The Journal of Experimental Biology 200, 2609–2616 (1997)
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JEB0920

SULFIDE ACQUISITION BY THE VENT WORM *RIFTIA PACHYPTILA* APPEARS TO BE *VIA* UPTAKE OF HS^-, RATHER THAN H_2S

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Accepted 23 July 1997

Summary

Deep-sea hydrothermal vents are home to a variety of invertebrate species, many of which host chemosynthetic bacteria in unusual symbiotic arrangements. The vent tubeworm *Riftia pachyptila* (Vestimentifera) relies upon internal chemolithoautotrophic bacterial symbionts to support its large size and high growth rates. Because of this, *R. pachyptila* must supply sulfide to the bacteria, which are far removed from the external medium. Internal ΣH_2S ($[H_2S+HS^-+S^{2-}]$) can reach very high levels in *R. pachyptila* (2–12 mmol l^{-1} in the vascular blood), most of which is bound to extracellular hemoglobins. The animal can potentially take up sulfide from the environment *via* H_2S diffusion or *via* mediated uptake of HS^-, or both. It was expected that H_2S diffusion would be the primary sulfide acquisition mechanism, paralleling the previously demonstrated preferential uptake of CO_2. Our data show, however, that the uptake of HS^- is the primary mechanism used by *R. pachyptila* to obtain sulfide and that H_2S diffusion into the worm apparently proceeds at a much slower rate than expected. This unusual mechanism may have evolved because HS^- is less toxic than H_2S and because HS^- uptake decouples sulfide and inorganic carbon acquisition. The latter occurs *via* the diffusion of CO_2 at very high rates due to the maintenance of an alkaline extracellular fluid pH. ΣH_2S accumulation is limited, however, to sulfide that can be bound by the hemoglobins, protecting the animal from sulfide toxicity and the symbionts from sulfide inhibition of carbon fixation.

Key words: tubeworm, *Riftia pachyptila*, sulfide, symbiosis, hydrothermal vent, diffusion, mediated transport, vestimentiferan.

Introduction

The hydrothermal vent tubeworm *Riftia pachyptila* was first found to be symbiotic with intracellular carbon-fixing sulfide-oxidizing bacteria in 1981 (Cavanaugh *et al.* 1981; Felbeck *et al.* 1981). Because *R. pachyptila* relies upon these internal bacterial symbionts for its nutrition, it must supply them with carbon dioxide, oxygen, hydrogen sulfide and other nutrients (Fisher, 1990; Childress and Fisher, 1992). These compounds are taken up from the environment across the plume and transported, *via* a well-developed vascular system, to the bacteria, found in a highly vascularized organ known as the trophosome (Jones, 1981; Arp *et al.* 1985; Childress and Fisher, 1992). This organ is located within the trunk of the worm, surrounded by non-circulating coelomic fluid, which is apparently in equilibrium with the circulating vascular blood for smaller molecules such as CO_2, H^+ and H_2S (Childress *et al.* 1984, 1991).

These worms have two extracellular hemoglobins in the vascular blood and another in the coelomic fluid that bind and transport both oxygen and hydrogen sulfide to the symbiont (Arp *et al.* 1985, 1987; Childress *et al.* 1991; Zal *et al.* 1996*a*,*b*). Vascular blood has a higher capacity for sulfide and contains more total sulfide (ΣH_2S) than coelomic fluid because the larger of the two vascular hemoglobins has a higher capacity (approximately threefold higher) for sulfide binding and overall hemoglobin concentration in the vascular blood is higher (Arp *et al.* 1987; Childress *et al.* 1991). *R. pachyptila* body fluids can reach extremely high concentrations of ΣH_2S (including H_2S, HS^- and S^{2-}), up to 6 mmol l^{-1} in the coelomic fluid and 12 mmol l^{-1} in the vascular blood (Childress *et al.* 1991). Other than the binding of sulfide by the hemoglobins, however, the mechanism of sulfide uptake has not been studied.

Goffredi *et al.* (1997) proposed that the mechanism for inorganic carbon acquisition in these worms is diffusion of the undissociated CO_2 species, which is supported by effective control of body fluid pH by proton-equivalent export, rather than mediated uptake of HCO_3^-. Both carbon dioxide and hydrogen sulfide demonstrate strong pH-dependent dissociation. The pK values, or dissociation constants (i.e. the pH values at which the ratios CO_2:HCO_3^- and H_2S:HS^- are 1:1), for CO_2 and H_2S are 6.1 and 6.6, respectively (Dickson

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and Millero, 1987; Millero *et al.* 1988), at 10 °C and 101.3 kPa. Thus, H_2S, like CO_2, is the dominant chemical species *in situ* owing to the acidic pH (near 6.0) around the worms. H_2S diffusion into the worms would be a function of the external and internal (intra- and extracellular) concentrations of free H_2S, which are functions of ΣH_2S, pH and sulfide binding by the hemoglobins in the blood. Hypothetically, if sulfide were acquired *via* H_2S diffusion, sulfide would be concentrated in the blood, as is the case for inorganic carbon because, at the physiological pH of 7.1–7.5 of *R. pachyptila*, H_2S dissociates into HS^- and H^+ and the protons would normally be eliminated (Childress *et al.* 1984, 1991; Goffredi *et al.* 1997). One would predict in this case that, all else being unchanged, a lower external pH would increase the rate of sulfide uptake while a lower internal (extracellular) pH would decrease uptake.

