Cah. Biol. Mar. (2002) 43 : 371-376

Chemoautotrophy as a possible nutritional source in the hydrothermal vent limpet *Lepetodrilus fucensis*

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Introduction

Metazoans acquire nutrition from hydrothermal vent bacteria by filter feeding or grazing, hosting chemoautotrophic symbionts, or some combination of the two. Obligate endosymbioses characterize the vent vestimentifera while vent clams (Felbeck et al., 1981) and mussels (Felbeck et al., 1981; Fisher et al., 1987) have well developed endosymbioses but still retain a functional gut and the ability to filter feed. Several putative ectosymbioses have been identified, such as in the case of the polychaete *Alivinella spp*. from the East Pacific Rise, or the Mid Atlantic Ridge shrimp *Rimicaris exoculata* (Gebruk et al., 1993), but there is so far no conclusive evidence of important nutritional benefit to the host animals. In this paper we re-examine an apparent ectosymbiose in the Juan de Fuca Ridge vent limpet *Lepetodrilus fucensis* McLean, 1988, first described by deBurgh & Singla (1985).

Lepetodrilus fucensis can comprise up to 50% of the total faunal biomass at Juan de Fuca Ridge vents (Sarrazin, 1999). While this limpet has been suggested to be primarily a suspension feeder (Tunnicliffe, 1991) its anatomy suggests that it is capable of grazing the tubeworm and rock surfaces that it colonizes (Fretter, 1988). Bacteria colonize the gills of *L. fucensis* and are phagocytosed by the gill epithelium. De Burgh & Singla (1985) proposed these bacteria are symbiotic, providing nutrition to the limpet. The goals of the work presented here were to:

1) use enzyme assays to determine whether the gill surface bacteria were chemoautotrophic

2) evaluate the quantitative significance of the external symbiosis through a detailed survey of microbial colonization of the gill of *Lepetodrilus fucensis* and the occurrence and processes of bacterial endocytosis and degradation by gill tissues.

Material and methods

Samples were collected at five sites located on the Endeavour Segment of the Juan De Fuca Ridge by the submersible *Alvin* at 2250 m in September 2000. Limpets from the first four sites were sampled from tubeworm bushes while the fifth sample came from a sulphide chimney colonized by bunched and stacked limpets. On the ship, limpets were removed from their shells and the gills carefully dissected. All shell, foot and gill tissues were kept. Twenty specimens from each location were immediately frozen at -80 °C and transported to the laboratory on dry ice. These samples were used in the enzyme assays (ATP sulphurylase, nitrate reductase and ribulose-1,5-biphosphate carboxylase-oxygenase). Another 10 animals from each site were placed in 3% glutaraldehyde solution and refrigerated at 4 °C for microscopy. A further 20 were frozen at -20 °C for stable isotope analysis.

Enzyme activities were measured in tissue extracts taken from the gills, which contain bacteria, and the tissues from the foot of the limpet which have no bacterial colonization (de Burgh & Singla, 1985). Trophosome tissues of the symbiont containing vestimentiferan *Ridgeia piscesae* Jones, 1985 were used as positive controls for the enzyme assays.

Gills from ten individual limpets from each site were homogenized together for ATP sulphurylase assays and the remaining ten were used for nitrate reductase assays. This preparation procedure was repeated for the foot tissues. Tissue samples were minced and homogenized using a ground-glass homogenizer according to Lee & Childress (1994) in volumes of 3 ml of ice-cold buffer containing 100 mmol l^{-1} Tris, 2.5 mmol l^{-1} $MgCl_2$, and 1 mmol l^{-1} ß-mercaptoethanol at a pH of 7.5. The homogenates were then vortexed three times for 15 s, and then centrifuged at

6400 rpm in a microcentrifuge for 10 min at 4 °C. Only the supernatants were used in the enzyme assays. ATP sulphurylase was assayed using the method of Lee & Childress (1994), and the method of Felbeck (1983) was used for the nitrate reductase assays. Protein levels in the soluble tissue extracts were measured using the bicinchonic acid protein assay (Sigma procedure TPRO-562) using bovine serum albumin as the standard. The RuBPC/O assay was based on the procedure of Tuttle (1985). Tissues were ground in distilled water using a glass homogenizer, then sonicated for 30 seconds to further separate tissues and symbionts. There was not enough tissue to run the RuBPC/O assays for each site, so twenty limpets from one site were dissected and pooled, and gill and foot tissues were used in the assay.

