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Authors for correspondence:

Martin Tresguerres

e-mail: mtresguerres@ucsd.edu

Greg W. Rouse

e-mail: grouse@ucsd.edu

How to get into bones: proton pump and carbonic anhydrase in *Osedax* boneworms

Martin Tresguerres, Sigrid Katz and Greg W. Rouse

Marine Biology Research Division, Scripps Institution of Oceanography, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 93092-0202, USA

Osedax are gutless siboglinid worms that thrive on vertebrate bones lying on the ocean floor, mainly those of whales. The posterior body of female *Osedax* penetrates into the bone forming extensions known as 'roots', which host heterotrophic symbiotic bacteria in bacteriocytes beneath the epidermis. The *Osedax* root epithelium presumably absorbs bone collagen and/or lipids, which are metabolized by the symbiotic bacteria that in turn serve for *Osedax*'s nutrition. Here, we show that *Osedax* roots express extremely high amounts of vacuolar-H⁺-ATPase (VHA), which is located in the apical membrane and in cytoplasmic vesicles of root and ovisac epithelial cells. The enzyme carbonic anhydrase (CA), which catalyses the hydration of CO₂ into H⁺ and HCO₃⁻, is also expressed in roots and throughout *Osedax* body. These results suggest *Osedax* roots have massive acid-secreting capacity *via* VHA, fuelled by H⁺ derived from the CA-catalysed hydration of CO₂ produced by aerobic metabolism. We propose the secreted acid dissolves the bone carbonate matrix to then allow the absorption of bone-derived nutrients across the skin. In an exciting example of convergent evolution, this model for acid secretion is remarkably similar to mammalian osteoclast cells. However, while osteoclasts dissolve bone for repairing and remodelling, the *Osedax* root epithelium secretes acid to dissolve foreign bone to access nutrients.

1. Introduction

Osedax is a genus of peculiar siboglinid worms that were originally discovered living on whale bones on the deep-ocean floor [1], and subsequently found to also survive on human-deployed cow and fish bones [2,3]. Female *Osedax* lack a mouth and a gut, and presumably feed by absorbing bone nutrients across extensive ramifications at the posterior end of their body known as 'roots' [1,4] (figure 1). The *Osedax* roots host endosymbiotic aerobic heterotrophic bacteria of the order Oceanospirillales in specialized bacteriocytes. The symbionts appear to be involved in metabolizing bone-derived complex organic compounds and in turn are digested by the host worm [1,4]. This contrasts with most other siboglinids, which obtain their nutrients via endosymbiotic thiotrophic (sulfur-oxidizing) bacteria. *Osedax* males are significantly smaller than females, ranging from 0.3 to 1 mm in length, and, depending on the species, hundreds of 'dwarf' males may inhabit the tube of a single female with the only purpose of producing sperm to fertilize her eggs. The males lack symbiotic bacteria and seem to rely on yolk reserves only for sperm production [1,5,6].

In order to access bone nutrients, female *Osedax* must first dissolve the inorganic bone matrix, which largely consists of calcium phosphate in the form of hydroxyapatite [7]. Other invertebrate animals bore into calcium carbonate structures using a combination of chemical and mechanical methods; for example, some sponges excavate into corals by secreting acid or enzymes followed by removal of etched chips of calcium carbonate [8,9]; some snails drill through shells of prey gastropods, bivalves or barnacles using a combination of acid/enzymes and radulae action [10,11]; and a range of polychaetes secrete calcium-chelating mucus containing acidic mucopolysaccharides to burrow into lime rocks, aided by removal of loosened sediments by chaetae [12,13].

