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ANAEROBIC SULFUR METABOLISM IN THIOTROPHIC SYMBIOSES

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Summary

Hydrogen sulfide is generally accepted to be the energy source for the establishment of sulfur-oxidizing symbiotic communities. Here, we show that sulfur-storing symbioses not only consume but also produce large amounts of hydrogen sulfide. The prerequisite for this process appears to be the absence of oxygen. Anaerobic sulfide production is widespread among different thiotrophic symbioses from vent and non-vent sites (*Riftia pachyptila*, *Calyptogena magnifica*, *Bathymodiolus thermophilus*, *Lucinoma aequizonata* and *Calyptogena elongata*). The extent of H_2S generation correlates positively with the amount of elemental sulfur stored in the symbiont-bearing tissues of the hosts. Sulfide production starts a few hours after anoxia sets in, with H_2S initially accumulating in the circulatory system before it is excreted into the surrounding environment. We propose that not sulfate but the elemental sulfur deposited in the symbionts serves as a terminal electron acceptor during anoxia and is reduced to sulfide. In anoxia-tolerant symbioses such as *L. aequizonata*, anaerobic sulfur respiration may be important for producing maintenance energy to help the species survive several months without oxygen. The increased levels of cysteine in the gills of *L. aequizonata* may be caused by a lack of reoxidation due to the absence of oxygen.

Key words: anoxia, anaerobic sulphide production, chemoautotrophic symbioses, *Lucinoma aequizonata*, *Riftia pachyptila*, *Calyptogena magnifica*, *Bathymodiolus thermophilus*, hydrogen sulphide, elemental sulphur, cysteine, glutathione, thiosulphate, sulphur respiration.

Introduction

Sulfur compounds are an important energy source for symbiotic associations between invertebrates and chemoautotrophic bacteria (Nelson and Fisher, 1995; Fisher, 1990). The energy released from the oxidation of reduced sulfur compounds by the intracellular symbionts can be trapped in the form of ATP and used for carbon fixation through the Calvin–Benson cycle. This process is the nutritional basis for these symbioses (Fisher, 1990).

Animals with intracellular, sulfur-metabolizing endosymbionts are found in a wide range of environments. Since their discovery at the deep-sea hydrothermal vents in 1977 (Cavanaugh et al., 1981; Felbeck, 1981; Corliss et al., 1979), many new species with symbionts have been described every year (Bernhard et al., 2000; Dubilier et al., 1998; Bauer-Nebelsick and Ott, 1996; Felbeck and Distel, 1992). Sewage outfalls, mangrove swamps, eelgrass beds and stagnant ocean basins are common habitats where sulfur-metabolizing symbioses thrive. The conditions that support the establishment of these bacteria–invertebrate associations require the presence of a redox boundary where reduced sulfur compounds are in close proximity to oxidized reagents such as oxygen or nitrate.

Symbiont-bearing organisms often have to deal with extreme environmental situations such as the depletion of oxygen in their immediate surrounding. At the hydrothermal vents, the ‘source end-member water’ is devoid of oxidized compounds (Corliss et al., 1979) and, depending on the degree of mixing with the surrounding sea water, vent fauna settling nearby may experience periods of severe hypoxia (Johnson et al., 1988). First investigations on the anaerobic capacity of the hydrothermal vent tubeworm *Riftia pachyptila* showed that this species can survive oxygen deficiency for at least 60 h (Arndt et al., 1998a; Arndt et al., 1998b). In non-vent habitats, symbiont-containing animals such as lucinid bivalves often thrive in environments that are periodically depleted of oxygen (Bernhard and Reimers, 1991). The clam *Lucinoma aequizonata* inhabits the dysoxic mud ($10\,\mu mol\,l^{-1}$ O_2) of the Santa Barbara Basin (California, USA) at a depth of 500±10 m and is the dominant macrofaunal species in this zone (Cary et al., 1989).

Studies on carbon-based energy metabolism have given a first insight into the potential anaerobic pathways of symbiotic organisms (Arndt et al., 1998a; Arndt et al., 1998b; C. Arndt, J.-P. Lechaire, H. Felbeck, in preparation). There is, however, very little information to date about how their sulfur metabolism changes when no oxidant is available. An unusual phenomenon that may be attributed to the sulfur-metabolizing activity of the symbiosis is the generation of hydrogen sulfide

under anaerobic conditions. It has frequently been observed that the incubation medium of *Lucinoma aequizonata* develops a strong sulfidic odor when the clams are incubated under anoxia even when no sulfide is offered during the experiment. Anoxic sulfide production has previously been reported in the bivalve *Calyptogena elongata* (Childress et al., 1993). These authors assumed that either sulfate from the sea water or the elemental sulfur stored in the symbionts of the clam was the substrate for this process. Because they failed to detect similar findings in other symbiotic systems, it was suggested that anaerobic sulfide production was unique to *C. elongata*.

In the present study, we gain insight into the metabolic mechanisms leading to the production of sulfide in chemoautotrophic symbioses. This anaerobic process is investigated in various thiotrophic symbioses to describe its distribution among different classes of organism. Using the example of the lucinid clam *Lucinoma aequizonata*, we provide indications about the sulfur source responsible for H_2S accumulation and discuss its overall significance for the clam's metabolism. Furthermore, sulfur-containing metabolites in the bacteria-containing gill tissues are analyzed to estimate the impact of long-term anoxia on the general sulfur metabolism of *L. aequizonata*.

