UNIVERSITÉ DU QUÉBEC À MONTRÉAL

COLONIES DE CILIÉS TAPIS BLEUS EN PROVENANCE DE SOURCES HYDROTHERMALES DU PACIFIQUE NORD-EST: SYMBIOSE MICROBIENNE ET ÉCOLOGIE

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COLONIAL "BLUE MAT" CILIATES FROM NORTHEAST PACIFIC HYDROTHERMAL VENTS: MICROBIAL SYMBIOSIS AND ECOLOGY

THESIS
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BY
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AVANT-PROPOS

Au cours de mes études de doctorat et dans le but de compléter le travail présenté dans cette thèse, j'ai eu la chance de bénéficier de l'expertise, des conseils, de la formation, des installations et de l'exceptionnelle communauté intellectuelle offerte par trois établissements de recherche :
GEOTOP, Montréal, Canada; l'unité *Adaptations aux milieux extrêmes* du Centre National de la Recherche Scientifique (CNRS), Paris, France; ainsi que du « Max Planck-Institute for Marine Microbiology » (MPIMM), Brême, Allemagne. Les trois articles présentés dans cette thèse sont le résultat de ces efforts de recherche collaborative internationale.

1. Kouris, Angela, S. Kim Juniper, Ghislaine Frébourg et Françoise Gaill. 2007. «Protozoan–bacterial symbiosis in a deep-sea hydrothermal vent folliculinid ciliate (*Folliculinopsis* sp.) from the Juan de Fuca Ridge». *Marine Ecology* 28: 63–71.

2. Kouris, Angela, S. Kim Juniper et Nicole Dubilier. «Identification and characterization of blue mat (*Folliculinopsis* sp.) ciliate symbionts from northeast Pacific hydrothermal vents». En préparation.

3. Kouris, Angela, Helene Limén, Catherine J. Stevens et S. Kim Juniper. «Faunal composition and food web structure in colonial ciliate (*Folliculinopsis* sp.) mats at northeast Pacific hydrothermal vents» *Marine Ecology Progress Series* (accepté).

Le premier article intitulé en français « Symbiose protozoaire-bactérienne dans un cilié folliculine (*Folliculinopsis* sp.) de la dorsale Juan de Fuca » constitue le premier chapitre de la thèse. L'article est le résultat d'un stage de recherche de 3 mois (2005) au sein de l'unité de recherche *Adaptations aux milieux extrêmes* du Dr. Françoise Gaill de l'Université Pierre et Marie Curie, CNRS, (Paris, France). Le laboratoire de Dr. Gaill se spécialise dans l'étude ultrastructurale des microorganismes, en particulier sur les symbioses entre bactéries et autres organismes des milieux marins extrêmes. L'article a été publié dans un numéro spécial de *Marine Ecology* intitulé « Advances in Vent, Seep, Whale- and Wood-Fall Biology ».

Pour cet article, comme pour l'ensemble des articles dans ma thèse, j'ai réalisé moi-même l'échantillonnage, la fixation et la préparation des échantillons, l'expérimentation, la rédaction de l'article, l'analyse des données et la création des figures et des tableaux, la recherche bibliographique et l'interprétation des résultats. Comme pour tous les autres articles, mon directeur de thèse, Dr. S. Kim Juniper m'a aidée avec l'interprétation des résultats et la correction des ébauches de l'article. Ghislaine Frébourg, la technicienne du laboratoire de Dr. Gaill, m'a fournie les protocoles de préparation des échantillons pour la microscopie électronique et m'a formée aux méthodes d'observation pour la microscopie électronique à balayage (MEB) et à transmission (MET). Dr. Françoise Gaill m'a accueillie dans son laboratoire, a mis à ma disposition les techniciennes et l'équipement nécessaire pour le travail et a aidé à l'interprétation des résultats et à la correction des ébauches.

Le deuxième article, intitulé en français « Caractérisation moléculaire de symbiotes des ciliés tapis bleus (*Folliculinopsis* sp.) des sources hydrothermales du Pacifique Nord-est » constitue le deuxième chapitre de la thèse. L'article reflète une partie du travail réalisé durant les deux stages de recherche de 3 mois (2006 et puis 2007) et un stage de recherche de 5 mois (2008) dans le groupe Symbiose du Dr. Nicole Dubilier du département d'écologie moléculaire du MPIMM. Le laboratoire de Dr. Dubilier se spécialise dans l'étude de la distribution, la diversité et la phylogénie des bactéries symbiotiques en association avec des organismes provenant d'environnement chimiosynthétique. Dr. Dubilier m'a accueillie dans son laboratoire, a mis à ma disposition les techniciennes et l'équipement nécessaire pour le travail, a aidé avec l'interprétation des résultats et à la correction des ébauches.

Le troisième article, intitulé en français « Composition faunistique et réseaux trophiques dans des tapis de colonies de ciliés (*Folliculinopsis* sp.) des sources hydrothermales du Pacifique Nord-est » constitue le troisième chapitre de cette thèse. Le travail présenté dans ce troisième article a été réalisé au GEOTOP (à l'UQAM), dans les laboratoires de microscopie optique (analyse des espèces) et de spectrométrie de masse (relations trophiques). La docteure Helene Limén m'a fourni les protocoles de préparation des échantillons pour les analyses des isotopes stables et m'a formée à l'identification de la méiofaune et la macrofaune, a aidé à l'interprétation des résultats et à la correction des ébauches de l'article. La docteure Catherine J. Stevens a réalisé les analyses lipidiques, de même que l'interprétation et la rédaction des résultats concernant les lipides.

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The completion of this doctoral dissertation would not have been possible without the guidance, support, and help of many individuals.

Of all the people that have contributed to this intellectual endeavor there are two that, for very different reasons, I owe the most to. Kim Juniper has been everything that I could ever hope for in a supervisor. His continued support and eagerness to allow me the freedom and flexibility to pursue new aspects of my research, learn new methodologies and participate in the intellectual settings of different research centers have truly made this doctorate an incredibly enriching experience. Despite often great geographic distance, I always felt that Kim was present to guide, advise and discuss.

Chantal Nadeau, my partner and confidant, has acted as a constant flow of motivation, care, and advice. Her unwavering encouragement made the commencement and subsequent completion of this dissertation a reality. To you, ma Chantal, I dedicate this work.

Beatrix Beisner has supported my research from day one ultimately becoming my adoptive (co) supervisor at UQÀM. I am very grateful for the input, often difficult questions and discussions.

I wish to extend a heartfelt thank you to the mentors that I have collaborated with and learned so much from: Françoise Gaill (CNRS, Paris) and Nicole Dubilier (MPIMM). Participating in their laboratories peaked my research interests in the most exhilarating intellectual ways.

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I am grateful to the external and internal members of my Dissertation Evaluation Committee (Connie Lovejoy - Université Laval, Lyle White – McGill University, Philip Spear – UQÀM,

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J'aimerais remercier celles qui, dans l'ombre, m'ont aidée de mille et une façons durant mon séjour à l'UQÀM, Ginette Lozeau-Lacroix (Département des sciences biologiques) and Josée Savard (GEOTOP).

Finally, my parents, Christine and Tom Kouris and sister, Joanna Kouris have helped and guided me in innumerable ways. I am truly grateful for having them share this moment of completion with me.

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RÉSUMÉ

Ma thèse de doctorat a pour titre « Colonial "blue mat" ciliates from northeast Pacific hydrothermal vents: microbial symbiosis and ecology ». Les colonies de ciliés folliculines forment d'épais et éclatants tapis bleus en périphérie de plusieurs sources hydrothermales de la dorsale Juan de Fuca dans le Pacifique Nord-est et ailleurs. Ces tapis peuvent couvrir jusqu'a 70% des substrats basaltiques auxquels ils sont attachés. À ce jour, nous connaissons très peu de l'écologie et de la biologie des ciliés folliculines en provenance des sources hydrothermales ni de leur importance pour d'autres espèces. Ma thèse se veut une investigation d'une symbiose potentielle entre les ciliés folliculines et les procaryotes aux sources hydrothermales. Elle considère aussi l'écologie trophique des tapis bleus ainsi que les invertébrés qui y sont associés.

Les symbioses sont un trait commun aux sources hydrothermales ainsi qu'aux protozoaires ciliés. Le succès écologique des tapis bleus, démontré notamment par leur abondance et leur distribution répandue autour des sources, pourrait être attribué aux symbiotes procaryotiques contenus dans les folliculines-hôte. L'ultrastructure des ciliés tapis bleus a été explorée en utilisant la microscopie électronique de balayage [MEB] et la microscopie électronique à transmission [MET]. En nous basant sur nos observations morphologiques et ultrastructurales, nous avons assigné ces ciliés au genre folliculine *Folliculinopsis*. Les ciliés folliculines secrètent et vivent dans des coques ou tubes (loricae). Nous avons trouvé que les loricae ont été colonisés à la fois par des bactéries coccoïdes et des procaryotes filamenteux. De plus grandes densités de coccoïdes et de microorganismes en bâtonnets ont été trouvées entre les rangs de cils sur le corps du cilié (zooïde), surtout sur les lobes du péristome (les extensions similaires à des bras typiques des ciliés folliculines). Le morphotype coccoïde (dedans ou indépendant des vacuoles) se retrouvait à travers tout le cytoplasme du cilié. Des groupes de cet organisme rassemblé dans des vacuoles étaient distribués régulièrement le long du cortex du cilié.

Des traits denses aux électrons, bornés par des vacuoles et caractérisés par des membranelles superposées, ont été également trouvés dans le cytoplasme du cilié. Des méthodes moléculaires complémentaires [comparaison des séquences d'ARN ribosomique 16S et l'hybridation *in situ* avec fluorescence (FISH)] ont été utilisées pour identifier les types de microorganismes associés symbiotiquement aux tapis bleus et pour déterminer leur répartition dans la cellule du cilié. Nos résultats indiquent que le *Folliculinopsis* sp. héberge des endosymbiotes euryarchées. Ceux-ci se situent dans un clade d'organismes qui sont étroitement apparentés à des séquences environnementales de fluides froids qui ont des quantités élevées de méthanogènes.

Sous microscope à épifluorescence équipé d'un filtre DAPI, la présence de l'enzyme 420 autofluorescente, typique aux méthanogènes, a été observée dans les cellules du cyploplasme du cilié. En plus d'archées, une haute densité de bactéries a été trouvée dans le cytoplasme du cilié de même que sur la surface du loricae. Quelques-unes de ces séquences

bactériennes ont été phylogénétiquement reliées aux ecto and endo symbiotes connues d'autres types d'hôtes. Les ciliés folliculines des sources hydrothermales du Pacifique Nord-est apparaissent dès lors héberger de multiples symbioses phylogénétiquement distinctes et localisées dans diverses parties de leurs cellules.

Puisque les tapis bleus sont physiquement dominants aux sources hydrothermales, ils forment un habitat physique substantiel dans lequel les espèces de méiofaune et de macrofaune peuvent trouver nourriture et refuge, comme c'est le cas avec les buissons de vestimentifères et les moulières de mytilidés de la sous-famille Bathymodiolinae qui croissent autour des sources hydrothermales et des sources de fluides froids.

Afin de déterminer l'importance probable de ces ciliés pour les autres métazoaires provenant des sources, les invertébrés associés aux échantillons de tapis bleus ont été identifiés et quantifiés et des analyses d'isotopes stables de carbone et de l'azote ont été réalisées. De plus, des analyses de lipides des tapis bleus ont été faites. Nous avons trouvé une récurrence d'invertébrés associés aux tapis bleus et dans tous les échantillons, la méiofaune était numériquement dominante. Les copépodes harpaticoïdes, *Amphiascus* sp. étaient fort plus abondants que les autres espèces dans les tapis bleus. Alors que certains invertébrés (ex. *Amphiascus* sp.) dans cet assemblage semblent exclusivement liés aux tapis bleus, d'autres proviennent de sources hydrothermales différentes à proximité. Les ciliés *Folliculinopsis* sp. étaient davantage réduits dans $\delta^{13}C$ que les invertébrés dans l'assemblage de tapis bleus indiquant du coup que ces derniers ne se nourrissent pas uniquement des premiers. Au moins deux niveaux trophiques existent dans cet assemblage. La macrofaune juvénile, les ostracodes et les nématodes occupent des niveaux trophiques supérieurs. Les analyses de lipides bactériennes indiquent que 16:1ω7 et 18:1ω7 (typiques aux sulfo-oxidantes) constituent plus de la moitié des acides gras des tapis bleus. Les tapis bleus apparaissent ainsi jouer un rôle indirect, mais important pour l'écologie des organismes qui leur sont associés.

Mots clés: folliculines; sources hydrothermales; isotopes stable; lipides; méiofauna; macrofauna; symbiose; microscopie électronique; ultrastructure

ABSTRACT

The title of my doctoral dissertation is *Colonial "blue mat" ciliates from northeast Pacific hydrothermal vents: microbial symbiosis and ecology*. Colonial folliculinid ciliates create dense bright blue carpets, termed blue mats, on the periphery of many northeast Pacific hydrothermal vents on the Juan de Fuca Ridge and elsewhere, and can cover as much as 70% of the basaltic substratum to which they attach. To date, very little is known about the ecology and biology of folliculinid ciliates at hydrothermal vents and their potential importance for other species. My dissertation investigates a potential symbiosis between folliculinid ciliates and prokaryotes and considers the trophic ecology of blue mats and related invertebrates and at hydrothermal vents.

Symbioses are a common feature at hydrothermal vents and also in ciliated protozoa. The ecological success of the blue mats, as evidenced by their abundance and widespread distribution around vents, may be attributed to prokaryotic symbionts hosted by the folliculinids. The ultrastructure of the blue mat ciliates was investigated using conventional scanning electron microscopy and thin section transmission electron microscopy. Based on our morphological and ultrastructural observations, we were able to assign these vent ciliates to the folliculinid genus *Folliculinopsis*. Folliculinid ciliates secrete and dwell in tubes (loricae). We found the loricae were colonized by both coccoid and filamentous prokaryotes. Greater densities of coccoid- and short-rod-shaped microorganisms were found between rows of cilia on the ciliate body (zooid) and especially on the peristomal lobes (arm-like extensions typical to folliculinid ciliates). A coccoid morphotype (within and independent of a vacuole) was located throughout the ciliate cytoplasm. Groups of this organism clustered within vacuoles were regularly distributed along the ciliate cortex. Electron dense, vacuole-bound features characterized by stacked membranous structures were also found within the ciliate cytoplasm.

Complementary molecular methods (16S rRNA gene sequence analysis and fluorescence *in situ* hybridization) were used to identify the types of microorganisms symbiotically associated with the blue mats and to determine their distribution in the ciliate cell. Our results indicate that *Folliculinopsis* sp. harbors euryarchaeal endosymbionts. These fall within a clade of closely related environmental sequences from seeps with high abundances of methanogens. When examined using a DAPI filter on an epifluorescent microscope, enzyme 420 autofluorescence typical to methanogens was observed in cells within the ciliate cytoplasm. In addition to archaea, a high diversity of bacteria was found within the ciliate cytoplasm as well as on the surface of the ciliate loricae. Some of these bacterial sequences were phylogenetically related to known ecto- and endosymbionts from other host phyla. Folliculinid ciliates at northeast Pacific hydrothermal vents therefore appear to harbor multiple phylogenetically distinct symbionts located in different parts of their cell.

Since blue mats are physically dominant at vents, they form a substantial physical habitat within which meiofauna and macrofauna species may find food and shelter, as is the case with vestimentiferan tubeworm bushes and Bathymodiolin mussel beds at hydrothermal vents and cold seeps. To determine the potential importance of these ciliates for other vent metazoans, invertebrates associated with sampled blue mats were identified, quantified and analyzed for stable carbon and nitrogen isotopes. In addition, polar lipid fatty acid analyses of blue mats were performed. We found a recurrent assemblage of invertebrates associated with the blue mats and, in all samples, meiofauna were numerically dominant. The harpacticoid copepod, *Amphiascus* sp. was far more abundant than any other species within the mats. While some of the invertebrates (ex. *Amphiascus* sp.) within this assemblage seem exclusively linked to blue mats, others are known from other nearby hydrothermal vent habitats. *Folliculinopsis* sp. ciliates were far more depleted in $\delta^{13}C$ than invertebrates within the blue mat assemblage indicating that the latter do not feed exclusively on the former. At least two trophic levels exist within this assemblage, with juvenile macrofauna, ostracod and nematode species occupying higher trophic levels. The bacterial polar lipid profiles indicate that 16:1ω7 and 18:1ω7 (typical to sulphur oxidizers) make up over half the blue mat fatty acids. Blue mats appear to play an indirect but important role in the ecology of organisms associated to them.