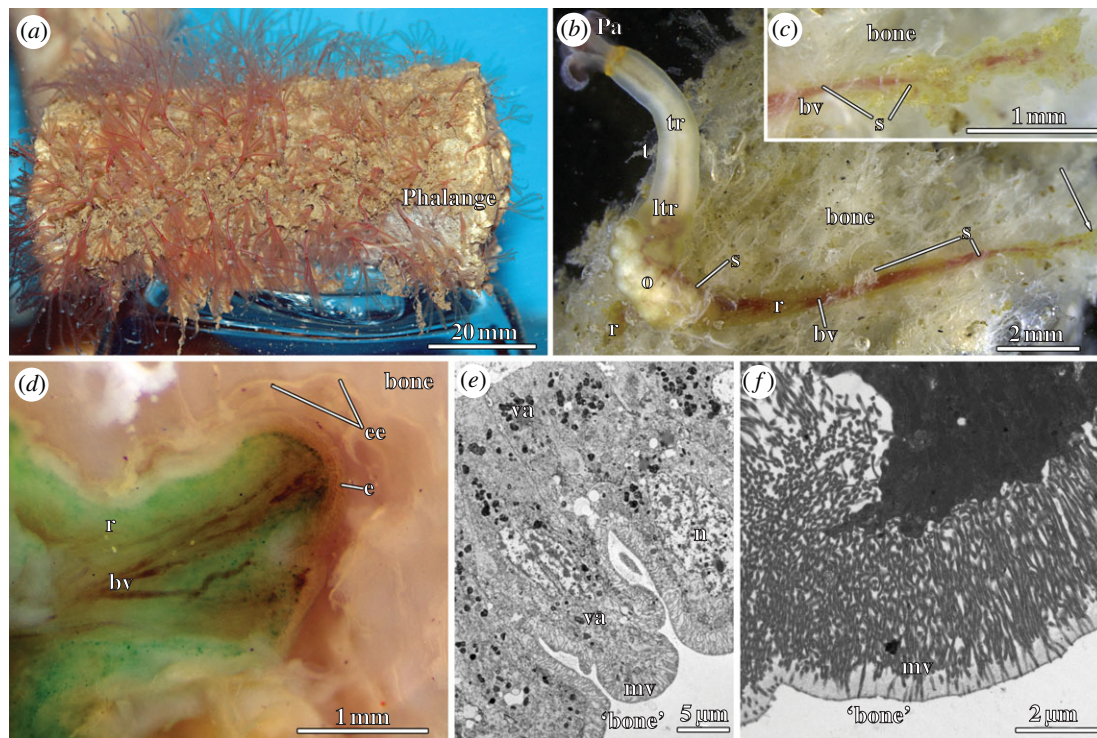


Figure 1. *Osedax* female anatomy. (a) More than 100 female specimens of *Osedax* 'orange collar' on a phalange of a grey whale (*Eschrichtius robustus*), collected at 633 m from the Monterey Submarine Canyon. (b) Highly contracted female specimen of *Osedax* 'yellow collar' with some of the whale bone surrounding the worm removed to show the base of the trunk and lower trunk (with green bacteriocyte-bearing tissue), the ovisac and part of the 'root' system. Blood vessels and white oocytes are visible in the ovisac, with the roots system showing large blood vessels and patches of green bacteriocyte-bearing tissue. A sheath of material that surrounds much of the ovisac and root system is continuous with the tube that surrounds the worm's trunk. Arrow indicates the extreme tip of one of the roots. (c) Detail of the tip of the root shown in (b). Blood vessels extend to near the tip, surrounded by bacteriocyte-bearing tissue. The sheath does not appear to extend to the very end of the root. (d) Lightly fixed and decalcified bone penetrated by *Osedax* 'nudepalp' sliced with a scalpel. Note blood vessels extending to root tip. The root tissue does not extend to the eroded edge of the bone tissue, but this may be a fixation artefact or represent a lacuna for secreted acid. No sheath is apparent around this region of the root. (e) Transmission electron micrograph of several root epidermis cells of *Osedax roseus* showing extensive microvilli and numerous electron-lucent and electron-dense vacuoles. (f) Transmission electron micrograph of root epidermis of *Osedax frankpressi* showing extensive microvilli. bv, Blood vessel; e, epidermis of roots; ee, eroded edge of bone; ltr, lower trunk; o, ovisac; pa, palps; r, roots; s, sheath; tr, upper trunk.

The epithelium of *Osedax* roots comprises cells displaying typical acid-secreting features, such as an extensive ruffled apical membrane and abundant mitochondria in the apical region [14,15]. Since *Osedax* do not have any obvious bioabrasive structures, Katz *et al.* [14,15] suggested *Osedax* bored into bones by secreting acid. This possibility was also discussed by Higgs *et al.* [16], who additionally found acidic mucopolysaccharides in the mucus of the root tissue and proposed that this material is important in the boring mechanism by acting as a chelating agent. However, the specific mechanisms by which *Osedax* secretes acid remain unknown.

In addition to dissolving the bone, *Osedax* must absorb nutrients, presumably collagen and possibly lipids [4,17], from the very material they erode into. The dual 'drilling and feeding' function does not occur in the boring invertebrate animals mentioned earlier, but it is analogous to the mechanism for bone resorption in osteoclast cells of vertebrate animals [18]. The potential similarities between marine boring organisms and osteoclasts have previously been recognized for sponges [8] and snails [10], and also *Osedax* [16].

Osteoclasts dissolve the bone carbonate matrix by secreting acid using vacuolar- H^+ -ATPases (VHA) [19]. This holoenzyme comprises multiple subunits, and it uses energy derived from ATP hydrolysis to pump H^+ against the concentration gradient (reviewed in [20]). VHAs are multifunctional enzymes since they also function in acid/base regulation, vesicular transport,

endosomal acidification and energizing ion and amino acid transport (reviewed in [20–22]).

In this study, we demonstrate that root epithelial cells express very high amounts of VHA, indicating massive capacity for acid secretion. We also establish that the enzyme carbonic anhydrase (CA) is also abundantly expressed in *Osedax* roots, suggesting the secreted acid is derived from CO_2 produced by aerobic metabolism at the roots. This bioeroding mechanism is essentially identical to the one used by osteoclasts [19,23]. However, while osteoclasts secrete acid for bone remodelling, *Osedax* secretes acid as part of a unique feeding mechanism that allows them to thrive on an otherwise difficult to access resource.