Materials and methods

Collection of specimens

The tubeworm *Riftia pachyptila* Jones 1980, the clam *Calyptogena magnifica* Boss and Turner 1980 and the mussel *Bathymodiolus thermophilus* Kenk and Wilson 1985 were collected by the submersible *Alvin* during hydrothermal vent cruises to the East Pacific Rise (9°N) in 1998 and 1999. Animals were held in a temperature-insulated container during recovery and transferred to a cooler with chilled sea water once on board. Specimens that were used in experiments were dissected immediately. The tissues were weighed on a motion-compensated shipboard balance system (Childress and Mickel, 1980).

Lucinoma aequizonata Stearns 1890 was collected from the Santa Barbara Basin at a depth of 500±10 m using an otter trawl from the R/V *Robert Gordon Sproul*. The clams were kept in chilled sea water until return to the Scripps Institution of Oceanography, where they were transferred to a flow-through seawater aquarium (10 °C) containing fresh mud from their habitat. They were maintained there until used in experiments.

Anaerobic sulfide production in live specimens

Specimens of the hydrothermal vent tubeworm *Riftia pachyptila* were incubated at 15 °C in high-pressure aquaria under anoxic conditions (<1 μmol l^{-1} O_2) (Arndt et al., 1998a). The sulfide concentrations in the circulatory system of the worms were analyzed after different incubation periods. To lower the initial sulfide level in the blood, worms were first incubated in O_2 saturated sulfide-free sea water for 1 day. Before starting an anoxic experiment, a plastic catheter was inserted into a blood vessel in the vestimentum of the worms; this was connected to a needle valve, allowing blood to be sampled while the worm was maintained under pressure (H. Felbeck, C. Arndt, M. Wells, U. Hertschel and J. J. Childress, in preparation). Sulfide, representing the sum of free H_2S and the amount bound to the hemoglobins in the blood, was determined *via* gas chromatography according to the method of Childress et al. (Childress et al., 1984). At the end of an experiment, the worms were killed. The coloration of the trophosome, which is an indication of the S^0 content (yellow represents a high content, black represents a low content; Fisher, 1990), was noted.

In the experiments with *Lucinoma aequizonata*, sulfide excretion was investigated by incubating the specimens in the dark in 500 ml jars containing anoxic sea water (<1 % air saturation) (10 °C). A small-diameter plastic tube was carefully inserted into the mantle cavity of the clam and connected to a syringe pierced through a septum in the lid of the jar. By opening a two-way valve attached to the syringe, mantle water samples could be taken repeatedly. The sulfide concentration of the surrounding sea water was monitored at the same time (Gilboa-Garber, 1971). To exclude sulfide production by sources other than *L. aequizonata*, jars without clams or with boiled clams were incubated as controls. The blue mussel *Mytilus edulis* and the oyster *Crassostrea virginica* were used to investigate whether anoxic sulfide production occurred in non-symbiotic bivalves.

Experiments on isolated tissues

Anaerobic incubation of isolated tissue samples from symbiotic specimens (*Lucinoma aequizonata*, *Calyptogena magnifica*, *Bathymodiolus thermophilus* and *Riftia pachyptila*) allowed us to identify the type of tissue responsible for H_2S generation. It was found that only symbiont-containing tissues (gill and trophosome) have the ability to produce anaerobic sulfide. A time series was performed to evaluate the extent of H_2S generation in each species. For each specimen, six tissue samples of approximately 0.7 g fresh mass were dissected, rinsed and incubated separately (for 0, 2, 6, 12, 24 or 48 h) in Vacutainers containing 10 ml of imidazole-buffered saline (IBS; Fisher et al., 1988). The color of the gills of *L. aequizonata* was noted because it is an indication of the S^0 content (light color represents a high content, black represents a low content; Vetter, 1985). The Vacutainers were bubbled with nitrogen gas for 15 min and then closed with a rubber stopper to establish anoxic conditions prior to incubations. At different times, the incubation vials were opened and samples of IBS were taken for sulfide analysis (Gilboa-Garber, 1971). The incubated tissues were rinsed, blotted dry and frozen in liquid nitrogen, where they remained stored until bimane analysis as described below.

A control experiment was performed by the addition of the bacterial growth inhibitor Kanamycin (50 μg ml^{-1}) to the medium to evaluate whether microbes contribute to anaerobic H_2S generation during the experiments.

Sulfide production under hypoxic conditions

Pieces of trophosome tissue of the hydrothermal vent tubeworm *Riftia pachyptila* were incubated in a hypoxic

environment to investigate whether sulfide production also occurs after saturation with a mix of 2 % oxygen and nitrogen. Before the tissues were placed into 10 ml of IBS medium, they were carefully rinsed to remove blood. To maintain constant conditions, a gas mixture (2 % O_2, 98 % N_2) was continuously bubbled through the medium starting 20 min before the incubations. After the tissue had been placed into the IBS, the tube was closed with a rubber stopper, which was pierced with two needles, one for gas inflow and one for outflow. The outflowing gas was captured in a container containing alkaline zinc acetate, which was exchanged every 15 min. At the end of the experiment, the sulfide content of the zinc acetate vials was analyzed according to the method of Gilboa-Garber (Gilboa-Garber, 1971). A control experiment was performed with trophosome incubated under anoxic conditions (<1 % air saturation) after bubbling N_2 gas for 15 min through the incubation vials.