In contrast, if the mechanism for sulfide acquisition in *R. pachyptila* were HS^- uptake, the pH-dependence of this mechanism would be expected to differ from that of CO_2 and H_2S uptake. HS^- uptake would probably be mediated by the negative charge of the ion. If it were to occur *via* facilitated diffusion, uptake of HS^- might increase with decreases in extracellular pH, because of effects on the equilibrium in the body fluids. It is possible, however, that the uptake of HS^- may not be affected much by changes in internal hydrogen ion concentration.

The purpose of the present study was to determine the mechanism used by *R. pachyptila* to acquire sulfide from the environment. This involved the measurement of internal sulfide concentrations from freshly captured worms and the execution of live animal experiments in pressure systems on board ship. To differentiate between H_2S diffusion and mediated uptake of HS^-, we measured coelomic fluid and vascular blood ΣH_2S of worms in two types of experiments: in one, we varied external H_2S and HS^- levels around the worms; in the other, we forced a decrease in the extracellular pH of the worms by exposing them either to hypoxic water or to a non-specific inhibitor of H^+-ATPases, *N*-ethylmaleimide.

Materials and methods

Collections

Riftia pachyptila Jones were collected at a mean depth of 2600 m by submersible (D.S.R.V. *Alvin* and *Nautile*) during research expeditions to 9°N (9°50′N, 104°18′W) and 13°N (12°48′N, 103°57′W) along the East Pacific Rise in 1994 and 1996. In 1994, hot venting water and warm water samples around the tubeworms were collected using titanium samplers from the *Alvin*. Animals were brought to the surface in a temperature-insulated container and transferred to cold sea water (5 °C) in a refrigerated van on board ship. Worms were then sorted to be used either for experiments on living animals or for immediate measurements of physiological parameters. Live animal experiments were initiated within 2 h of surfacing.

Pressure aquaria

All experiments were conducted inside a refrigerated van. Sea water was chilled by moving the water through polypropylene tubing past a refrigeration unit, after which it was pumped by high-pressure, Teflon diaphragm, metering pumps through stainless-steel vessels at flow rates ranging from 4 to 12 l h^{-1}, at a pressure of approximately 21.5 MPa. Pressure gauges and sample ports were placed in-line immediately after flow through the vessels to allow monitoring of pressure and water conditions. All worms were kept in these pressurized flowing-water aquaria, in which we were able to re-create many aspects of the vent environment, such as temperature, pressure and a variety of chemical conditions, including pH, ΣCO_2, ΣH_2S, O_2 and N_2 concentrations (Quetin and Childress, 1980; Goffredi *et al.* 1997). Water ΣCO_2, O_2 and N_2 levels and P_{CO_2} were varied by bubbling CO_2, O_2 and N_2 gas directly into a gas equilibration column, which supplied water to the high pressure pumps (see diagram in Kochevar *et al.* 1992). Sulfide concentrations were controlled by continuously pumping anaerobic solutions of sodium sulfide (30–50 mmol l^{-1}) into the gas equilibration column at rates dependent upon the desired final concentrations of sulfide. At the end of each experiment, the animals were quickly removed from the pressure vessel and dissected.

Experiments

In one type of experiment (‘sulfide series’), worms were placed in experimental vessels immediately upon collection from the sea floor. Thirty worms were kept at 8 °C and exposed to 4.0±0.5 mmol l^{-1} ΣCO_2 (mean ± S.E.M. for all values given) and 191±18 μmol l^{-1} O_2 for 17–20.5 h. To stabilize the pH, 10 mmol l^{-1} Mops or Mes buffer was added to the sulfide solutions. In some cases, depending on the desired final pH value, it was necessary to alter the pH of the buffered solutions by titration with hydrochloric acid. In this particular experiment, we achieved external [H_2S] values between 0 and 362 μmol l^{-1} and [HS^-] values between 0 and 265 μmol l^{-1} by controlling the external pH between 5.59 and 7.21 and ΣH_2S values between 63 and 511 μmol l^{-1}.

