The Journal of Experimental Biology 205, 3055–3066 (2002)
Printed in Great Britain © The Company of Biologists Limited
JEB4315

Effects of metabolite uptake on proton-equivalent elimination by two species of deep-sea vestimentiferan tubeworm, *Riftia pachyptila* and *Lamellibrachia* cf *luymesi*: proton elimination is a necessary adaptation to sulfide-oxidizing chemoautotrophic symbionts

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Accepted 9 July 2002

Summary

Intracellular symbiosis requires that the host satisfy the symbiont's metabolic requirements, including the elimination of waste products. The hydrothermal vent tubeworm *Riftia pachyptila* and the hydrocarbon seep worm *Lamellibrachia* cf *luymesi* are symbiotic with chemolithoautotrophic bacteria that produce sulfate and protons as end-products. In this report, we examine the relationship between symbiont metabolism and host proton equivalent elimination in *R. pachyptila* and *L.* cf *luymesi*, and the effects of sulfide exposure on proton-equivalent elimination by *Urechis caupo*, an echiuran worm that lacks intracellular symbionts (for brevity, we will hereafter refer to proton-equivalent elimination as 'proton elimination'). Proton elimination by *R. pachyptila* and *L.* cf *luymesi* constitutes the worms' largest mass-specific metabolite flux, and *R. pachyptila* proton elimination is, to our knowledge, the most rapid reported for any metazoan. Proton elimination rates by *R. pachyptila* and *L.* cf *luymesi* correlated primarily with the rate of sulfide oxidation. Prolonged exposure to low environmental oxygen concentrations completely inhibited the majority of proton elimination by *R. pachyptila*, demonstrating that proton elimination does not result primarily from anaerobic metabolism. Large and rapid increases in environmental inorganic carbon concentrations led to short-lived proton elimination by *R. pachyptila*, as a result of the equilibration between internal and external inorganic carbon pools. *U. caupo* consistently exhibited proton elimination rates 5–20 times lower than those of *L.* cf *luymesi* and *R. pachyptila* upon exposure to sulfide. Treatment with specific ATPase inhibitors completely inhibited a fraction of proton elimination and sulfide and inorganic carbon uptake by *R. pachyptila*, suggesting that proton elimination occurs in large part *via* K^+/H^+-ATPases and Na^+/H^+-ATPases. In the light of these results, we suggest that protons are the primary waste product of the symbioses of *R. pachyptila* and *L.* cf *luymesi*, and that proton elimination is driven by symbiont metabolism, and may be the largest energetic cost incurred by the worms.

Key words: metabolite uptake, proton-equivalent, tubeworm, *Riftia pachyptila*, *Lamellibrachia* cf *luymesi*, *Urechis caupo*, sulfide, oxidation, chemoautotrophy, symbiosis, vestimentiferan, hydrothermal vent.

Introduction

The discovery of chemoautotrophic symbionts in the hydrothermal vent tubeworm *Riftia pachyptila* expanded our concept of symbioses (Cavanaugh et al., 1981; Felbeck, 1981). In these associations, the bacteria fix inorganic carbon and oxidize reduced inorganic substrates, such as reduced sulfur compounds, to produce energy. Members of the class Vestimentifera, such as *R. pachyptila* and other tubeworms found at hydrothermal vents, hydrocarbon seeps and other chemically reduced deep-sea environments, exhibit a suite of morphological and biochemical adaptations to their association (Childress and Fisher, 1992). When mature, these worms do not possess a digestive tract. Instead, the symbionts are housed within host cells in a vascularized organ called the trophosome (Jones, 1981). The symbionts have no direct contact with the external milieu, and the host acquires all the metabolites required for chemoautotrophic metabolism.

Both vent and seep vestimentiferan symbionts are sulfide specialists that specifically utilize hydrogen sulfide to produce energy (Wilmot and Vetter, 1990). This metabolic process yields two primary end-products, an oxidized sulfur compound

(such as thiosulfate or sulfate) and protons (Nelson and Hagen, 1995). Although a small proportion of these protons may be utilized in other microbial reductive metabolic processes, e.g. the reduction of inorganic carbon and nitrate (Girguis et al., 2000), the surplus protons must be eliminated from the symbionts and their host into the environment.

In a previous paper, we described extremely high rates of net proton elimination into the environment by the tubeworm *R. pachyptila*, the dominant tubeworm at the hydrothermal vent communities along the East Pacific Rise (Girguis and Childress, 1998). However, we did not measure the quantitative relationship between proton elimination and host or symbiont metabolism. In the present study, we address these relationships, in particular the effects of sulfide, inorganic carbon and oxygen uptake on net proton elimination by the deep-sea vestimentiferan tubeworms *R. pachyptila* and *Lamellibrachia* cf *luymesi* (Kennicutt et al., 1985), and by the echiuran worm *Urechis caupo*. *L.* cf *luymesi* flourishes at the hydrocarbon seeps in the Gulf of Mexico (Macdonald et al., 1989). *U. caupo* is an echiuran worm that inhabits sulfide-rich environments and possesses mechanisms for oxidizing sulfide to prevent metabolic poisoning (Menon and Arp, 1998). Because *U. caupo* does not possess symbionts to which it is metabolically coupled, any observed proton elimination by *U. caupo* during sulfide exposure should be the result of sulfide detoxification. We chose to study *U. caupo* as a means of examining proton elimination that does not result from intracellular symbiont metabolism (note that vestimentiferans cannot survive without their symbionts, so they cannot be used for such experiments). In addition, we used four ATPase inhibitors to examine the mechanisms of proton elimination by *R. pachyptila*. We hypothesized that the proton elimination rates of *R. pachyptila* and *L.* cf *luymesi* would correlate primarily with symbiont metabolic processes and that any disruption to proton elimination (e.g. by the use of these inhibitors) would have negative repercussions on the metabolism of both the host and the symbiont.

Materials and methods

Animal collection and maintenance

Riftia pachyptila Jones tubeworms were collected from hydrothermal vent sites along the East Pacific Rise (12°48′N, 103°56′W and 9°50′N, 104°18′W), at a depth of approximately 2600 m, during expeditions in April 1996 (HOT 96), November 1997 (HOT 97) and November 1998 (LARVE 98). Worms were collected daily by the *DSV Alvin* and brought to the surface in a thermally insulated container (Mickel and Childress, 1982). Upon arrival at the surface, the worms were immediately placed into flow-through, high-pressure respirometer aquaria (Girguis et al., 2000). During our HOT 97 experiment on the relationship between oxygen uptake and proton elimination, three tubeworms that had been collected 3 days earlier were kept in maintenance aquaria and later used for experimentation. The maintenance aquaria (Goffredi et al., 1997) are distinct from our respirometer aquaria, and are capable of sustaining 16–20 worms under *in situ* vent conditions ($\sum CO_2$=5–6 mmol l^{-1}, $\sum H_2S$=250–600 μmol l^{-1}, [O_2]=100–400 μmol l^{-1}, [NO_3^-]=40 μmol l^{-1}, pH 6.5, temperature 12°C, pressure 20.6 MPa; $\sum$ is used to indicate the total concentrations of all ionic species of inorganic carbon or sulfide). In an earlier report, a comparison of freshly captured worms with maintenance worms showed no differences in $\sum H_2S$ or O_2 uptake rates when measured in respirometer aquaria (Girguis et al., 2000).

