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The influence of different incubation media on the carbon transfer from the bacterial symbionts to the hydrothermal vent tube-worm *Riftia pachyptila*

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Introduction

A large fraction of the carbon fixation in the hydrothermal vent community is achieved by symbiotic bacteria (see Childress & Fisher, 1992 for review). In *Riftia pachyptila* Jones, 1981, these bacteria are found intracellularly in a specialized tissue, the trophosome. The trophosome of the tube-worms comprises between 15 and 30% of the animal's fresh weight and fills most of the body cavity of the animal. It is composed of many lobules which are about 0.15 mm in diameter and are vascularized by the closed circulatory system of the worms. The lobules consist of an outer single layer of cells without bacteria and the central bacteriocyte cells containing the bacterial symbionts. The trophosome is heavily capillarized by small vessels (2-3 µm diameter). The outside is surrounded by a haemoglobin containing fluid filling the coelomic cavity of the worms.

Since the animal has no digestive tract, its nutritional needs have to be filled by other means than eating particulate organic food. Uptake of dissolved amino acids from the surrounding sea water could be demonstrated but their concentration appears to be too low to support the nutritional needs of these large animals. Possibly they can serve as additional nitrogen source.

The animal's carbon isotope ratio suggests that the host's organic carbon originates from the bacteria. Two possibilities have been proposed to transfer carbon between the symbiotic partners 1) a constant growth of the bacterial population with a concommitant digestion of excess bacteria by the worm and 2) a transfer of dissolved organics excreted by the bacteria to the host. It is generally assumed that digestion of symbionts is just used for internal housekeeping (Fisher, 1990). The most likely way for the bacteria to support the worm's needs is by transfer of dissolved organic material to the symbiotic partner.

The identity of the transferred compounds in *R. pachyptila* and the quantitative significance of this transfer has been elusive. We have attempted to identify a translocation product by using physically purified bacteria from trophosome tissues. If a metabolite is excreted by these suspensions, one should be able to find it in the supernatant of incubations with radiolabelled CO_2 after the bacteria incorporate label into their organic matter. The purpose of our research was to identify metabolites which are specifically excreted by the symbionts.

Material and methods

R. pachyptila used in these experiments were collected from 2600 m depth during two cruises to 12°49' N, 103°56'35"W on the East Pacific Rise. The animals were collected by a manned submersible (*Nautile,* IFREMER France in 1991, and *Alvin,* USA in 1992) and brought to the surface in a temperature-insulated container. Upon recovery, they were maintained under pressure as described earlier (Felbeck & Turner, 1995). Bacteria from trophosome tissue were purified anaerobically by centrifuging the suspension on a preformed 60% Percoll-gradient as described in Felbeck & Jarchow (1998).

Incubations of up to 70 min were used to determine the time course of label incorporation. The general methods used for these experiments were as described in Felbeck & Jarchow (1998). Since little is known about the physiology of the symbionts, we compared incubations in *Riftia* saline, axon buffer as commonly used in invertebrate physiology (Hodgkin, 1958), and diluted *R. pachyptila* coelomic fluid (2 parts coelomic fluid:1 part saline as final dilution). In addition, we tested a possible influence of 10 mM glutamate or 10 mM aspartate added to *Riftia* saline. Aspartate has been shown to be excreted by purified chemoautotrophic symbionts from *Lucinoma aequizonata* Stearns, 1890 (see Distel & Felbeck, 1988) and glutamate has been identified as becoming labelled in preliminary experiments with trophosome preparations (Felbeck, 1985).

Results

The average total carbon fixing activity calculated from the overall regression lines of all experiments was 13.3 ± 4.0 µmol CO_2 g^{-1} Prot h^{-1} (n = 9, ± SE) in saline, 7.1 ± 2.2 µmol CO_2 g^{-1} Prot h^{-1} (n = 3) in saline with glutamate, 22.3 ± 11.4 µmol CO_2 g^{-1} Prot h^{-1} (n = 5) in saline with asparate, and 23.6 ± 7.1 µmol CO_2 g^{-1} Prot h^{-1} (n = 5) in coelomic fluid. Incubations showing extensive lysis were not considered for any results reported here. Lysis was assumed when labelled aspartate, which is usually only found in the pellet, appeared in measurable quantities in the supernatant.

Succinate, aspartate, and 3-phosphoglyceric acid (3-PGA) could be identified in the extract of the bacterial pellet of all 16 animals analysed. Labelled glutamate was found in rather large amounts in the extracts of the bacterial pellets. The total quantity of radioactivity present in these compounds increased linearly over time. Labelled alanine and malate were frequently present in small amounts.

Succinate could be detected in the supernatant of the incubations in all cases. Glutamate was found in small amounts in the supernatant in one third of the experiments (data not shown). Due to its dilution in the supernatant, the label identified in glutamate approached the detection limits of the flow-through radioactivity detector used in the HPLC system. The absence of glutamate in the supernatant of several incubations could, therefore, be due to masking of the glutamate peak by background noise of the detector.