In another experiment (‘hypoxia’ experiments), we exposed twelve worms to hypoxic conditions (at 15 °C) with external oxygen levels no greater than 42 μmol l^{-1} O_2 for 13 h, while control worms were kept at oxygen levels of 316±23 μmol l^{-1}. *In situ* concentrations of O_2 around the worms fluctuate due to the mixing of vent waters (0 μmol l^{-1}) with ambient water (110 μmol l^{-1}). Data on the metabolism of the worms and the distribution of O_2 around them, however, show that they take up O_2 primarily from concentrations approaching 100 μmol l^{-1} (Johnson *et al.* 1988; Childress *et al.* 1991). In our experience, O_2 concentrations between 100 and 400 μmol l^{-1} do not appear to affect the symbioses (P. Girguis, personal communication). All worms in these experiments were kept in the aquaria described above and exposed to typical vent water concentrations of ΣCO_2 (4.4±0.2 mmol l^{-1}), ΣH_2S (156±21 μmol l^{-1}) and pH (6.23±0.05).

In additional experiments (‘inhibitor’ experiments), we initially kept worms in flowing-water maintenance aquaria, also at 8 °C and 21.5 MPa, supplied with surface sea water

(ΣCO_2 levels of 2.1 mmol l^{-1}, pH 8.2, and no sulfide). This experiment, in which we needed a uniform starting point for all of the worms, was directed at measuring the rates of uptake of CO_2 and sulfide over time; thus, we needed these worms to have low internal levels of ΣCO_2 and ΣH_2S, which resulted from maintenance in surface sea water. In order to observe uptake rates over time, the animals were then transferred to experimental vessels (for 0–20 h), and water conditions (ΣCO_2 4.8±0.1 mmol l^{-1}, O_2 230±9 μmol l^{-1}, ΣH_2S 311±39 μmol l^{-1} and pH 5.96±0.05) were controlled in the same manner as described for the sulfide series and hypoxia experiments. 15 worms were used as controls and 9 worms were exposed to *N*-ethylmaleimide (NEM), a non-specific inhibitor of H^+-ATPases, at concentrations between 1.1 and 1.6 mmol l^{-1} for 1–2 h (Marver, 1984; Stone *et al.* 1984).

It is important to note that during the 1–12 h of these inhibitor experiments, it is likely that the worms were not in autotrophic balance. It has been suggested that it takes at least 14 h in the presence of sulfide for these worms to become autotrophic (net uptake of CO_2; Childress *et al.* 1991). Another study has shown that, at 12 h, and under conditions similar to ours, there is some assimilation of both $^{13}CO_2$ and $^{15}NO_3^-$ by *R. pachyptila*, indicating that the symbionts are functioning (Lee and Childress, 1994). Regardless, time points before 12 h are meaningful in terms of the functioning of the animal, and for our studies it was more important to isolate the physiology of the worms from that of the symbionts.

Analytical techniques

All worm dissection techniques were similar to those described in Childress *et al.* (1991) and Goffredi *et al.* (1997). ΣCO_2 and ΣH_2S of coelomic fluid, vascular blood and water samples were measured on 0.5 ml samples using a Hewlett Packard 5880A series gas chromatograph (Childress *et al.* 1984). 'ΣH_2S', as measured by the gas chromatograph, represents the sum of H_2S, HS^-, S^{2-} concentrations and the amount of sulfide bound to the hemoglobins. 'ΣCO_2', as measured by the gas chromatograph, represents the sum of CO_2, HCO_3^- and CO_3^{2-} concentrations. Body fluid and water pH were measured using a thermostatted Radiometer BMS-2 blood pH analyzer equipped with a G299A capillary pH electrode and connected to a PHM73 pH meter. Additional water pH measurements were made using a double-junction combination electrode (Broadley-James) connected to a PHM93 pH meter (Radiometer).

Statistics

The Kendall rank correlation was used to test for correlations. The Mann–Whitney *U*-test was used to test for differences in distribution between data sets. Simple regressions were used to show linear relationships, and multiple regressions were used to compare influences of various parameters. The analysis of covariance (ANCOVA) was used to compare slopes and magnitudes of different data sets.

Results

Both freshly collected and experimental worms showed an increase in internal sulfide levels as the surrounding water pH increased. For freshly collected worms, the ΣH_2S values in both coelomic fluid and vascular blood increased as the surrounding water pH increased (P=0.0025 and P=0.0151, respectively, Kendall rank correlation). For 'sulfide series' worms, the coelomic fluid and vascular blood ΣH_2S levels also increased as we increased the surrounding water pH (P=0.0007 and P=0.0013, respectively, Kendall rank correlation). In addition, the internal ΣH_2S levels of these worms were positively correlated with the extracellular pH (y=3.451x–23.676, P=0.0006, and y=6.661x–43.014, P<0.0001 for the regressions of coelomic fluid and vascular blood of 'sulfide series' worms, respectively, where y is ΣH_2S and x is extracellular pH).