A clump of *Lamellibrachia* cf *luymesi* (van de Land and Nørrevang) tubeworms were collected by the *DSV Johnson Sea Link* from the outskirts of the Brine Pool NR1 hydrocarbon seep site (27°43′24″N, 91°16′30″W) at a depth of approximately 650 m during an expedition to the Gulf of Mexico in July 1998. *L.* cf *luymesi* were brought to the surface in a temperature-insulated box. Upon reaching the surface, they were immediately transferred to large buckets containing seawater chilled to 7°C and later maintained at atmospheric pressure in a large cooler that contained circulating, aerated seawater chilled to 7°C. Upon return to port, an intact clump of *L.* cf *luymesi* was transported in a large cooler containing ice-cold seawater to the University of California at Santa Barbara. The worms were immediately placed in a flow-through aquarium containing seawater at 5°C (pumped from offshore, coarse-filtered and chilled prior to flowing into the aquarium) and a layer of anoxic mud on the bottom of the aquarium. Worms were maintained in this aquarium for several days prior to experimentation. Care was taken to use healthy, active, undamaged worms that had intact ‘roots’ for experimentation (Julian et al., 1999). For experimentation, *L.* cf *luymesi* were placed into specially built two-compartment respiration chambers that allowed the posterior and anterior halves of the tubeworms to be isolated into different streams of flowing water (Freytag et al., 2001). All experiments were conducted within 17 days of collection.

Urechis caupo (Fisher and MacGinitie) were collected in November 1996 from the Morro Bay mudflats (35°40′12″N, 120°79′93″W) by a suction gun, consisting of a polyvinylchloride (PVC) tube with an o-ring-sealed plunger designed to extract worms from their burrows. Worms were transported to Santa Barbara in ice-cold seawater and, upon arrival, immediately placed into flowing seawater at 15° (pumped from offshore to our seawater tables). Worms were allowed to acclimate to the water tables for 3 days before experiments began. All experiments on *U. caupo* were conducted within 8 days of collection.

Measuring metabolite flux by R. pachyptila, L. luymesi *and* U. caupo

In all experiments, a worm or worms were placed into two respirometry aquaria. A third aquarium always served as a control and was devoid of worms.

In all experiments, seawater was filtered (0.2 μm diameter) and pumped *via* a metering pump (Cole-Parmer, Inc.) into an acrylic gas equilibration column and bubbled with a combination of CO_2, 5% H_2S/95% N_2, O_2 and N_2 or He to

achieve *in situ* dissolved gas concentrations (conditions used in experiments are described below). Mass flow controllers (Sierra Instruments, Inc.) regulated the gas flow into the equilibration column. A proportional pH controller and two metering pumps were used to regulate seawater pH (Prominent Industries, Inc.). A sodium nitrate solution (5 mmol l^{-1}) in 0.2 μm filter-sterilized seawater was pumped into the equilibration column at a rate that produced *in situ* seawater nitrate concentrations. The resulting seawater was then pumped from the equilibration column into each aquarium using three high-pressure pumps (for *R. pachyptila* experiments; Lewa America, Inc.) or three metering pumps (for *L.* cf *luymesi* and *U. caupo* experiments; Prominent Industries). Aquarium temperature was maintained by immersion in a circulating waterbath (Fisher Inc.). *R. pachyptila* aquarium pressure was maintained at 27.5 MPa *via* pneumatically charged or spring-loaded backpressure valves (Circle Seal, Inc.). Aquarium effluents passed through computer-driven stream selection valves (Valco, Inc.), allowing automated control of effluent analysis.

To determine metabolite flux rates, one seawater stream at a time was directed towards a gas extractor (Fig. 1) that stripped the gases dissolved in the seawater and routed them for analysis by a membrane-inlet mass spectrometer. The extractor is based on the principles of our gas chromatograph seawater inlet (Childress et al., 1984) and allows us to run dissolved gas analyses continuously with higher resolution (3–5 times the sensitivity of analyzing seawater directly; data not shown) and with reduced maintenance of the membrane inlet. To our knowledge, this device is unique in both its design and application in mass spectrometry. In the extractor, seawater was bubbled with helium while being mixed with helium-sparged 20% *o*-phosphoric acid/80% deionized water. The addition of the degassed phosphoric acid mixture dramatically reduced the pH, converting both inorganic carbon and sulfide species to carbon dioxide and hydrogen sulfide, respectively. A quartz-tipped optical level controller (Levelite Inc.) maintained the fluid level in the extractor. As dissolved gases are extracted from the seawater, they are carried to the membrane-inlet mass spectrometer (Hiden Analytical Inc.) to measure changes in the partial pressures of CO_2, H_2S, O_2 and N_2 (Kochevar et al., 1992). The mass spectrometer is capable of detecting extremely small changes in partial pressure but, for quantitative determination of metabolite flux, these data were converted into changes in concentration by calibrating the mass spectrometer with a Hewlett-Packard 5890A gas chromatograph (Childress et al., 1984). For the calibrations, 500 μl gas-tight syringes with 30 gauge sideport needles (Hamilton, Inc.) were used to collect seawater samples from water- and gas-tight septa just before the extractor. Regression plots of partial pressure *versus* total concentration were used to produce standard curves. In all cases, the concentrations used for calibration spanned at least one order of magnitude and encompassed the range of concentrations used in our experiments. In addition, at least 25 samples were collected and used for each chemical parameter; in all cases, $r^2 \geq 0.90$.

