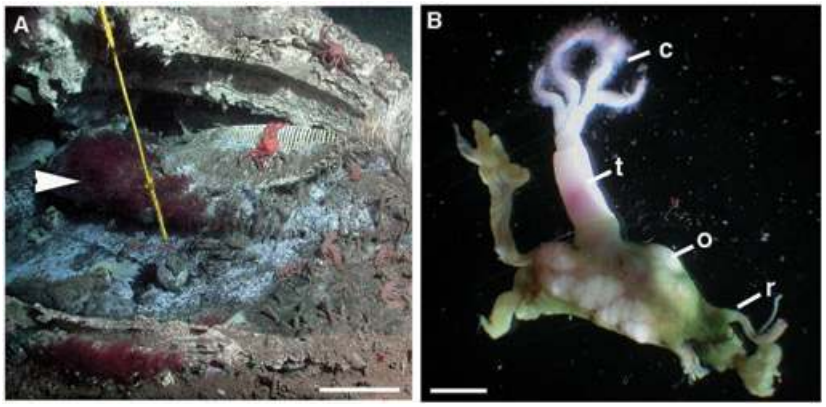


Fig. 1. *Osedax* spp. anatomy.
A. Whale skull showing abundant worms (*Osedax rubiplumus*, arrowhead) on jaw bone. Scale bar, 0.5 m.
B. *Osedax frankpressi* showing crown (c), trunk (t), ovisac (o) and roots (r). Scale bar, 2.5 mm.

ous trophosome, a highly vascularized internal organ that houses endosymbionts in all other siboglinids. Instead, female *Osedax* possess a large posterior ovisac covered by a sheath of green-coloured tissue that intricately branches into a vascularized 'root' system and invades the bone marrow (Fig. 1B). This report describes the novel association between bacteria housed within the root tissue of the genus *Osedax* that allows for exploitation of decomposing mammalian bones on the sea floor. This article focuses on the *O. frankpressi* symbiosis but compares, when possible, similarities and differences with the symbiotic association observed in *O. rubiplumus*.

Results and discussion

Osedax frankpressi possesses unique, highly vascularized roots that originate from the posterior part of a large eggsac (ovisac). Histology, epifluorescence microscopy and transmission electron microscopy of the ovisac and root system of *O. frankpressi* revealed bacteriocytes (20–50 μm maximum dimension; Fig. 2A) that contained large pleomorphic rod-shaped bacteria (2.0 × 6.0 μm diameter; Fig. 2B), many of which appeared to be dividing. Symbionts are enclosed in secondary vacuoles (up to five bacteria per vacuole; Fig. 2A and B). *Osedax rubiplumus* possesses the same ultrastructural morphology (including ovisac and root system), with the only notable difference being the slightly larger size of the bacterial symbionts (3.0 × 8.0 μm diameter; not shown).

The phylogenetic identity of the bacteria within the ovisac and roots from both species of *Osedax* was determined via 16S ribosomal DNA sequences (Fig. 3; Table 1). Symbiont phylotypes differed greatly from all other known chemoautotrophic symbionts found in siboglinid worms.

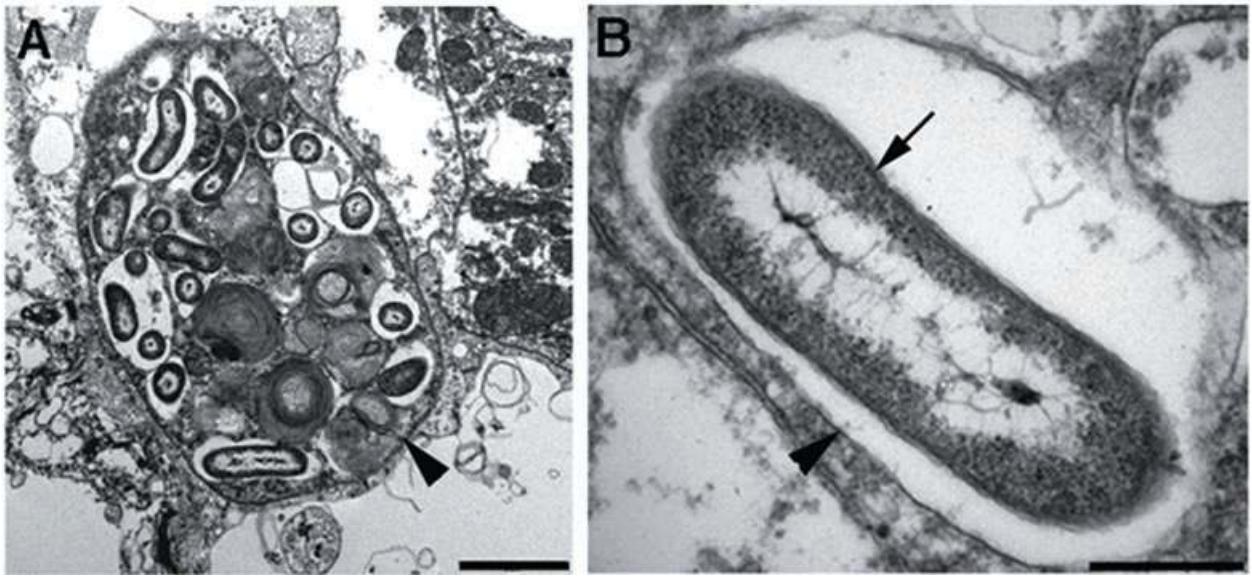


Fig. 2. *Osedax frankpressi* symbionts.
A. Transmission electron micrograph of bacteriocyte (arrowhead), containing bacterial symbionts within the ovisac. Scale bar, 2 μm.
B. Transmission electron micrograph of bacterial symbiont within secondary vacuoles (arrowhead). Note presence of Gram-negative double membrane (arrow). Scale bar, 0.5 μm.