Worms collected from the sea floor and placed immediately into 'sulfide series' experiments had initial internal ΣH_2S levels of 0.18±0.12 mmol l^{-1} and 0.43±0.17 mmol l^{-1} for coelomic fluid and vascular blood, respectively. In order to determine which external species of sulfide influenced internal ΣH_2S levels, we controlled the free H_2S and HS^- concentrations in the surrounding water for 17–20.5 h. It should be noted that there was no correlation between water H_2S and HS^- levels during these experiments (P=0.55, Kendall rank correlation). In order to determine whether this 3.5 h variation among experiments played an important role in the values measured, we plotted internal sulfide concentration *versus* the incubation time of these experiments. Both graphs (data not shown) showed no dependence of internal sulfide levels on incubation time over the limited range of times used in our experiments (P=0.95 and P=0.74 for the regressions of vascular blood and coelomic fluid, respectively). This time, however, far exceeds the time necessary to reach sulfide equilibrium with the surrounding water, as considered below.

Fig. 1 shows that both coelomic fluid and vascular blood ΣH_2S levels correlated well with external HS^- (P<0.0001), but not with H_2S (P=1). A multiple regression was used to determine whether H_2S or HS^- level in the external medium had a greater influence on internal ΣH_2S values. It was apparent from this test that HS^- plays a greater role (P=0.0001) in predicting coelomic fluid ΣH_2S than does H_2S (P=0.0901) and also in predicting vascular blood ΣH_2S values (P=0.001 for HS^- and P=0.81 and H_2S). Both the coelomic fluid and vascular blood pH increased as water HS^- increased (y=1.875x+7.287, P=0.0058, and y=2.411x+7.182; P=0.0018, respectively, for the regression equations, where y is pH and x is water [HS^-]). A multiple regression analysis was used to determine whether H_2S or HS^- level in the external medium had a greater influence on extracellular pH. Again, HS^- plays a greater role in predicting coelomic fluid and vascular blood pH (P=0.0124 and P=0.0052, respectively) than does H_2S (P=0.39 and P=0.21, respectively).

To test the effect of body fluid pH upon sulfide uptake, we conducted two types of whole-animal experiments in which we forced a decrease in the extracellular pH of the worms. In the