Stable carbon and nitrogen isotope analyses were performed to further characterize the limpet's nutritional sources. Twenty individual limpets were dissected and gill and foot tissues from each site were acidified using 1N HCl and then twice rinsed with distilled water before freeze-drying. The samples were then ground using a mortar and pestle and analysed individually using the Micromass Isoprime Isotope Ratio mass spectrometer at the GEOTOP research center, at the Université du Québec à Montréal.

Gill and foot tissues were prepared for the electron microscope by fixation in 3% glutaraldehyde and phosphate buffer pH 7.4 during 24 hours. They were rinsed and post-fixed in 1% osmium tetraoxide for one hour. The tissues were dehydrated using a graded acetone series and embedded in Epon 812. Thin sections were cut using a diamond knife and stained with uranyl acetate and lead citrate. The tissues were examined using a Jeol 2000 transmission electron microscope (TEM) at McGill University.

For the survey, the gills were divided into three regions: anterior, mid and posterior. Individual lamellae were removed from each region to be observed separately. The lamellae were sectioned vertically, and horizontally along the top (Fig. 1), and embedded in EPON. The sections were examined for the presence of bacteria and phagocytosis. Fifty epithelial cells were counted from each lamellae section and the amount of phagocytosis was recorded. Cells were then classified as having no phagocytosis (no bacterial cells being absorbed), some phagocytosis (1-10 bacteria per epithelial cell) and abundant phagocytosis (more than 10 bacteria per epithelial cell). Cells that were not undergoing phagocytosis were further divided into 4 categories; 1) ciliated cells (which had no bacteria associated with them), 2) non-ciliated cells with bacteria on the outside but none actively being endocytosed or present inside the cell, 3) non-ciliated cells where bacteria were inside, being degraded, but none visible outside or undergoing endocytosis, and 4) non-ciliated cells with no bacteria either inside or outside.

The frequencies of phagocytosis in the different gill sections were compared using the Chi-squared and Fisher tests.

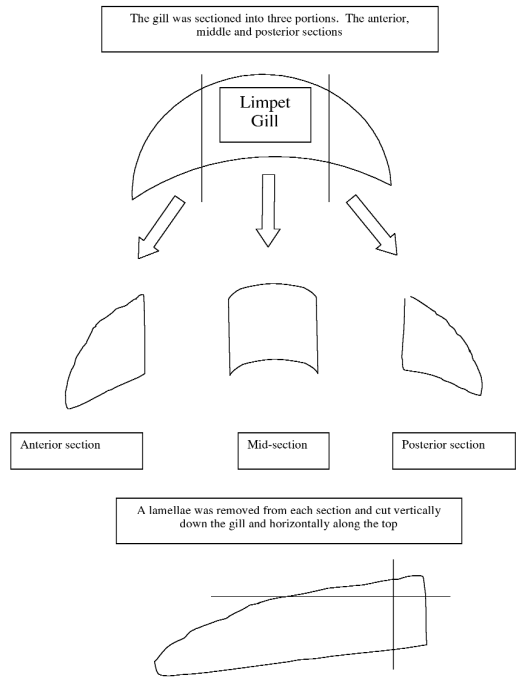

Figure 1. Preparation of gill samples for the Transmission Electron Microscopy survey.