2. Material and methods

(a) Specimens

Female *Osedax* spp. (*O.* 'yellow collar', *O. frankpressi*, *O. roseus*, *O.* 'orange collar', and *O. rubiplumus*; [24]) were obtained from whale or cow bones at depths of 382 (whale), 633 m (whale), 1820 m (whale and cow) and 2893 m (whale and cow) in the Monterey Bay Canyon, California [25]. The remotely operated submersibles *Tiburon*, *Ventana* and *Doc Ricketts* were used both for deploying the cow bones and for collecting all bones. Soon after reaching the surface, *Osedax* specimens were dissected from bones and fixed for light microscopy (3% glutaraldehyde in 0.2 mol l⁻¹ cacodylate buffer with 10% w/v sucrose, pH 7.4),

immunofluorescence (2% paraformaldehyde in 0.1 M phosphate buffer with 10% w/v sucrose, pH 7.8) or transmission electron microscopy (TEM) (3% glutaraldehyde in 0.1 mol l⁻¹ phosphate buffer with 10% w/v sucrose, pH 7.4). After fixation, specimens were rinsed several times in buffer and stored at 4°C in buffer or 70 per cent ethanol. Some pieces of bone containing *Osedax* were decalcified using RDO Gold (Apex Engineering Products Corporation) and then sliced with a scalpel to show the bone/'root' interface. Additional specimens were flash-frozen in liquid N₂ for immunoblotting.

(b) TEM and light microscopy

Specimens were post-fixed for 80 min in 1 per cent osmium tetroxide in 0.1 mol l⁻¹ phosphate buffer with 10 per cent w/v sucrose, pH 7.4 after several buffer rinses, dehydrated in a graded ethanol series, and embedded in Spurr's epoxy resin. Semi-thin (1 µm) and ultra-thin (80 nm) sections were cut with a Leica Ultracut S microtome. Semi-thin sections were stained with toluidine blue and examined with a Leica DMR compound microscope. Ultra-thin sections were stained with uranyl acetate and lead citrate and viewed with a Philips CM100 TEM.

(c) Antibodies

Polyclonal antibodies against two VHA subunits were used for immunofluorescence and immunoblots. Polyclonal antibodies against the epitope 'AREEVPGRRGFPGY' in the VHA B subunit were produced in rabbit. BLAST searches of various databases reveal this epitope is conserved in VHA B from all animals examined, including several annelid worms. Preliminary transcriptomic analyses demonstrate this epitope is 100 per cent conserved in VHA B from *Osedax frankpressi* (H. Tresguerres 2012, unpublished observation). The antibodies specifically recognize VHA B from fish, shark and mollusc tissues by Western blot, as well as 'VHA-rich cells' in shark gills by immunohistochemistry [26].

Antibodies against mouse VHA A were a kind gift from Dr Dennis Brown (Harvard University, MA, USA). These antibodies recognize the epitope 'MQNAFRSLED' [27], which is more than 80 per cent conserved from cnidarians to mammals. These antibodies specifically cross-react with VHA A from a wide range of species, including mammals [28], fish [26] and mollusc tissues (M. Tresguerres 2012, personal observation).

Antibodies against human CA2 were purchased from Rockland Inc. (Gilbertsville, PA, USA). These antibodies specifically detect an approximately 30 KDa protein by immunoblotting in mammals, fishes [29] and molluscs (M.T. 2012, personal observation).

(d) Immunofluorescence

Some samples were dehydrated in an increasing ethanol series, embedded in paraffin and sectioned at 5 µm. Other samples were cryosectioned at 5–10 µm after embedding in Optimal Cutting Temperature compound. Both methods yielded identical results. Sections were attached to glass slides, rehydrated and processed for immunofluorescence as previously described [26]. After blocking non-specific binding sites (2% normal goat serum in 10 mM PBS, pH 7.4), sections were incubated overnight at 4°C with antibodies against VHA or CA2 (3 µg ml⁻¹ in PBS). Secondary Alexa-fluor goat anti-rabbit antibodies (Life Technologies Inc.; 1:500 dilution) were applied for 1 h at room temperature. Nuclei were stained with Hoechst v. 33342 (1 µg ml⁻¹ in phosphate buffered saline (PBS), 5 min at room temperature). In between steps, sections were profusely rinsed in PBS.

Immunofluorescence was detected using an epifluorescence microscope (Zeiss AxioObserver Z1) connected to a metal halide lamp and appropriate filters. Localization of VHA in the

membrane was confirmed using structured illumination (Zeiss Apotome2) and confocal microscopy (Zeiss LSM-700).

Digital images were adjusted, for brightness and contrast only, using ZEISS AXIOVISION software and ADOBE PHOTOSHOP.