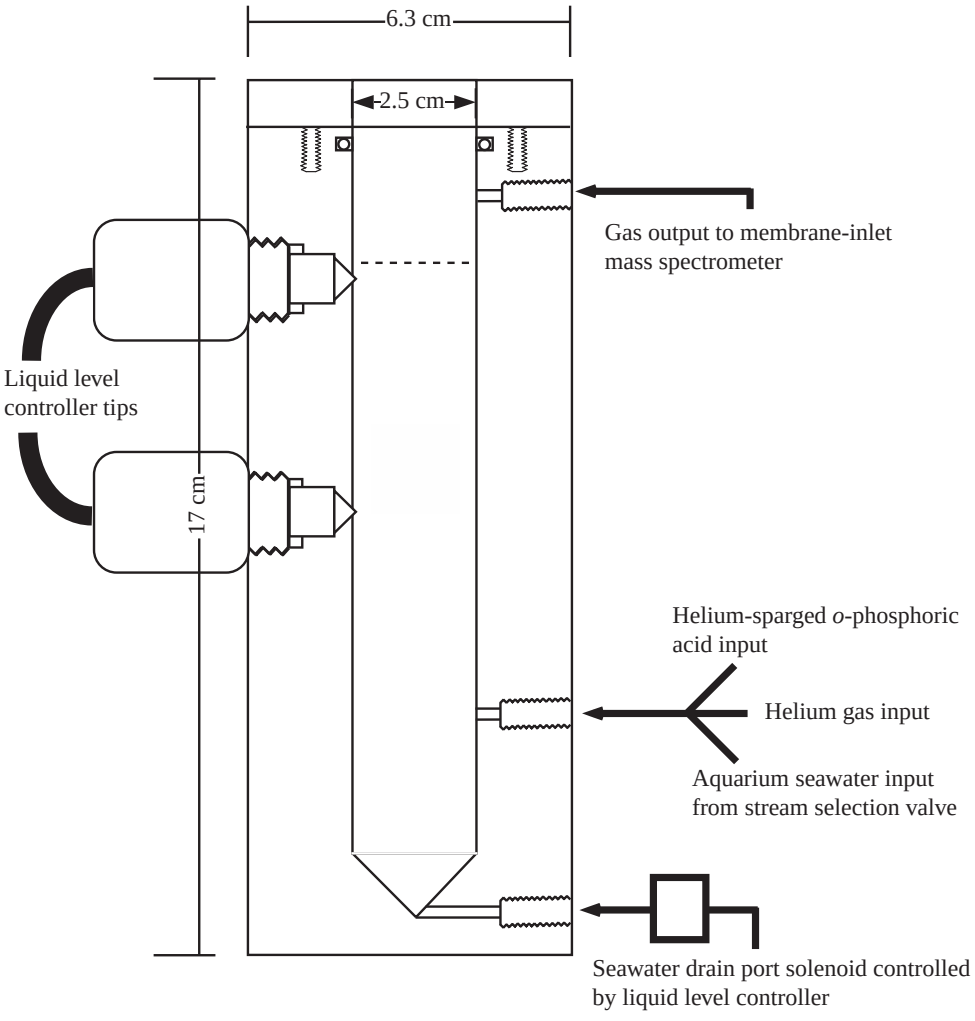

Fig. 1. Diagram of the custom-built polysulfone gas extractor. Seawater is directed by a stream selection valve into the extractor, where it is mixed with phosphoric acid, bubbled with helium and sent for analysis by a quadropole mass spectrometer. The liquid level is maintained between two quartz optical liquid detectors. The seawater/acid mixture is periodically drained off through the drain port when its level reaches the top detector.

Calibrated data, as well as the flow rate of the effluent and the total mass of the organisms, were used to determine mass-specific metabolite flux rates.

For all shipboard experiments, *R. pachyptila* tubeworms were maintained in respirometer aquaria (Kochevar et al., 1992) under *in situ* conditions ($\sum CO_2$=5–6 mmol l^{-1}, $\sum H_2S$=250–300 μmol l^{-1}, $[O_2]$=100–210 μmol l^{-1}, $[NO_3^-]$=40–50 μmol l^{-1}, pH 6.5, 12°C, 27.5 MPa) until autotrophy was established. Autotrophy describes a worm that exhibits net inorganic carbon, oxygen and sulfide uptake from the environment, and net elimination of protons into the environment. Autotrophy typically commenced after 12–24 h.

For all experiments with *L.* cf *luymesi*, tubeworms were placed in the split-vessel respirometer aquaria (Freytag et al., 2001) and maintained under *in situ* conditions until autotrophy was established ($\sum CO_2$=2 mmol l^{-1} in both top and bottom chambers, $\sum H_2S$=500 μmol l^{-1} in the bottom chamber, $[O_2]$ in the top chamber=100–210 μmol l^{-1}, $[NO_3^-]$=40–50 μmol l^{-1} in both top and bottom chambers, pH 6.5 in the bottom chamber, pH 8.0 in the top chamber, 12°C, 206 Pa). Autotrophy typically commenced after 48–72 h.

For all experiments with *U. caupo*, two worms were placed into 38 cm inner diameter Tygon tubing. Both ends of the tubing were fitted with reducing connectors to allow coupling to 0.3 cm diameter polyethylene tubing. Polypropylene plastic mesh was used to prevent the worms from occluding the incurrent and excurrent openings. Metering pumps (Prominent Industries) were used to flush the tubing with chilled, air-saturated seawater from the equilibration column. The entire assembly was placed into a circulating waterbath to maintain the temperature at 15°C. Worms were kept in this tubing (hereafter described as tubing aquaria) with flowing seawater for 12 h prior to experimental manipulations. One tubing aquarium was maintained without worms and served as our control.

To calculate changes in oxygen and sulfide concentration in the seawater caused by *U. caupo* pre- and post-sulfide exposure, 500 μl gas-tight glass syringes with 30 gauge sideport needles (Hamilton, Inc.) were used to collect seawater samples through gas-tight septa from both experimental and control tubing aquaria. Total dissolved oxygen and sulfide concentrations in each sample were determined by gas chromatography using a Hewlett Packard 5890 gas chromatograph modified for analyzing dissolved gases in seawater (Childress et al., 1984).

After completing *in vivo* experiments, *R. pachyptila*, *L.* cf *luymesi* or *U. caupo* were removed from the aquaria, weighed on a motion-compensated shipboard balance when at sea (Childress and Mickel, 1980) or an electronic balance (Mettler, Inc.) when on shore, quickly dissected on ice and frozen in liquid nitrogen for further analyses. In most cases, the empty worm tubes were left in the pressure aquaria for several hours and subjected to the same experimental conditions to determine the fraction of the observed flux rates attributable to bacterial growth or to other phenomena associated with the tubes. In this study, as well as previous reports, we have demonstrated no significant contribution of free-living bacteria to our observed metabolite flux rates (Girguis et al., 2000).

Determination of proton elimination rates by R. pachyptila, L. luymesi *and* U. caupo

To determine the proton elimination rates by *R. pachyptila*, *L.* cf *luymesi* or *U. caupo*, the seawater pH of the excurrent flows of the experimental and control aquaria was measured by a double-junction pH electrode resistant to interference from sulfide (Broadley-James, Inc.) and an Orion (model 920A) or Radiometer PHM 93 pH meter. In the *R. pachyptila* and *L.* cf *luymesi* experiments, the electrode was housed in an o-ring-sealed acrylic flow-through cell (volume 1.35 ml) with offset inlet and outlet ports to aid in clearing gas bubbles. The effluent stream in the flow-through cell was maintained at 206 kPa to reduce off-gassing, and the assembly was positioned after our automated stream selection valves. pH was measured every 0.25 s, recorded by a computer and averaged over 7.5 min.

In the *U. caupo* experiments, two worms were placed into the tubing aquaria, one per aquarium, as described above. The entire assembly was placed into a circulating waterbath to maintain the temperature at 15°C. Worms were kept in these tubing aquaria with flowing seawater for 12 h prior to sulfide exposure. One tubing aquarium was maintained without worms and served as our control. For the sulfide exposure experiments, seawater in the equilibration column was bubbled with hydrogen sulfide, to bring the dissolved sulfide concentration up to 100 μmol l^{-1}, and was pumped into the tubing aquaria. Exposure to sulfide continued for 7 h. During this time, the pH of the seawater from the control and experimental aquaria was measured by collecting samples of control and experimental effluent seawater in 60 ml disposable gas-tight syringes every 8–10 min. The effluent was transferred to 125 ml beakers, and the pH was measured with the aforementioned pH electrode and meter.

In seawater, there is considerable buffering by bicarbonate and other inorganic anions. To calculate the organisms' proton elimination rates accurately during the experiments, the buffering of protons by inorganic acid anions had to be considered (because of their relatively low abundance in our seawater, organic acid anions were not considered; Johnson et al., 1988). Equations for the dissociation of carbonic acid, water, boric acid and hydrogen sulfide in seawater as a function of temperature, salinity and pressure were used with our effluent pH measurements, temperature and gas chromatographic measurements of $\sum CO_2$ and $\sum H_2S$ (as described above) to calculate total alkalinity. The general form of this expression is:

$$TA = [HCO_3^-] + [2CO_3^{2-}] + [B(OH)_4^-] + [HS^-] + [OH^-] - [H^+],$$

where TA is the total alkalinity. A complete derivation, including the determination of the dissociation constants as a function of pressure, may be found in Millero (1995).

Total alkalinity was then used to calculate the hydrogen ion concentration required to produce the observed differences in the pH between experimental and control aquaria effluents. Total proton elimination rates were then calculated from the hydrogen ion concentrations, the effluent flow rates and the mass of the worms.