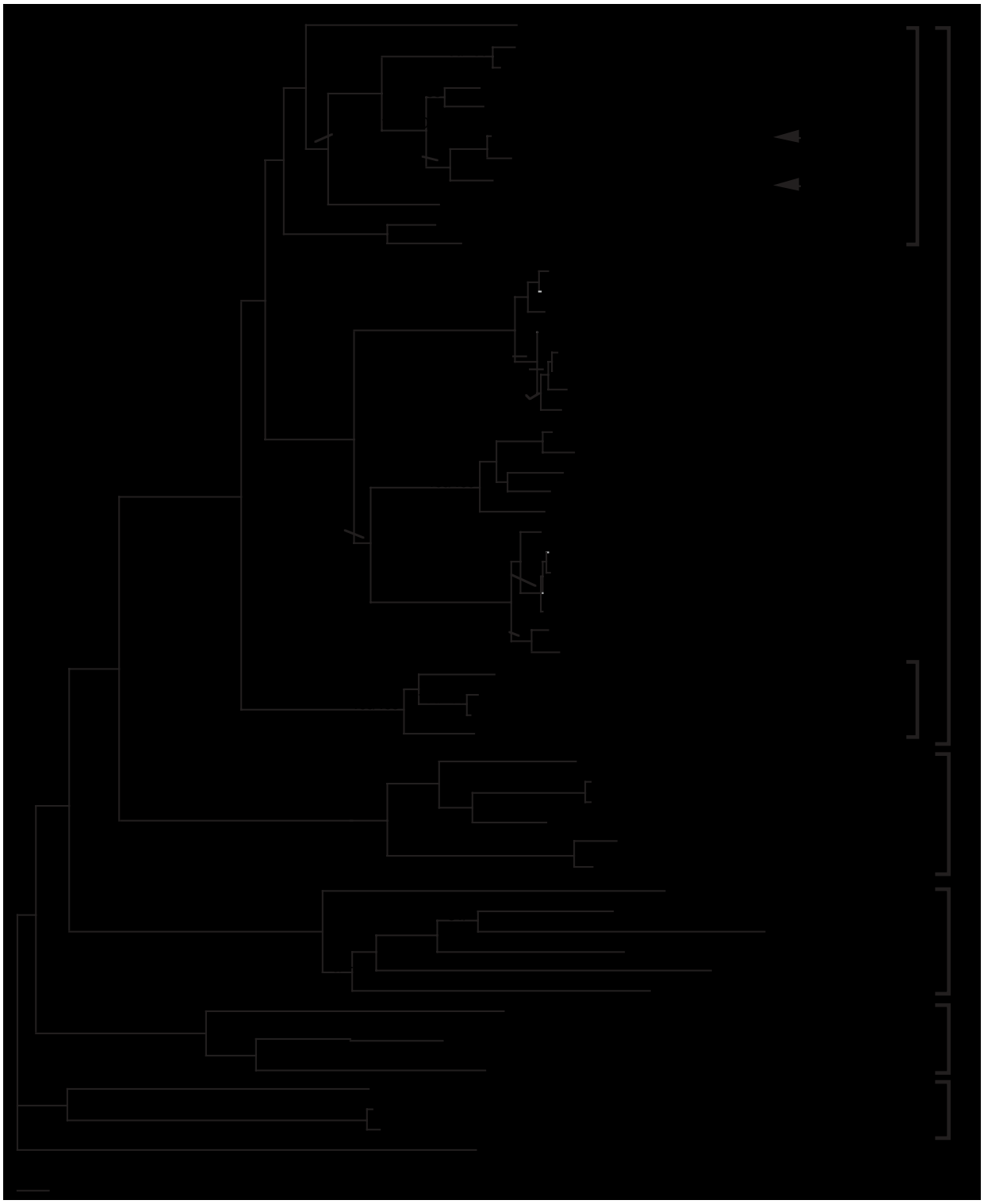


Fig. 3. Phylogenetic relationships, based on 16S rRNA (~1500 bp), of *Osedax* endosymbionts (arrowheads), whalefall-associated microflora and bacteria recovered from enrichment experiments (all in bold). A neighbour-joining tree using Kimura 2-parameter distances is shown. Ribotypes recovered are referenced in Table 1. 'enrich' indicates ribotypes recovered from symbiont enrichment attempts (from ovisac/root homogenates) using a variety of substrates, including fish oil, collagen, cholesterol and fatty acids supplements. The numbers at the nodes represent bootstrap values (as percentage) from both neighbour-joining (first number) and parsimony (second number) methods obtained from 1000 and 100 replicate samplings respectively. '--' indicates that no bootstrap value was available. GenBank accession numbers for sequences acquired during this study are AY548975–AY549004, AY577895–AY577900. Additional sequences were obtained from GenBank as noted.