KEY WORDS: folliculinid; hydrothermal vents; stable isotopes; fatty acid; meiofauna; macrofauna; symbiosis; electron microscopy; ultrastructure

INTRODUCTION

SUBJECT

Hydrothermal vents were first discovered in 1976 on the Galapagos Rift in the eastern Pacific and have since been found at centres of both converging and diverging plate boundaries (spreading centers) as well as where back arc-spreading occurs (Gage and Tyler 1991, Tunnicliffe et al. 2003).

Volcanic and tectonic activity result in the 'extreme', unstable and unpredictable environmental conditions found at hydrothermal vents. Deep-sea hydrothermal vents are devoid of sunlight and characterized by high pressure (2×10^4- 3×10^4 kPa) (Micheli et al. 2002). Vent temperatures vary widely (diffuse vent fluids are 2°C- 100°C while subsurface solutions and discharge from sulfide edifices can reach up to 400°C), as does pH (2.8-8.0) as do dissolved oxygen concentrations (0-110 μmol/L) as do levels of generally toxic sulfides and heavy metals (Micheli et al. 2002, Tunnicliffe et al. 2003). An abundant and highly specialized fauna colonizes newly formed and existing vents and thrives in these areas of the deep sea.

Communities of chemolithoautotrophic bacteria form the base of hydrothermal vent food chains, oxidizing reducing compounds such as H_2S and CH_4. They can be found as either symbionts living within host tissues, as free-living cells surrounding the vents, or as free-living cells attached to solid substrates (Gage and Tyler 1991, Colaço et al. 2002). These microbial communities are amongst the most biologically productive groups of organisms in known marine ecosystems (Gage and Tyler 1991).

Although abiotic factors may determine the ultimate community structure of hydrothermal vents, biotic factors (predation, competition and recruitment) can also play a large role in determining abundances of organisms (Voight 2000, Micheli et al. 2002). Habitats in close proximity to hydrothermal vent flow such as those dominated by bushes of symbiont hosting vestimentiferan tube worms and beds of symbiont bearing Bathymodiolin

mussels create habitats for other organisms (Tunnicliffe and Juniper 1990, Desbruyeres et al. 2000, Van-Dover 2002, Bergquist et al. 2003, Van Dover 2003). Interactions between fauna in these habitats have received attention and demonstrate the biological complexity of hydrothermal vent ecosystems (Levesque et al. 2006, Limén and Juniper 2006, Zekely et al. 2006, Colaço et al. 2007, Gollner et al. 2007). Habitats on the periphery of hydrothermal vent flow and those not created or dominated by mega and macrofauna have received little attention.

Endemic protozoans have yet to be reported from hydrothermal vents. The first summary of protistan organisms at vents revealed sessile peritrich and folliculinid ciliates to be most abundant (Small and Gross 1985). Tunnicliffe et al (1985) described folliculinid ciliates that formed dense blue-green colored protozoan mats adjacent to hydrothermal venting on Axial Volcano of the Juan de Fuca Ridge. Twenty years later, dense carpets of bright blue-green folliculinids still occur on Axial Volcano, and are now known from other vent sites in the northeast Pacific indicating that these ciliates are a recurrent component of the vent fauna in this region. A symbiosis involving folliculinids from vents has been proposed to explain their extensive distribution but remains unconfirmed (Small and Gross 1985, Rosati 2002)

Though symbiotic relationships between prokaryotes and protozoa are widespread in marine, freshwater and terrestrial environments, there is yet no direct evidence for a protozoan-prokaryote symbiosis at hydrothermal vents. Symbioses between chemolithoautotrophic sulfur-oxidizing bacteria and protists are common in other sulphidic environments (Ott et al. 1998). Ciliates from reducing environments that host chemosynthetic symbioses include the *Kentrophoros* (Ciliophora, Karyotelictida) sand ciliate and the *Zoothamnium niveum* (Ciliophora, Peritrichida), a sessile, colonial ciliate growing on mangrove peat, decaying leaves and rootlets and sunken wood in mangrove swamps of the Caribbean (Laurent et al. 2009).

CURRENT STATE OF KNOWLEDGE

The blue mat ciliate

Both freshwater and marine species of folliculinid ciliates exist (Andrews 1923) and can be found colonizing solid substrata, as epibionts on aquatic plants and mobile invertebrates (Andrews 1914; Aladro-Lubel and Martínez-Murillo 1999; Fernandez-Leborans 2003; Prime-Habdija and Matoničkin 2005). Folliculinid ciliates secrete and dwell in tube-like structures made of chitin (the loricae). During a folliculinids mobile life stage (swarmer) it is shaped like a small, cylindrical tube. Once prepared to settle onto a surface, the ciliate secretes and shapes the loricae and the ciliate body (zooid) partially splits in two, forming arm-like extensions (the peristomal lobes) that can stretch out passed the uppermost lip of the loricae. Initial attachment draws other swarming folliculinids that subsequently settle and form colonies (Fig. 1). At Axial Volcano vents we have mostly observed folliculinids as extensive blue mats colonizing basalt surfaces (Fig. 2) but in samples we have found them attached to pycnogonids, vestimentiferan tubes and limpet shells.

A recent study on eukaryotic diversity and microcolonizers at the Mid-Atlantic Ridge hydrothermal 'niches' offered the idea that ciliates and flagellates may initiate the colonization process in hydrothermal vent systems (Lopez-Garcia et al. 2003). It has been suggested elsewhere that marine folliculinids (and at least one freshwater folliculinid species) are early colonizers of virgin surfaces (Prime-Habdija and Matoničkin 2005).

Symbiosis

Symbioses are persistent, close associations between pairs of species. All major lineages of animals have long-term associations with microorganisms. Symbionts can trigger major adaptive radiation, can be the source of phenotypic complexity in potential hosts and can be a source of short-term adaptation to changing environments (Moran 2007). Traditionally symbiotic interactions have been compartmentalized into the following categories: Mutualism – where both host and symbiont benefit directly from the association; commensalism – where one symbiotic partner gains benefit while conferring no direct benefit or detriment to the other partner; parasitism – where a symbiont takes advantage of, but does

not necessarily cause death to, its host. In contrast to symbiotic interactions are pathogenic or predatory interactions where one organism kills the other.

Symbionts generally share one or more common characteristics. Most symbionts:

- Are host-specific
- Are closely related to other known symbionts
- Occur in specific, predictable locations or compartments within the host (example: the trophosome in gutless worms, bacteriocytes in mussels, crib spaces in basal metazoans)
- Are highly enriched and regularly distributed within (endosymbiosis), or on the surface of or within the epithelium of the host (ectosymbiosis)

Advances in the field of genomics and newly designed tools used to study DNA have shed new light on the intricacies and complexities of symbioses including the diversity and evolution of symbiotic interactions and how host and symbiont metabolisms are related. As a result, research on symbiosis has become more prominent in recent years and the very concept of the existence of the biological individual is being questioned.

Amongst the most common symbiotic interactions studied are those involving prokaryotes and either unicellular or multicellular hosts. Prokaryotes have a greater range of metabolic capabilities than do eukaryotes and, when associated with a host, can therefore enhance the latter's fitness either by sharing metabolites or by directly serving as a food source. Symbionts can be transferred to a host either vertically (transmitted from parent to offspring) or horizontally (taken up from the environment). One well studied case of vertical transmission is the Buchnera (gamma proteobacteria)-pea aphid symbiosis, where the bacteria are vertically transmitted and lose a large number of genes no longer necessary to them once integrated into the host's environment (Moran 2003). Buchnera surrenders its genes becoming dependent on the pea aphid host, and the pea aphid's genes potentially compensate for essential biosynthetic pathways missing in Buchnera (Moran 2003). Horizontally transmitted symbionts initially infect the host cell but are not immediately lysed. The location of infection is generally restricted and usually occurs either in oral openings, gills, root hairs, or in host trunks (Bright and Bulgheresi Forthcoming).

There are key recognition factors at play between the host and symbiont mediating the initial contact and subsequent inclusion of the symbiont into (or onto) the cell. For example, the

onset of cell-cell surface recognition can lead to endocytosis-phagocytosis of symbionts. A host may also have structures that facilitate uptake. Subsequent stabilization of the symbiosis may take years to occur such that the symbionts initially taken up from the environment are not necessarily the ones that persist over time with the host. In a coral reef environment, there are many free-living potential symbionts available but each host harbors either one or more specific symbionts (Coffroth et al. 2006; Thornhill et al. 2009). Infection of the host by eventual symbionts occurs during early ontogeny. Mature symbiosis between corals and microorganisms is not based on the initial symbiosis since it can take between 3.5 - 4 years for a lasting change of symbionts to occur (Coffroth et al. 2006).

Different animal taxa have developed different methods to deal with detection of symbionts and defense against other microorganisms. Hosts may have specific peptides that mediate and structure prokaryotic communities over time. The basal metazoan Hydra (a cnidarian) is essentially like a tube that exists to digest food entering in from the environment. Different species of Hydra host different bacterial guilds (Fraune and Bosch 2007). The closest relatives of Hydra symbiotic partners are other known symbionts. This host has both recognition and defense mechanisms. For example, effector genes kill all bacteria that are not Hydra specific (Bosch et al. 2009). Thus, in the case of Hydra, the host, and not the environment, decides what microbes are associated with it (Bosch et al. 2009).

In squid-vibrio symbioses, *Vibrio fischeri* are packed into specific areas of the host (light organ). They are horizontally transmitted ectosymbionts found in the epithelial cells. While many types of bacteria can adhere to crib spaces (the initial place of contact between potential symbionts and host epitheliem), within two hours of initial attachment *V. fisheri* become dominant (McFall-Ngai 2008). In this symbiosis, symbionts communicate with host to mediate integration into the epithelium.

Protozoan symbioses

Recent interest in theories of symbiogenesis and the common occurrence of symbioses at hydrothermal vents have prompted extensive investigations of symbioses in protozoa (Fokin 2004). The widespread occurrence of ciliate-prokaryote symbioses may have previously been overlooked although studies are now indicating that these associations have important

"ecological effects" and "evolutionary implications" (Vannini et al. 2003). Fokin (2004) reports that ciliates without bacterial symbionts are more the exception than the rule and that ciliates are "preadapted" to be suitable hosts. Ingestion by phagocytosis, for example, means that microorganisms can be ingested as food particles and may then escape digestion (Fisher 1996).

Advantages to the ciliates (hosts) include using bacteria as a food source, as a chemical defense against potential predators repelled by a 'shell' of bacteria, as a source of reduced organic carbon or as a means to reduce toxicity in their immediate surroundings (Rosati 2002; Kicklighter et al. 2004).

Ciliate hosts are known to harbor prokaryotic symbionts from various phyla. To date only few protozoan-prokaryote symbioses have been reported from chemosynthetic environments. These include the shallow water sand-dwelling ciliate *Kentrophoros* (unidentified symbionts) and the mangrove root inhabiting *Zoothamnium niveum* that hosts sulphur-oxidizing gammaproteobacterial symbionts (Rinke et al. 2006; Dubilier et al. 2008). Unlike metazoan hosts, protozoans in anaerobic environments (e.g. the rumen, anoxic freshwater environments, sewage sludge, sapropel and anoxic sands) are also known to host archaeal symbionts (Fenchel and Finlay 1991; Görtz 2006). Methanogens are the only types of archaea thus far found in symbioses with ciliates.

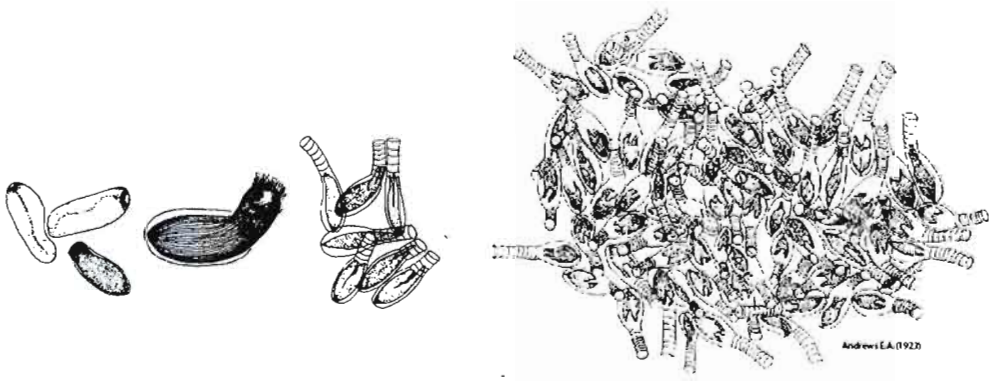

Figure 1. Folliculinid life cycle showing (from left to right) the swarmer stage, the initial attachment to a substrate and secretion of the loricae, initiation of colony formation and a fully formed aggregation of folliculinids.

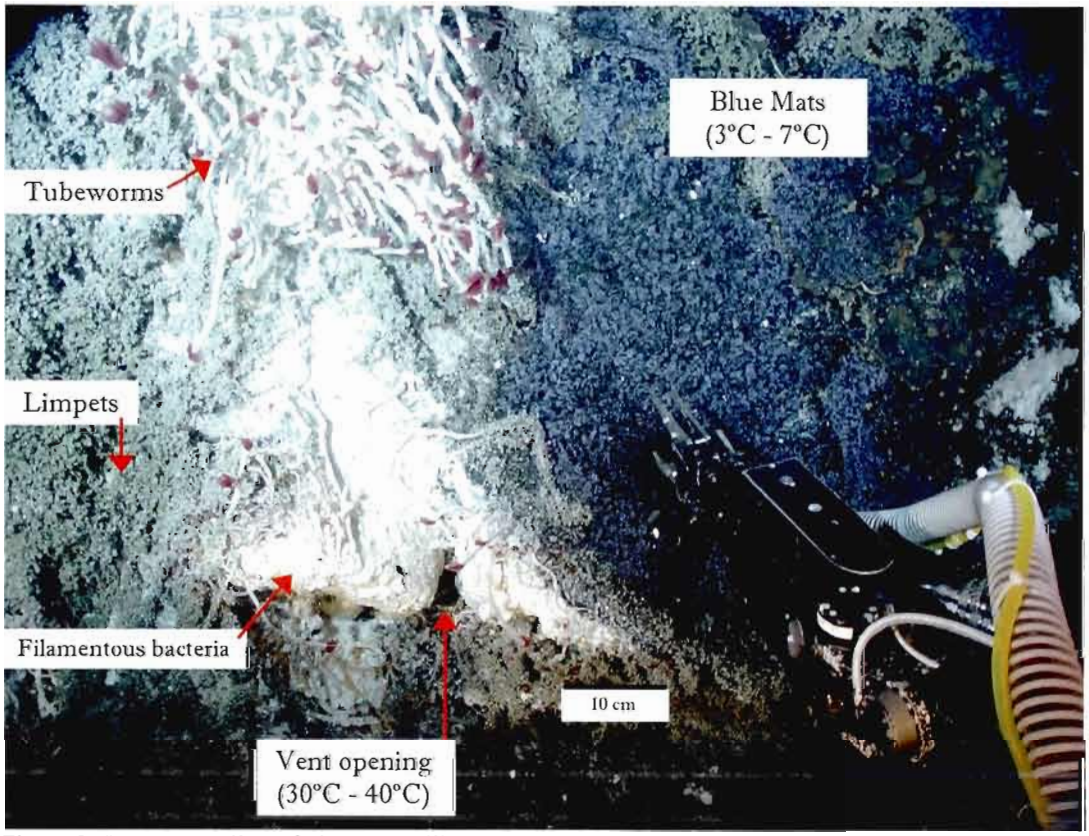

Figure 2. *In situ* sampling of blue mats ciliates. Common characteristics of diffuse flow basaltic sampling areas including the presence of vent limpets, tubeworms and bacterial mat.

Electron microscopy and molecular biology

Electron microscopic observations of blue mat folliculinids can provide visual evidence of the potential existence of symbiosis, including the abundance and location of prokaryotes associated with the ciliates. Using scanning electron microscopy whole ciliates can be viewed at magnifications of 80 – 4500 X while using transmission electron microscopy ciliate thin sections (4μm) can be viewed at 1000 – 100 00 X.