Sulfide uptake and proton elimination by R. pachyptila *and* L. luymesi

During the HOT 96 expedition, three autotrophic *R. pachyptila* tubeworms, weighing 7–16 g each, were placed into two of the high-pressure respirometry aquaria (one in one aquarium, two in the other aquarium). Dissolved gaseous hydrogen sulfide concentration was changed in the aquarium seawater at specific times over several hours to achieve a series of final seawater sulfide concentrations between 0 and 700 μmol l^{-1}. Worms were kept at each incremental hydrogen sulfide concentration until their sulfide and proton flux rates stabilized (typically 4–7 h). Other than experimental variation in external sulfide concentrations, worms were kept under constant *in situ* conditions for the duration of the experiment ($\sum CO_2$=5 mmol l^{-1}, [O_2]=150 μmol l^{-1}, [NO_3^-]=40–65 μmol l^{-1}, pH 6.2, 12°C, 27.5 MPa). The above experiment was repeated during the HOT 97 expedition using four freshly collected worms (two in each aquarium).

To determine the effect of sulfide uptake on rates of proton elimination by *L.* cf *luymesi*, two tubeworms, weighing 4–6 g each, were placed into one two-chamber respirometry vessel. Although simulating the conditions found *in situ* utilized the same equipment as in the *R. pachyptila* experiments, the seawater physico-chemical conditions differed ($\sum CO_2$=1.8 mmol l^{-1}, $\sum O_2$=300 μmol l^{-1}, $\sum H_2S$=0 μmol l^{-1}, temperature, 5°C, pressure, 200 Pa). When the oxygen uptake of *L.* cf *luymesi* stabilized, dissolved gaseous hydrogen sulfide was added to the posterior chamber fluid of the respirometer vessels in two increments over several hours to achieve final seawater sulfide concentrations of first 238 μmol l^{-1} and then 515 μmol l^{-1}. Aquaria conditions were kept at each incremental sulfide concentration until the worms' sulfide and proton flux rates stabilized, typically 2–5 h. Other than experimental variation in external sulfide concentrations, worms were kept under constant *in situ* conditions for the duration of the experiment.

Inorganic carbon uptake and proton elimination by R. pachyptila

During the HOT 97 expedition, four autotrophic *R. pachyptila* tubeworms, weighing 4–15 g each, were placed into two high-pressure aquaria (two per aquarium). Inorganic carbon concentration in the seawater was increased from 3 to 10 mmol l^{-1} over 6 h. The inorganic carbon concentration was then reduced to 4.4±0.12 mmol l^{-1} and maintained for 11 h to accustom the tubeworms to the lower environmental inorganic carbon concentration. Finally, the inorganic carbon concentration in the seawater was increased to 8.7 mmol l^{-1}, kept at this concentration for 10 h and then decreased to 2.1 mmol l^{-1} over 4 h.

Oxygen uptake and proton elimination by R. pachyptila

During the HOT 96 expedition, four autotrophic *R. pachyptila* tubeworms, weighing 6–8 g each, were placed into two high-pressure aquaria (two per aquarium). Sulfide concentration in the aquaria was maintained at 210–250 μmol l^{-1}, while dissolved seawater oxygen concentration was decreased from 350 to 78 μmol l^{-1} over 8 h. During the HOT 97 and LARVE 98 expeditions, three autotrophic *R. pachyptila* tubeworms were placed into two aquaria (one in one aquarium, two in the other) and the dissolved oxygen concentration was then decreased from 394 and 314 μmol l^{-1}, respectively, to <3 μmol l^{-1} (Childress et al., 1984) over 11 and 13 h, respectively.

The effects of transport inhibitors on proton elimination by R. pachyptila

During the HOT 96, HOT 97 and LARVE 98 expeditions, four inhibitors of ATPases, *N*-ethylmaleimide (NEM), amiloride, lansoprazole and vanadate, were used in separate treatments on *R. pachyptila* to assess the mechanism by which protons are being eliminated into the environment. NEM non-selectively inhibits ATPases and is a general metabolic poison (Hilden and Madias, 1991). amiloride inhibits Na^+ exchange (Kleyman and Cragoe, 1988). Vanadate inhibits P-type ATPases (Chatterjee et al., 1992). Lansoprazole is highly specific to and inhibits P-type K^+/H^+-ATPases (Tomiyama et al., 1994). In each treatment, 2–5 autotrophic *R. pachyptila* worms, weighing 4–14 g each, were placed into two of the three high-pressure aquaria (one, two or three worms per aquarium). In all treatments, inhibitors were introduced into the system before the high-pressure pumps to prevent depressurization of the respirometer chambers. Inhibitors were dissolved in 10 ml of deionized water, except for amiloride, which was dissolved in 10% dimethylsulfoxide (DMSO) in deionized water. The seawater concentrations of NEM, amiloride, lansoprazole and vanadate in these respirometer chambers were 2 mmol l^{-1}, 1 mmol l^{-1}, 2 mmol l^{-1} and 750 μmol l^{-1}, respectively (Goffredi, 1998). After addition of the inhibitor, the worms were left uninterrupted for at least 6 h while seawater dissolved gas concentrations and pH were being measured. The aquaria were continuously flushed by seawater under *in situ* conditions.

Results

The effects of sulfide exposure on proton elimination by R. pachyptila, L. *cf* luymesi *and* U. caupo

Data from the HOT 96 and HOT 97 expeditions demonstrated that an increased seawater sulfide concentration resulted in increased sulfide uptake by *R. pachyptila* and established the relationship between environmental sulfide concentrations and sulfide uptake rate (y=1.04+0.0148x, r^2=0.91, P=0.825, where y is sulfide uptake rate in μmol g^{-1} h^{-1}

Table 1. *Comparison of the oxygen sulfide and proton flux rates of* Riftia pachyptila, Lamellibrachia *cf* luymesi *and* Urechis caupo

Species	Oxygen uptake rate (μmol g^{-1} h^{-1})		Sulfide uptake rate (μmol g^{-1} h^{-1})	Proton elimination rate (μequiv g^{-1} h^{-1})	
	Pre-sulfide exposure	Post-sulfide exposure		Pre-sulfide exposure	Post-sulfide exposure
Riftia pachyptila	4.81±0.42 (8)	13.63±0.44 (28)	6.96±0.13 (28)	2.18±0.44 (14)	40.49±0.42 (28)
Lamellibrachia cf *luymesi*	1.4±0.59 (15)	6.1±2.3 (14)	2.6±0.2 (14)	0.79±0.83 (4)	11.90±0.94 (14)
Urechis caupo	3.12±0.19 (12)	4.55±0.61 (12)	ND	0*	2.17±1.06 (5)

R. pachyptila and *L.* cf *luymesi* worms were maintained under *in situ* conditions (see Materials and methods), but in the absence of sulfide, for at least 12 h. Sulfide was then added to the seawater (*R. pachyptila*, 263 μmol l^{-1} $\sum H_2S$; *L.* cf *luymesi*, 500 μmol l^{-1} $\sum H_2S$; *U. caupo*, 100 μmol l^{-1} $\sum H_2S$).

*Below the limits of detection.

Rates are expressed in terms of wet mass.

Values are means ± 1 S.D. (*N*). *N* is the number of samples (measurements) used in determining mean values.

ND, not determined.

$\sum H_2S$, total sulfide concentration.