(e) Immunoblotting

Osedax females were either dissected into 'roots/ovisac', and 'trunk/palps' and then frozen or dissected into these fractions from frozen whole specimens. These were then homogenized in 1:10 w/v ice-cold buffer (250 mM sucrose, 1 mM EDTA, 30 mM Tris, protease inhibitor cocktail (Sigma), pH 7.4). Debris and nuclei were removed by a low-speed centrifugation step (3000g, 10 min, 4°C); an aliquot of the supernatant was separated as 'crude homogenate', the remainder was centrifuged (22 000g, 30 min, 4°C) to obtain a 'membrane-enriched fraction' (pellet) and a 'soluble fraction' (supernatant). Protein concentration in each sample was quantified by the Bradford method. VHA abundance was determined in 'crude homogenates', CA2 abundance was determined in supernatants. Equivalent amounts of total protein (approx. 20 µg) were separated in 10 per cent polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes. Following blocking (5% skimmed milk in 0.5 M Tris-buffered saline with 0.1% Triton X-100 (TBS-T), 30 min at room temperature), PVDF membranes were incubated with primary antibodies against VHA A (1:1000), VHA B (1:1000) or CAII (1:3333) with gentle rocking at 4°C overnight. After three washes (15 min each) in TBS-T, PVDF membranes were incubated with HRP-linked goat anti-rabbit secondary antibodies (1 h, room temperature), washed (three times, 15 min). Immunolabelled protein bands were developed by incubation in Luminol solution, and visualized and quantified in a BioRad Chemidoc Imaging system.

3. Results

(a) General morphology

As shown in figure 1a (also see [2,4,30]), whalebones can sustain a large *Osedax* population. The anterior portion of the body of female *Osedax*, consisting of the upper trunk and the four palps ('gills'), is exposed to sea water (figure 1a,b). However, the trunk and palps can retract into the mucous tube secreted by the animal, and even partially into the excavated cavity in the bone, probably to avoid predators. The female *Osedax* lower trunk, ovisac and roots lie in a cavity excavated in the bone (figure 1b). The roots may branch out to create an extensive network inside the bone, although the shape varies from female to female, such that there may be bulbous lobes extending from the ovisac or thin elongate roots (figure 1c). Roots comprise epithelial cells, muscle cells, bacteriocytes containing symbiotic bacteria, non-symbiotic cells, and a network of blood vessels and capillaries. The green-coloured tissue corresponds to epidermis and bacteriocytes [1,15]. In the ovisac and roots, the major dorsal and ventral blood vessels of the trunk branch into several larger vessels, some of which reach the tip of the roots (figure 1d). Larger blood vessels and epithelial cells are distinguishable at relatively low magnification (figure 1b,d). A sheath of material, which is similar to and continuous with the external mucus tube, covers the ovisac and much of the roots (figure 1b,c). The *Osedax* roots are lined with a typical ion-transporting epithelium consisting of apical microvilli and abundant mitochondria, as well as numerous electron-dense and electron-lucent vacuoles (figure 1e,f). This matches