Source of sulfide production

Gill tissue of *Lucinoma aequizonata* was incubated anoxically in 10 ml of IBS containing $^{35}SO_4^{2-}$ (ICN; 7.4×10^5 Bq, 5.5×10^{13} Bq mmol^{-1}) to determine whether anaerobic sulfate reduction is responsible for the accumulation of H_2S. During this experiment, IBS samples were taken repeatedly, and sulfide was immediately trapped with alkaline zinc acetate. To separate the radioactive label in the precipitated zinc sulfide from the ^{35}S label in sulfate in the incubation medium, the sample was washed several times, centrifuged, and the supernatant containing $^{35}SO_4^{2-}$ discarded. Sulfide was incorporated into Methylene Blue according to the method of Gilboa-Garber (Gilboa-Garber, 1971). Proportional amounts of Methylene Blue were added to 3 ml of scintillation cocktail (Monofluor; National Diagnostics) and counted in a Beckman scintillation counter. In a different experimental approach, gills were incubated anoxically in IBS which had been prepared without sulfate or with molybdate (20 mmol l^{-1}; Taylor and Oremland, 1979), which inhibits sulfate reduction. Sulfide levels were measured at various times throughout the experiments.

Sulfur-containing metabolites in Lucinoma aequizonata *gills*

Sulfur-containing compounds were identified in gill tissues of *Lucinoma aequizonata* exposed to anoxic conditions for 10.5 months. In this experiment, freshly collected clams were incubated in anoxic sea water (500 ml) in jars that were partially filled with mud from the Santa Barbara Basin. After bubbling the jars with nitrogen gas for 1 h inside an anaerobic glove bag to induce anoxia, the containers were sealed with polyvinylchloride lids and several layers of electrical tape. Anaerobic conditions were checked at the beginning and at the end of the incubation period using a VacuVial Kit (Chemetrics, 1984), and oxygen levels were found to be below 1 part per billion. After 10.5 months, the experiment was ended, and sulfide concentrations higher than 1 mmol l^{-1} were detected in the incubation jars. For the sulfur metabolite analysis, gill samples were homogenized in a mixture of 100 µl of buffer (50 mmol l^{-1} Hepes, 5 mmol l^{-1} EDTA, pH 8.5) and 10 µl of monobromobimane (46 mmol l^{-1}). The homogenates were allowed to incubate at room temperature (25 °C) in the dark for 30 min. The proteins were then denatured in a heatblock for 60 °C for 10 min. The bimane sulfur compounds were stabilized with 100 µl of 65 mmol l^{-1} methane sulfonic acid and stored at −80 °C until analysis by high-performance liquid chromatography (HPLC) (as described by Vetter et al., 1989).

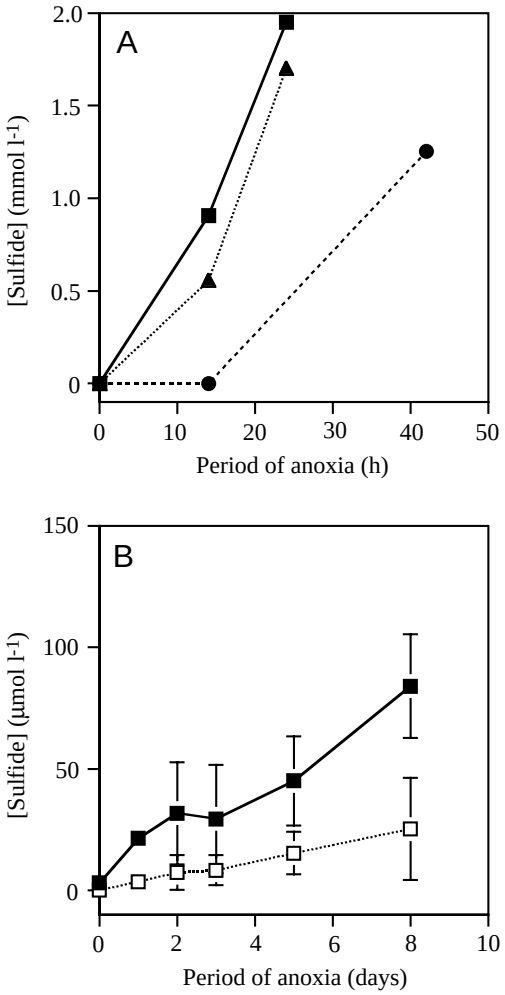

Fig. 1. Sulfide production in symbioses after different periods of anoxia. (A) Accumulation of sulfide in the blood system of the hydrothermal vent tubeworm *Riftia pachyptila* in three different specimens kept under high pressure at 15 °C. (B) Sulfide excretion by the Santa Barbara basin clam *Lucinoma aequizonata* (means ± S.D., *N*=3) into the mantle water (filled squares) and the surrounding incubation medium (open squares) at 10 °C.

Statistical analysis

Significant differences between data were determined using analysis of variance (ANOVA) and Scheffe *F*-tests, applying a confidence level of 95 %.

Terminology

Throughout this paper, the terms 'sulfide' and 'H_2S' refer to the sum of H_2S (gas), HS^- (bisulfide anion) and S^{2-} (sulfide

