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Evidence for a nitrate uptake mechanism in the hydrothermal vent tube-worm *Riftia pachyptila*

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Introduction

The vestimentiferan tube-worm *Riftia pachyptila* Jones, 1981 is found around hydrothermal vent areas in the deep sea. Intracellular bacterial chemoautotrophic symbionts use the oxidation of sulphide from the effluent of the vents as an energy source for CO_2 fixation (Nelson & Fisher, 1995). They apparently provide most or all of the nutritional requirements for their gutless hosts. A closed vascular system circulates haemoglobin containing blood and provides a vehicle for the transport of metabolites between the environment and the symbiotic bacteria. The trophosome, the internal organ harbouring the symbionts, is bathed in a coelomic fluid which also contains haemoglobin.

While the host respires oxygen in a heterotrophic metabolism, the bacteria require sulphide, CO_2 and an oxidant for their autotrophic metabolism. We present results that the symbiotic bacteria of *R. pachyptila* are capable of respiring nitrate and nitrite in addition to oxygen. Moreover, the vascular blood of *Riftia pachyptila* was shown to have nitrate concentrations of up to one hundred times that of ambient seawater. Blood nitrate levels reached concentrations of >1 mM with a maximum value of 4.5 mM, while nitrite was measured in the range of 400-700 µM with a maximum value of 2.2 mM. The concentrations of nitrate and nitrite in the coelomic fluids were 150-240 µM and < 20 µM respectively.

Material and methods

Because the symbionts cannot be cultured to date, we used physically purified bacteria for our experiments. *R. pachyptila* symbionts were purified anaerobically and incubated in *Riftia* saline containing sulphide and nitrate under anaerobic conditions. Aliquots were drawn in time intervals through a syringe, centrifuged and the concentration of nitrite was determined in the supernatant after the removal of sulphide (Hentschel & Felbeck, 1993).

Symbiont oxygen respiration was measured with a modified Clarke-type oxygen electrode (Hentschel & Felbeck, 1993). In order to distinguish between the respiratory oxygen consumption and the chemical reduction of oxygen by sulphide, oxygen consumption rates were determined in the following fashion: After a respiratory baseline had been established, sulphide was added and the "sulphide stimulated oxygen consumption rate" was measured. Oxygen was introduced in order to re-establish the oxygen concentration before the sulphide addition. Then azide was added as a respiratory inhibitor and the "azide insensitive oxygen consumption rate" was measured. The "azide insensitive rate" was subtracted from the "sulphide stimulated rate" to yield the "true oxygen consumption rate" which is based on the respiratory reduction of oxygen and not on chemical reduction of oxygen by sulphide. Nitrate respiration rates were measured in the same incubation after addition of 1 mM nitrate as described above. Oxygen and nitrate respiration rates of purified symbionts were measured in this fashion at various oxygen intervals ranging from 90-70%, 70-50%, 50-30%, 30-10% and < 10% oxygen saturation.

Blood and coelomic fluid samples were diluted, denatured, cooled, and centrifuged before nitrite and nitrate were determined as described previously (Pospesel et al., 1998). In order to determine the concentration of nitrate, an aliquot was combined with an *E. coli* culture, which had been grown overnight in nitrate-free M9 minimal media under oxygen limiting conditions. Samples were overlaid with mineral oil and incubated at 37°C in a heating block. After 2 hours, an aliquot was removed from under the mineral oil, centrifuged and the concentration of nitrite was determined in the supernatant. Nitrite and nitrate standard curves were prepared in vascular or coelomic blood and processed in parallel.

Results

When *R. pachyptila* symbionts were incubated under anaerobic conditions in the presence of nitrate, nitrite appeared in the medium at a linear rate for at least 90 min (Fig. 1) and was independent of the nitrate concentration between 50 µM and 1 mM (Hentschel & Felbeck, 1993). When the concentration of nitrate dropped below 20 µM, nitrite was reduced as well, indicating that nitrite can be respired further, presumably to nitrogen gas. There was no indication that the symbionts can respire nitrate to ammonia. Nitrate respiration was stimulated by sulphide with maximal rates at 500 µM sulphide, but not by thiosulphate. This supports previous results that the symbionts are sulphide specialists.

Because denitrification is usually a trait found in bacteria from anaerobic environments, the influence of oxygen on the reduction of nitrate by the symbionts had to be assessed.