Table 1. Bacterial ribosomal 16S rDNA clone library results for *Osedax* spp. tissues and whale samples.

Microbial group Representative (GenBank No.)	*Osedax frankpressi* ovisac/roots	*Osedax rubiplumus* ovisac/roots	Whale bone
γ-Proteobacteria	42	87	23
Osedax_sym1 (AY549004)	42	–	–
Osedax_sym2 (AY549005)	–	87	11
C3H1 (AY577900)	–	–	6
C3F9 (AY548975)	–	–	9
ε-Proteobacteria	2	10	56
C3A6 (AY548993)	1	–	22
C3F4 (AY548994)	1	–	4
C15A1 (AY548996)	–	–	13
C3E4 (AY548987)	–	–	16
Other	–	10	1
Cytophaga-Bacteroides	0	4	20
C3D9 (AY548988)	–	–	3
C13A5 (AY548999)	–	4	–
C15B1 (AY548995)	–	–	1
C15C1 (AY548974)	–	–	10
Other	–	–	6
Fusobacteria	2	2	52
C3C8 (AY548984)	2	2	46
C3E6 (AY548989)	–	–	1
Other	–	–	5
Firmicutes	0	4	8
C13A3 (AY549001)	–	4	–
C3C9 (AY548990)			8
Unknown/other	1		18
Total clones sequenced	47	107	180

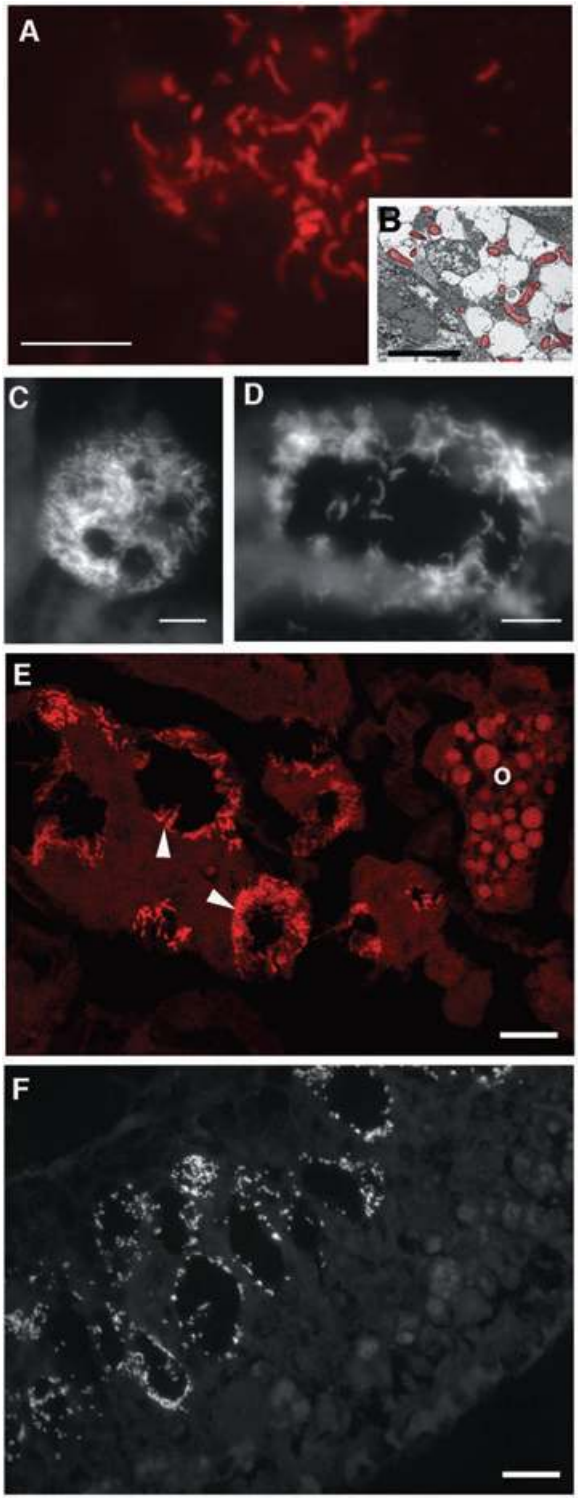
Representative ribotypes listed for each library type correspond to a representative in Fig. 3, unless specified as 'unknown' or 'other'.

The dominant 16S phylotype (*Osedax*_sym1; GenBank AY549004) amplified and cloned from *O. frankpressi* comprised 89% of 47 recovered sequences. *Osedax rubiplumus* possessed a related (~2% divergence in 16S rRNA from *Osedax*_sym1) dominant (81% of 107 sequence types) phylotype (designated *Osedax*_sym2; GenBank AY549005). Phylogenetic analyses (distance and parsimony methods) placed these *Osedax* microbes within a well-supported clade of γ-Proteobacteria (Fig. 3) that contains heterotrophic members of the Oceanospirillales. The hydrocarbon-degrading *Neptunomonas naphthovorans* (GenBank AF053734; Hedlund *et al*., 1999) is the closest cultured relative (93–94% 16S sequence similarity) to the *Osedax* symbionts, and a free-living γ-proteobacterium (UMB6E; GenBank AF505730) isolated from Boston Harbor is the closest environmental relative (96–97% 16S sequence similarity). Other microbial phylotypes associated with the ovisac and root tissues, but in much lower abundance in the *Osedax* spp. clone libraries, included ε-Proteobacteria (4–9%), *Cytophaga-Bacteroides* (0–4%) and the Fusobacteria (2–4%; Fig. 3, Table 1).