The possible changes of labelling pattern with increasing incubation periods were examined for each compound using the non-parametric Kendall test (Table1). A p-value of below 0.05 indicated a trend with a 95% significance. As an example, the increase of the label in succinate in the supernatant of incubations in coelomic fluid is shown in Fig. 1.

The distribution pattern of radioactivity in most metabolites stayed relatively constant under most conditions. Malate was never labelled when symbionts were incubated in “axon buffer”, and alanine was never labelled in the presence of aspartate. The fraction of label in succinate detected in the supernatant of bacterial incubations increased significantly during the tests (Fig. 1), except during incubations in “axon-buffer”. The total quantity of radioactivity in excreted succinate as well as the fraction of the total label increased. Relative label in bacterial 3-phosphoglyceric acid decreased during incubations in *Riftia* saline, buffer with glutamate, and in coelomic fluid. Relative label in bacterial aspartate increased in incubations in coelomic fluid, and the relative label in bacterial glutamate increased slightly but significantly during incubations in axon buffer.

Table 1. Relative radioactivity found in different metabolites after incubation with labelled bicarbonate under various conditions. The radioactivity is listed as fraction relative to the sum of all recovered fixed label given as 1. When the p-value of the Kendall statistical test was below 0.05 a trend was detected, otherwise the means and standard errors are given. * indicates a value which is significantly different from the others in this group (ANOVA, Scheffe F-Test). SE = standard error, na = not applicable, 3-PGA = 3 phosphoglyceric acid, # = see also Felbeck & Jarchow (1998).

metabolite		incubation media:				
		glu (n=16)	coel. fluid# (n=22)	saline# (n=27)	axon buffer (n=16)	asp (n=8)
succinate (supernatant)	p-value (Kendall)	0.002	0.045	0.0002	0.821	0.018
	mean	na	na	na	0.063	na
	SE				0.012	
succinate (bacteria)	p-value (Kendall)	0.096	0.096	0.067	0.048	0.266
	mean	0.265	0.275	0.273	na	0.257
	SE	0.03	0.017	0.016		0.044
3-PGA	p-value (Kendall)	0.002	0.001	0.011	0.300	0.902
	mean	na	na	na	0.183	0.088
	SE				0.028	0.015
malate	p-value (Kendall)	0.224	0.735	0.252	1	0.458
	mean	0.067	0.033	0.034	0	0.142
	SE	0.039	0.011	0.011		0.059
glucose	p-value (Kendall)	0.471	0.464	0.227	0.893	0.387
	mean	0.145	0.037	0.056	0.005	0.148
	SE	0.042	0.013	0.021	0.003	0.045
fructose	p-value (Kendall)	0.322	0.464	0.819	0.719	0.536
	mean	0.086	0.037	0.036	0.025	0.044
	SE	0.028	0.013	0.014	0.014	0.022
alanine	p-value (Kendall)	0.177	0.297	0.692	0.192	1
	mean	0.021	0.002	0.036	0.049	0
	SE	0.011	0.001	0.009	0.019	
aspartate	p-value (Kendall)	0.163	0.012	0.189	0.300	0.902
	mean	0.189	na	0.221	0.131	0.194
	SE	0.028		0.017	0.016	0.044
glutamate	p-value (Kendall)	0.392	0.933	0.739	0.048	0.902
	mean	0.139	0.385*	0.193	na	0.126
	SE	0.027	0.018	0.024		0.049

All of the other different incubation conditions resulted in temporally stable radioactivity incorporation patterns, i.e., the distribution of radioactivity in the various

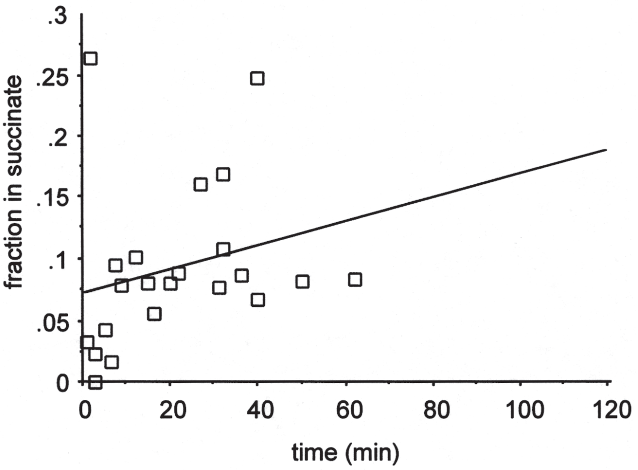

Figure 1. Radioactivity in succinate in the supernatant after incubation of purified symbionts of *Riftia pachyptil*a in coelomic fluid in the presence of $^{14}CO_2$. Radiolabel expressed as a fraction of the total label incorporated from labelled bicarbonate is plotted against incubation time.

metabolites did not change significantly after different incubation times. The fractions of radioactivity identified in the individual components were, therefore, averaged and compared to each other using one factor ANOVA (98%, Scheffe F-test). The only significant effect due to any incubation method was an increased incorporation of label into glutamate when the symbionts were incubated in coelomic fluid.