Result and Discussion

The enzyme assay results indicate the gill surface bacteria are capable of chemoautotrophy, with activity levels for the 3 measured enzymes comparable to those in gill tissues of endosymbiont-containing bivalves (Table 1). All three enzymes were found in higher concentrations in the gills than in the foot tissues. Nitrate reductase (Fig. 2) and ATP sulphurylase (Fig. 3) showed significantly higher levels of

Table 1. Comparison of the nitrate reductase, ATP sulphurylase and RuBPC/O activities in the gill cells of the limpet *L. fucensis* and other chemoautotrophic species. Enzyme activity is measured as nmols of substrate produced mg^{-1} protein $minute^{-1}$

Organism	Nitrate Reductase	ATP Sulfurylase	RuBPC/O
*Riftia pachyptila**	98 (115)	74.0	0.22
Ridgeia piscesae (this study)	0.78	47	0.41
Bathymodiolus thermophilus	4*	2.7**	0.4#
*Solemya velum**	0.33	77.0	0.2
*Calyptogena magnifica**	0.2	25.0	0.4
Lepetodrilus fucensis (this study)	0.57	37	0.24

*from Felbeck et al., 1981; **from Fisher et al., 1987; # from Felbeck, 1983.

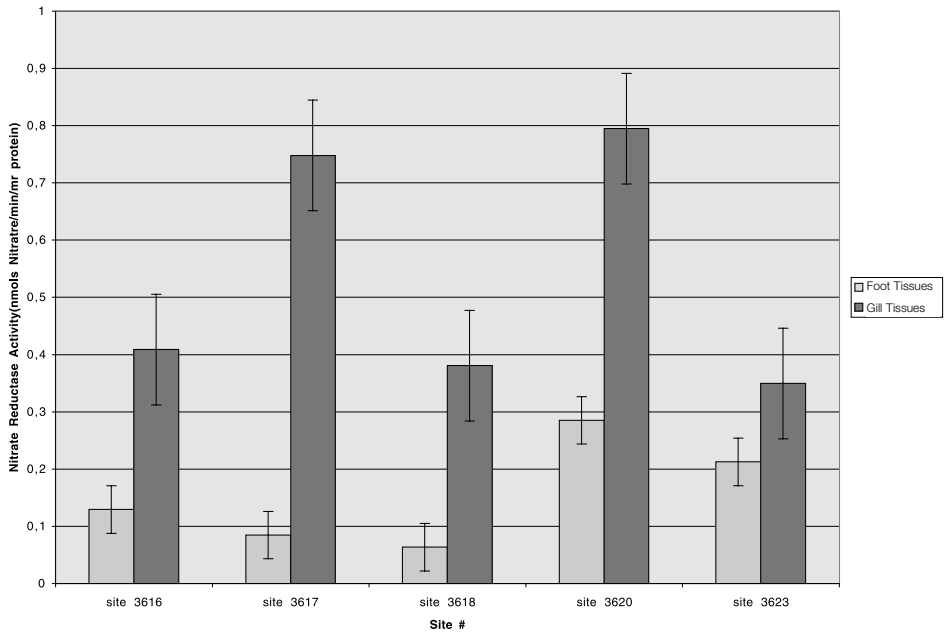

Figure 2. Nitrate reductase activity in the gill and foot tissues of a limpet from five hydrothermal vent sites.

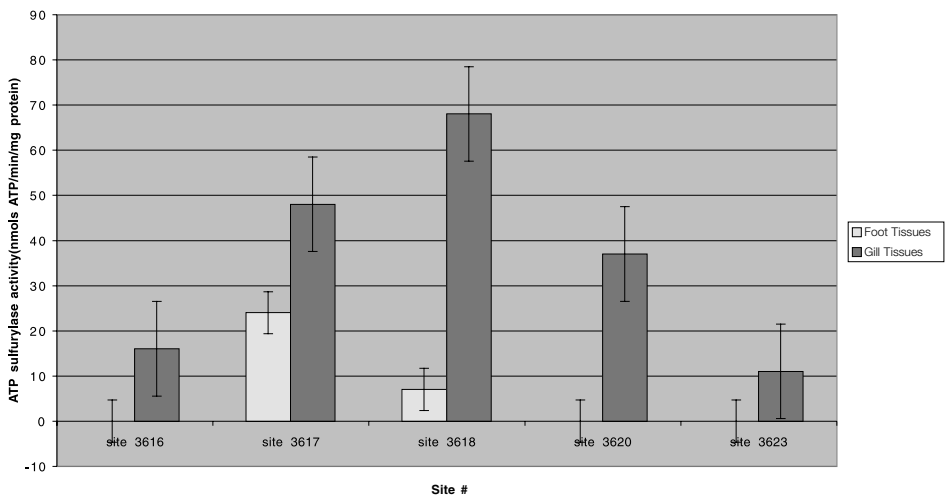

Figure 3. ATP sulphurylase activity of the foot and gill tissues of the limpet from five hydrothermal vent sites.