Attempts to culture the *Osedax* endosymbiont and characterize the cultivable bacterial community were made via growth of root homogenates on enrichments of cod liver oil, fatty acid supplements, cholesterol and collagen. Ribotypes isolated in this manner included members of the *Psychromonas* and *Pseudoalteromononas* genera (Fig. 3).

To determine which of these microbial components might be specific to the worms, we examined 16S sequences ($n = 180$) from bone samples that appeared to be free of *Osedax* tissues. A diverse microflora was recovered (Fig. 3, Table 1), including: ε-Proteobacteria (31% of the clones), Fusobacteria (29%), *Cytophaga-Bacteroides* (11%) and γ-Proteobacteria (other than members of the Oceanospirillales, 5%). Representatives of these groups recovered in lower abundance from the ovisac and roots probably resulted from contamination of these tissues during removal from the bone matrix. This was confirmed by fluorescent *in situ* hybridization (FISH) microscopy (described below) in which, for example, a *Cytophaga*-specific probe (CF319) did not hit any targets within the *Osedax* root tissue. Phylotypes closely related to the *Osedax*_sym phylotypes (< 2.8% difference in 16S sequence) were also found in the bone (3% of the bone libraries), and may have resulted from *Osedax* root tissue present within the bone. Despite the potential for cross-contamination during our dissections, marked enrichment of the *Osedax*_sym1 and *Osedax*_sym2 phylotypes in root tissues of *O. frankpressi* and *O rubiplumus*, respectively, compared with bone samples, suggests that these phylotypes are the endosymbionts.

To test this hypothesis we conducted FISH microscopy with an oligonucleotide probe (*sym435_I*) targeted against a specific region of the *O. frankpressi* bacterial phylotype (Fig. 4). The *sym435_I* probe hybridized strongly with bacteria that densely populated the ovisac and root tissues and appeared to be concentrated in bacteriocytes (Fig. 4C–F), confirming their intracellular location observed via transmission electron microscopy (TEM) (Figs 2A and 4B). The bacteria were mostly concentrated in a distinct zone along the inner margins of the root tissue (Fig. 4F) and within the ovisac itself, immediately adjacent to developing oocytes (Fig. 4E).



This dense internal population of bacteria coupled with the lack of a gut suggested the existence of a nutritional endosymbiosis in these worms. Support for this hypothesis was observed in bulk stable carbon and nitrogen isotopic values. The $\delta^{13}C$ values for *O. frankpressi* tissues [$-12.5 \pm 0.8‰$ for symbiont-free crown/trunk ($n = 4$) and $-13.5 \pm 0.2‰$ for symbiont-containing ovisac/root tissue ($n = 9$)] were similar to values observed for whale bones ($-14.2 \pm 0.1‰$, $n = 3$), both in this study and in others on modern and fossil whale bones (Stott *et al.*, 1997), suggesting a potentially heterotrophic reliance on the bone for nutrition (DeNiro and Epstein, 1978). Similarly, a 3–4‰ offset in $\delta^{15}N$ values between the whale bone ($12.8 \pm 0.3‰$) and *O. frankpressi* tissues ($16.0 \pm 0.03‰$ and $16.6 \pm 0.4‰$ for crown/trunk and ovisac/root respectively) supports a trophic enrichment from the nitrogen-rich whale bone (8.8% nitrogen by weight) to the worm symbionts (DeNiro and Epstein, 1981). Heterotrophy, which is a distinct possibility for the *Osedax* symbionts (as all close relatives are known heterotrophs), was additionally supported by a failure to detect [via polymerase chain reaction (PCR)] the gene that codes for ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBPCo) in the symbiont-containing tissues of *O. frankpressi* (data not shown). RuBPCo is a key microbial enzyme in the autotrophic Calvin Benson cycle, commonly observed in chemoautotrophic siboglinid/bacterial symbioses (reviewed in Fisher, 1990).

Lipid composition analyses supported a trophic interaction between the symbiont and worm host (Jahnke *et al.*, 1995; Jahnke *et al.*, 2004). Extracts of fatty acid methyl esters (FAMEs) were compared from several sample types, including whale flesh, whale bone and *Osedax*

Fig. 4. *Osedax* spp. symbionts. Fluorescent *in situ* hybridization (FISH) microscopy of bacteria within the ovisac and roots of *O. frankpressi* (except panel 1B is TEM of *O. rubiplumus*). All are hybridizations with the *sym435_I* probe labelled with Cy3. All scale bars, 10 μm.
A. Fluorescent *in situ* hybridization (FISH) image showing bacteria within homogenized roots. Images were coloured with Image-Pro Plus (Media Cybernetics, Silver Springs, MD).
B. Transmission electron microscopy (TEM) with false red colour to emphasize symbiont morphology.
C and D. Epifluorescent FISH images of paraffin-embedded sections (6 μm thick) hybridized with the *sym435_I* probe.
E. Confocal FISH image of paraffin-embedded sections showing intact bacteriocytes (arrowheads) within the ovisac, immediately adjacent to developing oocytes (o).
F. CARD-FISH image of paraffin-embedded sections showing placement of bacteriocyte layer 40–50 μm from external surface of root tissue.