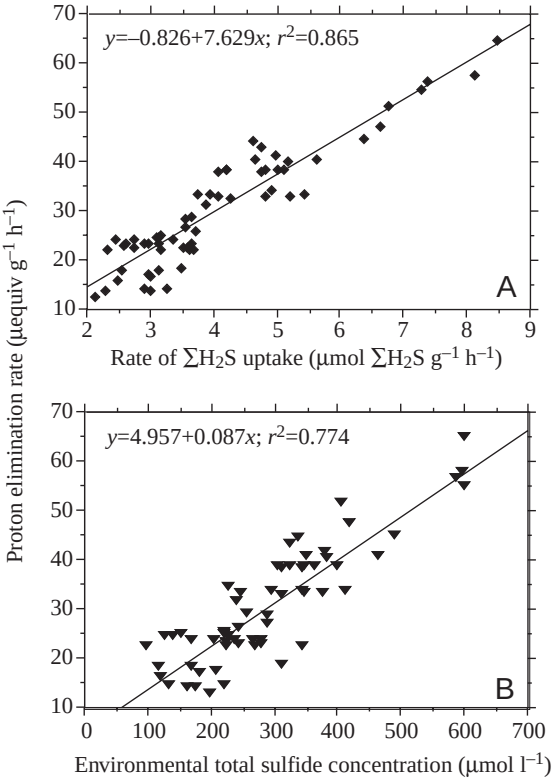

Fig. 2. Plots of proton elimination rate (μequiv g^{-1} h^{-1}) *versus* (A) total sulfide uptake rate (μmol $\sum H_2S$ g^{-1} h^{-1}) and (B) total sulfide concentration (μmol l^{-1}) by *Riftia pachyptila*. The regression lines for the relationship between proton elimination rate and total sulfide uptake rate (A), and the relationship between proton elimination rate and environmental total sulfide (B) are highly significant ($P<0.0001$ and $P<0.005$; ANOVA). In this experiment, the total dissolved sulfide concentration in the seawater was increased incrementally from 100 to 408 μmol l^{-1}, while sulfide uptake and proton elimination rates were determined simultaneously. All other conditions were held under approximately *in situ* conditions ($\sum CO_2$=5.5 mmol l^{-1}, [O_2]=250 μmol l^{-1}, [NO_3^-]=40–65 μmol l^{-1}, pH 6.2; temperature, 12°C, pressure, 27.5 MPa). Rates are expressed in terms of wet mass.

and *x* is environmental total sulfide concentration in μmol l^{-1}; data from Fig. 2). In addition, increased proton elimination rate by *R. pachyptila* correlated strongly with increased sulfide uptake (r^2=0.87; Fig. 2A) for seawater sulfide concentrations of 0–700 μmol l^{-1} (Fig. 2B). The correlation between sulfide uptake and proton elimination rates appeared to remain linear between 0 and 700 μmol l^{-1}. Proton elimination rate changed rapidly in response to changes in environmental sulfide concentration, with proton elimination increasing within 20 min of an increase in seawater sulfide concentration (Table 1). Oxygen uptake rate prior to exposure to sulfide was approximately 4.81 μmol g^{-1} h^{-1}, and increased by 280% after sulfide had been introduced to the aquaria (Table 1). Removing sulfide from the aquarium seawater stopped inorganic carbon uptake and reduced oxygen uptake and proton elimination by approximately 50% and 90%, respectively (Fig. 3).

In our experiments on *L.* cf *luymesi*, exposure to hydrogen sulfide induced proton elimination from nearly undetectable rates to 11.90±0.94 μequiv g^{-1} h^{-1} (mean ± S.D., *N*=14) (Table 1). Increasing the total sulfide concentration in the bottom chamber water from 238±44 μmol l^{-1} to 515±71 μmol l^{-1} total H_2S resulted in a concomitant increase in proton elimination in the top chamber from 5.31±1.39 μmol g^{-1} h^{-1} to 11.90±0.94 μmol g^{-1} h^{-1}, respectively. In addition, the increased total sulfide in the bottom chamber led to a corresponding increase in sulfide uptake by *L.* cf *luymesi* in the bottom chamber (from 0.91±1.2 μmol g^{-1} h^{-1} to 2.6±0.2 μmol g^{-1} h^{-1}, respectively). Due to technical difficulties, proton elimination by *L.* cf *luymesi* into the bottom chamber of the aquaria was not measured.

Prior to sulfide exposure, *U. caupo* exhibited no proton elimination (Table 1). Upon exposure to 100 μmol l^{-1} sulfide, *U. caupo* exhibited a modest rate of proton elimination into the environment for approximately 45 min (2.17±1.06 μmol g^{-1} h^{-1}; mean ± S.D., *N*=5) (Table 1). The rate of proton elimination was five- and 20-fold lower than that of *L.* cf *luymesi* and *R. pachyptila* respectively. The sulfide oxidation rate by the worm,

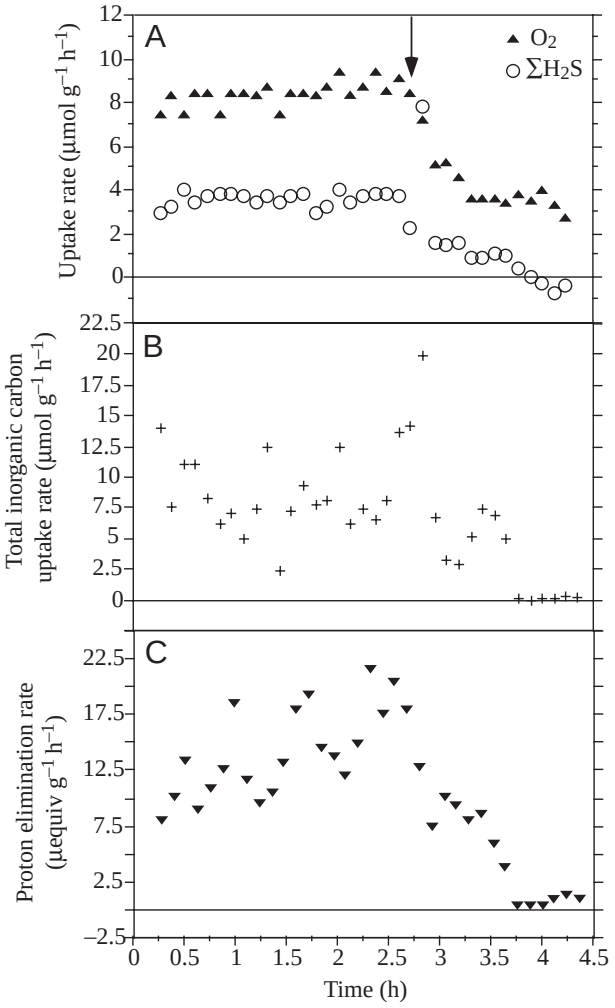

Fig. 3. Effects of decreased environmental sulfide on metabolite uptake rates (A,B) (μmol $g^{-1} h^{-1}$) and proton elimination rates (C) (μequiv $g^{-1} h^{-1}$) by *Riftia pachyptila.* Worms were kept at *in situ* conditions (see Materials and methods) in the aquaria until autotrophy. Gaseous hydrogen sulfide flow into the equilibration column was turned off, and the aquarium seawater sulfide concentrations dropped to levels below our limits of detection (approximately 5 μmol l^{-1}; Childress et al., 1984). The arrow indicates the time at which gaseous sulfide flow was stopped in the equilibration column. Rates are expressed in terms of wet mass. $\sum H_2S$, total sulfide.

more specifically the quantity of sulfide detoxified by the worm, was not determined. A comparison of oxygen uptake rates for the two *U. caupo* groups, one group maintained in seawater and the other exposed to 100 μmol l^{-1} sulfide in seawater, showed a significant difference: 3.12±0.19 and 4.55±0.61 μmol $g^{-1} h^{-1}$, respectively (means ± S.D., $P<0.0001$; $N=4$ worms per group; Mann–Whitney *U*-test).