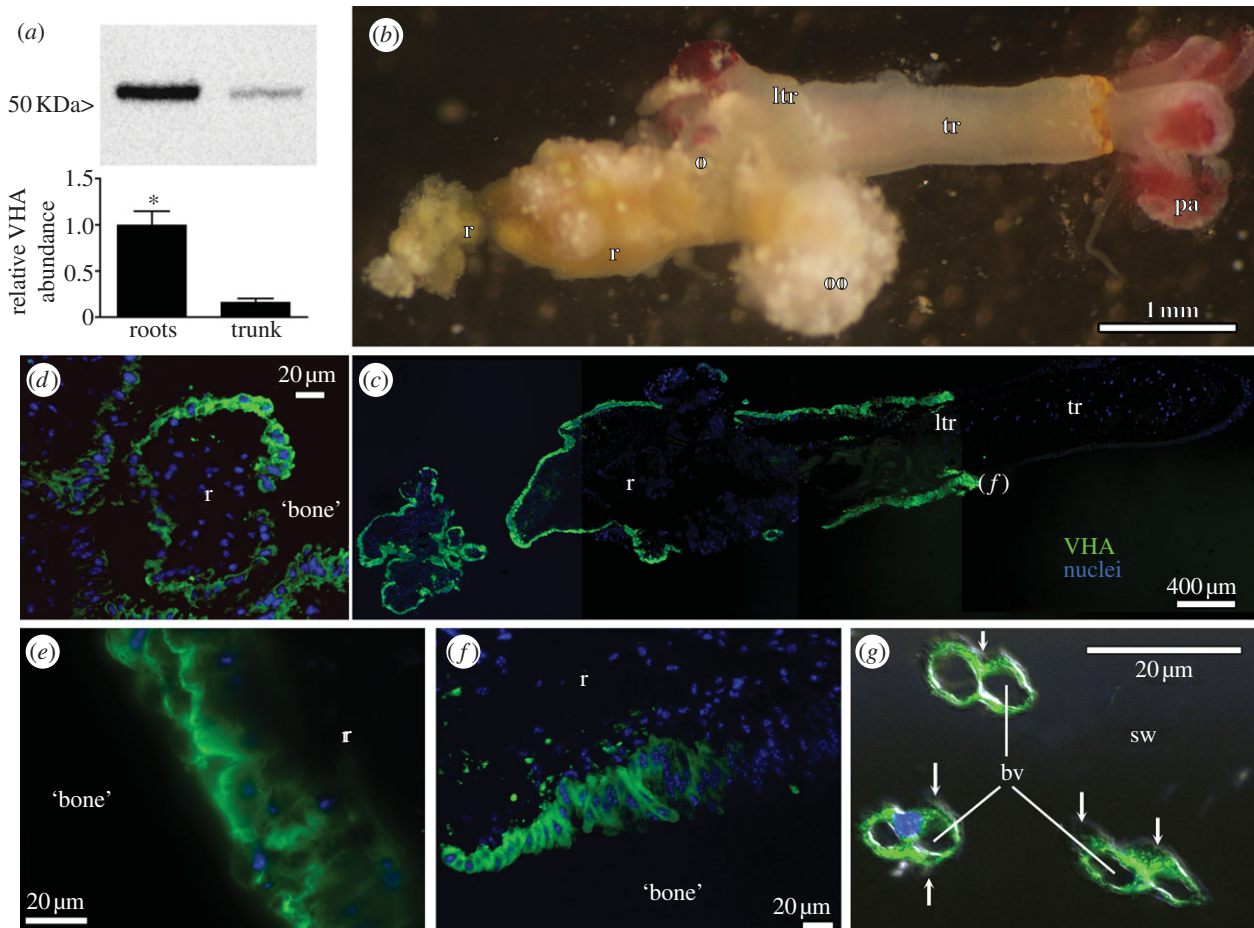


Figure 2. Vacuolar-H⁺-ATPase (VHA) in female *Osedax* spp. (a) Western blot showing VHA is 6.2-fold more abundant in root and ovisac ('roots') compared with trunk and palps ('trunk') tissues ($p < 0.05$, paired t -test, denoted with an asterisk). (b) External view of an *Osedax* 'yellow collar' specimen with the trunk (especially the lower trunk) and palps very contracted; the ovisac has been damaged with some oocytes bursting out. (c) Epifluorescence image of a sagittal section of the specimen shown in figure (b) immunostained against the VHA A subunit (green), nuclei were stained with Hoechst (blue). (d) Detail of the roots. (e) Confocal microcopy image of the root cells of an *O. frankpressi* specimen showing VHA immunostaining in the apical area and membrane. (f) Epifluorescence image of the lower trunk/upper trunk boundary showing the abrupt transition from cells expressing abundant VHA to cells without detectable VHA signal (using the same exposure time). (g) Structured illumination image of palp cells showing VHA immunostaining in cytoplasm and basolateral area (using longer exposure times compared with images from root tissue). Note the absence of VHA signal in the apical membrane (arrows). b, blood vessel; o, ovarian tissue; pa, palps; r, root tissue; sw, sea water; t, trunk.

previous ultrastructural descriptions for other *Osedax* species [14,15].

(b) VHA abundance and localization

The anti-VHA B antibodies specifically immunodetected VHA B in *Osedax* tissues, based on the size of the band (approx. 54 KDa) and on lack of bands in controls (omission of primary antibodies and preabsorption of anti-VHA B antibodies with antigen peptide). Quantitative Western blots demonstrated VHA B is 6.2-fold more abundant in *Osedax* 'roots/ovisac' compared with 'trunk/palps' ($p < 0.05$, paired t -test, $n = 5$; figure 2a). Anti-VHA A antibodies yielded similar results (not shown).

Immunofluorescence revealed VHA B is abundantly present in epithelial cells of the roots and lower trunk, both in putative cytoplasmic vesicles and in the cell apical membrane (figure 2c–f). There is an abrupt transition between cells expressing abundant VHA in the lower trunk region and cells without detectable VHA immunosignal in the upper trunk (figure 2f); this region presumably delimits the epithelial cells that actively secrete acid. VHA B immunostaining was also detected in *Osedax* palp cells. However,

since the signal in palps was much dimmer compared with roots and ovisac much longer exposure times were required in order to visualize the VHA immunofluorescent signal. VHA B intracellular localization in palps is also different as it is absent from the apical membrane and seems preferentially located in cytoplasmic vesicles and in the basolateral ('blood-side') membrane of cells (figure 2g). Anti-VHA A antibodies yielded identical results (not shown).

(c) CA abundance and localization

The anti-human CA2 antibodies specifically immunodetected an approximately 35 KDa band in *Osedax* tissues (figure 3a) which was not present in preabsorption controls. Although roots had higher abundance of CA2-like protein compared with the main trunk and palps, no statistically significant differences were found between body regions.

Immunolabelling of *Osedax* sections matched the immunoblot results, as CA2 signal was present throughout the worm (figure 3b). However, some areas of the roots, blood vessel cells, muscle cells and palp cells (figure 3c–f) demonstrated more intense staining. CA2 intracellular localization was generally cytoplasmic, although it appeared stronger in