Table 2. Fatty acid composition for whale samples and *Osedax* spp. tissues (as percentage of dry weight).

Lipid category	Whale flesh	Whale bone[a]	Whale bone[b]	Symbiont fraction[c]	*Osedax frankpressi* ovisac/root[d]	*Osedax frankpressi* crown/trunk[e]	*Osedax rubiplumus* whole
Fatty acids[f]							
Total C15	**29.1**	**nd**	**5.2**	nd	nd	0.5	0.5
Branched 15:1	**2.3**	**nd**	**0.2**	nd	nd	0.4	nd
Total C16	25.0	26.9	31.7	23.3	30.1	26.4	30.4
Total C17	**12.6**	**7.5**	**3.3**	0.8	0.3	0.3	nd
Branched 17	**10.6**	**4.5**	**0.7**	nd	nd	nd	nd
17:1	**6.2**[g]	**5.7**[g]	**1.6**[g]	0.5	0.3	nd	nd
18:0	**1.9**	**13.0**	**2.5**	1.9	1.1	1.3	1.1
18:1Δ11	4.7	16.6	23.7	**21.8**	**25.8**	**14.3**	**13.0**
20:5ω-3	3.0	2.9	2.1	**20.0**	**8.6**	**15.3**	**16.9**
20:4ω-6	3.2	1.1	0.9	**1.7**	**11.5**	**11.4**	**9.4**
Total ω-3 FAs	6.6	4.0	4.2	**24.1**	**22.3**	**30.7**	**29.6**
NMI[h]	0.9	nd	2.8	**5.1**	**4.5**	**6.1**	**4.9**

a. Bone in which *Osedax* were not observed *in situ*.
b. Bone that contained *Osedax in situ* (removed before analysis).
c. Symbiont-enriched fraction was produced from overlaying homogenized *O. frankpressi* ovisac/root tissue over a 2% Nycodenz density gradient.
d. *Osedax frankpressi* ovisac/roots = symbiont-containing tissue.
e. *Osedax frankpressi* crown/trunk tissue was separated from the ovisac/roots, thus preventing symbiont contamination.
f. Total fatty acid abundance measured 253, 73, 1024, 103, 3365, 5839, 3726 μg per gram dry weight for whale flesh, whale bone[a], whale bone[b], symbiont fraction (no dry weight available), *O. frankpressi* ovisac/root, *O. frankpressi* crown/trunk and *O. rubiplumus* whole animal respectively.
g. 4.1% of flesh and all of whale bone[a] samples as cycloproyl 17:1.
h. Non-methylene interrupted (NMI) 20:2, bond positions Δs (5,13) and (7,11) identified but not inclusive; 22:2 bond positions identified Δs (5,13), (7,13), (5,15), (7,15).
Bold values are intended to illustrate the difference in diversity between bone and *Osedax*/symbiont composition.
nd, not detected or < 0.1%.

crown/trunk (symbiont-free) tissue, ovisac/root (symbiont-containing) tissue and gradient-separated symbiont enrichments (Table 2). There were marked differences in fatty acid composition between whale samples and *Osedax* spp. samples. A large and diverse bacterial signal [mostly C15 and C17 short-chain fatty acids, some (9 wt%) branched] was observed in both the whale flesh and whale bone (Table 2). This signature was not observed for *Osedax* tissues or the symbiont enrichment, confirming the presence of a distinct microflora associated with the whale flesh and bone samples.

Conversely, even though *Osedax* tissues contained 3–6× higher fatty acid levels than bone samples, two of the most abundant FAMEs recovered from the symbiont-containing *Osedax* tissues were the bacteria-specific biomarker vaccenic acid (18:1Δ11) and the long-chain poly unsaturated fatty acid eicosapentaenoic acid (EPA, 20:5ω-3; Table 2). Vaccenic acid [which comprised 22–26 wt% of the *O. frankpressi* symbiont-related samples (ovisac/root and symbiont enrichment) and 14% of the worm host tissue] is a common end-product of fatty acid synthesis in certain microbes (Jackowski *et al.*, 1991). Eicosapentaenoic acid (which comprised 20 wt% of the symbiont enrichment and 9–17 wt% of *Osedax* tissues) is produced by several γ-Proteobacteria species (Nichols *et al.*, 1992; Russel and Nichols, 1999; Metz *et al.*, 2001), including barophilic and psychrophilic bacteria, and is thought to be involved in the maintenance of membrane fluidity in high-pressure, low-temperature environments (Lechevalier, 1977; Delong *et al.*, 1997). The presence of high levels of vaccenic acid in the gutless marine oligochaete *Inanidrilus leukodermatus* has been interpreted in support of a metabolic role of the bacterial endosymbiont in this species (Giere *et al.*, 1991). The presence of these biomarkers within worm tissues and the symbiont enrichment strongly suggests that the endosymbiotic γ-Proteobacteria are responsible for their synthesis, and that these fatty acids are effectively transferred to the worm, which is presumed to be incapable of producing them *de novo*. We believe the worm is getting bacterial fatty acids from the symbionts rather than from free-living microbes in the environment because there is an enormous difference in the diversity and make-up of bone versus symbiont fatty acids. Only the symbiont fatty acid pattern is reflected in host tissues (bold in Table 2).