The effects of inorganic carbon uptake on proton elimination by R. pachyptila

In our experiments, proton elimination rates correlated with changes in environmental inorganic carbon concentrations only while environmental inorganic carbon concentrations were in flux (Fig. 4). Continuously increasing the inorganic carbon concentration in the respirometer aquaria resulted in short-lived, but continuously increasing, proton elimination rates (Fig. 4A), while continuously decreasing the environmental inorganic carbon concentration decreased proton elimination rates by *R. pachyptila* (Fig. 4C). When environmental inorganic carbon concentrations were held constant, there was no correlation between inorganic carbon uptake and proton elimination rates (Fig. 4B). During all three inorganic carbon regimes, seawater sulfide concentrations and *R. pachyptila* sulfide uptake rates remained constant.

The effects of oxygen uptake on proton elimination by R. pachyptila

One of three experiments demonstrated a near-cessation of proton elimination when environmental oxygen tensions decreased from 350 to 78 μmol l^{-1} (Table 2). In this experiment, oxygen uptake was diminished from 12.4±3.6 μmol $g^{-1} h^{-1}$ to 1.9±4.3 μmol $g^{-1} h^{-1}$ (means ± S.D., N=17–19). In two later experiments, proton elimination rates nearly ceased when oxygen tension was reduced to 5 μmol l^{-1} or below (Childress et al., 1984). In these two experiments, the reduced oxygen tensions eliminated sulfide uptake by *R. pachyptila* (Table 2).

The effects of transport inhibitors on proton elimination by R. pachyptila

With the exception of lansoprazole, exposure to all ATPase inhibitors reduced proton elimination rates by at least 96% (Table 3). Amiloride, vanadate and NEM all led to the cessation of proton elimination by *R. pachyptila*, as well as eliminating inorganic carbon uptake and drastically reducing sulfide and oxygen uptake rates (for example, see Fig. 5). Exposure of *R. pachyptila* to lansoprazole reduced proton elimination rate by 17.8% (Table 3), and reduced sulfide uptake rates by 26%. Lansoprazole did not, however, affect the inorganic carbon or oxygen uptake rates of *R. pachyptila*.

Discussion

The necessity to maintain an intracellular pH appropriate for enzyme function mandates that organisms eliminate excess protons. The buffering of protons is an effective mechanism for preventing the onset of acidosis during transient proton production, e.g. during anaerobic metabolism (Heisler, 1993). However, the symbionts of *R. pachyptila* and *L.* cf *luymesi* continuously oxidize sulfide when the organisms are maintained in typical vent conditions (approximately 5 mmol l^{-1} $\sum CO_2$, 110 μmol l^{-1} $\sum H_2S$, 100 μmol l^{-1} O_2, $[NO_3^-]$=40 μmol l^{-1}, pH 5.9, 12.5°C), and this leads to sustained proton production (Fig. 2) and elimination. Because proton elimination rates correlate strongly with sulfide uptake rates and are drastically reduced when oxygen or sulfide levels are experimentally depleted in the aquarium seawater, we suggest that proton elimination is driven by the oxygen-dependent sulfide oxidation of the symbiont. Our *in vivo*

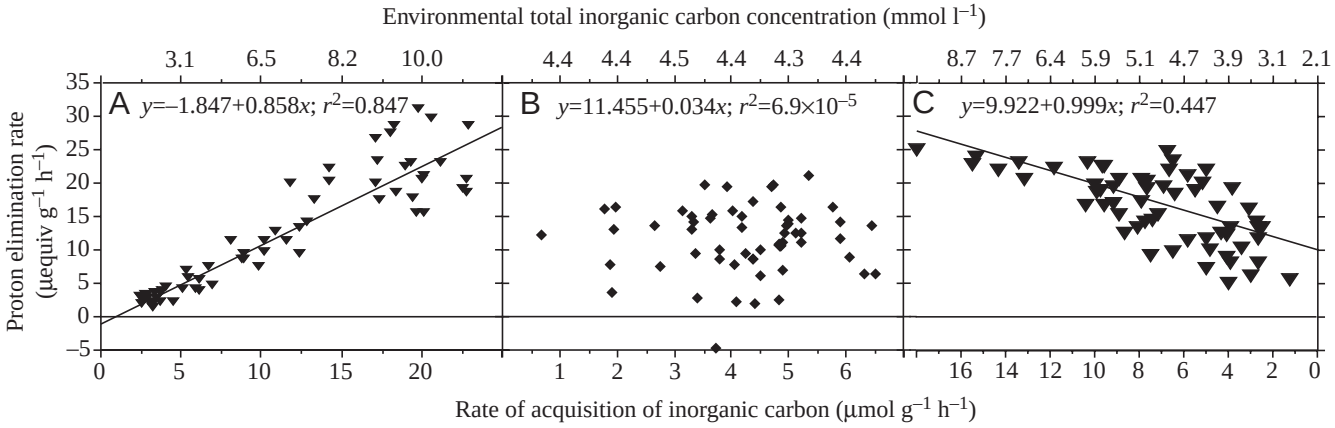

Fig. 4. (A–C) Plots of proton elimination rates (μequiv g^{-1} h^{-1}) *versus* total inorganic carbon uptake rates (μmol g^{-1} h^{-1}) by *Riftia pachyptila* during exposure to three different seawater inorganic carbon regimes. Worms were maintained for several hours to establish autotrophy. Total dissolved seawater inorganic carbon concentration was then varied to produce (A) continuously increasing, (B) steady or (C) continuously decreasing environmental inorganic carbon concentrations, while proton elimination rates were measured. All other conditions were held under approximately *in situ* conditions (see Materials and methods). Rates are expressed in terms of wet mass.

experiments, conducted under environmentally relevant conditions, support the results of previous *in vitro* studies (Childress et al., 1991; Scott et al., 1998). Proton elimination by *R. pachyptila* constitutes the largest mass-specific metabolite flux measured for this species. Proton elimination by *L.* cf *luymesi* also appears to be the highest metabolite flux for the species, although a full respirometric study of *L.* cf *luymesi* remains to be completed.

R. pachyptila, however, does experience anoxia *in situ*, and we chose to examine the contribution of anaerobic metabolism to proton elimination. In two of our hypoxia experiments, *R. pachyptila* maintained at an oxygen concentration below 5 μmol l^{-1} demonstrated drastically reduced proton elimination rates and undetectable inorganic carbon and sulfide uptake rates (Table 2). Protons eliminated by *R. pachyptila* during these experiments are probably the result of anaerobic metabolism, i.e. glycolysis, and are 1–14% of proton elimination rates observed during aerobic sulfide oxidation. Although previous studies have shown that *R. pachyptila* can survive anoxic conditions for 60 h (Arndt et al., 1998; Goffredi,

Table 2. *Proton elimination rates and oxygen, sulfide and inorganic carbon uptake rates by* Riftia pachyptila *during exposure to high and low seawater dissolved oxygen concentrations*

Cruise	Dissolved oxygen concentration (μmol l^{-1})	Proton-equivalent elimination rate (μmol g^{-1} h^{-1})	*P*-value	Oxygen uptake rate (μmol g^{-1} h^{-1})	Sulfide uptake rate (μmol g^{-1} h^{-1})	Inorganic carbon uptake rate (μmol g^{-1} h^{-1})
HOT 96						
High	350±14 (17)	50.6±2.4 (17)	<0.0001	12.4±3.6 (17)	6.75±1.2 (17)	12.45±0.93 (17)
Low	78±6 (19)	0.3±0.52 (19)		1.9±4.3 (19)	0.911±0.14 (19)	0*
HOT 97						
High	394±22 (9)	42.1±4.2 (9)	<0.0001	9.7±2.3 (9)	5.82±0.67 (9)	9.67±2.5 (9)
Low	0*	6.1±2.1 (13)		ND	0*	0*
LARVE 98						
High	314±11 (14)	45.8±6.1 (14)	<0.0001	11.9±1.7 (14)	5.90±1.73 (14)	10.24±1.9 (14)
Low	0*	3.2±0.35 (8)		ND	0*	0*

Worms were maintained until autotrophy had been established (see Materials and methods).