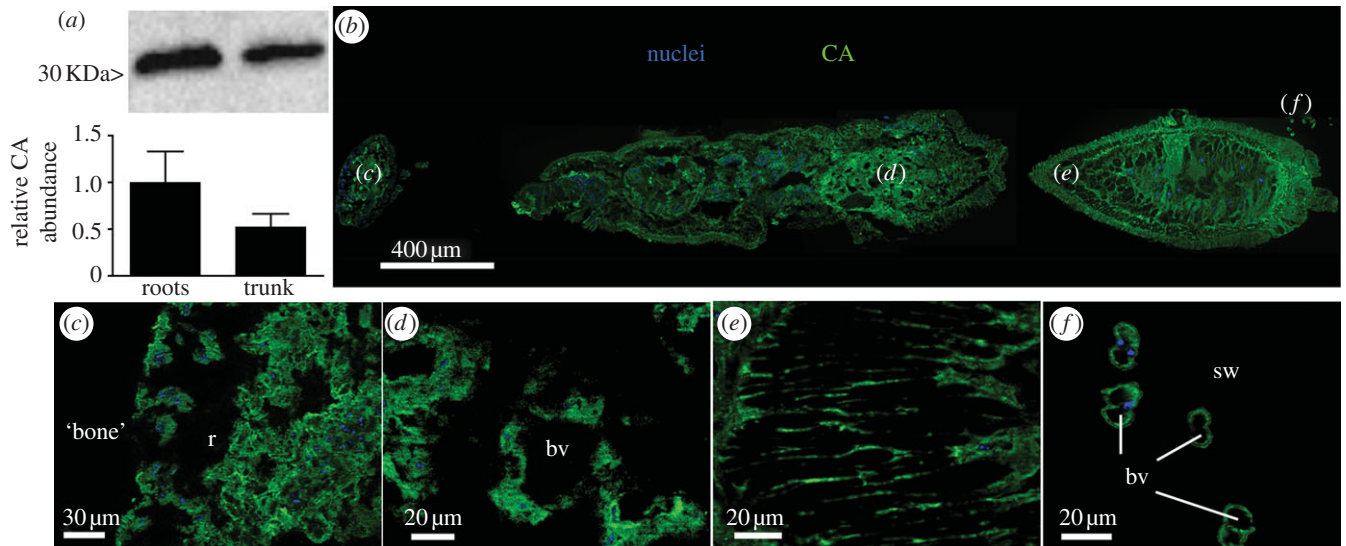


Figure 3. Carbonic anhydrase 2 (CA2) in female *Osedax* spp. (a) Western blot showing CA2 abundance in root and ovisac ('roots') and in trunk and palps ('trunk') tissues of *O. 'yellow collar'*. No statistical significant differences were found ($p > 0.05$, paired t -test). (b) Epifluorescence image of a sagittal section of the specimen shown in figure 1b immunostained against CA2 (green), nuclei were stained with Hoechst (blue). (c) Structured illumination image of the roots. (d) Structured illumination image of a blood vessel. (e) Structured illumination image of longitudinal muscle. (f) Structured illumination image of palp cells. bv, blood vessel; r, root tissue; sw, sea water.

the apical region of root cells (figure 3c) and in apical and basolateral regions of palp cells (figure 3f).

4. Discussion

Osedax feeding is a two-step challenge: bone demineralization and nutrient absorption. Confirming the previous hypothesis that *Osedax* dissolves the bone by secreting acid [14–16], we found that VHA is abundantly present at the root epithelium, thus solving the first step of the supposed feeding mechanism.

Female *Osedax* are clearly specialized for generating acid and drilling into bone (figure 4). We propose the mechanism of bone demineralization involves: (i) transport of oxygen from sea water to the roots *via* the extensive vascular system; (ii) production of CO_2 by aerobic metabolism in root epithelial cells; (iii) hydration of CO_2 into H^+ and HCO_3^- by intracellular CA; (iv) secretion of H^+ via VHA across the apical membrane directly onto the bone surface, and absorption of HCO_3^- into blood by as yet unknown mechanisms; (v) transport of HCO_3^- in blood to the palps; and (vi) excretion to sea water by cells expressing basolateral VHA and CA.

The most likely source of acid in *Osedax* root epithelial cells is hydration of metabolic CO_2 by CA. This would result in both H^+ and HCO_3^- , which have to be differentially transported across apical and basolateral membranes, respectively. The acid is secreted by VHAs directly onto the bone surface to dissolve bone hydroxyapatite, thus exposing trapped collagen. *Osedax* root epithelial cells have abundant mitochondria and extensive apical microvilli. These cells also express high levels of VHA throughout the cell and directly in the apical membrane, as well as intracellular CA2-like proteins. These features are typical of other cells specialized for acid secretion, such as mammalian kidney α -intercalated cells, clear cells in the epididymis, osteoclasts and mitochondrion-rich cells in turtle and amphibian urinary bladder, frog skin and insect salivary gland (reviewed in [21]).