activity in the gill compared to foot tissues at the four sites where limpets were collected from tubeworm bushes. RuBPC/O levels (.22 nmols CO_2 fixed mg^{-1} protein min^{-1}) were particularly higher in the gill tissues compared to the foot, where activity was negligible. At the fifth site, where stacked limpets were collected from a sulphide chimney, there were high activities of nitrate reductase in both gill and foot tissues (Fig. 2). This may have been caused by contamination of foot tissue by stomach contents in this experiment. The small size of the limpets resulted in the entire body being homogenized along with foot tissues, once the gills were removed. It is possible that the stacked limpets at this site were filter feeding or grazing at a high rate and had undigested bacteria in their stomachs as well as on their gills. The bacteria in the stomach could have given positive results in the enzyme assays despite there being negligible levels in the foot tissue.

Bacteria were observed in all areas of the gills of *L. fucensis* but most bacterial colonization and endocytotic activity occurred in the middle and posterior areas of the gill (Table 2). The top of the gill (horizontal sections), where phagocytosis was most common (compared to vertical

Table 2. Numbers of epithelial cells undergoing some, abundant, or no phagocytosis, per section, both vertical (V) and horizontal (H) cuts. The cells where no phagocytosis was observed were further divided into 4 categories (n=50 for each gill section).

	No Phagocytosis				Phagocytosis	
Gill Section	Ciliated cells	Non-ciliated with bacteria outside gill cells	Non-ciliated with bacteria inside gill cells	Non-ciliated with No bacteria outside or inside gill cells	Some phagocytosis	Abundant phagocytosis
Anterior (V)	22	1	1	10	6	10
Middle (V)	19	3	4	0	10	14
Posterior (V)	11	3	3	0	5	28
Anterior (H)	27	0	0	3	5	15
Middle (H)	5	1	0	0	12	32
Posterior (H)	15	2	1	0	8	24

sections), has the largest surface area and is the most accessible and most exposed to inhalant water movement.

The concentration of endocytosis in the middle and posterior regions of the gill may be due to the distribution of ciliated cells, which primarily occurred in the anterior portion of the gill where there were the fewest bacteria. Bacteria were never observed being phagocytosed or associated with cells that had cilia (Fig. 4D). The limpets use ciliated cells to move water over the gills to oxygenate them (Fretter, 1988). They also use the same movement to transfer food towards the digestive tract when filter feeding. The beating of the cilia would tend move inflowing bacteria back along the gill and probably also prevent any bacteria from colonizing ciliated cell.

The types and quantities of bacteria on the gills provide some indication of whether the bacteria phagocytosed are replaced by local bacterial cell division at the surface of the gills or by filtration of water moving over the gills. Almost all bacteria seen on the gill tissues consisted of two morphotypes, and occasionally a third, but only one type was phagocytosed. This suggests that bacteria selectively multiply directly on the gill surface and are then phagocytosed by the epithelial cells. If the gills were simply filtering bacteria from inflowing water, we would expect to see a greater morphological diversity along and within the gill cells.