During the HOT 96 expedition, oxygen flow into the equilibration column was reduced to yield 78 μmol l^{-1} dissolved oxygen in seawater. During the HOT 97 and LARVE 98 expeditions, oxygen flow was halted, and oxygen concentrations dropped below our level of detection.

Values are means ± 1 S.D. (*N*). *N* is the number of samples (measurements) used in determining mean values.

Statistical significance was determined using Mann–Whitney non-parametric two-group test.

*Below the limits of detection.

Rates are expressed in terms of wet mass.

For dissolved oxygen concentrations, values are means ± 1 S.E.M.

ND, not determined.

1998), our experiments demonstrate that symbiont function ceases very quickly and that symbiont metabolism cannot be sustained in the absence of oxygen (Fig. 3).

In one of our oxygen experiments, lowering environmental oxygen concentrations to 78 μmol l^{-1} resulted in the cessation of proton elimination, inorganic carbon uptake and sulfide uptake. It is possible that net proton elimination is an oxygen-dependent process (such as a redox proton pump; Steinmetz and Andersen, 1982). However, we suggest that it is more likely that proton elimination is indirectly dependent on energy from aerobic metabolism. Proton elimination by the intertidal worm *Sipunculus nudus* was induced by anaerobic conditions, but the subsequent proton elimination correlated with overall metabolic rate, in particular with aerobic respiration rate (Portner et al., 1991). We agree with these authors that there is a correlation between energy-consuming ion translocation and energy availability, which is primarily derived from aerobic metabolism.

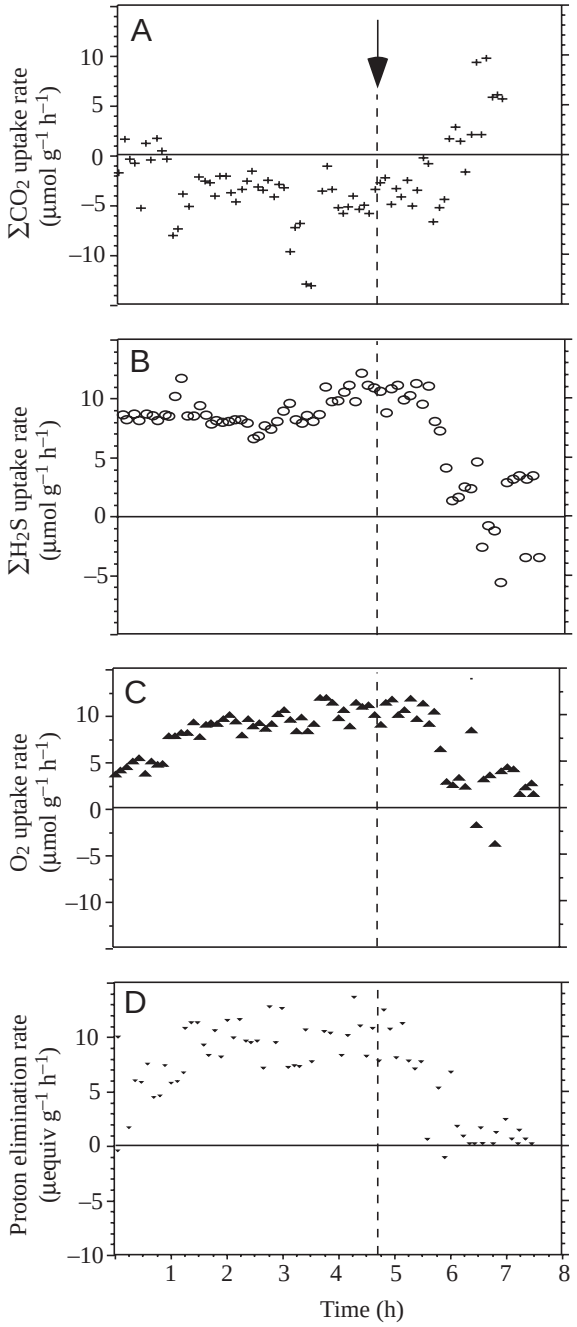

Fig. 5. Plots of the effects of amiloride on metabolite uptake rates (A–C) (μmol g^{-1} h^{-1}) and proton elimination rate (D) (μequiv g^{-1} h^{-1}) by *Riftia pachyptila*. Worms were maintained under *in situ* conditions (see Materials and methods) until they exhibited signs of autotrophy. The arrow denotes the time at which amiloride was added to the seawater to achieve a final concentration of 1 mmol l^{-1}. A flow of fresh seawater was maintained through the aquaria after exposure to amiloride. Rates are expressed in terms of wet mass.

In situ, *R. pachyptila* will also encounter large fluctuations in environmental CO_2 concentrations (from 2.1 to 7.1 mmol l^{-1}; Childress et al., 1993; Goffredi et al., 1997; Johnson et al., 1988). Inorganic carbon acquisition by *R. pachyptila* occurs by diffusion of CO_2 from the environment into the vascular blood, so maintaining the internal pH alkaline relative to the environment is paramount to sustaining inorganic carbon acquisition (Goffredi et al., 1997). Our experiments showed that proton elimination by *R. pachyptila* was not correlated with inorganic carbon concentrations when all environmental metabolite concentrations were maintained at constant levels (Fig. 4). Under these conditions, there is no net production of protons from the acquisition and transport of inorganic carbon by *R. pachyptila* because CO_2 is converted to bicarbonate in the vascular blood and is reconverted to CO_2 for use by the symbionts (Scott et al., 1999). However, transient increases in CO_2 concentration induced proton elimination (Fig. 4), and*R. pachyptila* appeared to eliminate protons while the internal and environmental pools of inorganic carbon pools were equilibrating. We suggest that protons are eliminated to control pH as the bicarbonate concentration increases in the vascular blood. This response is similar to that of organisms experiencing hypercapnia (Arndt et al., 1998; Goffredi et al., 1999; Kochevar et al., 1991).

Interestingly, proton elimination by *R. pachyptila* will also effectively reduce the pH of the seawater in contact with the gill, further favoring the passive influx of carbon dioxide. In addition to the large surface area and the presence of abundant carbonic anhydrase (Goffredi et al., 1999; Kochevar et al., 1991), this mechanism may further enhance the ability of *R. pachyptila* to acquire inorganic carbon for its symbionts.