Unlike free-living bacteria, intracellular symbiotic prokaryotes cannot be maintained or cultivated outside the host cell (Fokin 2004). Culture-independent molecular techniques such as comparative sequence analyses of 16S rRNA and fluorescence in situ hybridization (FISH) can be used to determine the microbial diversity, location in the host cell and phylogenetic position of these microorganisms (Amann et al. 1990; Amann 1995; Hugenholtz et al. 1998; Fokin 2004). Gene sequences are used to construct molecular phylogenetic trees that relate organisms (Hugenholtz et al. 1998). These methods are useful tools for the study of symbioses and have been extensively applied in recent years,. For example, comparative 16S rRNA analyses showed that Bathymodiolin mussels dominating a methane seep community in the southeast Atlantic host dual symbionts while FISH analyses revealed the distribution patterns of these microorganisms in the gill epithelium (Duperron et al. 2005). Similarly, based on 16S rRNA sequence analysis, endosymbionts of a gutless marine oligochaete *Inanidrilus leukodermatus* were shown to be phylogenetically related to chemoautotrophic free living bacteria and endo and ecto symbionts from other host phyla while FISH confirmed that the microbial gene sequence obtained from the worms originated from the symbionts (Dubilier et al. 1995). In the case of protozoa, molecular biological methods can also be used to determine the nature, diversity and phylogenetic positions of symbiotic microorganisms. In several cases, findings have contradicted earlier results obtained from traditional morphological and biological studies (Fokin 2004). Beier et al. (2002) used molecular biological analyses to showed that two morpho-biologically similar species of *Caedibacter* bacterial endosymbionts hosted in different ciliate species can be attributed to separate bacterial phyla (alpha and gamma Proteobacteria). In contrast to what was previously assumed, the genus *Caedibacter* is therefore polyphyletic and comprises two groups of unrelated bacteria (Beier et al. 2002; Fokin 2004).

Food web structure and faunal composition

Dense clusters of blue mat colonies may create habitats where organic matter, detritus and bacteria are accumulated. Epstein et al (1992) suggest that ciliate species selectively feed on cyanobacteria, eggs of meiobenthic organisms, and other debris in a temperate sandy tidal flat zone. Accumulations of organic debris could therefore serve as nourishment for the blue mat folliculinid ciliates as well as for meio and macrobenthic invertebrates living within the blue mats. The blue mats themselves may also serve as a food source for other species. Van Dover Folliculinids, for example, are found in digestive tracts of galatheid crabs (Small and Gross 1985).

Feeding experiments on vent ciliates, meio and macrobenthic organisms are technically challenging and, for most intents and purposes, simply not feasible. Stable isotope and polar lipid fatty acid analyses offer powerful tools for the study of the blue mat food webs. Examining gut contents may underestimate the proportion of quickly digested prey (bacteria, small protozoans) while overestimating the importance of larger, more slowly digested prey (diatoms) (Epstein et al. 1992). The advantage of stable isotope analyses is that the measurements are done on animal tissues and therefore reflect assimilated food (Vander Zanden and Rasmussen 1999; Colaço et al. 2002; Post 2002; Levesque et al. 2003).

Hydrothermal vent meiofauna

Meiofauna are benthic invertebrates and protists that can pass through a 1mm mesh-sieve and be retained on a 63 μm mesh-sieve (Giere 1993). The ecological role of meiofauna at hydrothermal vents has only been explored in a handful of recent publications (e.g. Limén and Juniper 2006; Limén et al. 2006; Zekely et al. 2006; Gollner et al. 2007; Limén et al. 2007; Limén et al. 2008). Interactions between protozoa and meiofauna are commonly studied in other aquatic environments (e.g. Epstein and Gallagher 1992; Hamels et al. 2001; Calbet and Saiz 2005; Reiss and Schmid-Araya 2008), but have yet to be explored at hydrothermal vents. In the same way that polychaetes may create safe havens (patches) for harpacticoid copepods (Thistle et al. 1993), blue mat colonies may provide small invertebrates with refuges from predation.

Meiofauna species composition differs between vent fields. For example, the dominant copepod species found in habitats close to vents were *Stygiopontius quadrispinosus* whereas *Aphotopontius forcipatus* copepods are most abundant at new vents and *Benthoxynus spiculifer* at old vents along the Juan de Fuca ridge (Tsurumi et al. 2003). Further, meiobenthic species found within detrital matter from the base of the vents differed from those associated with megafauna such as *Alvinella pompejana* polychaetes, *Calyptogena magnifica* clams and *Riftia* pogonophores (Dinet et al. 1988).

At the 21°N vent field on the East Pacific Rise, nematode species composition was different between hydrothermal vents as well as from surrounding oxic environments, cold seeps, and subsurface anoxic sediments (Vanreusel et al. 1997). It is thus reasonable to expect that in addition to known macrofaunal species at northeast Pacific hydrothermal vents, a distinct community of meiofauna may exist within colonies of blue mat ciliates.

AIMS AND HYPOTHESES

A resurgent interest in symbiosis resulted from the discovery that this type of interaction underpins the ecological success of communities at hydrothermal vents and cold seeps. The overall aim of this dissertation was to consider two aspects of symbioses at hydrothermal vents: first as an interaction between a eukaryotic host (folliculinid ciliate) and its prokaryotic symbionts and second as an interaction between the ciliate host and the hydrothermal community for which it provides a physical habitat. More specifically this dissertation had three objectives:

The first aim was to determine whether blue mat folliculinids from northeast Pacific hydrothermal vents host symbiotic bacteria and, if so, where the symbionts were located (within or on the surface of the host cell or both). To determine this, scanning and transmission electron microscopy (SEM and TEM) were used to examine the ultrastructure of vent folliculinid ciliates. The principle hypotheses were: 1) Because of their widespread distribution and local abundance at deep-sea northeast Pacific hydrothermal vents, folliculinid ciliates host symbiotic prokaryotes; 2) Symbionts are located within the ciliate cytoplasm (endo) and on the surface of (ecto) the ciliate.

The dissertation's second aim was to identify prokaryotic symbionts and to determine their phylogeny. Comparative 16S rRNA gene sequence analysis and catalysed reported deposition–fluorescence *in situ* hybridization (CARD-FISH) were the methods used to characterize the folliculinid symbionts. The 16S rRNA gene is highly conserved and present in all individuals. It is therefore a commonly used and reliable marker in phylogenetic studies. CARD-FISH is a method with improved FISH sensitivity that results in a stronger, more stable fluorescence signal. The ciliate cytoplasm autofluorescence generally impedes detection of more commonly used mono-labeled oligonucleotide FISH probes rendering CARD-FISH necessary for this study. The principal hypothesis was that blue mat symbionts were related to symbiotic microorganisms from reducing environments such as hydrothermal vents and cold seeps.

The third aim was to identify the faunal composition and to determine the food-web structure of organisms associated to blue mats. Carbon and nitrogen stable isotope and polar lipid fatty acid analyses were used to meet this objective. The principle hypotheses were: 1) There is a repeated, consistent and predictable group of invertebrates found within the blue mat colonies; 2) There are organisms specifically confined to blue mats; 3) Folliculinid ciliates are a food source for organisms associated to the colonies of blue mats; 4) There are observable predator-prey relationships between species in the blue mat communities; 5) Folliculinids derive nutrition from organisms associated to them.

CHAPTER I

PROTOZOAN-BACTERIAL SYMBIOSIS IN A DEEP-SEA HYDROTHERMAL VENT FOLLICULINID CILIATE (FOLLICULINOPSIS SP.) FROM THE JUAN DE FUCA RIDGE

1.1 ABSTRACT

This study provides a first description of the morphology of Blue Mats: sessile, colonial folliculinid ciliates (*Folliculinopsis* sp.) that create dense bright blue carpets in certain Juan de Fuca Ridge vent fields and at vents elsewhere. In one area of widespread venting, for example, Blue Mats occupied approximately 70% of the substratum. The ultrastructure of the Blue Mat ciliates was investigated in samples from Axial Volcano on the Juan de Fuca Ridge using conventional scanning electron microscopy (SEM) and thin section transmission electron microscopy (TEM). These *Folliculinopsis* sp. ciliates secrete and dwell in tubes (loricae). The loricae were colonized by both coccoid and filamentous bacteria-like structures. Greater densities of coccoid and short-rod shaped bacteria were found between rows of cilia on the ciliate body (zooid) and especially on the peristomal lobes (arm like extensions typical to folliculinid ciliates). A coccoid bacterial morphotype (within and independent of a vacuole) was located throughout the ciliate cytoplasm. Groups of this organism clustered within vacuoles were regularly distributed along the ciliate cortex. Electron dense, vacuole bound features characterized by stacked membranous structures were also found within the ciliate cytoplasm. These results suggest the existence of at least an endosymbiosis between *Folliculinopsis* sp. ciliates and bacteria at hydrothermal vents. The chemolithoautotrophic nature of these symbiotic bacteria remains to be confirmed. To our knowledge, this is the first report of a protozoan-bacterial symbiosis at vents as well as the first reported symbiosis in folliculinid ciliates.

KEY WORDS: Folliculinid ciliate, hydrothermal vent, endosymbiosis, ectosymbiosis, Juan de Fuca, protozoa

1.2 INTRODUCTION

Protozoa have a cosmopolitan distribution and play an integral role in the decomposition of organic matter, in nutrient cycling and in the maintenance of energy flow within both terrestrial and aquatic ecosystems (Anderson 1988; Atkins *et al.* 2000). They can be of low abundance but widespread in environments that only marginally suit their survival or can rapidly colonize and exploit microenvironments that more optimally satisfy their needs (Anderson 1988). Though relatively little is known about the potential ecological significance of protozoan assemblages in hydrothermal vent environments, many protozoa can tolerate reducing conditions and are likely candidates for survival in these marine ecosystems. Ciliated protozoa have been studied at shallow water vents (Medvedev 1991a,b) and a few studies have explored the diversity of foraminifera and flagellates as well as flagellate tolerance to pressure, sulfide and metal concentrations at deep sea hydrothermal vents (Jonasson *et al.* 1995; Jonasson and Schröder-Adams 1996; Atkins *et al.* 1998; Atkins *et al.* 2000; Atkins *et al.* 2002).

To date, there are no reported endemic protozoa species at hydrothermal vents. Small and Gross (1985) published the first summary of protistan organisms at 21°N on the East Pacific Rise hydrothermal vents in which, based on Small and Lynn's recognized classes (1985), the authors found specimens representing 14 families, 15 genera and at least 20 species. The most abundant protozoa reported in their study were sessile peritrich and folliculinid ciliates (Small and Gross 1985). Tunnicliffe *et al.* (1985) described folliculinids that formed dense blue coloured protozoan mats adjacent to hydrothermal venting on Axial Volcano of the Juan de Fuca Ridge. Twenty years later, dense carpets of bright blue folliculinids still occur on Axial Volcano, and are now known from other vent sites in the northeast Pacific indicating that these ciliates are a recurrent component of the vent fauna in this region. Van Dover (1988) reports that folliculinid ciliates and foraminifera were the most abundant taxa found on long term recruitment arrays (3.3 years) left at Clam Acres on the East Pacific Rise and concluded that these protozoa must play an important role in the vent community. A recent study on eukaryotic diversity and microcolonizers at the Mid-Atlantic Ridge hydrothermal 'niches' offers the idea that ciliates and flagellates may initiate the colonization process in hydrothermal vent systems (Lopez-Garcia *et al.* 2003). It has

been suggested elsewhere that marine folliculinids (and at least one freshwater folliculinid species) are early colonizers of virgin surfaces (Prime-Habdija and Matoničkin 2005).

Both freshwater and marine species of folliculinid ciliates exist (Anderson 1923) and can be found colonizing solid substrata or as epibionts on aquatic plants and mobile invertebrates (Andrews 1914; Aladro-Lubel and Martínez-Murillo 1999; Fernandez-Leborans 2003; Prime-Habdija and Matoničkin 2005). In the Indo-Pacific and Gulf of Aqaba (Red Sea) dense clusters of the only known pathogenic folliculinid, *Hallofolliculina corallasia* cause a coral-killing disease, Skeleton Eroding Band (SEB), by settling onto and becoming embedded in living coral skeleton while secreting their black-grey loricae (Antonius and Lipscomb 2000; Winkler et al. 2004). At Axial Volcano vents, while we have mostly observed folliculinids as extensive blue mats colonizing basalt surfaces, we have also found the blue loricated folliculinids attached to pycnogonids, vestimentiferan tubes and limpet shells (A. Kouris unpublished observations).

Recent interest in theories of symbiogenesis and the common occurrence of symbioses at hydrothermal vent environments have prompted extensive investigations of endosymbioses (or endocytobioses) in protozoa (Fokin 2004). The widespread distribution, local abundance and depleted carbon isotopic signature ($\delta^{13}C$ -33‰) (Angela Kouris unpublished results) of the Blue Mat ciliates in hydrothermal vent fields along the Juan de Fuca Ridge suggest that these folliculinids may have a symbiotic food source (See Childress *et al.* 1987). Though symbiotic relationships between prokaryotes and protozoa are widespread in marine, freshwater and terrestrial environments, there is yet no direct evidence for symbiosis between protozoa and bacteria at hydrothermal vents. Such symbioses have been suggested in reference to the Blue Mats (Small and Gross 1985; Rosati 2002) but remain unconfirmed. Symbioses between chemolithoautotrophic sulfur-oxidizing bacteria and protists are common in other environments characterized by abundant reduced sulfur compounds (Ott et al. 2005). The primary aim of the present study was to use scanning electron microscopy (SEM) and transmission electron microscopy (TEM) to determine whether or not the Blue Mat folliculinids hosted symbiotic bacteria. The possibilities of both external and internal symbioses were investigated. We also used TEM to characterize the macronucleus ultrastructure in order to identify organisms to the genus level (Fauré-Fremiet 1936). Relatively few folliculinids have been studied using electron microscopy (Mulish *et*

al. 1993) and, to our knowledge, no other folliculinid ciliate is reported to host symbiotic bacteria.

1.3 MATERIALS AND METHODS

Samples were collected during a 2004 New Millennium Observatory (NeMO) submersible expedition to the hydrothermal vent fields of Axial Volcano on the Juan de Fuca Ridge. The pump-operated suction sampler on the ROPOS ROV was used to sample folliculinid ciliates at Marker N3 vent (depth = 1550 meters). Samples were immediately fixed in 3% glutaraldehyde-cacodylate buffer (2.0M Cacodylic Acid adjusted to pH 7.4, 12.5ml glutaraldehyde and 0.45M saccharose per 100ml of buffer) on shipboard and stored in this solution until needed. Samples were post fixated in osmium and dehydrated in a series of alcohol baths up to 100%. For SEM, samples were gold coated and viewed in a Jeol JSM 840A. For TEM, samples were embedded in Epon resin and serial sections were made with a diamond knife. Ultrathin sections were stained with uranyl acetate for seven minutes, removed and rinsed with 50% alcohol, placed on droplets of lead citrate for seven minutes and again rinsed with ultra pure water. Ultrathin sections were viewed in a Phillips 201 TEM.

1.4 RESULTS

Distribution and general morphology

The blue colour typical of many folliculinid loricae was pronounced and vibrant around hydrothermal vents because these ciliates were densely packed beside each other in large colonies. In an area of widespread venting, for example, where vent openings were 50-100cm apart over an area of 10-20m^2 or more, Blue Mats occupied approximately 70% of the substratum (Fig.1a). Like other folliculinids, the blue mat ciliates secrete and dwell in chitinous sheaths, the loricae, attached to the substratum and to each other (Figs.1 & 2).

They initially secrete an ampula (sac like structure) that adheres to a solid substratum and then construct the tube-like lorica that is arranged in a series of concentric spirals ending with a terminal everted lip (Andrews 1923). At the posterior end, the ciliate cell (zooid) splits

into two arm-like extensions (peristomal lobes) that extend out of the lorica (Andrews 1923) (Fig. 3a). Under a light microscope, the zooid was purplish-red while the lorica was blue-green. The loricae ranged from 300-2000 μm in length.

SEM - Extracellular bacteria

Irregularly scattered filamentous bacteria that were several tens of micrometers long and slightly less than one micrometer in diameter fouled the lorica surface (Fig. 3b). Occasionally, loricae were heavily encrusted with bacterial filaments and probably mineral deposits. No apparent attachment structures for bacteria were visible on the loricae. The oblong shaped peristomal lobes were covered by rows of somatic cilia (kineties) (Fig. 3a and 3c). Short-rod and coccoid shaped bacteria (average size is 0.9 μm in length) were densely piled between rows of kineties on the peristomal lobes (Fig. 3c). The concentration of these bacterial morphotypes was so high that the surface of the zooid and the base of the cilia were not visible in SEM (Fig. 3d).