Hydration of CO_2 in epithelial cells also produces equimolar amounts of HCO_3^- , which must be removed from the epidermal cells to maintain an elevated CO_2 hydration rate and avoid acid/base homeostasis disturbances. Because secretion of HCO_3^- across the apical membrane into the bone cavity would titrate the secreted H^+ , thus slowing down bone dissolution, we propose that HCO_3^- must instead be secreted across the basolateral membrane. After making their way across muscle layers and bacteriocytes, the absorbed HCO_3^- ions must reach the coelom and blood to eventually be secreted to the surrounding sea water across the palps. The network of blood capillaries and larger vessels (figure 1b–d) ensures efficient removal of HCO_3^- as well as oxygen supply. The large amounts of cytoplasmic CA2-like present throughout the worm may function (together with extracellular CAs and membrane ion transporters) to bring $\text{CO}_2/\text{HCO}_3^-$ to the palps. In a mirror image of the ion-transporting processes that take place in root epithelial cells, the branchial cellular mechanism for HCO_3^- secretion seems to rely on cytoplasmic CA, and basolateral (instead of apical) VHAs. This mechanism resembles other specialized HCO_3^- -secreting, H^+ -absorbing cells such as gill cells from hagfish [31,32], shark [26,33–35] and trout [36], and β -intercalated cells from mammalian kidney [21,37,38].

Osedax roots are often partially covered by a sheath that is continuous with the tube that protects the worm's trunk. For *Osedax mucrofloris* this has been reported as consisting of mucus containing acidic mucopolysaccharides [16]. This sheath was also noted here in our examination of some *Osedax* females, though it did not appear to be continuous all the way to the end of the roots (figure 1b). While it has been speculated that the mucous sheath helps dissolve the bone by acting as a chelating agent [13], it could have at least three other, not mutually exclusive, functions. First, it could prevent damage of *Osedax* skin by soaking up excess acid. Second, it could act to direct acid to the site of most active erosion. Third, it could help prevent regions of the bone matrix around the worm from dissolving, thus stabilizing the position of the worm in the bone. In any case, figure 1b–d shows the *Osedax*

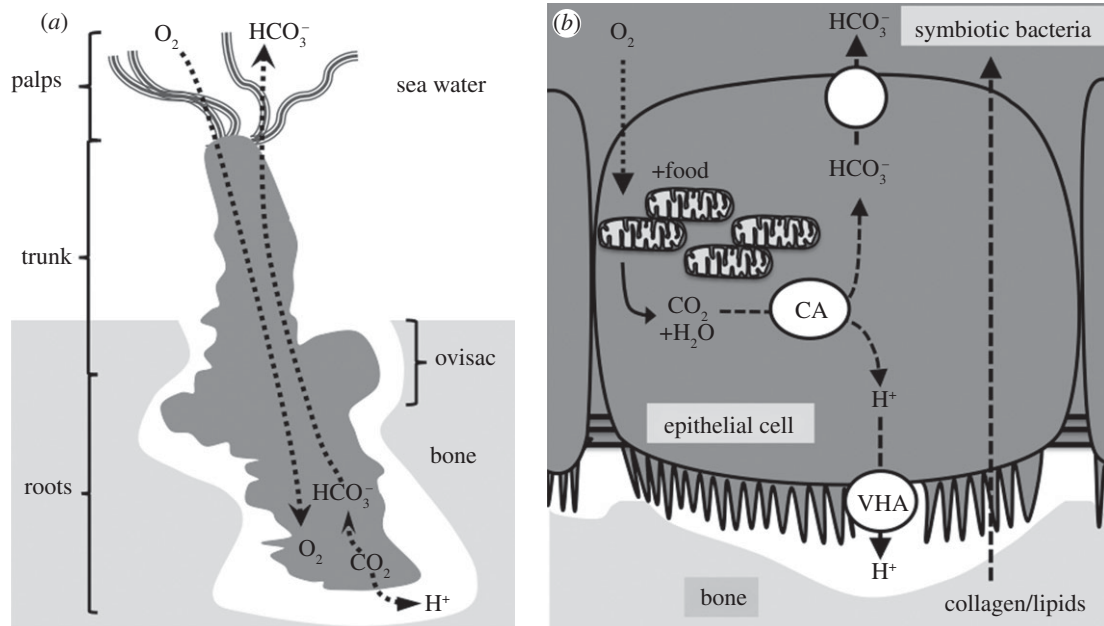


Figure 4. Proposed model of acid secretion for bone dissolution in female *Osedax*. (a) Whole animal fluxes. Oxygen (O_2) is absorbed from sea water across the palps and transported in blood and coelomic fluid to roots, where carbon dioxide (CO_2) is produced by aerobic respiration. Hydrogen (H^+) and bicarbonate (HCO_3^-) ions are produced and secreted or absorbed by the cellular mechanisms depicted in (b). The secreted H^+ dissolve bone hydroxyapatite, while the absorbed HCO_3^- is transported in blood and coelomic fluids to the palps and secreted to sea water. (b) Proposed cellular mechanism for H^+ secretion and HCO_3^- absorption in root epithelial cells. Metabolic CO_2 is hydrated by intracellular carbonic anhydrases (CA). H^+ are secreted directly onto the bone by apical Vacuolar- H^+ -ATPases (VHA), dissolving bone hydroxyapatite and releasing trapped collagen and lipids that are absorbed and transported to symbiotic bacteria located in sub-epidermal bacteriocytes in the roots. HCO_3^- ions are absorbed and transported to the palps for secretion.

epidermis can be in direct contact with the bone (except for a thin area analogous to the resorption lacuna of osteoclasts [19,23]), demonstrating direct secretion of H^+ onto the bone can occur.