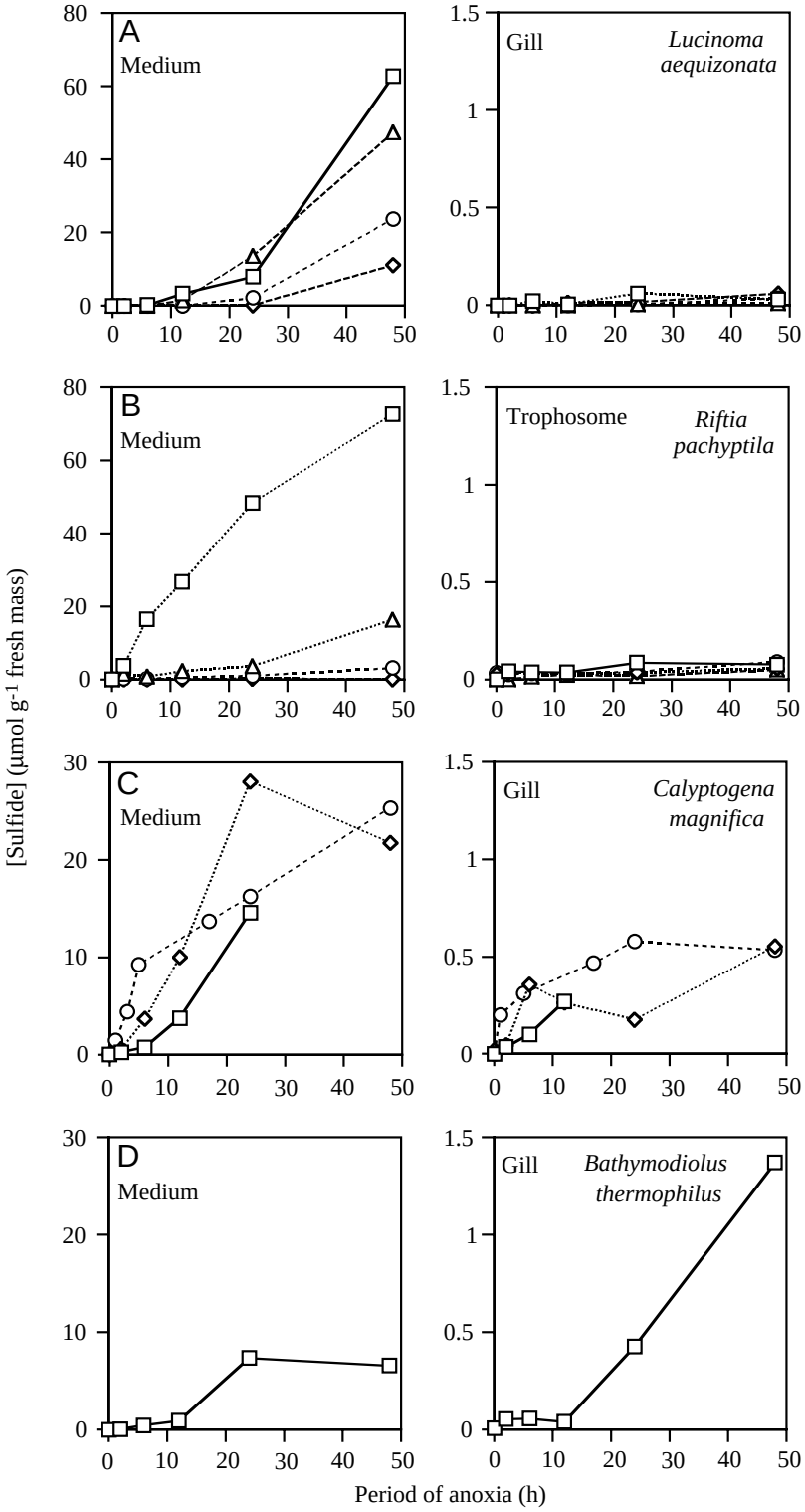

Fig. 2. Sulfide generation by different symbiont-bearing tissues during anoxic incubation. (A) *Lucinoma aequizonata* gills of yellow (triangles), light-gray (squares), gray (circles) and brown (diamonds) coloration. (B) *Riftia pachyptila* trophosome of yellow (squares), yellow-green (triangles), green (circles) and black (diamonds) coloration. (C) *Calyptogena magnifica* gills of light-gray color (three different specimens). (D) *Bathymodiolus thermophilus* gill (gray, one specimen). In each pair of graphs, similar symbols represent tissues from the same specimen.

anion) because the chemical forms of sulfide change depending on the environmental pH (Miller et al., 1988).

Results

Anaerobic sulfide production in live symbioses

When catheterized *Riftia pachyptila* were incubated under anoxia, sulfide accumulated in the vascular blood of the worm. At the beginning of the experiment, no sulfide could be detected in the blood, but the sulfide level increased to between 1 and 2 mmol l^{-1} after a 24–42 h period of anoxic incubation (Fig. 1A). The trophosome of all incubated specimens was of green coloration, indicating the presence of elemental sulfur.

In the clam *Lucinoma aequizonata*, sulfide generation was

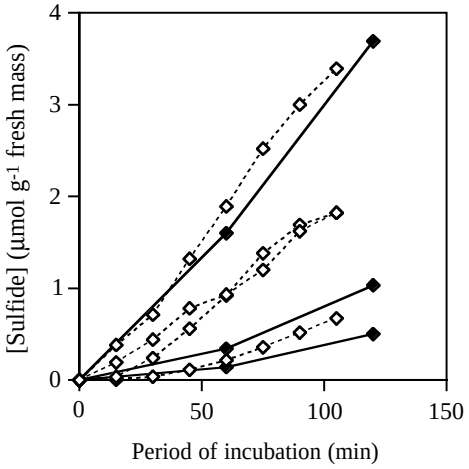

Fig. 3. Sulfide production by *Riftia pachyptila* trophosome incubated in imidazole-buffered saline (IBS; Fisher et al., 1988) containing 2 % oxygen (10 % air saturation) (open diamonds) and in anoxic IBS buffer (filled diamonds) after various incubation periods.

found in approximately 50 % of all specimens incubated under anoxia. The clams started excreting sulfide into their mantle cavity within the first day of anoxic exposure (Fig. 1B). The sulfide concentration increased continuously over a period of 8 days, reaching a mean value of 84 μmol l^{-1} in the mantle cavity. During the same time, a slower accumulation to approximately 25 μmol l^{-1} sulfide was detected in the surrounding incubation water. The extent of H_2S production varied, with the strongest accumulation in clams with white-yellow gills. No sulfide production was found in *L. aequizonata* with black gills. Specimens with gray gills produced intermediate amounts of H_2S.