Additionally, a high storage capability in the form of wax ester production (C34, C36 and C38 wax esters) was observed in *O. frankpressi* (Table 3). Wax esters, a group of compounds composed of C18 fatty acid and alcohol moieties, measured in the *Osedax* host tissue, have the same carbon number and bond positions as the symbiont vaccenic acid observed in the ovisac/roots (data not shown), providing support for the incorporation of this bacterially produced C18 fatty acid into wax esters within the worm. Furthermore, $\delta^{13}C$ values for C18 fatty acids from both the *Osedax* symbiont-free and symbiont-containing tissues (–20.4‰ to –20.6‰; Table 3) closely approximate those obtained for C34 and C36 wax esters

Table 3. Stable carbon isotope composition ($\delta^{13}C‰$) for *Osedax frankpressi* tissues and whalebone samples, including specific fatty acids and wax esters (WXE).

Lipid category	Whale bone[a]	Whale bone[b]	Symbiont fraction[c]	*Osedax frankpressi* ovisac/root[d]	*Osedax frankpressi* crown/trunk[e]
Fatty acids					
18:1Δ11[f]	–24.6	nm	–20.6	nm	–20.4
20:4ω-6[g]	nm	nm	–20.1	nm	–22.8
20:5ω-3[g]	nm	nm	–20.1	nm	–22.8
Wax esters					
C32 WXE	nm	–20.5 (80)	nm	–19.8 (1421)	–20.3 (253)
C34 WXE	nm	–20.2 (149)	nm	–19.5 (2432)	–19.9 (566)
C36 WXE	nm	–20.9 (92)	nm	–19.6 (415)	–20.6 (407)

a. Bone in which *Osedax* were not observed *in situ*.
b. Bone that contained *Osedax in situ* (removed before analysis).
c. Symbiont-enriched fraction was produced from overlaying homogenized *O. frankpressi* ovisac/root tissue over a Nycodenz density gradient.
d. *Osedax frankpressi* ovisac/roots = symbiont-containing tissue.
e. *Osedax frankpressi* crown/trunk tissue was separated from the ovisac/roots, thus preventing symbiont contamination.
f. $\delta^{13}C$ value is Σ18:2 + 18:1.
g. $\delta^{13}C$ value is Σ20:4 + 20:5. $n = 1$ for all fatty acid isotope values shown; however, all values are the average of at least two runs with standard deviations <0.7.
Values listed in parens following WXE $\delta^{13}C$ values are total wax ester biomass as $\mu g\ g^{-1}$.

in the worm (–19.9‰ to –20.6‰; Table 3). These values are different from the isotopic signature of vaccenic acid in the bone samples (–24.6‰; Table 3), providing additional negative evidence for the uptake of fatty acids from the bone, but rather transfer of fatty acids from the symbionts to the worm host.

Additionally, we observed an isotopic difference of up to 7‰ between bulk (–13.5‰) and individual fatty acid values (–17.6‰ to –20.6‰) for *Osedax* symbionts. Although rare, some bacteria do show significant depletion in ^{13}C values between fatty acids and biomass. The most relevant comparison with *Osedax* symbionts is *Shewanella putrefaciens*, another member of the Oceanospirillales, which demonstrated a similar difference in ^{13}C values for fatty acids and biomass of 5–9‰ (Teece *et al*., 1999). Data of this type (both specific lipid and bulk biomass values) are limited, but clearly there are groups of bacteria that differ in fractionation patterns, depending on biochemical pathways and potential fractionation points.

Conclusion

The *Osedax* symbiosis differs markedly from other marine symbioses. The vast majority of bacteria known to form obligate nutritional symbioses with more than 200 marine invertebrate species are autotrophic. One exception may be the cellulolytic bacteria found in wood-boring bivalves within the family Teredinidae; however, the hosts of these symbionts possess functional guts (Distel and Roberts, 1997; Coan *et al*., 2000). The *Osedax* symbionts belong to the Oceanospirillales, a diverse bacterial group that has been successfully cultured in the laboratory and is known for heterotrophic aerobic degradation of complex organic compounds, suggesting a potentially similar strategy for the *Osedax* symbioses. More studies are needed to fully understand the specific nutritional integration between *Osedax* worms and their endosymbionts, as well as the nature of organic carbon utilized by the symbiosis overall. Potential sources include collagen (proteins) and cholesterol (hydrocarbons), the major constituents in bone. Additional studies to investigate these possibilities are ongoing.

The remarkable local abundance, large effective population sizes and reproductive prolificacy of the worm host are testament to the successful nature of this unusual symbiotic arrangement. The recent discovery of this symbiotic system strongly illustrates our vast underestimation of the scope and significance of the process of symbiosis.