TEM - Intra- and extra- cellular bacteria and macronuclei

The microorganisms that were growing between rows of cilia did not appear to be inserted in the cortex, that region of the cell that is in contact with the environment on one side and the endoplasm on the other (Fisher 1996). TEM did not show any structures on the outer cortex that may serve as attachment sites for epibiotic bacteria.

In the cytoplasm, immediately adjacent to the cortex, regularly distributed vacuoles were filled with multiple (from two to over thirty) coccoid shaped bacterial morphotypes (Fig. 4a & 4c). In general, the zooid was highly vacuolated. Many vacuoles near the axis of the cell appeared empty though a few were filled with digested matter (Fig. 4a). Intact individual bacterial morphotypes (both vacuolated and non vacuolated) were ubiquitously distributed in the cell but not necessarily interconnected. Unlike mitochondria and nuclei, intact individual bacterial morphotypes were, in the majority of instances, surrounded by a halo (Fig. 4). Another vacuole bound feature within the cell was much more electron dense

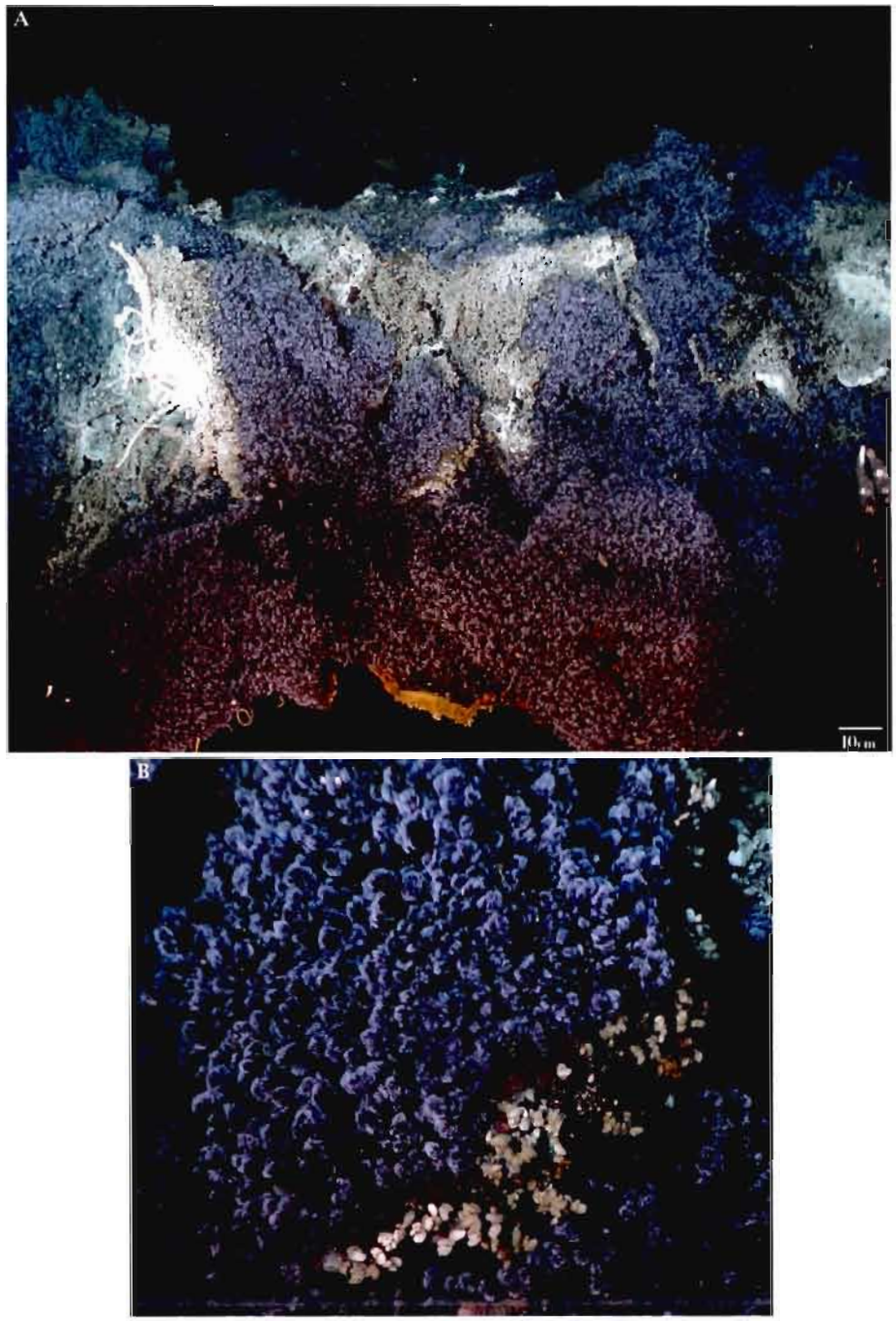

Figure 1A-B. *Folliculinopsis* sp. *in situ* Digital Still Camera (DSC) photographes: Blue Mat folliculinids attached to the basalt and to each other at Marker N3 vent, Axial Volcano, Juan de Fuca Ridge; A Bare basalt visible in the background while *Folliculinopsis* sp. ciliates carpet the foreground; B Folliculinids stacked closely together in typical 'bouquet' type formations.

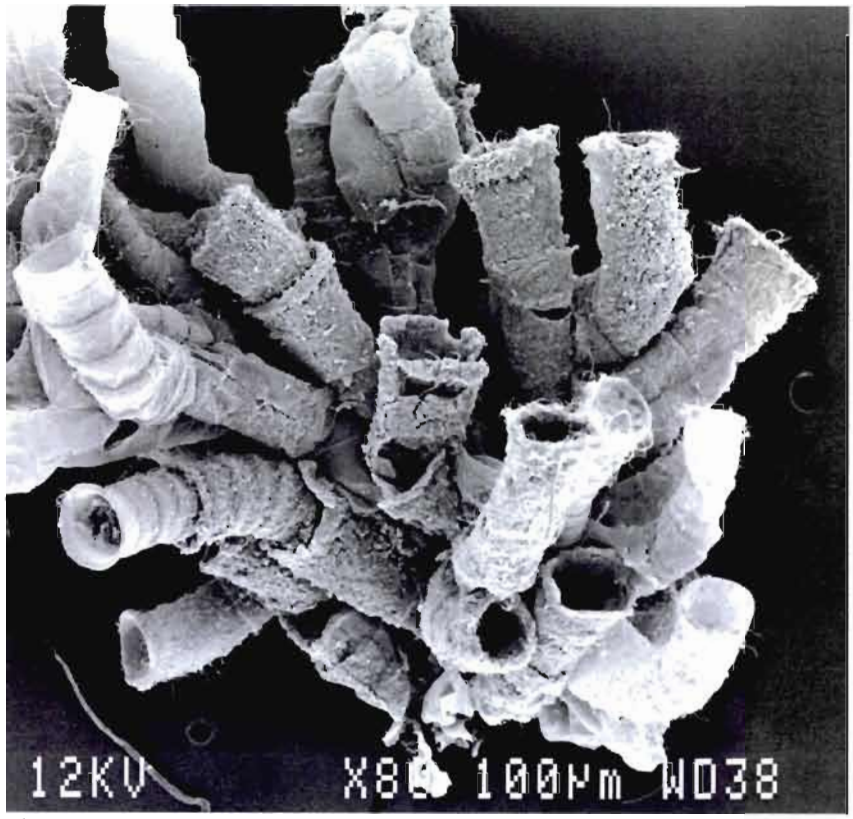

Figure 2. *Folliculinopsis* sp. SEM. A cluster of blue mat folliculinids.

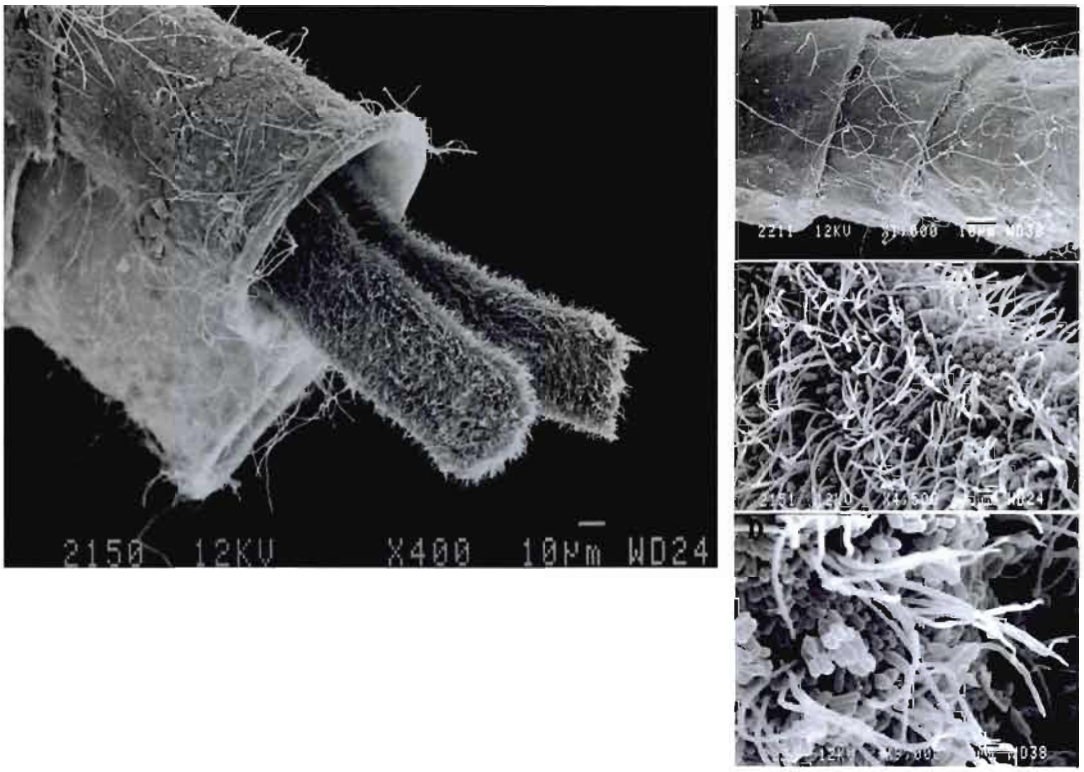

Figure 3A-D. *Folliculinopsis* sp. SEM. A Extended *Folliculinopsis* sp. with peristomal lobes protruding from the mouth of the lorica; B Scattered filamentous and coccoid bacterial morphotypes on the ridged lorica surface; C Longitudinal rows of cilia on the peristomal lobes. Coccoid and short-rod bacterial morphotypes fill the spaces between the rows of cilia; D High magnification of densely clustered coccoid and short-rod bacterial morphotypes between rows of cilia on the peristomal lobes.

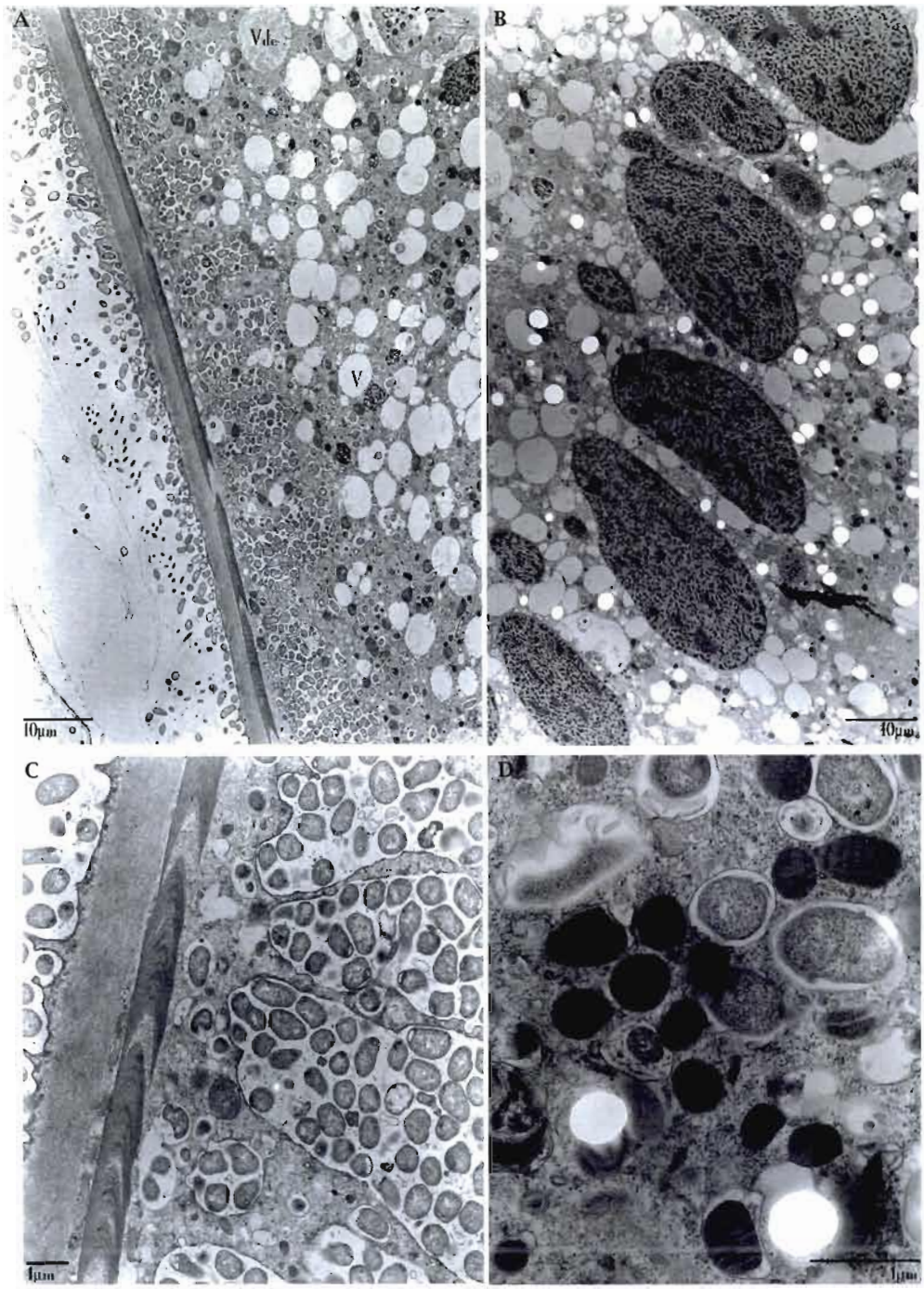

Figure 4A-D. *Folliculinopsis* sp. TEM. A General view: Cortex separating the ciliate cytoplasm from external bacterial morphotypes. Inside cortex, along the row of somatic ciliature, vacuoles are filled with intact coccoid bacterial morphotypes. Empty vacuoles (V) and vacuoles with degraded material (Vde); B Oblong beaded macronuclei; C Coccoid bacteria laden vacuoles along somatic ciliature and cortex; D Individually vacuolated, electron dense features with stacked membranes.

than the vacuole encased coccoid microorganisms (Fig. 4d). These electron dense membranes were organized in stacked structures. They were slightly smaller than individual vacuolated bacteria and only occurred singly within their encasing vacuole.
In ultra-thin sections, the ciliate macronuclei were large, generally oval shaped and beaded (Fig. 4b).

1.5 DISCUSSION

Identification of folliculinid ciliates can be complicated by the several transformations that these organisms undergo during their life history. Individual studies may allocate different species names to the same ciliate. The single well-preserved folliculinid in Small and Gross' study was classified as *Metafolliculina* sp. based on the morphology of its lorica (Small and Gross 1985). Lorica length alone however, is not enough to taxonomically classify a ciliate. Fauré-Fremiet (1936) proposed a classification system that divides the folliculinids into two groups based on the shape of their macronucleus. Since their macronucleus is beaded, the blue mat folliculinids reported in this study would, by Fauré-Fremiet's system, belong to the genus *Folliculinopsis* (Protoctista - Ciliophora - Polyhymenophora - Spirotricha - Heterotrichida - Coliphorina - Folliculinidae - Folliculinopsis) (Costello *et al.* 2001; Costello *et al.* 2004). This genus has been accepted (See Mulisch 2001; Costello *et al.* 2004). Mulisch *et al.* (1993) argue that the organization of the peristome is a more conservative trait than lorica and macronucleus shape. We agree with the latter authors that ultrastructure studies on type species of folliculinids are needed to address differing opinions on their classification. While our study considers the blue mat folliculinid from an ecological (symbiotic) point of view, a purely morphologically descriptive study may reveal that this vent ciliate is a new species. Here we will refer to the blue mat ciliate as *Folliculinopsis* sp. based on our observation of the beaded macronucleus.