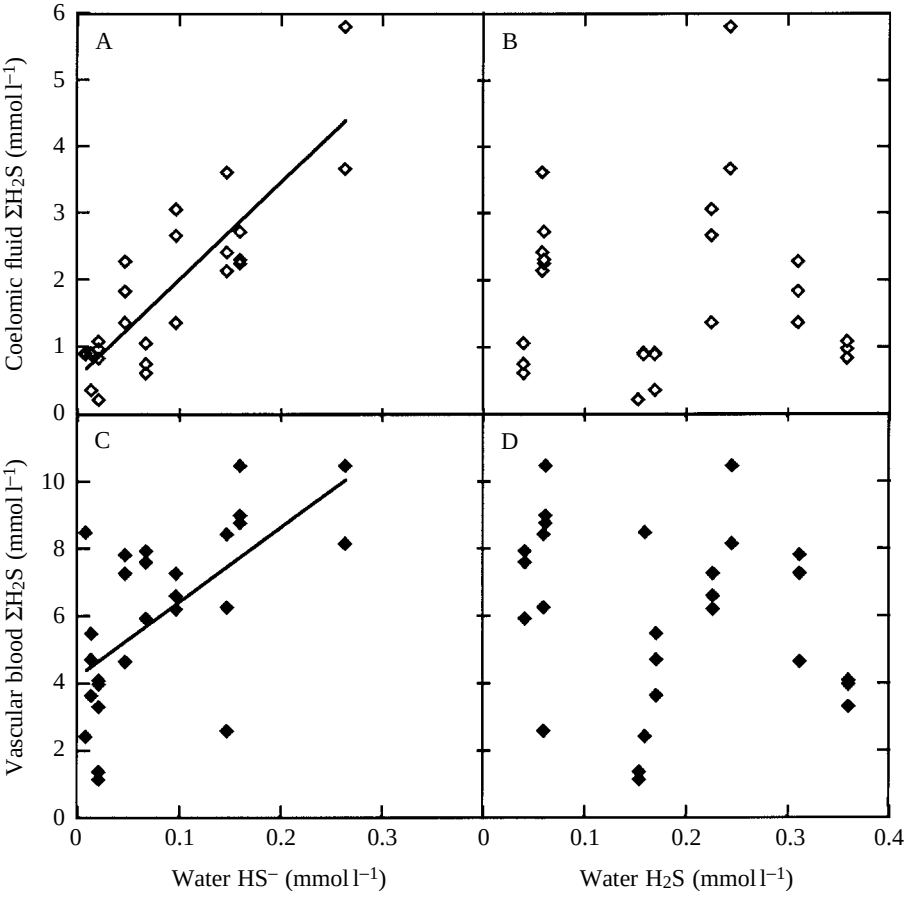

Fig. 1. Relationship between total inorganic sulfide concentration, ΣH_2S, in coelomic fluid and vascular blood from the tubeworm *Riftia pachyptila* and the HS^- (A,C) and H_2S (B,D) levels of the surrounding water during 12 shipboard experiments. Experimental vessel conditions were controlled at fixed combinations of pH and ΣH_2S. ΣH_2S values between 63 and 511 μmol l^{-1}, and pH values between 5.6 and 7.2 were achieved (N=30). (A) y=0.515+14.585x, r=0.86, P<0.0001; (B) y=1.789+0.030x, r=0.003, P=1, not significant; (C) y=4.164+22.177x, r=0.63, P=0.0004; and (D) y=7.049+5.617x, r=0.23, P=1, not significant.

first experiment, twelve worms were exposed to hypoxic conditions with external oxygen levels no greater than 42 μmol l^{-1} O_2. Exposure to these low oxygen levels resulted in significantly decreased extracellular pH (Table 1), probably because of the build up of end-products of anaerobic metabolism. However, this decrease in extracellular pH failed to significantly affect internal ΣH_2S levels in these worms (Table 1). The situation for internal ΣCO_2 was reversed in that the decrease in extracellular pH caused a significant decrease in body fluid ΣCO_2 levels (Table 1). This was expected for ΣCO_2 because inorganic carbon is concentrated into the worms as a result of the pH difference maintained between the internal and external fluids (Goffredi *et al.* 1997). More importantly, the mechanism for sulfide acquisition appears to have a different pH-dependence from that for inorganic carbon acquisition.

In the second experiment, in which 9 worms were exposed to *N*-ethylmaleimide and 15 were used as controls, we followed the accumulation of sulfide and inorganic carbon in the worms over time. After 2–4 days in water with a low P_{CO_2} and no sulfide, coelomic fluid ΣCO_2 levels were 3.8±0.7 mmol l^{-1} and ΣH_2S levels were 0.002±0.001 mmol l^{-1}

Table 1. *Body fluid pH, ΣH_2S and ΣCO_2 of control and hypoxic* Riftia pachyptila

Group	Coelomic fluid pH	Coelomic fluid ΣH_2S (mmol l^{-1})	Coelomic fluid ΣCO_2 (mmol l^{-1})	Vascular blood pH	Vascular blood ΣH_2S (mmol l^{-1})	Vascular blood ΣCO_2 (mmol l^{-1})
Control worms	7.14±0.05	1.10±0.23	17.81±1.98	7.09±0.04	5.76±0.89	16.16±1.71
Hypoxic worms	6.64±0.03	0.99±0.22	6.34±0.72	6.79±0.06	4.98±1.69	5.56±0.89
P	0.0012	0.9326	0.0004	0.0253	0.9035	0.0036

Mean (± S.E.M.) extracellular pH, ΣH_2S and ΣCO_2 for control (N=14) and hypoxic (N=12) tubeworms kept in high-pressure flowing water aquaria at 15 °C, 21.5 MPa, and exposed to external ΣH_2S concentrations of 156±21 μmol l^{-1}, ΣCO_2 values of 4.4±0.2 mmol l^{-1}, and pH values of 6.23±0.05 for 13 h.

Hypoxic worms were kept at oxygen levels below 42 μmol l^{-1} and control worms were kept at oxygen levels of 316±23 μmol l^{-1}.

P values are for the Mann–Whitney U-test for differences in internal parameters between the control and hypoxic worms.