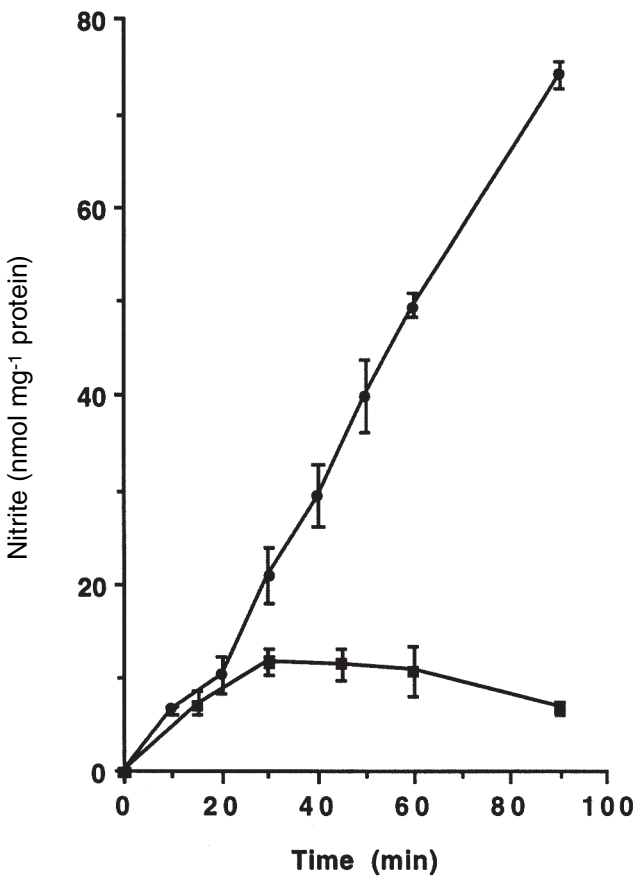

Figure 1. Production of nitrite by purified *Riftia pachyptila* symbionts. The symbols represent incubations in 50 µM (▪) or 1 mM (•) nitrate. Data are presented as means ± S.E., n = 3. Reprinted by permission from *Nature (Nature*, 366: 338-340) copyright (1993) Macmillan Magazines Ltd.

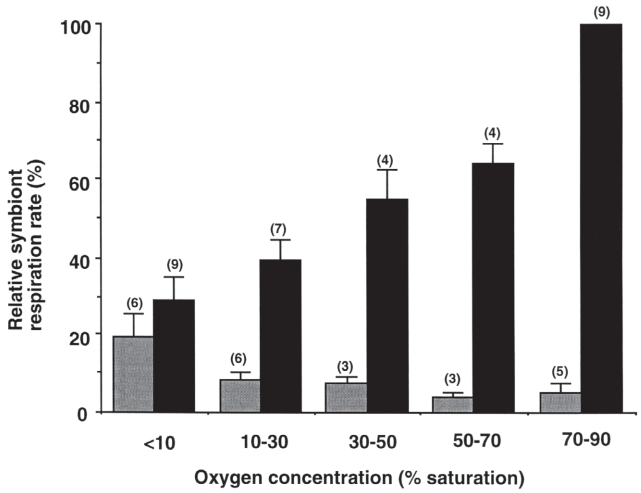

Figure 2. Oxygen and nitrate respiration rates at different oxygen concentrations. Black bars represent oxygen respiration rates, grey bars represent nitrate respiration rates (means ± S.E., n given above for each error bar). The respective rates are expressed relative to the sulphide-stimulated oxygen respiration rate at oxygen saturation. Reprinted by permission from *Nature (Nature*, 366: 338-340) copyright (1993) Macmillan Magazines Ltd.

Therefore, we designed an experiment in which the rates of symbiont oxygen and nitrate respiration were measured simultaneously at various oxygen concentrations. Although oxygen respiration decreased with decreasing oxygen concentrations, nitrate respiration increased (Fig. 2). At 100% saturation, oxygen respiration was significantly higher than nitrate respiration. At the lowest oxygen concentration (< 10 % saturation equal to < 26 µM) the rates were statistically equivalent.

Discussion

In vivo, the concentration of free oxygen in the immediate vicinity of the symbionts is likely to be very low. A rough estimate of the oxygen concentration inside the bacteriocyte can be obtained from the haemoglobin properties of the coelomic blood, which fills the coelomic cavity and which bathes the trophosome. Both, the vascular and coelomic haemoglobin molecules have an extremely high affinity for oxygen (P50 = 0.1- 0.3 Torr at pH 7), and therefore the concentration of free oxygen is only about 2 µM in the coelomic blood. Considering that the concentration of free oxygen inside the tissue is determined by the blood properties, the oxygen concentration in the bacteriocytes should be maximally around 2 µM (<1% saturation). This is a conservative estimate, because factors such as oxygen consumption in the cytosol or diffusion barriers could lower the oxygen levels even more.

We can extrapolate now that under estimated in vivo conditions at least half of the oxidative capacity of the symbionts can be provided by nitrate. Nitrate respiration can also be measured in the intact symbiosis. Small animals were incubated under pressure in closed vials in seawater containing nitrate. They excreted nitrite at rates of up to 250 nmol g worm fresh weight^{-1} hr^{-1} (data not shown). These rates are corrected for activities of epibiotic bacteria on the tubes by removing the worm from its tube at the end of each incubation and continuing to measure nitrite production by the empty tube. Because the animals were probably stressed during these experiments, these results are therefore only indicative of their ability to reduce nitrate, not of their in situ metabolic rate.

In the hydrothermal vent environment both the presence of nitrate and its consumption near tubeworm clusters have been demonstrated (Johnson et al., 1988). The rate of nitrate removal was five times higher than necessary if it was due to nitrate assimilation. In this area, characterized by silicate concentrations of 500-600 µM, the animals can be exposed to conditions where the concentration of oxygen is only double that of nitrate (around 30 µM). The sulphide consumption rate of the tubeworms in situ was highest under these conditions.