Extracellular bacteria

Rosati (2002) suggests that since ectosymbiotic associations between ciliates (both aerobic and anaerobic) and bacteria are frequently observed, these relationships must be mutually advantageous for both organisms. Advantages to the ciliates (hosts) include using bacteria as a food source, as a chemical defense against potential predators repelled by a 'shell' of bacteria, as a source of reduced organic carbon or as a means to reduce toxicity in the immediate surroundings of the ciliates (Rosati 2002; Kicklighter *et al.* 2004). Desbruyères *et al.* (1998) rank both a "biological interface" such as a tube and epibiotic bacteria amongst the top defense mechanisms against the toxicity of the hydrothermal vent environment, in this case for the polychaete *Alvinella pompeja*, found at vents on the East Pacific Rise.

Examination of morphological adaptations by the host to accommodate epibiotic bacteria can provide a basis for distinguishing between biofouling and an epibiotic association that may be advantageous to the host. At hydrothermal vents, *Rimicaris exoculate* bresiliid shrimp (Mid-Atlantic Ridge) are extensively covered by populations of episymbiont filamentous bacteria attached to their hosts by holdfast organelles (Gebruk *et al.* 1993; Wirsen *et al.* 1993). Similarly, *Alvinella pompejana* have morphological adaptations such as dorsal epidermal expansions and cuticular protrusions that appear to facilitate epibiont attachment to the host (Gaill and Hunt 1991; Desbruyères et al. 1998). The filamentous bacteria distributed on the *Folliculinopsis* sp. lorica appear to be more a case of biofouling than symbioses. The number of filamentous bacteria is not greater on the surface of the ciliate lorica than it would be on any other surface within a venting environment. Colonization of the lorica is patchy and irregular and there are no apparent morphological adaptations of the lorica surface to accommodate ectosymbionts.

More equivocal diagnostic features of epibiosis (versus biofouling), are attributes of the epibionts themselves, such as the regularity of microbial colonization of metazoan or protozoan surfaces. In contrast with apparent biofouling of the *Folliculinopsis* sp. lorica, microbial growth on the ciliate body (zooid) is more characteristic (in terms of density of bacterial epibionts) of other proposed ectosymbioses such as in *Kentrophoros* spp. ciliates. Large clusters of regularly distributed coccoid shaped bacteria-like organisms found between rows of *Folliculinopsis* sp. cilia and especially on the peristomal lobes suggest an

ectosymbiosis in these vent folliculinids. In part, the short-rod shaped bacteria-like organisms stand on end like in the ciliate *Kentrophoros* spp. and the in the nematode *Laxus* spp. (Ott et al. 1995; Ott et al. 2005). Free-living bacteria could attach to the peristomal lobes when the zooid is extended outside the lorica. Metachronism (coordinated beating of cilia that creates wave) may result in the circulation of nutrients from the environment to the ectosymbiotic bacterial "guests" on the host ciliates. *Zoothamnium niveum*, a sessile, colonial peritrich ciliate colonizing mangrove roots in the Carribean hosts bacterial epibionts (Ott *et al.* 1998) on the surface of the zooid that are identical to bacterial morphotypes found within food vacuoles inside the host cytoplasm. As in the case of *Z. niveum*, the bacterial epibionts on the blue mat zooid may contribute to the nutrition of the Blue Mat ciliates (Bauer-Nebelsick *et al.* 1996a; Ott *et al.* 1998).

Intracellular bacteria

The simple occurrence of bacteria inside the cells of colonial or other ciliates is itself not diagnostic of any symbiotic relationship. Folliculinids, like other ciliates, are known bacterivores and like other large protozoa can ingest up to 50% of their cell volume per hour (Fenchel 1987). They are capable of greatly extending their bodies outside of their loricae and can create currents using their peristomal lobes to gather bacteria or other food items for ingestion. Food particles, including bacteria, are shunted into the cytostome which is found at the base of the peristomal lobes. They are then packaged into a vacuole secreted by the ciliate host. The vacuole pinches off at the cytostome and enters into the cytoplasm along a canal (Andrews 1946). Potential bacterial endosymbionts can also enter the cytoplasm by this pathway (Fokin 2004). The widespread occurrence of ciliate-prokaryote mutualisms may have previously been overlooked though studies are now indicating that these associations have important "ecological effects" and "evolutionary implications" (Vannini *et al.* 2003). Fokin (2004) reports that ciliates without bacterial symbionts are more the exception than the rule and that ciliates are "preadapted" to be suitable hosts. Ingestion by phagocytosis, for example, means that microorganisms can be ingested as food particles and then escape digestion (Fisher 1996).

According to Fokin *et al.* (2003), to date, nearly 60 types of bacteria have been found in ciliates including those distributed in the cytoplasm (34), macronuclei (14), micronuclei (5) or perinuclear space (6). Symbionts can satisfy their nutritional needs as well as gain shelter within the host ciliate intracellular environment (Fisher 1996).

Several characteristics of the occurrence of intracellular bacteria in *Folliculinopsis* sp. lead us to propose the existence of an endosymbiosis. The first of these characteristics is the occurrence of bacteria in regularly distributed vacuoles. Görtz (2002) reviews ciliate-bacterial symbiotic relationships reported in the literature and explains that once phagocytosed, bacteria are "attacked by acidification, oxidative burst and lysosomal enzymes" but that they can nevertheless "prevail" in the ciliate cytoplasm either naked or in host secreted vesicles (known as either a symbiontophorous vesicle or a symbiontrophic vacuole). Though bacterial endosymbionts can occupy any area of the cytoplasm, they would be 'safest' in compartments that are devoid of the digestive activity of lysozomes such as the cortex, the nuclear apparatus the perinuclear space and the mitochondria (Fokin 2004). As shown in our TEM images, most vacuolated bacterial endosymbionts are evenly distributed along the *Folliculinopsis* sp. ciliate cortex. Though digestion of bacterial food sources is also evident (due to the presence of lysozomes), the majority of bacteria in the *Folliculinopsis* sp. cytoplasm are intact.

Given that the stacked membranous, electron dense features in the *Folliculinopsis* sp. cytoplasm are similar in size to the vacuolated bacterial endosymbionts, they may in fact be methanotrophs. Similar electron dense features observed in the folliculinid *Lagotia minor*'s cytoplasm have been interpreted as pigment granules (Mulisch *et al.* 1993). These latter features, however, lack the stacked membranous structure. Methanotrophic bacterial symbionts found in Bathymodiolus mussels at deep-sea hydrothermal vents and cold seeps have characteristic stacked membranous structures inside their symbionts' cells (Fujiwara *et al.* 2000). Methanotrophic endosymbionts and dual symbioses are known in vent invertebrates (Endow & Ohta 1989; Fisher *et al.* 1993; Distel *et al.* 1995; Duperron *et al.* 2006). The presence of a coccoid bacterial morphotype within the *Folliculinopsis* sp. cytoplasm as well as a possible second methanotrophic form is not unusual since ciliate cells are know to exhibit multiple bacterial infections and multiple symbioses (Görtz 2002; Fokin 2004). The chemolithoautotrophic nature of these symbiotic bacteria remains to be

confirmed. Complementary molecular analysis could be performed to identify the types of microorganisms associated to the Blue Mats.

1.6 SUMMARY

Symbioses are a common feature at hydrothermal vents and also in ciliated protozoa. While bacteria appear to foul the Blue Mat lorica surface, regular colonization of microorganisms between rows of somatic cilia and especially on the peristomal lobes may indicate an ectosymbiosis. Within the *Folliculinopsis* sp. cytoplasm, the regular distribution of coccoid bacteria adjacent to the cortex, an area devoid of lytic degradation of food bacteria, suggests an endosybiosis. Electron dense, vacuole bound features characterized by stacked membranous structures were also found within the ciliate cytoplasm. Morphological examination alone cannot confirm whether these latter features are methanotrophs or pigment granules. To our knowledge, this is the first report of a protozoan-bacterial symbiosis at vents as well as the first reported symbiosis in folliculinid ciliates.

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CHAPTER II

MOLECULAR CHARACTERIZATION OF BLUE MAT (*FOLICULINOPSIS* SP.) CILIATE SYMBIONTS FROM NORTHEAST HYDROTHERMAL VENTS

2.1 ABSTRACT

Symbioses between protozoans and prokaryotes are widespread in marine, freshwater and terrestrial environments yet little is known about these types of associations at hydrothermal vents. Colonial folliculinid ciliates (*Folliculinopsis* sp.) create dense bright blue carpets, called blue mats, at hydrothermal vents on the Juan de Fuca Ridge and elsewhere in the East Pacific, and can cover as much as 70% of the basaltic substratum to which they attach. Bacterial lipid analysis and a recent ultrastructure study suggested that sulfur-oxidizing autotrophic symbionts are the primary source of carbon for the blue mats. This study aimed to identify the *Folliculinopsis* sp. ciliate symbionts from the Axial Volcano on the Juan de Fuca Ridge and to determine their distribution in the ciliate cell using 16S rRNA gene sequence analysis and fluorescence *in situ* hybridization. Our results showed that *Folliculinopsis* sp. harbored euryarchaeal endosymbionts. These fell within a clade of closely related environmental sequences from seeps with high abundances of methanogens. When examined using a DAPI filter on an epifluorescent microscope, enzyme 420 autofluorescence typical to methanogens was observed in cells within the ciliate cytoplasm. In addition to archaea, a high diversity of bacteria was found within the ciliate cytoplasm as well as on the surface of the ciliate loricae. Some of these bacterial sequences were phylogenetically related to known ecto- and endosymbionts from other chemosynthetic hosts. Folliculinid ciliates at northeast Pacific hydrothermal vents therefore appear to harbor multiple phylogenetically distinct symbionts located in different parts of their cell.

Key words: Folliculinid, symbiosis, methanogen, hydrothermal vents

2.2 INTRODUCTION

Chemosynthetic symbioses frequently dominate the faunal biomass at hydrothermal vents, and nearly all studies have focused on associations between invertebrates such as vestimentiferan tube worms, bathymodiolin mussels, vesicomyid clams and shrimps and their bacterial symbionts (Dubilier et al. 2008). In contrast, little is known about symbiotic relationships between prokaryotes and protozoa at vents, although these types of associations are widespread in marine, freshwater and terrestrial environments. The only known examples of chemosynthetic symbioses involving protozoan hosts are marine ciliate colonies with sulfur-oxidizing epibionts from shallow water mangrove swamps (Bauer-Nebelsick et al. 1996; Ott and Bright 2004; Laurent et al. 2009) and non-colonial ciliates from anoxic marine sediments (Fenchel and Finlay 1989). *Folliculinopsis* sp. ciliates form blue mats that carpet basalt surfaces around hydrothermal vent openings on the Juan de Fuca Ridge (Chapter 1). Recent electron microscopic observations of these *Folliculinopsis* sp. ciliates suggested that these protozoa host prokaryotic symbionts (Chapter 1). Stable isotopic and lipid biomarker profiles of the ciliates indicated a nutritional dependence on chemosynthetic microorganisms (Chapter 3).

Electron microscopy of *Folliculinopsis* sp. revealed coccoid and filamentous prokaryotes covering the ciliate loricae while coccoid and short-rod microorganisms were densely stacked between rows of cilia (in particular on the peristomal lobes) (Chapter 1). More importantly, individually vacuolated coccoid microorganisms were distributed throughout the ciliate cytoplasm suggesting an endosymbiosis. Morphologically similar prokaryotes were also found clustered within larger vacuoles along the length of the ciliate cortex. Slightly smaller, single, vacuole-bound electron dense features were found exclusively within the ciliate cytoplasm. While degradation of food bacteria can be observed in the *Folliculinopsis* sp., the vast majority of microorganisms contained within the ciliate cytoplasm were intact. Molecular analysis of the identity of these potential prokaryotic symbionts is currently lacking. To our knowledge, no other study has investigated the molecular biology of prokaryotic symbionts in folliculinid ciliates. The purpose of this study was to identify the hydrothermal vent *Folliculinopsis* sp. ciliate symbionts and to determine their distribution within the ciliate cell.

2.3 MATERIALS AND METHODS

Specimen collection.

The pump-operated suction sampler on the remotely-operated submersible ROPOS was used to sample blue mats during a New Millennium Observatory (NeMO) expedition to Marker N3 hydrothermal vent on Axial Volcano on the Juan de Fuca Ridge (August – September, 2006). Samples for DNA analyses were fixed in liquid nitrogen immediately after collection and stored at -8o°C. Samples for FISH were fixed in 4% paraformaldehyde in 1x phosphate-buffered saline and stored at 4°C.

DNA preparation and PCR amplification.

Samples were thawed on ice and individuals or clusters of specimens were removed from the bulk samples under the light microscope and rinsed three times with Milli-Q water. Ciliate DNA isolation and purification was performed using the QIAgen DNAeasy Blood & Tissue Kit. Polymerase chain reaction (PCR) was used to amplify DNA with primers GM3F and GM4R for bacteria (Muyzer et al. 1995) and archaeal specific primers ARC20F and ARC958R for archaea (DeLong 1992). Template DNA (1-2 µl) was added to the PCR master mix (total volume, 20 µl). The following thermocycling protocol was used: 1 cycle at 95°C for 10 min; 30 cycles at 95°C for 1min, 44°C (bacterial primers) or 55°C (archaeal primers) for 1 min 30 sec, 72°C for 2 min; and 1 cycle at 72°C for 1hour. Archaeal and bacterial PCR products were separately pooled and subsequently purified using the QIAgen PCR-product-purification-Kit.

Cloning and sequencing

Archaeal and bacterial PCR products were cloned separately using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, California). The ligation and transformation steps were performed according to the user manual specifications. To screen both archaeal and bacterial 16S genes, 96 clones were randomly picked for each transformation product and individually placed into liquid medium (100 µl LB- ampicillin per well of 96 well MTP plates). Clones

grew overnight at 37°C. A screening PCR using M13F and M13R primers was used to check for transformation and insert size. Template DNA (1 µl of clones in medium) was amplified as described above though with 36 cycles and an annealing temperature of 55°C. PCR products were purified using a MultiScreen HV-plate with Sephadex G-50 superfine. Purified PCR products with the correct insert size (~ 1 500 bp) were screened by partial sequencing of 600-900 bp using the vector primer 907R. An in-house capillary DNA Sequencer was used for sequencing of DNA fragments. Sequencing reactions were performed using Big Dye according to the following thermocycling protocol: 1 cycle at 95°C for 1 min; 60 cycles at 95°C for 10 sec, 56°C for 5 sec, 60°C for 4 min. A rapid thermal amp was programmed for all steps. Sequencing reactions were purified using Sephadex G-50. Sequences were aligned and compared using the BioEdit program (www.mbio.ncsu.edu/BioEdit/BioEdit.html). Clones were grouped together if they shared $\geq$ 99% partial sequence identity (percentage of identical nucleotides). A representative clone from each clone group was selected and nearly fully sequenced in both directions (1 478 bp for bacterial sequences and 920 bp for archaeal sequences).

Phylogenetic analysis.

For tree reconstruction only nearly full sequences ($\geq$ 1,400 bp in the case of bacteria and $\geq$ 920 for archaea) were used except in the case of one deltaproteobacterial partial sequence. 16S rRNA sequences were checked for similarity against sequences in the NCBI database using BLAST (Altschul et al. 1990). The 16S rRNA sequence data were analyzed using the ARB software package (www.arb-home.de) combined with SILVA 93 dataset (Pruesse et al. 2007). Sequences were automatically aligned using the ARB tool Fast_Aligner and then manually refined. Phylogenetic trees were calculated by performing parsimony, distance, and maximum-likelihood analyses with different sets of filters.

Probe design.

The ARB Probe Design tool was used to design probes targeted specifically to sequences from each clone group. Two probes were designed for the gammaproteobacterial symbionts. ARB's Probe Match tool was used to check the designed probes against all 16S rRNA sequences in the SILVA 93 database. The nucleotide sequences of all the newly designed probes are given in Table 1.

Catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH).