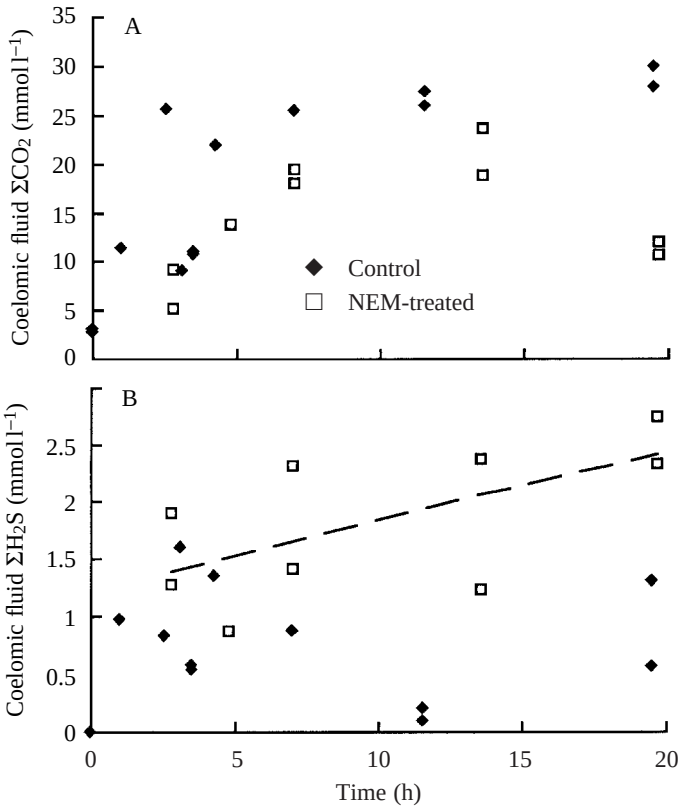

Fig. 2. Coelomic fluid ΣCO_2 (A) and ΣH_2S (B) values over time for both control (filled symbols, *N*=15) and NEM-treated (open symbols, *N*=9) *Riftia pachyptila* kept in high-pressure flowing-water aquaria and exposed to external ΣCO_2 concentrations between 4.6 and 5.5 mmol l^{-1}, a P_{CO_2} of 6.2 kPa, pH between 5.9 and 6.2, and ΣH_2S between 0.1 and 0.6 mmol l^{-1}. NEM-treated worms were exposed to *N*-ethylmaleimide at concentrations between 1.1 and 1.6 mmol l^{-1} for approximately 1–2 h. In B, the regression line for NEM-treated worms is y=1.223+0.061x, r=0.63, P=0.0211. The regression for control worms (y=0.485+0.019x, r=0.24, P=0.5339) was not significant.

(worms at time zero). When subsequently exposed to flowing water at typical vent conditions (4.8±0.1 mmol l^{-1} ΣCO_2 and 0.31±0.04 mmol l^{-1} ΣH_2S), these worms demonstrated increases in total extracellular fluid inorganic carbon and sulfide. Between 0 and 7 h, the rate of increase in coelomic fluid ΣCO_2 in control worms was 3.22 mmol l^{-1} h^{-1}, with the rate leveling off after 7 h at a mean ΣCO_2 concentration of 27.8 mmol l^{-1} (Fig. 2A) (see also Goffredi *et al.* 1997). NEM-treated worms, however, did not accumulate inorganic carbon in the blood as rapidly (2.2 mmol l^{-1} h^{-1}) and ΣCO_2 did not plateau at the same level (17.1 mmol l^{-1}; Fig. 2A). In addition, the control worms demonstrated a sulfide uptake rate of 0.97 mmol l^{-1} h^{-1} within the first hour (Fig. 2B). There appeared to be no further increase in the internal ΣH_2S of control worms (mean 0.81±0.14 mmol l^{-1}) after 1 h. There was, however, a slight increase (from 1.59 to 2.55 mmol l^{-1} at a rate of 57 μmol l^{-1} h^{-1}) in the internal ΣH_2S of NEM-treated worms over time (P=0.0211; Fig. 2B). The mean external sulfide concentrations for the two groups were not significantly different (Mann-Whitney test, 0.27±0.05 mmol l^{-1} for control and 0.37±0.07 mmol l^{-1} for NEM-treated worms); however, internal ΣH_2S and external ΣH_2S for both groups of worms are not correlated over the limited range in this data set (P=0.28, Kendall rank correlation). Thus, the difference in external ΣH_2S levels cannot explain the difference in internal ΣH_2S levels measured between the two groups. After 4 h, the increase in coelomic fluid ΣH_2S in the NEM-treated worms was not significantly different from that in the control worms (ANCOVA, P=0.1650); however, they were significantly different in overall magnitude (ANCOVA, P=0.0013).

It has been proposed that regulation of extracellular pH in *R. pachyptila* occurs primarily through proton-equivalent ion transport *via* an ATP-requiring process, specifically *via* H^+-ATPases (Goffredi *et al.* 1997). The apparent inhibition of proton-equivalent ion transport by NEM resulted in significant decreases of approximately 0.4 pH units in both coelomic fluid and vascular blood pH (Table 2). This decrease in extracellular pH, however, did not result in a decrease in internal ΣH_2S levels, as expected in the case of H_2S diffusion, but rather a significant increase in internal sulfide levels (Table 2). Again, this result is contrary to that seen for internal ΣCO_2, in which a lower extracellular pH caused a reduction in the diffusion gradient for CO_2, resulting in significant decreases in internal ΣCO_2 (Table 2). These contrasting results support different modes of acquisition for inorganic carbon and sulfide.