The strong correlation between gill color and anaerobic sulfide production in *Lucinoma aequizonata* suggests that the gills are the tissues responsible for sulfide production. To verify this, isolated tissues of *L. aequizonata* were dissected and incubated under anoxic conditions. As expected, no tissue other than the gill had the ability to produce H_2S. Sulfide production could not be detected when *Mytilus edulis*, *Crassostrea virginica*, boiled specimens of *L. aequizonata* or sea water without specimens were incubated anaerobically.

Anaerobic sulfide production in sulfur-storing tissues

When different tissues of the vent clam *Calyptogena magnifica*, the vent mussel *Bathymodiolus thermophilus* and the vent tubeworm *Riftia pachyptila* were used in the investigations, sulfide production was, as in *Lucinoma aequizonata*, detected only in symbiont-containing tissues (bivalves, gill; tubeworm, trophosome; Fig. 2). In *R. pachyptila* trophosome, the extent of H_2S excretion was dependent on the coloration of the tissue, which is influenced by the sulfur content of the symbionts (Fig. 2B). The strongest sulfide production rates were found in tissues with the highest S^0 concentration. In specimens of *C. magnifica*, no variation in the light-gray gill coloration was apparent. A difference was observed among specimens in the

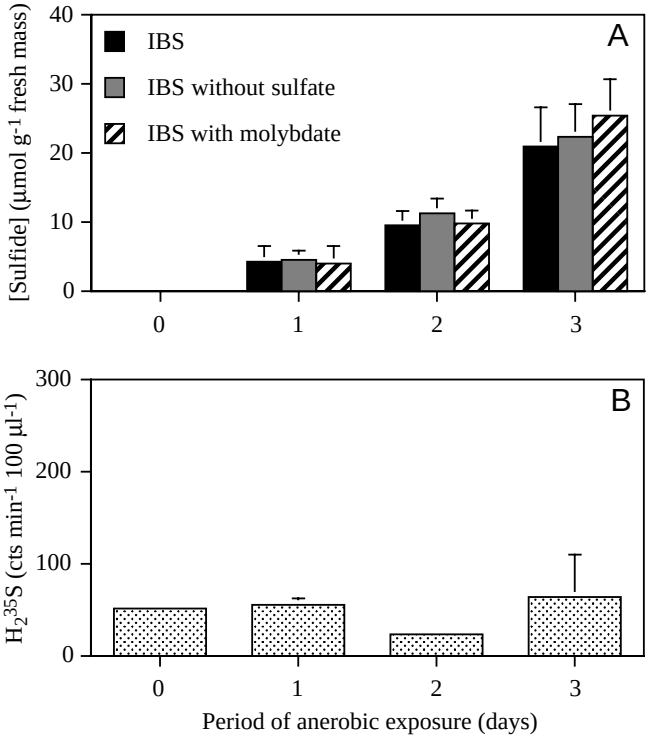

Fig. 4. Sulfate as a possible source of anaerobically produced sulfide in *Lucinoma aequizonata*. (A) Analysis of sulfide concentration in the incubation medium of yellow gills of *L. aequizonata*. Samples were incubated in imidazole-buffered saline (IBS; Fisher et al., 1988), in IBS without sulfate or in IBS supplemented with 20 mmol l^{-1} molybdate. The sulfide concentration in the incubation medium was analyzed after different times (means + S.D., *N*=3). (B) Incubation of yellow *L. aequizonata* gills with $^{35}SO_4^{2-}$ under anaerobic conditions. Sulfide samples were taken from the incubation medium and analyzed for ^{35}S label (means + S.D., *N*=3).

accumulation of H_2S inside the symbiont-containing tissue. Both *L. aequizonata* and *R. pachyptila* excreted most of the H_2S produced into the surrounding medium and never accumulated more than 0.09 μmol g^{-1} fresh mass in the tissue (Fig. 2A,B). *C. magnifica* and *B. thermophilus*, however, were able to concentrate sulfide to more than 0.5 and 1.3 μmol g^{-1} fresh mass, respectively, in their gills (Fig. 2C,D).

When symbiont-containing tissues were incubated anaerobically in the presence or absence of Kanamycin, sulfide production was unaffected, indicating the absence of free-living sulfide-generating microbes. This result also indicates that Kanamycin did not have any inhibitory effects on the H_2S-generating ability of the tissues (results not shown).

Sulfide production under hypoxic conditions

Sulfur-containing *Riftia pachyptila* trophosome was incubated in a 2 % oxygen atmosphere to investigate whether hypoxia would trigger sulfide production. The results show clearly that H_2S is produced at a rate similar to those found under fully anoxic conditions. A positive correlation between