Individuals and clusters of up to 30 ciliates were embedded in Tissue Tek O.G.T. embedding medium and placed at 4°C overnight. Samples were placed in pre-formed plastic moulds, covered with embedding medium and placed at -20°C for 6 hours. Serial thin sections (3-4 µm) were made using a cryo-microtome (Microm HM 505 E), immobilized on glass slides and kept at -20°C until needed. Symbionts were detected by CARD (catalyzed reporter deposition) FISH with horseradish peroxidase (HRP)-labeled probes and tyramide signal amplification as described before with slight modifications (Pernthaler and Pernthaler 2007). Tissue sections were hybridized with the HRP-labeled probe for 4 h at 46°C. Hybridizations were performed at the highest possible formamide concentrations to ensure specificity (See: Table 1). After washing for 15 min at 48°C in washing buffer, the sections were equilibrated for 20 min at room temperature in phosphate-buffered saline (PBS, pH 8.0). The moist tissue sections were incubated with amplification solution (1x PBS pH 8.0, 2M NaCl, 0.1% Blocking Reagent in 100 mM maleic acid buffer pH 7.5, 0.0015% H_2O_2; and 1% Alexa Fluor 488, 546, or 633 or Fluorescein isothiocyanate (FITC) dye [Molecular Probes, Leiden, The Netherlands]) for 20 min at 37°C in the dark and rinsed in PBS buffer for at least 20 min at room temperature. After air drying, tissue sections were DAPI stained, embedded in a 1:4 mix of Citifluor (Citifluor Ltd, London, U.K) and VECTASHIELD (Vector Laboratories, Inc., Burlingame, CA) mounting fluid and stored for microscopic evaluation at -20°C until analysis.

The NON 338 probe was used as a negative control for all hybridization experiments. Positive controls were EUBI-III probe (targeting all Bacteria) and ARCH915 probe (targeting

all Archaea). Hybridized sections were examined using a ZEISS Laser Scanning Microscope (LSM) 510 equipped with two helium-neon lasers (543 and 633 nm), an argon-laser (458, 488 and 514 nm) and a UV-laser (351 and 364 nm). Images were analyzed with the ZEISS LSM Image Browser software.

2.4 RESULTS

Phylogenetic analyses

Phylogenetically diverse 16S rRNA sequences belonging to the alpha, delta, gamma and epsilon subdivisions of the Proteobacteria were found in the *Folliculinopsis* sp. bacterial clone libraries. In total, nine nearly full-length sequences representing different clone groups and one partial sequence were used to reconstruct phylogenetic trees. Four of the gammaproteobacterial sequences clustered together and were closely related to the filamentous sulfur oxidizer *Lucothrix mucor*, (Fig 1). One of the sequences from *Folliculinopsis* sp. fell within a clade of Cytophaga/Flavobacteria/Bacteroides. Two epsilonproteobacterial sequences were phylogenetically distinct but fell within the same clade and were closely related to epibionts from two hydrothermal vent hosts, the deep-sea vent barnacle *Vulcanolepas osheai* from the South Pacific and the Atlantic deep-sea hydrothermal vent shrimp *Rimicaris exoculata* (Fig 1). The deltaproteobacterial sequences from *Folliculinopsis* sp. fell within a clade of uncultivated microorganisms, while the alphaproteobacterial sequences were closely related to free-living *Roseobacter* bacteria and symbionts of the brittle star, *Ophiopholis aculeata*. NCBI-BLAST searches indicated that the *Folliculinopsis* sp. bacterial symbiont sequences are similar (96-99%) to uncultured microorganism sequences usually from hydrothermal vents or cold seeps and in several cases, to vent invertebrate symbiont sequences such as a vent gastropod from Indian Ocean and *Ridgeia piscesae* endosymbionts (Table 2).

All archaeal sequences clustered within the same clone group. Three nearly full-length sequences were used to reconstruct a phylogenetic tree from the archaeal clone library. *Folliculinopsis* sp. archaeal sequences belonged to the euryarchaea and fell within a clade of closely related environmental sequences from seeps with high abundances of methanogens

and more distantly related (but still in the same cluster) to several sequences from activated sludge samples (Fig 2). When UV exalted using a DAPI filter on an epifluorescent microscope, enzyme 420 autofluorescence typical to methanogens was observed in cells within the ciliate cytoplasm. Based on sequence information alone we could not, however, assign the corresponding ciliate symbionts to the methanogens since all of the closest relatives remain uncultured. NCBI-BLAST searches showed that the archaeal *Folliculinopsis* sp. sequences compare most closely related (98-99%) to uncultured archaeal sequences from cold seeps and brackish marine sediments with diffusive methane fluxes (Table 2).

CARD-FISH

The newly designed gammaproteobacterial probes (FolliGam21434 and FolliGam1644) hybridized to filamentous and short-rod shaped bacteria within the cytoplasm and to the exterior of the ciliate cortex (Fig 3C-D). The newly designed epsilonproteobacterial probes hybridized to cells on the ciliate loricae (FolliEpsi67, Fig 4E) and within the ciliate cytoplasm (FolliEpsi193). The newly designed alphaproteobacterial probe hybridized to cells on the loricae. The general archaeal probe (ARC915) and newly designed ArcFolli53 probe hybridized to cells exclusively within the ciliate cytoplasm (Fig 4A-B).

The general bacterial probe (EUB338I-III) hybridized to bacteria throughout the ciliate cytoplasm as well as on the loricae though not within the ciliate's beaded macronuclei (Appendix B, Fig 1). The number of hybridized cells with the general bacterial probe was far greater than the total number of hybridized cells with all other bacterial probes combined suggesting that there may be bacteria belonging to other groups that were not represented in our clone libraries. We did, however, apply a total of 19 other general probes from different groups of Bacteria to the ciliate sections (see Appendix B) and none of these showed a positive signal. The general EUB338 bacterial probe has been known to bind to eukaryotic organelle rRNA and it is possible that this might have caused false-positive signals in the ciliate sections (Knapp and Graham 2004).

Table 1. Oligonucleotide probes used in this study

Probe	Specificity	Probe sequence (5'-3')	Position[a]	FA [%][b]	Literature reference
NON338	Antisense	ACT CCT ACG GGA GGC AGC	338-355	10	Wallner et al. (1993)
EUB1-III	*Bacteria* covered by probe EUB338, EUB338II (*Planctomycetes*) and EUB338III (*Verucomicrobia*)	GCW GCC WCC CGT AGG WGT	338-355	35	Amann et al. (1990), Daims et al. 1999
ARCH915	Archaea	GTG CTC CCC CGC CAA TTC CT	915-934	0	Stahl and Amann (1991) Daims et al. (1999)
FolliGam644	*Folliculinopsis* sp. Gamma symbiont	CCC AAA CTC TAG TCT ACC	644-661	20	This study
FolliGam21434	*Folliculinopsis* sp. Gamma symbiont	GAA GGT TCG CCT AGC TAC	1434-1451	20	This study
FolliAlph63	*Folliculinopsis* sp. Alpha symbiont	AAG ATC ATC ACT GCG CTC	63-80	20	This study
FolliEpsi193	*Folliculinopsis* sp. Epsilon symbiont	GTG TTT CCC TAT CAT CAC	193-210	20	This study
FolliEpsi67	*Folliculinopsis* sp. Epsilon symbiont	GCA AGC AGT TGC TTC ATC	67-84	20	This study
ArcFolli53	*Folliculinopsis* sp. Archacal endosymbiont	AGC CCC CTG ACT CGC ATG	53-70	0	This study

a. Position in the 16S rRNA of *E. coli*.
b. Formamide concentrations used in CARD-FISH hybridization buffer in percentage (v/v)

Table 2. Results of NCBI BLAST (Basic Local Alignment Search Tool) searches for all almost full length sequences from *Folliculinopsis* sp. bacterial and archaeal symbiont clone libraries.

Sequence	Type	Top BLAST search results including % similarity and publication title
AKO163	Alpha	98% uncultured bacteria Rainbow vents MAR; 97% uncultured alpha proteobacteria from vents
AKO214	Alpha	98% uncultured bacteria carbonate ritch metalliferous sediments from Rainbow vent MAR; 96% uncultured alpha vents
AKO217[a]	Delta	97% uncultured delta clone (Microbial diversity in dead chimney structures found in deep-sea hydrothermal systems, Mid-Okinawa Trough and Indian Ocean Ridge); 96% uncultured delta (Comparison of the microbial diversity in cold-seep sediments from different depths in the Nankai Trough)
AKO147	Epsilon	96% uncultured Sulfurovum sp. clone (*Identification of Ectosymbiont on the Hair of Shinkaia crosnieri Inhabiting Hydrothermal Vents of Okinawa Trough*); Uncultured bacteria 88% methane and sulfur metabolizing microbes at Lost City
AKO170	Epsilon	98% uncultured bacteria Rainbow vents MAR; 97% uncultured from vents; 97% uncultured clone (*Novel Forms of Structural Integration between Microbes and a Hydrothermal Vent Gastropod from the Indian Ocean*)
AKO141	Gamma	96% uncultured gamma bacterium from vent gastropod Indian Ocean; 96% methane and sulfur metabolizing microbes at Lost City
AKO185	Gamma	98% uncultured *Leucothrix* sp. clone (*Identification of Ectosymbiont on the Hair of Shinkaia crosnieri Inhabiting Hydrothermal Vents of Okinawa Trough*); 96% uncultured gamma from a vent gastropod Indian Ocean
AKO186	Gamma	98% uncultured clone (*GeoChip-based analysis of metabolic diversity of microbial communities at the Juan de Fuca Ridge hydrothermal vent*); 98% uncultured *Leucothrix* sp. clone (*Identification of Ectosymbiont on the Hair of Shinkaia crosnieri Inhabiting Hydrothermal Vents of Okinawa Trough*); 96% uncultured gamma from vent gastropod Indian Ocean
AKO197	Gamma	98% uncultured *Leucothrix* sp. clone; 98% uncultured clone from Juan de Fuca vents; 95% uncultured gamma early bacterial colonizers Qingdao coast; 95% uncultured gammas from vent gastropod Indian Ocean
AKO208	Gamma	Uncultured Bacteroidetes; 99% community on Ridgea piscesae; 99% *Ridgeia piscesae* endosymbiont; 99% uncultured bacteria from Juan de Fuca Ridge hydrothermal mound; 98% environmental bacteria (*Microbial communities colonizing mineral surfaces within the sulfide-microbial incubator*)
F6	Archaea	Unclutured archaea partial sequence 99% (*Biomarker indicators for anaerobic oxidizers of methane in brackish-marine sediments with diffusive methane fluxes*); Uncultured achaeal clone 98% (*Methanogen diversity evidenced by molecular characterization of McrA genes in hydrothermal sediments of the Guaymas Basin*)
F2	Archaea	Unclutured archaea partial sequence 99% (*Biomarker indicators for anaerobic oxidizers of methane in brackish-marine sediments with diffusive methane fluxes*); Uncultured achaeal clone 98% (*Methanogen diversity evidenced by molecular characterization of McrA genes in hydrothermal sediments of the Guaymas Basin*)
R14	Archaea	Unclutured archaea partial sequence 99% (*Biomarker indicators for anaerobic oxidizers of methane in brackish-marine sediments with diffusive methane fluxes*); Uncultured achaeal clone 98% (*Methanogen diversity evidenced by molecular characterization of McrA genes in hydrothermal sediments of the Guaymas Basin*)

a. Partial sequence (561 bp)

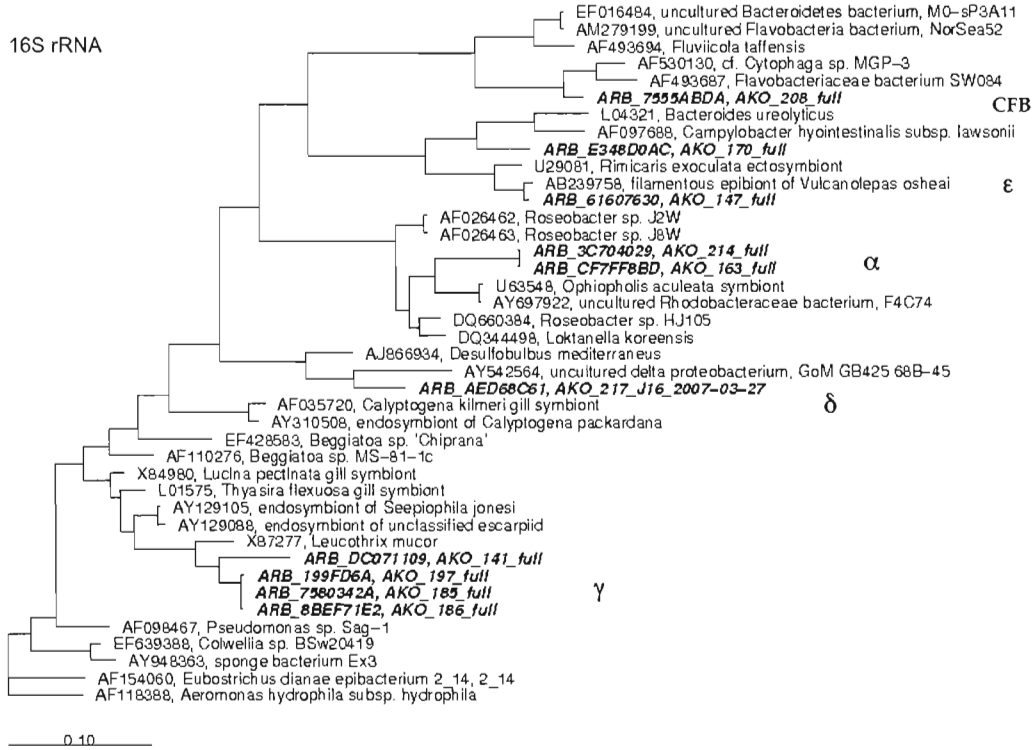

Figure 1. Phylogenetic placement of alphaproteobacterial (α), deltaproteobacterial (δ), gammaproteobacterial (γ), and epsilonproteobacterial (ε) symbionts from *Folliculinopsis* sp. based on maximum-likelihood analyses of 16S rRNA sequences (only the deltaproteobacterial sequence is a partial sequence). *Folliculinopsis* sp. symbionts are indicated in bold. Scale bar = 0.10 estimated substitutions per site. Alignments and phylogenetic analyses were performed with the ARB program (Ludwig *et al.*, 2004).

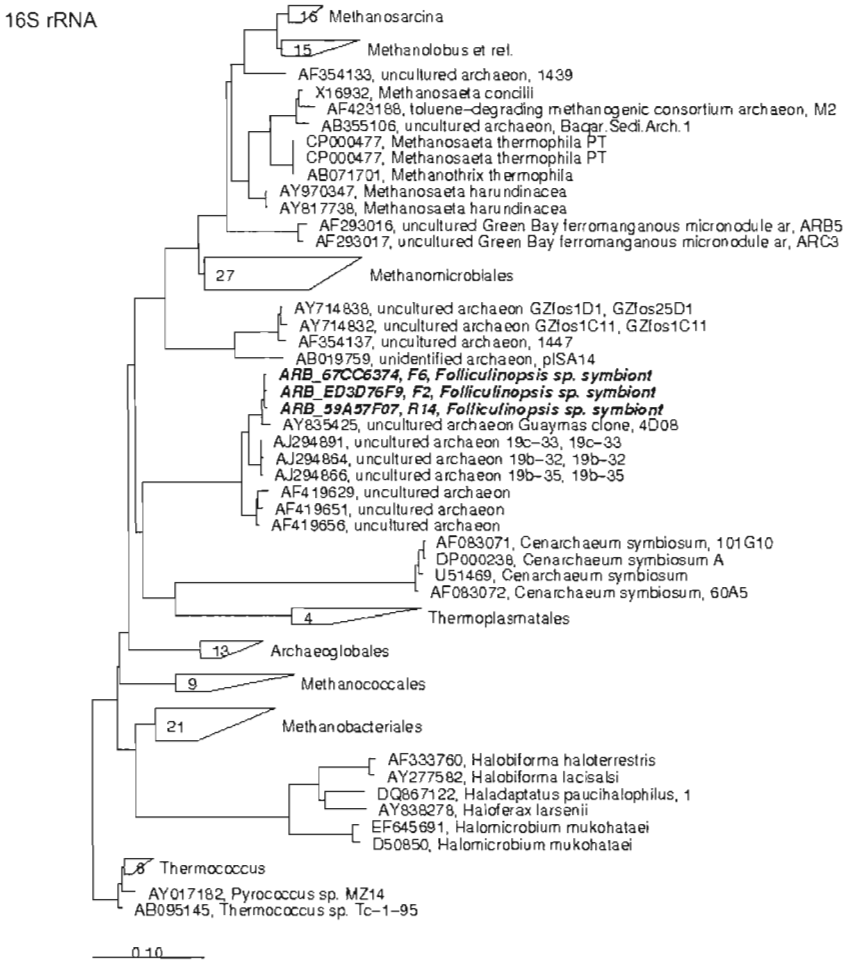

Figure 2. Phylogenetic placement of archaeal symbionts from *Folliculinopsis* sp. based on parsimony analyses of 16S rRNA sequences. *Folliculinopsis* sp. symbionts are indicated in bold. Scale bar = 0.10 estimated substitutions per site. Alignments and phylogenetic analyses were performed with the ARB program (Ludwig *et al.*, 2004).