Table 2. *Body fluid pH, ΣH_2S and ΣCO_2 of control and NEM-treated* Riftia pachyptila

Group	Coelomic fluid pH	Coelomic fluid ΣH_2S (mmol l^{-1})	Coelomic fluid ΣCO_2 (mmol l^{-1})	Vascular blood pH	Vascular blood ΣH_2S (mmol l^{-1})	Vascular blood ΣCO_2 (mmol l^{-1})
Control worms	7.26±0.05	0.81±0.14	27.83±0.74	7.15±0.05	4.63±0.38	25.65±1.08
Hypoxic worms	6.84±0.08	1.72±0.22	17.13±1.82	6.81±0.06	8.67±0.52	15.58±1.44
P	0.0022	0.0075	0.0105	0.0007	0.0002	0.0105

Mean (± S.E.M.) extracellular pH, ΣH_2S and ΣCO_2 for control (*N*=15) and inhibited (*N*=9) tubeworms kept in high-pressure flowing water aquaria at 8 °C, 21.5 MPa, and exposed to external ΣH_2S concentrations of 311±39 μmol l^{-1}, ΣCO_2 values of 4.8±0.1 mmol l^{-1}, and pH values of 5.96±0.05 for at least 4 h.

Inhibited worms were exposed to *N*-ethylmaleimide (NEM), at concentrations between 1.1 and 1.6 mmol l^{-1} for 1–2 h.

P values are for the Mann–Whitney *U*-test for differences in internal parameters between the control and the NEM-treated worms.

Discussion

On the basis of our studies, we propose a model for sulfide uptake in *R. pachyptila* in which sulfide acquisition from the environment is primarily *via* HS^- uptake facilitated by transporter or channel proteins. Once across the outer epithelium of the plume, HS^- must enter the vascular blood compartment, where it is bound by the sulfide-binding hemoglobins present in the blood and transported to the bacterial symbionts. H_2S movement into these worms, however, appears to be severely limited.

Both freshly collected and experimental worms had higher internal ΣH_2S levels as the surrounding water pH increased. This suggests that external HS^- is the most important factor affecting internal ΣH_2S values because HS^- is more abundant than H_2S at higher pH. In addition, at higher extracellular pH in experimental worms, the internal ΣH_2S levels were also higher, suggesting that the species of sulfide being accumulated increases the internal pH. Both multiple regressions indicate that internal ΣH_2S levels and extracellular pH are influenced more by external [HS^-] than by external [H_2S]. This suggests that external HS^- levels play a greater role in the uptake of sulfide in these animals than do H_2S levels.

Our experiments involving depressed extracellular pH also support the contention that HS^- is the primary species of sulfide moving into the worms. We observed a different pH-dependence for sulfide acquisition from that expected for H_2S diffusion and measured for CO_2 diffusion (Goffredi *et al.* 1997). As the extracellular pH in these animals was depressed (by exposure to hypoxic conditions or *N*-ethylmaleimide), no decrease in internal sulfide levels was observed. NEM inhibits enzymes by forming covalent bonds with sulfhydryl groups (SH^-), causing deleterious conformational changes in these enzymes (Stone *et al.* 1984; Lin and Randall, 1993). Thus, it is possible for NEM to react with a variety of enzymes and proteins possessing reactive sulfhydryl groups, including *R. pachyptila* hemoglobins, which have been shown to contain free cysteine residues (Zal *et al.* 1997). Experiments were conducted to determine whether NEM adversely affects sulfide binding by *R. pachyptila* hemoglobin (Zal *et al.* 1997). In summary, when *R. pachyptila* hemoglobin was pre-treated with NEM, prior to any exposure to sulfide, a 30 % decrease in sulfide binding resulted. However, if the hemoglobin was first exposed to sulfide, as in the case of our experiments, there was no effect of NEM on sulfide binding (Zal *et al.* 1997). The worms were given NEM and sulfide simultaneously; therefore, they were not pretreated with the inhibitor as sulfide would be expected to move into the worms faster than NEM. In addition, we feel that because there was no decrease in internal sulfide levels, adverse effects on other proteins did not create artifacts.

Our results also show that there is discrimination against H_2S movement into the extracellular fluids of these animals. In general, organisms are believed to be unable to block the diffusion of H_2S across membranes while still retaining permeability to other gases, such as CO_2 and O_2, both of which diffuse into *R. pachyptila* (Somero *et al.* 1989; Bagarinao, 1992; Völkel, 1995; Goffredi *et al.* 1997). For example, it has been shown that the shrimp *Crangon crangon*, which inhabits shallow sandy areas, is only permeable to H_2S and that there is no uptake of HS^- (Vismann, 1996). Researchers have also shown that sulfide penetration into the alga *Valonia macrophysa* increased as external pH decreased, indicating that for this alga H_2S is the more permeable of the two sulfide species (Jacques, 1936). Although *Urechis caupo*, the fat innkeeper worm, shows a higher H_2S permeability, HS^- permeability across the body wall has been demonstrated to be 37 % of the H_2S permeability (Julian and Arp, 1992). In contrast, it appears that H_2S movement into *R. pachyptila* is much lower than expected, limited by some currently unknown mechanism.