Experimental procedures

Specimens

All specimens were collected in Monterey Canyon (36°36.8′N;122°26.0′W) from February 2002 to August 2003 and either frozen at –80°C and transported back to the laboratory on dry ice (for molecular work) or preserved directly for FISH or TEM studies (see specifics below). The symbiont fraction was created by centrifugation of root homogenates through a 2% Nycodenz density gradient.

DNA analyses

The DNEASY kit (Qiagen, Valencia, CA) was used to extract total DNA from frozen tissue, following the manufacturer's protocol. A ~1500 bp fragment of 16S rRNA gene was generated by bacteria-specific 16S rRNA primers (27F, 5′-AGAGTTTGATCCTGGCTCAG-3′; 1492R, 5′-GGTTACCTTGTTACGACTT-3′; Lane, 1991). Polymerase chain reaction products were either cloned (Invitrogen Topo TA cloning technology) or sequenced directly (Applied Biosystems ABI 3100). Bacterial clone libraries were constructed from the

ovisac of three individual worms of *O. frankpressi*, two individuals *O. rubiplumus* and three unique bone samples (data in Table 1). Additional sequence data were obtained from the GenBank database and all sequences were compiled and aligned using ARB Fast Aligner. Phylogenetic analyses, including parsimony (maximum parsimony analysis was performed with the heuristic search option, with tree-bisection reconnection branch swapping algorithm) and neighbour-joining methods (distance matrix calculated with the algorithm of Kimura's two-parameter model), were performed using PAUP*4.0b10 (Swofford, 1998).

Additionally, we tested *Osedax* tissues with conserved PCR primers for Forms I and II of RuBPCo (*cbbL*F, 5′-GACTT CACCAAAGACGACGA-3′; *cbbL*R, 5′-TCGAACTTGATTTC TTTCCA-3′; *cbbM*F, 5′-ATCATCAARCCSAARCTSGGCC TGCGTCCC-3′; *cbbM*R, 5′-MGAGGTGACSGCRCCGTG RCCRGCMCGRTG-3′; Elsaied and Naganuma, 2001) and ATP-dependent citrate lyase (892F, 5′-TGGACMATGGTDGC YGGKGGT; 1204R, 5′-ATAGTTKGGSCCACCTCTTC-3′; Campbell *et al*., 2003), using vestimentiferan symbionts as our positive control for RuBPCo and *Alvinoconcha* symbionts as our control for *aclB*.

Electron microscopy

Samples for TEM were preserved in 2% glutaraldehyde in either 0.1 M cacodylate or 0.1 M phosphate buffer with 0.25 M sucrose (pH 7.8). After buffer rinses, tissues were post-fixed in 1% osmium tetroxide, dehydrated in a graded ethanol series and embedded in Spurr's epoxy resin. Ultrathin sections (80 nm thick) were stained with uranyl acetate and lead citrate and viewed using a Philips CM100 electron microscope.

Fluorescent in situ *hybridization (FISH) microscopy*

Osedax ovisac/root tissue was prepared for *in situ* hybridizations by an initial 3 h preservation at 4°C in phosphate-buffered 4% paraformaldehdye and subsequent transfer to 1× phosphate-buffered saline-ethanol (1:1). Tissue samples were then either homogenized and gently sonicated or embedded in paraffin and sectioned (6 μm thick, on 3-aminopropyltriethyloxysilane-coated slides). If homogenized, ~50 μl of samples were pulled onto a 0.2 μm polycarbonate filter. Filters were rinsed with phosphate-buffered saline-ethanol (1:1), dried and hybridized. If samples were sectioned, paraffin was first removed by three rinses in 100% xylene (10 min), followed by rehydration in a graded ethanol series (10 min each) and a final rinse in 0.2 m HCl (12 min). Hybridization buffers and wash buffers were made as described in Glockner and colleagues (1999), using 35% formamide in the hybridization buffer and 450 mM NaCl in the wash solution (Glockner *et al*., 1999). Our formamide stringency was determined empirically. Signal of our symbiont-specific probe breaks down at >40% formamide, thus we used 35% for maximum stringency (35% was used to accommodate multiple probes during hybridization). Hybridizations were conducted at 46°C for 2–8 h, followed by a 15 min wash at 48°C. Washed filters or thick sections were stained with a dilute 4′6′-diamidino-2-phenylindole (DAPI) solution (5 μg ml^{-1}) for 1 min and examined under epifluorescence microscopy with either a Zeiss Axiophot 2 microscope or a Zeiss 510 Axioplan 2 confocal microscope (excitation at 543 nm, emission >560 nm). Much better signal to background autoflourescence was achieved by using tyramide signalling and a probe labelled with heat stabilized horseradish peroxidase (CARD-FISH), following the protocol of Pernthaler and colleagues (2004).