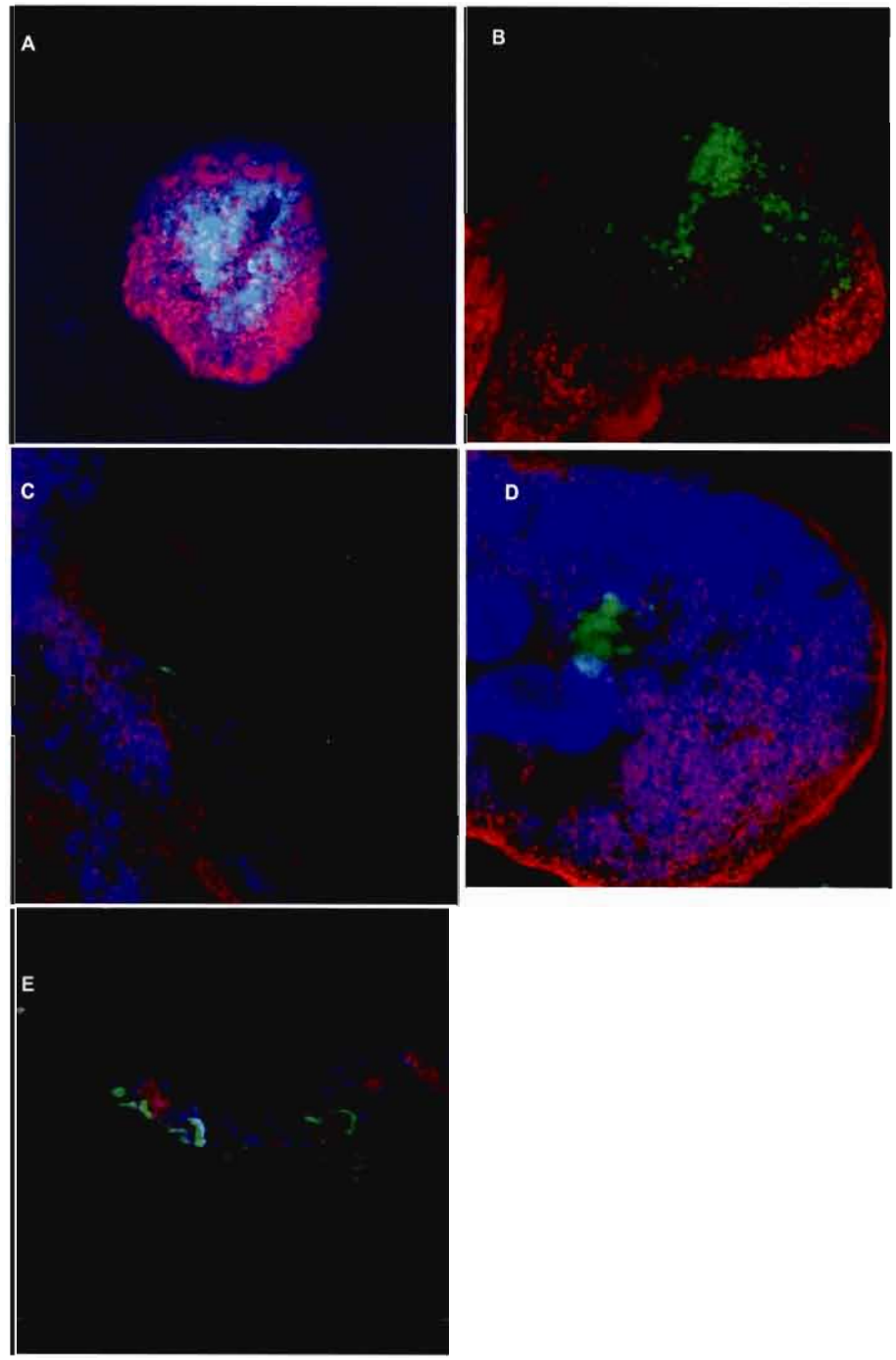

Figure 3. Fluorescence *in situ* hybridization (FISH) identification of archaeal symbionts (A-B), gammaproteobacterial (C-D) and epsilon proteobacterial symbionts (E) in *Folliculinopsis* sp. ciliates. Images B-D are frontal sections of an individual ciliate along an anterior-posterior axis (A-P axis). Image A is a transverse sections of a ciliate and image E is a transverse section of the ciliate loricae. Ciliate autofluorescence color is red. Hybridized cells are green. DAPI staining is visible in purple. Magnification = 100x.

2.5 DISCUSSION

Our results suggest that *Folliculinopsis* sp. ciliates at northeast Pacific hydrothermal vents harbor multiple phylogenetically distinct symbionts located in different parts of their cell. FISH results confirmed the presence of Archaea and Bacteria found in the *Folliculinopsis* sp. clone libraries. The cytoplasm is largely occupied by archaea and relatively few (though phylogenetically distinct) bacteria. In contrast, a larger number of bacteria appear on the ciliate loricae and to the exterior of the cortex, areas entirely devoid of archaea. A number of factors lead Kouris and colleagues (Chapter 1) to propose that the prokaryotes associated to *Folliculinopsis* and that we have identified in this study, are true symbionts (at least *sensu lato* as a regular, close association of unrelated pairs of species): the morphological placement, regular distribution and extreme enrichment of prokaryotic cells on the surface of the cortex (ectosymbionts) and within the cytoplasm (endosymbionts). Filamentous bacteria on the loricae were described as a case of biofouling (Chapter 1). We show here that epsilonproteobacteria found on the loricae are also found on the surface of the cortex as well as within the cytoplasm suggesting that at least some of the microorganisms present on the loricae are symbiotic epibionts. The phylogeny of these epsilonproteobacteria corroborates the idea of a true symbiosis since they are closely related to known *Rimicaris exoculata* symbionts.

The phylogeny of the *Folliculinopsis* sp. symbionts provides little to no clues about their metabolism because, in most cases, the closest known relatives were uncultured microorganisms. Ciliate fatty acid profiles reflected the blue mat's chemosynthetic environment where bacterial-synthesized lipids typical to sulfur oxidizers made up over half of the fatty acids in the blue mats (Chapter 3). Symbionts found in blue mat ciliates that might be sulfur oxidizers could be those with 16S rRNA sequences belonging to the Gammaproteobacteria (since these were related to the sulfur oxidizer *Leucothrix mucor*) or the Epsilonproteobacteria (related to *Rimicaris exoculata* ectosymbionts). Thiotrophic symbionts are mostly known from these two subdivisions of Proteobacteria although a thiotrophic alphaproteobacteria has recently been reported in symbiosis with a catenulid flatworm (Gruber-Vodicka et al. 2008).

The euryarchaeal endosymbionts hosted by *Folliculinopsis* sp. fall within a clade of closely related environmental sequences from seeps with high abundances of methanogens. Cell autofluorescence following excitation with ultraviolet light can be diagnostic for coenzyme F_{420}, an indicator of methanogenic archaea and methane production (van Bruggen et al. 1983). All cells associated with *Folliculinopsis* sp. that exhibited autofluorescence were endosymbiotic and all cells that hybridized with the general and specific archaeal FISH probes occurred exclusively inside the ciliate cytoplasm. The F_{420} signals and the archaeal hybridization signals were very comparable. These results suggest that most of the archaeal endosymbionts in *Folliculinopsis* sp. were methanogens. Archaeal symbionts have not been previously reported in metazoan hosts from hydrothermal vents. Ciliates from anaerobic environments such as the rumen, anoxic freshwater environments, sewage sludge, sapropel and anoxic sands are known to harbor methanogenic endosymbionts (Fenchel and Finlay 1991; Görtz 2006). In these environments, anaerobic ciliates contain hydrogen-producing organelles (hydrogenosomes) closely coupled to their symbiotic methanogens. The methanogens consume H_2 produced by these organelles and oxidize it to methane. Methanogens are the only types of archaea thus far found in symbioses with ciliates. Marine anaerobic ciliates hosting endosymbiotic methanogens can also host ectosymbiotic prokaryotes though the latter are never methanogens (Fenchel et al. 1977; Görtz 2006). The blue mats sampled in this study, are, however, not proliferating in anoxic environments and many methanogenic symbionts are 'extremely oxygen sensitive' (Schink 2006). It is possible that the archaeal symbionts are not obligate anaerobes and that the ciliate controls it's symbionts exposure to oxygen when extending and retracting from the loricae.

While $\delta^{13}C$ carbon signature (-30‰ to -35‰) reported for the host folliculinids is depleted (Chapter 3), the $\delta^{13}C$ signatures of methane oxidizers using biogenic methane are much more negative (-45‰ - 90‰). A mixed carbon source that might include biogenic methane could skew the folliculinid's $\delta^{13}C$ signatures into the -30‰ range. Alternatively, we can't exclude the possibility that bacteria are fixing carbon via the Rubisco Type I pathway ($\delta^{13}C$ -30‰ end member). In chemoautotrophic symbioses, form I Rubisco may fractionate against ^{13}C as much as 40‰ (Robinson and Cavanaugh 1995). This latter explanation was invoked to explain the -35‰ $\delta^{13}C$ signatures of hydrothermal vent mussels from the Eifuku volcano in the Mariannas (Limén and Juniper 2006).

Since bacterial *Folliculinopsis* sp. symbionts are present within the cytoplasm (endo), to the outside of the cortex (ecto) and on the loricae (ecto) it is reasonable to suggest that they are taken up from the environment. During feeding, a folliculinid ciliate can extend up to twice the length of its loricae projecting past the mouth and along the sides of this tube and reaching as far as the base of the substrate it is attached to (Andrews 1923). This extreme flexibility of its body could be a physical strategy used by *Folliculinopsis* sp. to sequester new symbionts from the environment and to supply symbionts with reductants and oxidants. Symbiont uptake is not dependent on movement but rather specific recognition factors between host and symbiont. By transferring prokaryotes from the outside environment into the cytoplasm, the ciliate host provides an opportunity for recognition to occur in the case of endosymbionts. Free-living microorganisms from chemosynthetic habitats are often the closest relatives to chemosynthetic symbionts (Dubilier et al. 2008). Free-living vestimentiferan tube worm endosymbionts have been detected in sea water and biofilms surrounding vents of the East Pacific Rise suggesting that symbionts can be acquired by hosts from the environment at hydrothermal vents (Harmer et al. 2008). Locally-adapted microbes taken up by symbiont-bearing hosts confer the latter with the *flexibility needed to exploit a wider range of geochemical regimes* (Won et al. 2003).

Advanced molecular methods and analyses have revealed an unsuspected phylogenetic diversity of co-occurring symbionts in chemosynthetic hosts such as Bathymodiolin mussels from vents, Idas mussels from cold seeps and gutless oligochaetes from shallow water marine sediments (Dubilier et al. 2008). Bathymodiolin mussels host thiotrophic symbionts, methanotrophic symbionts and, in some cases, both. The co-occurance of sulfur- and methane-oxidizing symbionts in mussles that host both 'improves the fitness of their host' in environments with unstable fluid regimes (Duperron et al. 2009). A recent genomic study has revealed the metabolic diversity of co-occurring symbionts in the gutless oligochaetes *Olavius algarvensis* results in a mutually beneficial syntrophic exchange of reduced and oxidized compounds that ultimately provides the host with multiple sources of carbon (Blazejak et al. 2005; Dubilier et al. 2008). A host with multiple symbionts such as the folliculinid ciliates, may be more adaptable to environments where conditions are variable because their different symbionts could exploit different conditions.

We cannot determine, based on the characterization of *Folliculinopsis* sp. symbionts, whether the vent ciliate's survival is dependant on its symbiosis (obligate). At vents and seeps, obligate symbioses are common in hosts that, like the folliculinids, dominate biomass. The Bathymodiolin mussel symbiosis is obligate – there are no known Bathymodiolin mussels without symbionts, the symbiosis is highly specific to a given host, and all members of the population harbor the symbionts. Adult vestimentiferan tube worms rely entirely on thiotrophic endosymbionts for their nutrition and Vesicomyid clams abundantly distributed at cold seeps vertically transmit their thiotrophic symbionts (Won et al. 2003; Chao et al. 2007). Ciliates from other environments are also know to host obligate symbionts. *Paramecium* ciliates have obligate endoymbionts of the genus *Caedibacter* that confer a killer trait to their hosts (Schrallhammer et al. 2006). Killer Paramecia release toxic particles produced by *Caedibacter* that kill sensitive Paramecia (ones that don't have the killer trait) (Kusch and Görtz 2002; Schrallhammer et al. 2006). *Euplotes* ciliates host obligate *Polynucleobacter* endosymbionts. Antibiotic treatment that killed the symbionts lead to subsequent death of the ciliates after 15-20 days (Heckmann 1975). A genomic study of the blue mat protozoan-prokaryote symbiosis would provide insight not only on the metabolic diversity of the symbionts but on whether the symbiosis is obligate. Reduced genome size (which would become apparent in a genomic analysis) is indicative of obligate associations since very small genomes (<1 Mb) are only found in bacteria involved in a "continuous association with a host" (Moran 2003).

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CHAPTER III

FAUNAL COMPOSITION AND FOOD WEB STRUCTURE IN COLONIAL (*FOLLICULINOPSIS* SP.) MATS AT NORTHEAST HYDROTHERMAL VENTS

3.1 ABSTRACT

This study provides a first description of the faunal composition and food web structure associated with blue mat ciliates (*Folliculinopsis* sp.) at hydrothermal vents of the Juan de Fuca and Explorer Ridges in the Northeast Pacific. Invertebrates associated with blue mats were identified, quantified and analyzed for stable carbon and nitrogen isotopes. In addition, polar lipid fatty acid analyses of blue mats were performed. We found a recurrent assemblage of invertebrates associated with the blue mats and, in all samples, meiofauna were numerically dominant. The harpacticoid copepod, *Amphiascus* sp. was far more abundant than any other species within the folliculinid mats. While some of the invertebrates (e.g. *Amphiascus* sp.) within this assemblage seem exclusively linked to blue mats, others are known from other nearby hydrothermal vent habitats. *Folliculinopsis* sp. ciliates were far more depleted in $\delta^{13}C$ than invertebrates within the blue mat assemblage indicating that the latter do not feed exclusively on the former. At least two trophic levels exist within this assemblage, with juvenile macrofauna, ostracod and nematode species occupying higher trophic levels. The polar lipid profiles indicate that 16:1ω7 and 18:1ω7 (typical to sulfur oxidizers) make up over half the blue mat fatty acids. Similarly to tube worm bushes at hydrothermal vents and mussel beds at cold seeps, blue mats may create a habitat within which meiofauna and juvenile macrobenthic species can find shelter and food thus playing an indirect but important role in the ecology of these organisms.

KEY WORDS: Food web; folliculinid; hydrothermal vents; stable isotopes; fatty acid; meiofauna; macrofauna.

3.2 INTRODUCTION

Hydrothermal vent habitats form on the walls of sulphide mineral edifices as well as around sites where vent fluids diffuse from cracks in the seafloor (Tunnicliffe et al. 2003). Many hydrothermal vent ecosystem studies have sought to identify and describe distinct, predictable communities related to specific hydrothermal conditions in order to understand overall hydrothermal vent ecology (Tunnicliffe et al. 1985; Dinet et al. 1988; Tunnicliffe 1988; Van Dover 1995; Sarrazin & Juniper 1999; Tsurumi & Tunnicliffe 2001; Levesque et al. 2003). Species assemblages inhabiting peripheral diffuse-flow hydrothermal vent habitats have received far less attention than those found on active sulphide edifices, or in vestimentiferan tube worm bushes and mytilid mussel beds. One peripheral assemblage, referred to here as 'blue mats', is formed by dense, blue-green sessile colonial folliculinid ciliates that individually range from 300 – 2000 μm in length (*Folliculinopsis* sp.) and that can cover extensive areas on basalt surfaces or patches on sulfide chimneys in some eastern Pacific Ocean hydrothermal vent fields and elsewhere (Tunnicliffe et al. 1985; Chapter 1). For example, in areas of widespread venting of 10 - 20m^2 or more on the Juan de Fuca Ridge, blue mats can occupy as much as 70% of the basaltic substratum (Chapter 1). Hydrothermal vent folliculinid ciliates have also been reported attached to mobile invertebrates (Chapter 1) as well as on the tubes of the vestimentiferan *Ridgeia piscesae* (Bergquist et al. 2007; Gollner 2007).