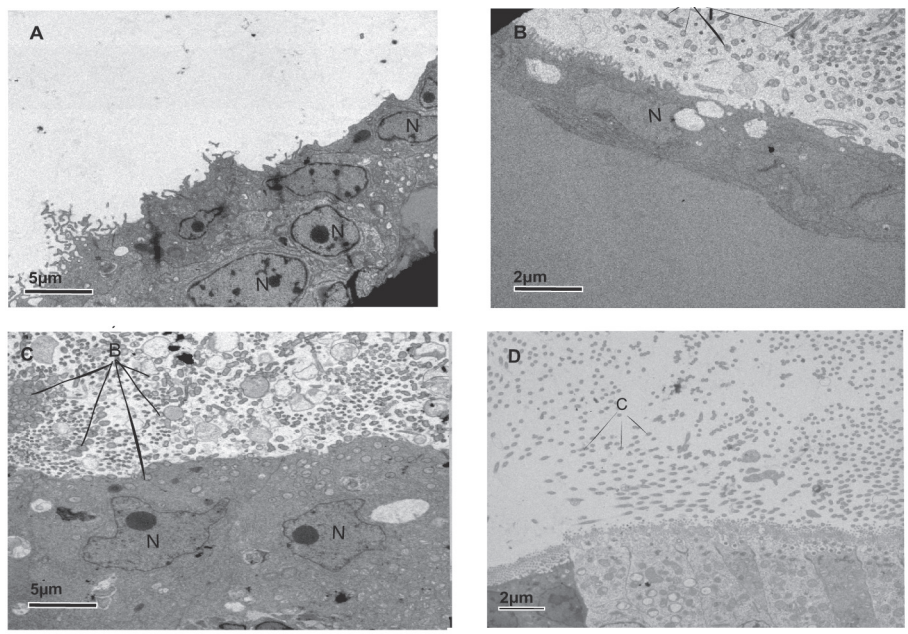

Figure 4. Some of the main types of gills cells observed during the TEM survey. **A.** No bacteria on the outside of the cell and thus no phagocytosis. **B.** Some cells were found to have bacteria on the exterior but little activity. **C.** Most of the cells that showed phagocytosis had numerous bacteria along the exterior regions as well as many being actively phagocytosed in various stages of degradation. **D.** For ciliated cells no bacteria were observed either inside or outside, and thus there was no phagocytosis. (*B*) Bacteria, (*N*) Nucleus, (*C*) Cilia (in cross and oblique sections).

Our TEM survey suggests that the process by which the gill cells undergo phagocytosis is cyclic, rather than the continuous process proposed by DeBurgh & Singla (1984). There appear to be 5 identifiable stages: 1) cells without bacteria either inside or outside (Fig. 4A); 2 cells where there are bacteria outside but not being endocytosed (Fig. 4B); 3) cells with abundant phagocytosis and many bacteria on the outside (Fig. 4C); 4) cells with little phagocytosis and