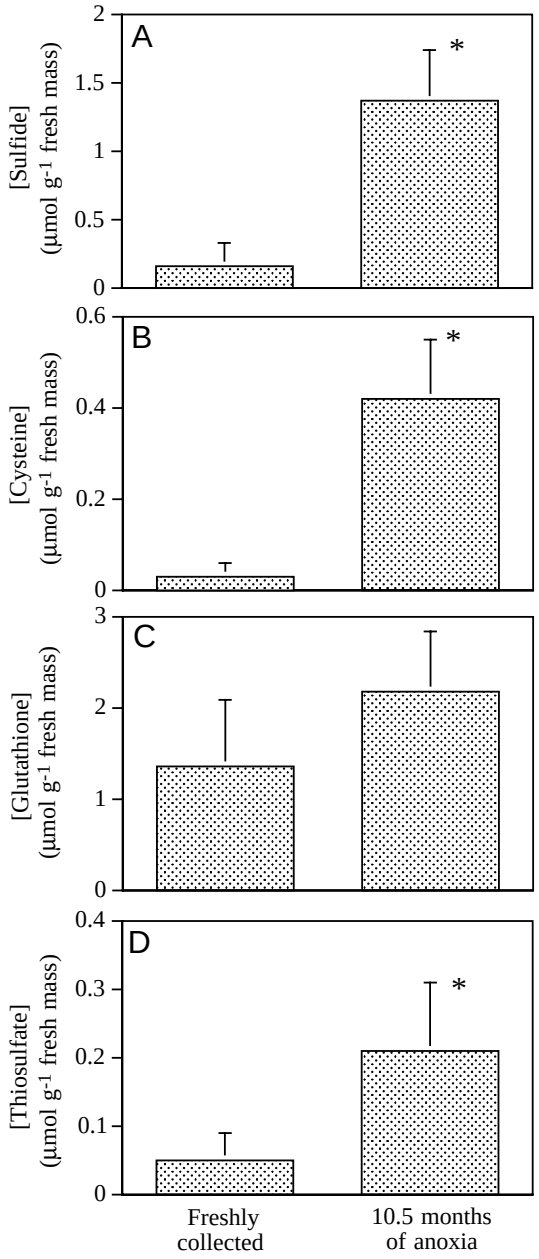

Fig. 5. Concentrations of the sulfur-containing metabolites sulfide (A), cysteine (B), glutathione (C) and thiosulfate (D) in the gill tissue of *Lucinoma aequizonata* in freshly collected (means + S.D., *N*=15) and long-term anoxic specimens (means + S.D., *N*=4). An asterisk indicates a significant difference between freshly collected and anoxic specimens (ANOVA, Scheffe *F*-test, 95 %).

the sulfur concentration of the trophosome and sulfide production was noted (Fig. 3).

Sulfate as substrate for sulfide production

Yellow gills of *Lucinoma aequizonata* (*N*=3) were exposed for 3 days to anoxic IBS containing $^{35}SO_4^{2-}$, and the incubation water was tested for $H_2{}^{35}S$ generation (Fig. 4B). The gills started to excrete sulfide during the first day of anoxic exposure. While the sulfide concentration in the incubation medium increased constantly throughout the run of the experiment, the ^{35}S label in sulfide was never significantly higher than that of the background. In another experiment, yellow gills of *L. aequizonata* were exposed to anoxic IBS lacking sulfate and to IBS supplemented with molybdate, an inhibitor of sulfate reduction. The results of these experiments show that sulfide is produced in large amounts under both conditions (Fig. 4A).

Sulfur metabolism of Lucinoma aequizonata *gills*

Sulfur-containing compounds were analyzed in the gills of freshly collected and long-term anoxic *Lucinoma aequizonata* (10.5 months, Fig. 5). Freshly collected clams had a relatively low concentration of hydrogen sulfide in their gills (0.2 µmol g^{-1} fresh mass). This increased significantly to 1.4 µmol g^{-1} fresh mass after the clams had been exposed to anoxia for 10.5 months. Similar results were obtained for the distribution of thiosulfate and cysteine, levels of which were significantly higher in the gills of specimens that had experienced long-term anoxic conditions than in the gills of freshly collected clams. Glutathione also tended to be more concentrated in anoxic gills. The increased levels, however, were not significantly different from those of freshly collected specimens.

Discussion

Anaerobic sulfide excretion by symbiont-bearing animals

Our study shows that the anaerobic generation of H_2S is a widespread phenomenon among thiotrophic symbioses. The process could be detected in specimens from both hydrothermal vents (*Riftia pachyptila*, *Calyptogena magnifica* and *Bathymodiolus thermophilus*) and non-vent sites (*Lucinoma aequizonata* and *Calyptogena elongata*; in the latter species by Childress et al., 1993; Fig. 2), but not in the non-symbiotic specimens investigated. Our results show that H_2S generation is initiated within the first few hours of anoxia. In the tubeworm *R. pachyptila*, oxygen depletion causes an accumulation of up to 2 mmol l^{-1} sulfide in the circulating blood system within 24 h (Fig. 1A). Initial data suggest that high levels of H_2S also build up in the hemolymph of the clam *L. aequizonata* only a few hours after anoxia sets in (data not shown).

Exposure to sulfide can cause severe physiological problems for most organisms. Well-described effects include the inhibition of oxidative phosphorylation as a result of a deactivation of cytochrome *c* oxidase or general metabolic impairments caused by changes in enzyme activities or levels of metabolites and cofactors (Khan et al., 1991; National Research Council, 1979). In the presence of an oxidant, thiotrophic bacterial symbionts can oxidize and, therefore, detoxify sulfide that is taken up from the environment into the host system (Nelson and Fisher, 1995). In addition, oxygen-dependent, mitochondrial sulfide oxidation in animal tissues causes sulfide to be converted to non-toxic thiosulfate (Powell and Somero, 1986).

Under anoxic conditions, sulfide accumulates in the body fluids of sulfur-storing symbioses (Fig. 1A). This may be dangerous because, in many non-symbiotic invertebrates, sulfidic anoxia causes premature death compared with simple anoxia (Theede et al., 1969; Vismann, 1990) and this could be due to an increase in anaerobic glycolysis, which has a comparatively inefficient ATP yield (Arndt and Schiedek, 1997). In some symbioses, exposure of the host tissues to sulfide can be minimized by binding sulfide to hemoglobin or non-protein substances in the blood (*Riftia pachyptila* and *Calyptogena* spp., respectively; Childress et al., 1993; Arp et al., 1984; Arp and Childress, 1983). These adaptations, however, were not found in *Lucinoma aequizonata* or *Bathymodiolus thermophilus*, indicating that anaerobically generated sulfide could diffuse into the tissues and possibly harm them.