In the low-pH vent environment (Johnson et al., 1988), proton elimination by *R. pachyptila* occurs against a concentration gradient, so the process must be coupled to ATP hydrolysis. The transport of protons may occur *via* a cation exchanger, e.g. a Na^+/H^+ exchanger, or *via* a proton-translocating ATPase (Tomiyama et al., 1994). During our inhibitor experiments, both vanadate and amiloride (Fig. 5) were very effective at inhibiting proton elimination, suggesting that P-type ATPases and possibly Na^+/H^+-ATPases are involved in net proton elimination. The rates of inorganic carbon and sulfide uptake were also reduced, suggesting that

Table 3. *Effects of four ATPase inhibitors on* Riftia pachyptila *proton-equivalent elimination rates*

Inhibitor	Number of worms exposed	Proton elimination rate (μmol l g^{-1} h^{-1})		Inhibitor targets	Percentage reduction in proton elimination rate post-exposure
		Pre-exposure	Post-exposure		
Amiloride	4	16.44±0.88 (14)	0.59±0.51(11)	All ATPases	96.5
Lansoprazole	5	14.3±3.9 (9)	11.9±5.2 (15)	K^+/H^+-ATPases	17.8
Vanadate	3	12.6±3.6 (19)	–0.05±0.05 (8)	All ATPases	100
N-ethyl-maleimide	4	93.7±13.0 (17)	3.33±0.49 (13)	All ATPases	96.4

Worms were maintained until autotrophy had been established. Inhibitors were then introduced into the seawater by injection prior to the high-pressure seawater pumps. Worms were exposed to inhibitors for at least 6 h. Changes in seawater pH (i.e. the difference in the seawater pH between the control and experimental aquaria) were used to calculate proton elimination rates before and after exposure to the inhibitors.

Values are means ± 1 S.E.M. (*N*). *N* is the number of seawater samples used in the determination of the mean.

All rates are expressed in terms of wet mass.

symbiont metabolic processes are disrupted by the cessation of proton elimination (Fig. 5). However, the potential of these inhibitors to inhibit Na^+/K^+-ATPases and disrupt cellular Na^+ concentrations is a confounding factor. Lansoprazole, a highly specific K^+/H^+-ATPase inhibitor (Sachs et al., 1995), inhibited 17.8% of proton elimination, demonstrating the role of K^+/H^+-ATPases in proton elimination (Table 3). These are the first live-animal experiments detailing the rates and mechanisms of proton exchange by any deep-sea organism, and they suggest that *R. pachyptila* possesses both K^+/H^+-ATPases and Na^+/H^+-ATPases that are involved in sulfide-driven proton elimination. A recent *in vitro* study of frozen *R. pachyptila* tissues has shown high activities of ATPases in the plume, as well as activities of both K^+/H^+-ATPases and Na^+/H^+-ATPases (Goffredi and Childress, 2001). Although that study estimated that 2-6% of the total ATPases are K^+/H^+-ATPases, the present results suggest that a larger percentage of the ATPases of *R. pachyptila* are K^+/H^+-ATPases. It is difficult to determine whether the discrepancy is due to incomplete efficacy of inhibitors in the whole animal or *in vitro* experiments. In addition, proton elimination by H^+-ATPases, e.g. electrogenic proton pumps, cannot be ruled out because they may also account for a large fraction of proton elimination.

The protons generated by symbiont sulfide oxidation are not coupled to oxidative phosphorylation (as are the proton by-products of anaerobic metabolism; Hochachka and Somero, 1984) and are primarily a waste product of symbiont metabolism. Disposing of these protons may represent a large fraction of the energetic costs of *R. pachyptila* and *L.* cf *luymesi*. H^+-translocating ATPases usually translocate 1–3 protons per ATP hydrolyzed (Steinmetz and Andersen, 1982) and at typical *R. pachyptila* proton elimination rates (Table 3), between 12 and 15 μmol g^{-1} h^{-1} ATP should be utilized in proton exchange. Using a ratio of 6.2 ATP per mole of O_2 for aerobic metabolism (Hochachka and Somero, 1984), 2.4 μmol O_2 g^{-1} h^{-1} is involved in ATP synthesis for proton elimination. This accounts for approximately 25% of the oxygen taken up by *R. pachyptila* and 60% of the host's oxygen consumption (determined by eliminating sulfide from the environment and stopping symbiont autotrophic metabolism).

Proton elimination and sulfide uptake rates by *R. pachyptila* are 4–7 times higher than the corresponding rates by *L.* cf *luymesi* when both are exposed to comparable environmental levels of sulfide (approximately 500 μmol l^{-1}; Figs 3, 4). The conservation in the stoichiometry of protons produced per sulfide oxidized by *R. pachyptila* and *L.* cf *luymesi* (5.82±0.17 and 3.84±0.59 protons per sulfide, respectively) suggests that the symbiont pathways of sulfide oxidation may be similar. Our experimental results fall within the range of theoretical and experimental models of the number of protons generated per sulfur oxidized (see fig. 3 in Nelson and Hagen, 1995). Individual variation in these values may result from differences in the rates of reduction of inorganic carbon and nitrogen (Girguis et al., 2000).

Our experiments with *U. caupo* illustrate the pronounced difference in proton elimination rates between chemoautotrophic symbioses and non-symbiotic metazoans. Although we did not quantify the rate of sulfide oxidation of *U. caupo*, we have demonstrated that exposure to sulfide induced proton elimination as well as a significant (P<0.05) change in oxygen consumption rates (Table 1). However, proton elimination by *U. caupo* was short-lived, lasting less than 45 min, and was typically 5–20 times less rapid than in *L.* cf *luymesi* and *R. pachyptila* (Table 1). Non-symbiotic organisms that reside in chemically reduced habitats may exhibit ephemeral proton elimination resulting from the oxidative detoxification of reduced substrates and environmentally induced hypercapnia (e.g. sipunculid worms; Pörtner et al., 1991) but do not require high sustained rates of proton elimination. A previous study found that proton elimination by *Sipunculus audus* averaged 0.08–0.32 μmol g^{-1} h^{-1} and lasted for nearly 3 days (Portner et al., 1991). *U. caupo*, however, exhibited much higher, albeit shorter-lived, proton elimination rates. Neither *S. audus* nor *U. caupo* exhibited rates comparable with those of *R. pachyptila* or *L.* cf *luymesi*.

Although proton concentrations in organisms are typically 3–5 orders of magnitude lower than those of the most prevalent cytoplasmic ions (Hochachka and Somero, 1984), the ability to regulate intracellular and extracellular pH is ubiquitous amongst organisms. Chemoautotrophic symbioses, in

particular those of *R. pachyptila* and others with a relatively high metabolite flux, must contend with the continuous net production of protons by the symbionts and of protons produced by their own metabolism. As the principal waste product of sulfide oxidation, the ability of the host rapidly and efficiently to eliminate protons produced by sulfur metabolism is a necessary adaptation to this mode of symbiosis. While the incurred metabolic costs of proton elimination by *R. pachyptila* and *L.* cf *luymesi* appear to be tremendous, protons are the primary end-product of sulfur oxidation. The rapid and copious elimination of protons by vestimentiferans is an essential adaptation to symbiosis with sulfide-oxidizing bacteria, the absence of which would result in the rapid acidification and eventual metabolic dysfunction of both host and symbiont.

The authors would like to thank Dr Charles Fisher for generously providing the *Lamellibrachia* used in this research, as well as the captains and crew of the *RV New Horizon*, *RV Atlantis II* and *RV Atlantis*, *DSRV Alvin*, *RV Wecoma*, *RV Nadir* and *DSRV Nautile*. We also thank Dr F. Zal and S. Goffredi for their tireless efforts, as well as Dr R. Trench and Dr R. Suarez for their reviews and revisions of this manuscript. We are indebted to Dr Qais Al-Awqati for his comments and insight. Special thanks go to Dr F. Gaill, Dr A. Chave and Dr D. Manahan, chief scientists of the 1996, 1997 and 1998 expeditions. Funding for this project was provided by NSF grants OCE-9301374 (J.J.C.), OCE-9632861 (J.J.C.) and OCE-002464 (J.J.C.).

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