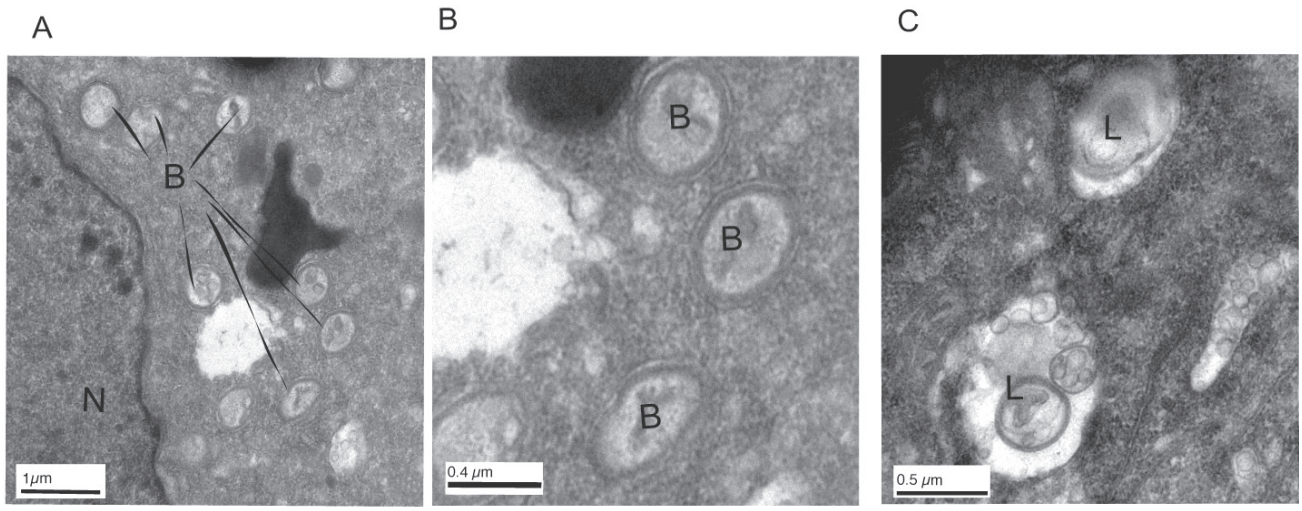

Figure 5. Close-up of a *Lepetodrilus fucensis* gill cells showing process of degradation of bacteria. **A.** General view of bacteria enclosed in membranes (lysosomes ?) near the nucleus. **B.** Lysosome-like bodies with residual material suggesting degradation. **C.** Lysome-like bodies closer to the basal region of the epithelial cell are further degraded. (*B*) Bacteria, (*N*) Nucleus, (*L*) Lysosome-like body.

fewer bacteria outside the cells (not shown); 5) cells that seem to have already taken up all the bacteria outside and are in the final processes of bacterial digestion (not shown).

Most of the cells undergoing phagocytosis showed high activity. This indicates that most of the cells can undergo phagocytosis when bacteria are present. We suggest that cells with bacteria accumulated on the outside, but with none being endocytosed, represent the beginning of the cycle (Fig. 4B). As more bacteria accumulate, endo-cytosis begins and increases to a point where it exceeds the rate of bacterial accumulation on the epithelial surface. In the following stage, where no bacteria are left on the outside of the gill, endocytosis has slowed but some bacteria can still be seen being degraded within lysosome-like bodies (Fig. 5A-C). Finally, when bacteria are absent from both the inside and the outside of the gill cell, the cell is re-colonized and the process begins again. The cycle we propose implies that endocytotic activity accelerates and decelerates in response to the accumulation and depletion of bacteria on the cell surface. This point requires further investigation.

In summary, the TEM survey showed that endocytosis and intracellular degradation of bacteria are widespread in the gill of *Lepetodrilus fucensis* and that there are identifiable stages of bacterial accumulation and endocytosis that suggest a cyclic process of colonization by bacteria and harvesting by the gill epithelial cells. The combined positive results for the enzyme assays (nitrate reductase, ATP sulphurylase and RuBPC/O) in gill tissues indicate that the bacteria on the gill are chemoautotrophic, and that the gill tissues have microbial enzyme activity levels similar to those reported for bivalves with endosymbionts. This suggests that the *L. fucensis* gill bacteria have the potential to serve as a significant source of nutrition for the animal. However, stable isotope data (δ^{13}C = -19.5 to -14.8‰; δ^{15}N = 2.5 to 5.0‰) situate *L. fucensis* within a group of known deposit feeding invertebrates at the Juan de Fuca Ridge vents, rather than in a separate grouping as is the case for the vestimentiferan worm *Ridgeia piscesae* that is nourished exclusively by chemoautotrophic symbionts (C. Levesque, personal communication). The limpet does have a well-developed radula and is capable of grazing (Hickman, 1983). Filter feeding has also been proposed for this limpet and may be related to the observed stacking behavior where several limpets attach to each other's shells forming stacks or hanging chains. An ability to use multiple methods of acquiring nutrition from grazing, or filter feeding, or the endocytosis and degradation of bacteria directly by the gill epithelium may be one of the reasons for the ecological success of the limpet at vents.

Acknowledgements

This research project was funded by NSERC Canada grants to SKJ. MF was supported by fellowships from the GEOTOP Research Centre and the Département des Sciences Biologiques at the Université du Québec à Montréal. Jeannie Mui and the technicians at McGill University are thanked for their help with the electron microscopy. Chief scientist John Delaney and the crew of the R/V *Atlantis* and *Alvin* enabled the collection of the limpet samples at sea.

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