The excretion of sulfide from the body into the surrounding environment immediately after its generation is obviously an important response to a metabolic toxin accumulating in the circulatory system of an animal. After its excretion from the gills, sulfide levels build up in the mantle cavity of catheterized *Lucinoma aequizonata* before it is transported with the water stream into the surrounding anoxic medium (Figs 1B, 2A). This process is probably similar in all species investigated (Fig. 2B–D; Childress et al., 1993). There seem, however, to be differences regarding H_2S accumulation inside the symbiont-bearing tissues. While no significant levels of sulfide could be detected in the gill and the trophosome of *L. aequizonata* and *Riftia pachyptila*, respectively, the gills of *Calyptogena magnifica* and *Bathymodiolus thermophilus* built up a relatively high sulfide level before it was released into the incubation medium (Fig. 2C,D). It is possible that *L. aequizonata* and *R. pachyptila* possess mechanisms of sulfide excretion that actively transport sulfide out of the tissue, but there is no proof for this hypothesis at this time.

Sources and significance of anaerobic sulfide production

Anoxic sulfide production in biological systems can have various sources. While metazoans do not produce sulfide as a metabolic end-product, microorganisms can use a variety of organic and inorganic sulfur-containing compounds, which they reduce to H_2S (Fauque et al., 1991). Microbial sulfate reduction is the most significant sulfide-generating process in anoxic marine sediments and brine seeps, decomposing organic matter and using sulfate as a terminal electron acceptor (Postgate, 1984). Our $^{35}SO_4^{2-}$ experiments on the clam *L. aequizonata* show that sulfate reduction is not the metabolic path leading to the generation of H_2S (Fig. 4). It is likely that these observations can be applied to all thiotrophic symbioses.

The extent of H_2S production in *Lucinoma aequizonata* and *Riftia pachyptila* correlates significantly with the color of the gill and trophosome, respectively, of the incubated specimens and, therefore, with the sulfur content of the symbiotic bacteria (Fig. 2A,B; Fisher, 1990; Vetter, 1985; Vetter and Fry, 1998). These results indicate that elemental sulfur is the substrate responsible for the anaerobic generation of H_2S. Variations in the sulfur content of symbiont-bearing tissues are often observed in freshly collected specimens and are suggested to reflect the balance between environmental sulfide supply and the rate of metabolic consumption of S^0 by the symbionts (Vetter and Fry, 1998).

S^0 occurs as liquid-crystalline droplets confined to the periplasma space of the symbionts, which have a similar appearance to those of the genera *Beggiatoa*, *Chromatium* and *Thiovulum* (Vetter, 1985). The particular pathways by which sulfur accumulates in the endosymbionts are not clear to date. Moreover, there appears to be no universal pattern of sulfur deposition in free-living microbes. Flavocytochrome *c* and sulfidequinone oxidoreductase are currently considered as possible catalysts in the formation of sulfur globules from H_2S and thiosulfate in *Chromatium* spp. and other photo- and chemotrophic sulfur oxidizers (Brune, 1989; Brune, 1995; Friedrich 1998). It remains to be shown whether similar sulfur-accumulating pathways exist in the sulfur-oxidizing endosymbionts.

Very little is known about the metabolic fate of sulfur deposited in both free-living and symbiotic sulfur-oxidizing bacteria. In endosymbionts, sulfur had been proposed to be an energy-storage compound that may be oxidized by the symbionts during the temporary absence of H_2S or thiosulfate. This assumption is supported by the sulfur loss of symbionts maintained aerobically (Vetter, 1985). Trüper (Trüper, 1984) suggests that sulfur in phototrophic bacteria is actively reduced and then oxidized to sulfite *via* a reverse sulfite reductase. Because the initial pathways of sulfide and thiosulfate oxidation, however, vary greatly between genera and among different species of the same genus (Nelson and Hagen, 1995), future research is needed to describe exactly the metabolism of S^0 in the symbionts.

The reduction of sulfur deposits under anaerobic conditions suggests a novel significance of the storage compound for chemoautotrophic symbioses. In many strictly anaerobic bacteria and archea, S^0 is used as a final electron acceptor, thereby supporting growth through sulfur respiration, while organic compounds (e.g. succinate, acetate, formate) are utilized as electron donors (Hedderich et al., 1999; Stetter et al., 1993; Fauque et al., 1991; Pfennig and Biebl, 1976). This metabolic process, to date, has received little attention in aerobic sulfide oxidizers. Early reports on *Chromatium vinosum* (van Gemerden, 1968) and *Beggiatoa* spp. (Schmidt et al., 1987; Nelson and Castenholz, 1981) describe H_2S production and the degradation of sulfur deposits in these species under anaerobic conditions. *Beggiatoa* spp. has been shown to grow under these conditions while oxidizing an organic compound (acetate).

It is possible that sulfur-oxidizing chemoautotrophic symbionts may respond in a similar way to oxygen depletion. Surrounded by an organic-rich environment (the bacteriocyte), the symbionts may take advantage of the rich pool of metabolites produced by the host animal. Initial experiments with purified *Riftia pachyptila* symbionts (Distel and Felbeck, 1988) have shown that the bacteria take up and metabolize

compounds such as succinate and alanine (H. Felbeck, unpublished data), indicating mixotrophy by the symbionts. If anaerobic sulfur reduction in the symbionts is associated with energy conservation, it could contribute to supplying the symbiosis with metabolic energy during anoxia.