A well-developed respiratory system [39] and high capacity for $O_2/CO_2/HCO_3^-$ transport in fluids are probable preadaptations for the *Osedax* unique 'dissolving and feeding' mechanism, as these features are also present in other siboglinid worms (reviewed in [40]). For example, *Riftia pachyptila* uses VHAs and CAs in the palps to secrete H^+ and absorb CO_2 (in addition to O_2 and H_2S) from hydrothermal vent fluids, followed by transport of HCO_3^- in blood and coelomic fluid to the trophosome where chemoautotrophic symbiotic bacteria generate organic compounds [41–45]. In fact, $[HCO_3^-]$ in *Riftia* blood and coelomic fluid can reach over 30 mM [45]. While Vestimentifera that live at hydrothermal vents, such as *Riftia* [46], take up sulfide via their anterior plume, seep-dwelling taxa such as *Lamellibrachia* use a single posterior extension of the tube (called a 'root') to reach deep into the sediment to take up sulfide from the interstitial water [47,48]. Similarly, the open posterior end of the tubes of Frenulata allows them to access sulfide from the interstitial water of the anoxic sediment their posterior body is buried in [49,50]. Compared with vent vestimentiferans such as *Riftia*, the H^+ and HCO_3^- fluxes are reversed in *Osedax*, however they may be similar in *Osedax*, seep vestimentiferans such as *Lamellibrachia* and possibly in frenulates. Altogether, this evidence suggests that our proposed mechanism for acid secretion across the roots has specialized from pre-existing mechanisms that relied on VHA and CA for other physiological functions.

Lamellibrachia release the majority of SO_4^{2-} and H^+ , both byproducts of the metabolism of their symbiotic bacteria, into the sediment via their posterior trunk and opisthosoma

that is contained within the 'root'. The sulfate can be recycled by being reduced to sulfide again, thus sustaining large tubeworm aggregations for long periods. The release of H^+ may enhance sulfide production and help limit carbonate precipitation onto their tubes [51–53]. Both SO_4^{2-} and H^+ excretion across the *Lamellibrachia* root seems to follow passive facilitated diffusion [53], but the specific ion transporters still need to be identified. Additionally, active H^+ secretion would possibly allow *Lamellibrachia* to initially penetrate through the carbonate rock, just as *Osedax* dissolve the mineral part of the bone. Clearly, the 'roots' in *Lamellibrachia* and *Osedax* serve a similar function in nutrient access and supply. However, whether or not VHA is involved in proton excretion in the *Lamellibrachia* root region, as in *Osedax* roots and whether or not *Lamellibrachia* and *Osedax* roots correspond to homologous body regions remains to be determined.

We are currently conducting experiments to elucidate the second step of the proposed feeding mechanism, which must start by absorption of bone-derived nutrients across the root epithelium. *Osedax* root tissue has robust collagenolytic activity and high concentration of Zn^{2+} (the metal cofactor for many collagenases) [17], suggesting *Osedax* secretes collagenases to degrade bone collagen at the root-bone interface followed by absorption of smaller organic molecules. However, it has not yet been determined whether the collagenolytic activity is derived from the host, the endosymbionts or both. The absorption of nutrients could take place via at least two mechanisms: amino acid transporters (as described across skin and gill epithelia of many marine invertebrates [54–57] and hagfish [58]), and transcytosis of macromolecules (as in osteoclasts [59]). Similar to previous reports [14,15], our TEM images show abundant vesicles in the *Osedax* root epithelium; however, we cannot establish whether they are transcytotic.

As host worm tissues are enriched in fatty acids with a distinct bacterial signature, Goffredi *et al.* [4] suggested at least part of the nutrients are transported to the symbionts, which eventually transfer metabolites back to the host. Also, Katz *et al.* [15] found evidence for bacteriocyte degradation in the ovisac region compared with the more distal roots, suggesting the worm eventually digests bacteriocytes for nutrition. However, other researchers have doubted that *Osedax* females host enough endosymbiotic bacteria to sustain all of their metabolic needs, though they did not provide an explanation for how *Osedax* might otherwise acquire nutrition [60]. We suggest that if *Osedax* females do not fully rely on bacterial symbionts, further nutrition could be acquired by direct absorption of bone amino acids, as described earlier, followed by digestion without the intermediate bacterial step.

Based on the worldwide distribution of *Osedax* and its utilization of mammal, bird, fish and reptile bones as substrates, they are probably substantial contributors to the recycling of vertebrate organic matter in oceanic ecosystems. In a remarkable case of convergent evolution, the main features of *Osedax*

'dissolving and feeding' mechanism resemble the mechanism for bone resorption in mammalian osteoclasts [18,19]. However, while osteoclasts are single (though multinucleate) cells present in internal fluids that regulate bone remodelling and blood acid/base homeostasis, the *Osedax* root system has an epithelium specialized for feeding. Understanding the process of bone demineralization in *Osedax* is only the first step in understanding this unusual feeding mode. Investigation of nutrient uptake and transport, as well as the nutritional relationships between the host and endosymbiotic bacteria in this symbiosis, will expand our knowledge on novel modes of nutrition.

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