The specific mechanism for HS^- uptake is also unknown at this time; however, we propose that HS^- enters *via* facilitated diffusion due to its charge and because of the strong correlation between internal ΣH_2S and external [HS^-]. Although [HS^-] is relatively low in the vent environment (50 $\mu mol\,l^{-1}$ at pH 6.0 and 300 $\mu mol\,l^{-1}$ ΣH_2S), a gradient for HS^- movement into the worms is created and maintained by the high concentrations of sulfide-binding hemoglobins present in the body fluids (Arp and Childress, 1983; Arp *et al.* 1987; Fisher *et al.* 1988). Sulfide binding by the hemoglobins has been shown to be maximal at pH 7.5, which suggests that the actual species of sulfide bound by the hemoglobins is HS^- (Childress *et al.* 1984).

For two reasons, we believe that HS^- uptake, as the mechanism for sulfide acquisition in *R. pachyptila*, acts as a protection against sulfide poisoning. The first is the fact that perhaps the two species of sulfide ([S^{2-}] is negligible, with a pK of 12–13) are not equally toxic to the animal (Bagarinao and Vetter, 1990). It has been suggested that H_2S is more toxic than HS^-, and that H_2S is actually the species of sulfide that binds to the cytochrome *c* oxidase complex (Smith *et al.* 1977; Powell and Somero, 1986; Bagarinao and Vetter, 1990; Oeschger and Vismann, 1994). Specifically, Powell and Somero (1986) have shown that cytochrome *c* oxidase activity in *R. pachyptila* plume tissue, in the presence of sulfide, decreases markedly with decreasing extracellular pH (from 7.0 to 6.0), suggesting that H_2S is the more inhibitory form of sulfide. In a similar experiment, it was shown that there was no HS^- inhibition of mitochondrial respiration of the killifish *Fundulus parvipinnis* and, again, that H_2S was the toxic form of sulfide (Bagarinao and Vetter, 1990). If this were the case for *R. pachyptila*, it would be advantageous for these worms to exclude H_2S while importing HS^-.

The second possibility is that if inorganic carbon and sulfide were acquired *via* the same mechanism, i.e. diffusion of the undissociated form (CO_2 and H_2S), *R. pachyptila* could not control sulfide uptake independently. Thus, the second way in which HS^- uptake could protect *R. pachyptila* from sulfide poisoning would be to decouple sulfide acquisition from inorganic carbon acquisition. Although *R. pachyptila* must maintain an alkaline extracellular pH in order to concentrate inorganic carbon internally, if sulfide were accumulated in the same way, *via* H_2S diffusion, free sulfide, like ΣCO_2, would

reach very high concentrations in the blood. This unlimited accumulation of sulfide internally could potentially poison the worm and its symbionts. However, with the proposed mechanism, sulfide uptake is expected to be largely limited by the binding capacity of the hemoglobins.

In contrast, the vesicomyid clam *Calyptogena elongata*, which also contains chemoautotrophic symbionts that it must supply with sulfide, does not maintain its extracellular pH constant in the face of changing external and internal ΣH_2S levels (Childress *et al.* 1993*a*). Specifically, when exposed to increasing amounts of internal ΣH_2S, the extracellular pH of *C. elongata* decreases (Childress *et al.* 1993*a*). *C. elongata* can potentially accumulate sulfide *via* H_2S diffusion because increasing levels of internal ΣH_2S cause the extracellular pH to decrease, which dissipates the sulfide gradient into the animal, acting as a self-limiting sulfide acquisition mechanism for the clam.

R. pachyptila, however, does not rely on the same self-limiting mechanism and has apparently evolved an alternative mode of sulfide acquisition, mediated transport of HS^-, as well as reduced permeability to, or some discrimination against, H_2S, apparently as a protection against sulfide poisoning. In this way, *R. pachyptila* is able to control sulfide movement, while keeping the extracellular pH stable and alkaline. Restricting the internal ΣH_2S level to that which can be bound by the hemoglobins ensures that, even at high external ΣH_2S levels, internal sulfide levels in *R. pachyptila* are not toxic to either partner but are still sufficient for the symbionts. This mechanism appears to be a further specialization of *R. pachyptila* for successfully supporting autotrophic endosymbionts and thriving in such a hostile environment.

Funding for this project was provided by NSF grants OCE-9301374 (J.J.C.) and OCE-9632861 (J.J.C.) and by Ifremer URM 7 (F.H.L.). The authors thank the captains and crew of the R.V. *New Horizon*, R.V. *Atlantis II*, R.V. *Nadir*, R.V. *Wecoma*, D.S.R.V. *Alvin* and D.S.R.V. *Nautile*. We also thank R. Lee, P. Girguis, S. Powell, J. Freytag and J. Smith for technical support at sea. Finally, we would like to thank L. Mullineaux and F. Gaill, chief scientists on the HERO 94 and HOT 96 expeditions, respectively.

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