The Cy3-labelled oligonucleotide probe specific to the *O. frankpressi* endosymbiont was designed from the 16S rRNA gene and designated *sym435_I* (sequence, 5′-CTTTCCTCA CAGCTGAA-3′; *Escerichia coli* positions 435–452). This probe was designed to be within a region (I) of the 16S ribosomal gene known for better *in situ* accessibility by fluorescently labelled oligonucleotides (Behrens *et al*., 2003). Additionally, *sym435_I* was designed to have a T_m of 61°C. The probe, when checked against sequences in GenBank and the Ribosomal Database Project, demonstrated only seven matches, including UMB6E, *Marinobacterium*, *Oceanospirillum* and four uncultured γ-Proteobacteria (found in an oil field, gas pipeline, and in wastewater). A bacterial universal probe set (EUB338, 5′-GCTGCCTCCCGTAGGAGT-3′, EUB338 II, 5′-GCAGCCACCCGTAGGTGT-3′, EUB338 III, 5′-GCTGCCACCCGTAGGTGT-3′, *E. coli* position 338–355) and a γ-Proteobacteria universal probe (Gam42a, 5′-GCCTT CCCACATCGTTT-3′) were used as a positive control (Amann *et al*., 1990; Manz *et al*., 1992). A *Cytophaga*-specific probe (CF319a, 5′-TGGTCCGTGTCTCAGTAC-3′) was used to test for the presence of *Cytophaga*-related bacteria (Manz *et al*., 1992). A complementary probe to EUB338 (NONEUB, 5′-ACTCCTACGGGAGGCAGC-3′) was used as negative control in hybridization experiments (Wallner *et al*., 1993). Additionally, a negative control probe was designed to target *Marinobacter* sp. This probe (marino, 5′-CTTTCCTC CTCGCTGAA-3′) had 3 bp mismatches with the *O. frankpressi* symbiont. Results were negative when the probe designed to target *Marinobacter* sp. was used (data not shown).

Enrichment cultures

Attempts to culture the *Osedax* endosymbiont and characterize the cultivable bacterial community were made via growth of root homogenates on enrichments of cod liver oil, fatty acid supplements, cholesterol and collagen. Comparisons were made between root homogenate enrichments and those attempted with inoculants from decaying whale bones and surrounding sediment. Liquid cultures were made with filtered seawater and one of the following substrates: 2.5 mM cholesterol (with 0.1% Tween 20), collagen (0.5% w/v, 0.1% Tween 20), fatty acids (1 mM) and fish oil (1:1000 v/v) (Smith *et al*., 1993; DSMZ, 2004). Agar plates were made with artificial seawater (30 g Instant Ocean l^{-1}) with 2% agarose and the following substrate levels: 2.5 mM cholesterol (with 0.1% Tween 20), collagen (0.1% w/v), fatty acids (5 mM) and fish oil (1:1000 v/v). Individual colonies (from agar plates) or 0.5 ml of liquid (from liquid cultures) were extracted directly using the Qiagen DNAeasy kit (for direct PCR and sequencing) or preserved in 10% formaldehyde and transferred to 95% ethanol (for subsequent FISH microscopy).

Isotopes

Carbon and nitrogen isotope values of bone and worm bulk materials were determined by continuous-flow isotope ratio mass spectrometry via two methods. (i) Dried samples were combusted using a Eurovector Elemental Analyzer. The resulting N_2 and CO_2 gases were analysed using a Micromass Isoprime mass spectrometer. Routine precision for reference materials was ±0.3‰ and ±0.1‰ for $\delta^{15}N$ and $\delta^{13}C$ respectively. (ii) The $\delta^{13}C$ and $\delta^{15}N$ of biomass samples were determined using a Carlo Erba CHN EA1108 elemental analyser coupled to a Finnigan Delta Plus XL isotope ratio mass spectrometer (EA-IRMS). Before analysis, samples were lyophilized, homogenized and acidified (for 24 h with 1 m phosphoric acid). Precision for biomass analyses was typically ±0.3‰ and ±0.2‰ for $\delta^{15}N$ and $\delta^{13}C$ respectively. Isotopic compositions are reported as delta (δ) values, defined as: $\delta(‰) = 1000 \times [(R_{sample}/R_{standard}) - 1]$ where R is the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratio. Nitrogen and carbon working standards were calibrated via reference standards against the primary isotope ratio standards atmospheric nitrogen (Air) and Vienna PeeDee Belemnite (VPDB) respectively.

Lipid analyses

Lipid extracts were prepared from lyophilized samples using a modified Bligh and Dyer (1959) technique. Phospholipids were precipitated using cold acetone and the fatty acid methyl esters (FAME) prepared by mild alkaline methanolysis. Wax esters and hydrocarbons remaining in the acetone supernatant were separated using thin layer chromatography. Samples were analysed by gas chromatography-mass spectrometry (GCMS) using a DB-5ms column. Abundance was determined from GCMS total ion current and should be considered semi-quantitative. An internal standard (C23 FAME) was added after initial compound identification. Identification was based on mass spectra and retention times of standard compounds. GC-isotope ratio-MS, as previously described, was used to determine compound $\delta^{13}C$ values (Jahnke *et al*., 1995; 2004). For FAME values where baseline separation was not always possible, an isotope value was integrated over sum of peaks (noted in Table 3). The carbon isotopic composition of individual lipids was determined using a Hewlett Packard 6890 GC coupled to a Finnigan Delta Plus XL IRMS (GC-C-IRMS). Precision for compound-specific analysis was typically ±0.5.

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