Discussion

Our results show that autotrophy is at least one of the incorporation mechanisms used by the purified bacteria, since our experiments yielded labelled 3-phosphoglycerate, a characteristic metabolite of the incorporation through the Calvin-Benson cycle (Table 1). Not surprisingly, the fraction of label in this compound decreased with longer incubation times as label was transferred to other metabolites in subsequent metabolic pathways.

Our purified symbionts fixed CO_2 at rates comparable to those described in trophosome preparations (Scott et al., 1994). The total incorporation of label into acid stable soluble substances always increased due to continuing incorporation of label from the medium. This total value was used for the calculation of fixation rates.

An incubation of purified symbionts in coelomic fluid caused a significant increase of the fraction of label in glutamate (to 38%) mostly in the bacterial pellet. Glutamate was also detected in the supernatant of bacterial incubations. Coelomic fluid is the most natural incubation medium. In contrast to the other incubation media which undoubtedly become anaerobic during the incubations, it is able to buffer the oxygen and sulphide supply to the bacteria instead of relying on the initially present amounts and on uptake of oxygen from the air. Glutamate could possibly serve as a transfer molecule for nitrogen and carbon during aerobic incubations of symbionts.

Succinate excretion accounted for up to a third of the overall fixation of label from $^{14}CO_2$ into nonvolatile compounds. Large fractions of fixed radioactivity have been detected in succinate after incubations of perfused isolated plumes, whole animals, and isolated trophosome (Felbeck 1985; Felbeck & Turner, 1995). It is also present in rather high concentrations of 8 - 10 mmol l^{-1} in the blood of tube-worms and accumulates to extremely high levels during hypoxic incubations of whole animals (Arndt et al., 1998).

The overall role of succinate in carbon transport is, therefore, proposed to be twofold (Fig. 2): to be a predominant carbon source for the nutrition of the host animal and, possibly, to serve as carrier for CO_2 from the

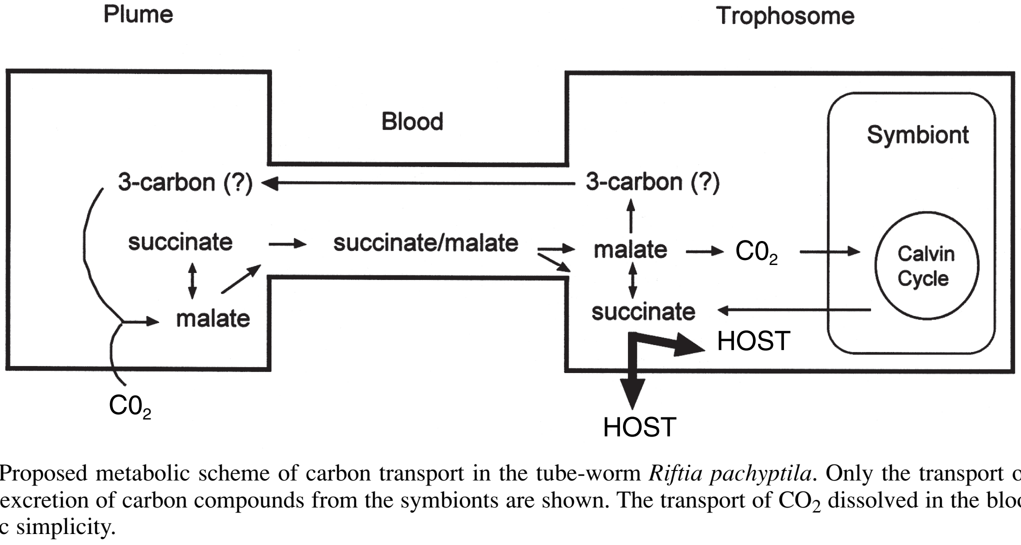

Figure 2. Proposed metabolic scheme of carbon transport in the tube-worm *Riftia pachyptila*. Only the transport of CO_2 as organic acids and the excretion of carbon compounds from the symbionts are shown. The transport of CO_2 dissolved in the blood has been omitted for graphic simplicity.

plume to the trophosome. For every four 4-carbon molecules transported from the plume to the trophosome via malate and succinate, one additional succinate could be made from the CO_2 released after decarboxylation of malate (Felbeck, 1985). Malate, which can be formed from succinate via an oxidation and a hydration, may be the actual molecule that is decarboxylated; therefore, this process could be regulated independently from the further metabolism of succinate. The 3-carbon compound deriving from this decarboxylation could be transported back to the plume via the blood (Felbeck, 1985; Felbeck & Turner, 1995). The host can incorporate any succinate released by the bacteria easily into its intermediary metabolism since it is an integral part of the citric acid cycle, the central metabolic turntable for most eucaryotic organisms.

Extrapolation of our results obtained with purified symbionts to quantitative statements about metabolic exchanges between tube-worms and their symbiotic bacteria must be cautious. However, our finding that purified symbiotic bacteria of *R. pachyptila* excreted succinate and glutamate after incubations with radiolabelled CO_2 strongly suggests that these metabolites serve as a supply of carbon and nitrogen to the tissues of the host tube-worm. Succinate and glutamate have not been identified as transfer components before, and together they constitute a supply of carbon as well as nitrogen, which may enable *R. pachyptila* to survive without a digestive tract.

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