Because the symbionts are located deep inside the trunk of the animal, nitrate would have to pass the host compartment first before it could reach the symbionts. Therefore we aimed to measure the concentrations of nitrate and nitrite in the vascular blood and the coelomic fluid of the animal. Because standard nitrate detection assays had previously failed, to determine nitrate concentrations in *R. pachyptila* blood we have developed a novel nitrate detection assay, which utilizes a nitrite reductase deficient strain of *E. coli* (Crooke & Cole, 1995) for the reduction of nitrate to nitrite which is then determined spectrophotometrically (Pospesel et al, 1998). This assay presented here is simple, applicable for laboratory and shipboard use and works reliably within the 0.5-2000 µM range.

Nitrite values in coelomic fluid and vascular blood of tubeworms collected from 9°N (12 ± 2.9 µM, (n = 44) and 420 ± 51 µM, (n = 34) respectively) and 13°N (17 ± 3.1 µM, (n= 46) and 680 ± 100 µM, (n = 45) respectively) were orders of magnitude higher than ambient nitrite (less < 1 µM). Nitrite in the blood is likely to be the metabolic end product of nitrate respiration by the symbionts. In most animals, nitrite has deleterious effects on haemoglobins, however *R. pachyptila* haemoglobin is unaffected by even extremely high levels of nitrite (Zal & Lallier, personal communication).

Nitrate values in coelomic fluid and vascular blood of animals collected from 9°N (240 ± 33 µM, (n = 44) and 1100 ± 89 µM, (n =34) respectively) and 13°N (150 ± 17 µM, (n = 45) and 1200 ± 150 µM (n = 45) respectively) were 50-100 times that of ambient sea water (40 µM). The high nitrate concentrations particularly in the vascular blood suggest that the animal may possess a nitrate uptake mechanism which, to our knowledge, would be novel in the animal kingdom.

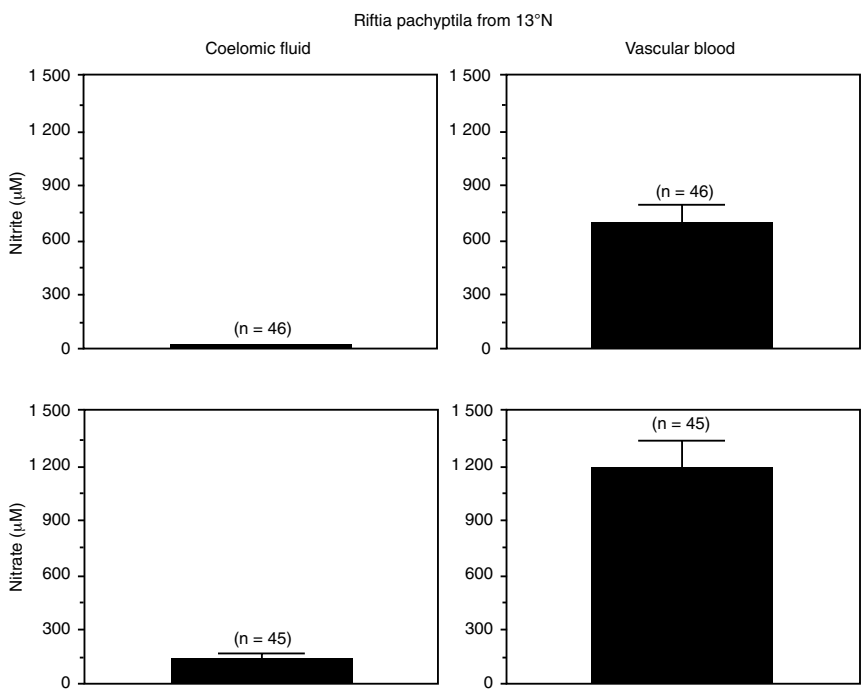

Figure 3. Evidence for or nitrate uptake mechanism.

Nitrite and nitrate concentrations in the blood had maxima of 2.2 mM and 4.5 mM, respectively. Both chemical species were not evenly distributed in both coelomic fluid and blood. In the 68 paired samples, nitrite and nitrate concentrations in the blood were correlated with, and significantly greater than, nitrite and nitrate concentrations in the coelomic fluid (Fig. 3, Kendall rank correlation coefficient, $p < 0.001$ in both cases and Wilcoxon signed ranks test, $p < 0.001$ in both cases).

R. pachyptila could be the first animal known to concentrate nitrate from the environment. Nitrate concentration in *R. pachyptila* might be achieved by a nitrate-binding molecule in the blood similar in function to that described for sulphide. Another possibility is an uptake mechanism, such as a protein ion pump, in the plume cells or membranes. Nitrate transporters of this type are known in bacteria, algae, and higher plants. Further experiments are underway to determine the precise mechanism by which *R. pachyptila* is able to take up and concentrate nitrate from the environment.

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