The observation that sulfide production in the trophosome tissue of *Riftia pachyptila* occurs even under hypoxia (Fig. 3) may be due to the metabolic activity of the bacteriocytes. It is likely that the low levels of oxygen bubbled through the incubation medium during the experiment did not enter and penetrate the entire trophosome sample. Oxygen could have been respired at the periphery of bacteriocytes before reaching the symbionts which, therefore, would experience local anoxia and produce sulfide. Whether hypoxic conditions cause the same reaction in intact *R. pachyptila* needs to be determined *in vivo*.

In addition to the activity of bacterial symbionts, the host tissue of sulfur-storing symbioses could also possibly convert elemental sulfur anaerobically to H_2S. This hypothesis is supported by the failure of all attempts to demonstrate anaerobic sulfide production in purified symbionts of the clam *Lucinoma aequizonata*. The potential of eukaryotic cells to reduce S^0 has been demonstrated in the past (Searcy and Lee, 1998) and is assumed to be an ancestral metabolic trait acquired after evolutionary fusion of archebacterial and eubacterial cells. Among the prokaryotes, sulfur-respiring archea are the most closely related to the eukaryotic nucleocytoplasm and have been shown to contribute substantially to the eukaryotic genome (Pace, 1997; Gupta and Golding, 1996). However, in the cases reported to date, H_2S generation from S^0 was never significant in the overall metabolism of the species investigated which, naturally, have no access to elemental sulfur (Searcy and Lee, 1998). In thiotrophic symbioses such as *L. aequizonata*, S^0 reduction may be a significant process because elemental sulfur and water-soluble polysulfides could be provided to the host cells by the intracellular bacteria. This process could play an important role, at least during the first 8 weeks of anaerobic incubation before the gills of incubated specimens become depleted of sulfur (C. Arndt, personal observation).

Sulfur metabolites in long-term anoxic gills of Lucinoma aequizonata

It has recently been discovered that *Lucinoma aequizonata* has an extraordinary capability to tolerate environmental anoxia (50 % mortality=262 days, 10 °C). While details on the anaerobiosis of this clam will be the subject of a separate study (C. Arndt, J.-P. Lechaire and H. Felbeck, in preparation), here, we investigate how the sulfur metabolism of the symbiont-bearing gill tissue is affected under long-term anoxia (10.5 months). Fig. 5A shows that, in contrast to the results presented in Fig. 2A, sulfide cannot be excluded from the gill tissue once the surroundings of the clams accumulate more than $1\,mmol\,l^{-1}$ sulfide, causing a concentration of between 1 and $2\,\mu mol\,H_2S\,g^{-1}$ gill. In addition to the clam's own ability to produce sulfide, free-living sulfate reducers in the incubation medium probably contribute to H_2S production in these experiments.

The high H_2S concentrations in the anoxic gills may have caused the more than 10-fold accumulation of cysteine in the same tissues (Fig. 5B). In *Escherichia coli*, the enzyme serine sulfhydrase converts serine and H_2S to cysteine (Schlossman and Lynen, 1957). In a different pathway, serine and acetyl-coenzyme A react *via* serine transacetylase to form *o*-acetylserine. This compound, in turn, combines with H_2S through the enzyme *o*-acetylserine sulfhydrase to form cysteine and acetate (Kredrich and Tomkins, 1966). The respective enzyme activities, however, remain to be determined in *Lucinoma aequizonata*. High levels of cysteine and glutathione may also be caused by the lack of reoxidation under anoxia. The oxidation of reduced organic disulfides is an important metabolic trait to protect cells from toxic oxygen species (Vina, 1990).

The significant increase in thiosulfate levels in the gill tissues of long-term anoxic incubation specimens of *Lucinoma aequizonata* is, however, difficult to explain (Fig. 5D). No known bacterial metabolic process produces significant amounts of thiosulfate under anoxic conditions. Microbial and animal thiosulfate generation is normally associated with aerobic processes, in which less-oxidized sulfur compounds (e.g. sulfide, sulfur, sulfite) are degraded in the presence of oxygen (Nelson and Fisher, 1995; Grieshaber and Völkel, 1998). In mammalian tissues, thiosulfate was found to be a by-product of cysteine catabolism (Sasaki and Otsuka, 1912). Large amounts of cysteine are present in the anoxic gill tissues of *L. aequizonata*, so a similar process may be active in this clam.

Outlook

Our study shows that the anaerobic sulfur metabolism of bacteria–invertebrate symbioses offers a large potential for future exploration. While much information exists concerning the uptake and oxidation processes of reduced sulfur compounds in these symbioses (Nelson and Hagen, 1995; Childress and Fisher, 1992), the potential of elemental sulfur as a storage compound has been largely neglected. The possibility that sulfur can serve as a final electron acceptor for maintenance purposes, even if only temporarily, may be important for the survival of symbioses during periods of low oxygen availability. In addition, H_2S excretion by symbiotic animals may be of ecological significance because it could affect any fauna living nearby, which would have to develop adaptations to the metabolic poison (Vetter et al., 1991; Grieshaber and Völkel, 1998).

Our data on the vent tubeworm *Riftia pachyptila* trigger the question of whether the often large amounts of sulfide found in the blood of the freshly collected tubeworms originate entirely from the environment. While there is no question that vent waters are the primary source of sulfide, a proportion of the sulfide circulating in the blood may also be produced by the trophosome once the oxygen level decreases. The conditions that permit sulfide from the trophosome to accumulate in the

tubeworm and the effects this has on the metabolic machinery of the symbiosis require further exploration.

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