To our knowledge, no other colonial ciliate has ever been reported to blanket such extensive areas in the marine environment. The ecological success of the blue mats, as evidenced by this abundance and widespread distribution around diffuse-flow vents, may be attributed to recently described potential prokaryotic symbionts hosted by the folliculinids. Abundant intra-cellular prokaryotes are apparent in transmission electron micrographs of the folliculinid cytoplasm and loricae (Chapter 1). These symbionts have also been the subject of 16S-rRNA and molecular probe (CARD-FISH) studies (Chapter 2).

To date, little is known about the potential importance of blue mat folliculinids for other hydrothermal vent species. Our digital still images and video observations do not show macrofaunal species to be abundant on the surface of the blue mat colonies. The densely stacked colonies of folliculinid ciliates can, however, form a substantial physical habitat within which meiofauna and smaller macrofauna species could find food and shelter, as is the

case with tube worm bushes at hydrothermal vents and with mussel beds at vents and cold seeps (Sarrazin & Juniper 1999; Bergquist et al. 2003; Gollner et al. 2006; Zekely et al. 2006; Bergquist et al. 2007; Gollner et al. 2007). The role of meiofauna at hydrothermal vents has been explored in a handful of recent publications (e.g. Gollner et al. 2006, Limén & Juniper 2006, Limén et al. 2006, Zekely et al. 2006, Limén et al. 2007, 2008). Interactions between protozoa and meiofauna are commonly studied in other aquatic environments (e.g. Epstein & Gallagher 1992, Hamels et al. 2001, Calbet & Saiz 2005, Reiss & Schmid-Araya 2008), but have yet to be explored at hydrothermal vents.

The aims of the present study were to describe the metazoan organisms living in association with the blue mats (*Folliculinopsis* sp.) at NE Pacific vents, to determine whether or not there was a recurrent 'blue mat fauna' and to delineate their trophic relationships. For the latter, we undertook stable carbon and nitrogen isotope analyses of organisms found in blue mat samples, and used fatty acid biomarker analysis to identify the dietary components of the blue mat ciliates.

3.3 METHODS

Sample collection

Two samples (R669 and R670) of blue mats were collected during a 2002 submersible expedition to the Tube worm vent (Magic Mountain vent field) on the Explorer Ridge in the northeast Pacific. At Axial Volcano on the adjacent Juan de Fuca Ridge, samples were collected at the Village (2003; sample R743) and Marker N3 (2004; sample R856) vents in the 1998 vent field (Embley et al. 1999) as well as at Phoenix vent (2004; sample R854) in the ASHES vent field. These samples were analyzed for faunal composition (Table 1), relative species abundance (Table 1), and stable isotope ratios (Table 2).

At Clam Bed on the Endeavour Segment of the Juan de Fuca Ridge (Z = 2190m), two samples (R682-0005 and R682-0006; approximately 1m apart) were collected in 2002 for lipid analyses (Table 3) from weak diffuse flow tube worm habitats. The pump-operated suction sampler on the remotely-operated submersible ROPOS was used for all sampling of blue mats and associated small organisms such as juvenile macrofauna and meiofauna.

Animals were retained on a double layer of 200μm Nitex mesh sieve fastened to the outflow of 2L suction jars. To prevent cross contamination between suction samples, the sampler hose was flushed with ambient bottom water before and after each collection. Suction samples were kept sealed during the ascent to the surface.

Faunal composition

Bulk samples for analyses of species composition and carbon and nitrogen isotopes were sorted on shipboard and frozen at -80°C until needed. In the laboratory, invertebrates within the blue mat samples were separated, identified and quantified under a dissecting microscope.

Stable isotope analyses

Gut contents and shells were removed from the macrofauna and gut content was examined under a light microscope. Prior to stable isotope analysis, all specimens were soaked in 0.1N HCl and MilliQ water each in turn for one minute to remove carbonates and dried at 55°C overnight. Samples were ground to a fine powder and distributed into tin capsules. In the case of meiofauna, individuals of the same species were pooled in tin capsules each containing a droplet of 0.1N HCl (enough to cover all the animals). All specimens were dried overnight at 55°C. Nitrogen and carbon stable isotopes were simultaneously analyzed by continuous-flow isotope ratio mass spectrometry using a Carlo Erba elemental analyzer connected to a GV Instruments Isoprime isotope ratio mass spectrometer. Isotopic ratios are reported on the delta scale:

$$\delta^{H}X = [(R_{sample} / R_{standard}) - 1] \times 1000\ (‰)$$

where the H notation is specified for the selected element (in this study X = carbon or nitrogen). The H superscript refers to the heavier stable isotope mass of the selected element (here ^{13}C or ^{15}N) and R is the ratio of the heavy isotope to the light isotope of the selected elements (in this case, $^{13}C/^{12}C$ or $^{15}N/^{14}N$) (Fry 2006). Standards used in our analyses were Pee-Dee Belemnite (PDB) for carbon and atmospheric N_2 for nitrogen.

Lipid analysis

The blue mat samples from Clam Bed were cleaned to remove adhering macrofauna and meiofauna and lyophilized for ca. 36 h. Dried tissues were then placed in 2 ml chloroform and extracted according to Parrish (1999). Total lipids were derivatized using BF_3-methanol (1.5 h, 85°C) and passed through copper columns to remove sulfur (S. Wakeham, unpublished). The resulting fatty acid methyl esters (FAME) were analyzed by flame-ionization detection on an Agilent Model 6890N gas chromatograph equipped with a DB5 column (30m x 0.32mm x 0.25μm). Helium was the carrier gas and the column was programmed as follows: 100°C (hold 1 min), 214°C at 4°C min^{-1}, 216°C at 0.5°C min^{-1}, 219°C at 4°C min^{-1}, 223°C (hold 3 min) at 0.5°C min^{-1}, 270°C at 30°C min^{-1}, 315°C (hold 10 min) at 1.5°C min^{-1}. FAME were identified following Ackman (1986) using the commercial standards '37-Component', 'PUFA No. 1' and 'Bacterial Acid Methyl Esters' (Supelco, Sigma-Aldrich). In this paper, we use the term 'odd and/or branched fatty acids' (OBFA) to describe those fatty acids that have odd-numbered carbon chains and/or iso (i-) and anteiso (ai-) branches. Polyunsaturated fatty acids (PUFA) are defined as those with three or more double bonds. The bacterial markers summed in the feeding index 'BACT' in Table 3 are those known to be synthesized by bacteria (Stevens et al. 2008 and references therein).

3.4 RESULTS

Faunal composition

Under the light microscope, all sampled invertebrates had a blue-greenish hue. When gut contents were removed from larger, macrofaunal invertebrates (ex. *Amphisamytha galapagensis*) the remaining epidermis and cuticle (as well as the removed gut contents) maintained the blue-green hue.

Macrofauna

The largest number of macrobenthic species was found in the Phoenix vent (ASHES vent field) sample (Table 1). At Phoenix, eight macrofaunal species were identified compared to four species each at Tube worm, Village and Marker N3. Three species, the

gastropods *Lepetodrilus fucensis* and *Depressigyra globulus*, and the polychaete *Amphisamytha galapagensis* co-occurred at all sites. Adult *Lepetodrilus fucensis* limpets were only recorded at Phoenix vent though juveniles of the same species outnumbered them at this site. Gut contents of polynoid polychaetes found in the Phoenix sample contained up to seven intact (juvenile) *Lepetodrilus* shells.

Meiofauna

The harpacticoid copepod *Amphiascus* sp. was the most numerically dominant organism in all of the blue mat samples and occurred at all sites (Table 1). It belongs to the 'minutus' group and resembles most *A. longarticulatus* Marcus. The blue mat *Amphiascus* is most likely new to science (Michel Clément, personal communication). Other unidentified copepods (ca. 4-5 types) were also present although not nearly as abundant. The nematodes *Chromadoridae* sp. and *Geomonhystera* sp. co-occurred at all sites except the Village vent (Axial Volcano). *Geomonhystera* sp. were more abundant than *Chromadoridae* sp. in all but one sample. The only other meiofaunal organism to be present at all sites (though in small numbers) was the ostracod *Podocopida* sp..

Stable isotope analyses

Stable carbon and nitrogen isototope values for individuals of the same species varied between sites and between samples (Table. 2). *Folliculinopsis* sp. ciliates were the most depleted in $\delta^{13}C$ (average values between sites ranged from -30‰ to -35‰) of all species analyzed.

At Phoenix, $\delta^{15}N$ values indicated that juvenile (mean shell length = 0.6 mm) and adult (mean shell length = 10.1 mm) *Lepetodrilus fucensis* occupied different trophic levels. The juvenile limpets had a mean $\delta^{15}N$ of 4.5‰ while for adult limpets, mean $\delta^{15}N$ was 7.5‰ [See Fry (2006) for explanation of trophic level differences inferences based on stable nitrogen isotope values]. The juvenile limpets at Phoenix were more depleted in both ^{15}N and ^{13}C than the adults. The latters' carbon and nitrogen stable isotope ratios tended to group with those of other macrofauna such as the gastropod *Depressigyra globulus,* pycnogonids and an

unidentified whelk. At all sites but Marker N3, juvenile *Lepetodrilus fucensis* $\delta^{15}N$ and $\delta^{13}C$ values were similar to those of the copepod *Amphiascus* sp.. The species with the greatest between-site variation in $\delta^{15}N$ and $\delta^{13}C$ was the polychaete *Amphisamytha galapagensis*, ranging from 4.9‰ to 8.2‰ for $\delta^{15}N$ and -24‰ to -29‰ for $\delta^{13}C$.

The numerically dominant copepod *Amphiascus* sp. had mean $\delta^{13}C$ values that ranged from -23‰ to -27‰ and mean $\delta^{15}N$ values ranged from 4‰ to 6‰ between sample sites. Individuals of the same species of unidentified copepods were too few in number to provide enough pooled biomass for stable isotope analyses. The two nematode species found within blue mat samples were only abundant enough at Tube worm and Marker N3 for isotope analyses. *Chromadoridae* sp. appear to occupy a higher trophic level with $\delta^{15}N$ for pooled specimens ranging 5.2‰ to 7.4‰. For the single pooled sample of *Geomonhystera* sp. (at Tube worm) $\delta^{15}N$ was 4.8‰. The $\delta^{13}C$ values for nematodes from pooled specimens from the two Tube worm vent samples ranged from –23.5‰ to -24.8‰.

A comparison of carbon and nitrogen isotopic signatures of metazoans found within the blue mats with values for the same species collected from other vent habitats on Axial Volcano indicates that there are significant differences between the carbon isotopic values of, for example, *Lepetodrilus fucensis* (T-test = $p < 0.001$) and *Amphisamytha galapagensis* (T-test = $p < 0.001$) within and outside of the folliculinid colonies (Fig. 1). Average *A. galapagensis* $\delta^{13}C$ values were -27.3‰ at blue mat sites and -20.3‰ in other vent habitats, while average *Lepetodrilus fucensis* $\delta^{13}C$ values were -21.8‰ at blue mat sites and -15.1‰ in other vent habitats (See Levesque et al 2006 for average isotope values outside of the blue mat).

Lipid composition

The blue mat fatty acids were predominantly saturated and monounsaturated and they contained very little PUFA. This is a common bacterial signature and indicates a dominance of bacterial-synthesized lipids in the samples. The lipid analyses also provide evidence of a major presence of sulfur-oxidizing bacteria. Sulfur oxidizers are mainly composed largely of

16:1ω7 and 18:1ω7 (Conway & Capuzzo 1991) and these make up over half of the fatty acids in the blue mats. 18:2ω4 is also a bacterial fatty acid (Pond et al. 1997) and the levels are notable in the blue mats. The simple fatty acid profiles of the blue mats reflect their chemosynthetic environment. The two samples were similar except that the one (R682-0005) has a higher proportion of bacterial fatty acids, especially 18:1ω7.

Table 1. Macrofaunal and meiofaunal abundance relative to one gram of blue mat (dry weight). Samples indicated according to sample site, year, depth (Z) and ROPOS dive number. Abundances are rounded to nearest whole number. Sample N3 R856 calculated from estimated sample dry weight.

	2002 Tubeworm R669 Z = 1780m	2002 Tubeworm R670 Z = 1780m	2004 N3 R856 Z = 1529m	2003 Village R743 Z = 1520m	2004 Phoenix R854 Z = 1500m
Macrofauna					
A. galapagensis	31	0	333	55	71
D. globulus	225	158	941	37	10
L. fucencis	0	0	0	0	313
L. fucencis (juvenile)	51	42	1016	1098	720
Unidendified polychaete	0	0	0	0	<1
Polynoidae (Polychaeta)	0	0	0	0	20
Whelks	5	0	0	0	20
Unidentified worm	0	0	0	0	24
Pycnogonids	0	0	0	0	27
Meiofauna					
Amphiascus sp.	2937	31749	2749	17914	8647
Unidentified copepods	102	3161	201	27	125
Chromadoridae sp.	868	1770	1423	0	10
Geomonhystera sp.	465	1897	5750	0	17
E. climax	0	443	6	0	0
Nereis sp. juvenile	0	0	0	9	0
Podocopida	5	0	11	55	7
Prinospio sp.	5	0	6	0	0

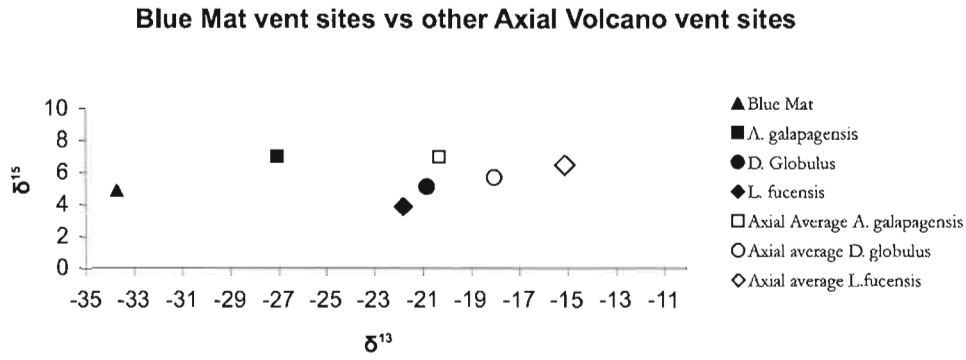

Figure 1. Average stable carbon and nitrogen isotope values of the same species at blue mat sites and at non- blue mat sites (Levesque et al. 2006) on the 1998 Axial Volcano vent field. (T-test for $\delta^{13}C$ differences - *A. galapagensis* $p < 0.001$; *D. globulus* $p = 0.07$; *L. fucensis* $p < 0.001$).

Table 2. Carbon and nitrogen stable isotope ratios for faunal species associated with blue mats at sample sites from Magic Mountain vent field (Tube worm) of the Explorer Ridge and the 1998 vent field (Marker N3, Village) and the Ashes vent field (Phoenix) on Axial Volcano, the Juan de Fuca Ridge.

Blue Mats R669/ 2002/ Tube Worm at Magic Mountain on the Explorer Ridge

Invertebrate	Mean δ13C	StDev	Mean δ15N	StDev
Amphiascus sp.	-23.3	0.04	4.3	0.07
D. globulus	-20.8	3.6	7.0	0.3
Folliculinopsis sp.	-30.2	0.1	4.7	0.1
Conical mollusk	-27.4		6.3	
A.galapagensis	-28.8		4.6	
Chromadoridae sp.	-23.8		7.4	
Geomonhystera sp.	-23.5		4.8	
L. fucensis (juvenille)	-20	3.1	5.1	0.4

Blue Mats R670/ 2002/ Tube Worm at Magic Mountain on the Explorer Ridge

Invertebrate	Mean δ13C	StDev	Mean δ15N	StDev
Amphiascus sp.	-26.3	0.4	4.9	0.4
w/t gravid	-26.2	0.1	4.8	0.1
Folliculinopsis sp.	-33.05	0.4	4.6	0.2
D.globulus	-24.0	1.6	6.8	0.5
L.fucensis	-28.8	0.2	6.0	0.7
E.climax	-26.3		8.4	0.7
Chromadoridae sp.	-24.8		6.9	

Blue Mats R856/ 2004/ N3 at Axial '98 Lava Flow

Invertebrate	Ave δ13C	StDev	Ave δ15N	StDev
Amphiascus sp.	-24.2	0.7	6.1	0.2
w/t gravid	-23.8	0.4	6.0	0.2
Folliculinopsis sp.	-33.4	0.3	4.8	0.1
D.globulus	-20.8	1.9	5.1	1.8
L.fucensis (juvenille)	-19.2		2.7	
Geomonhystera sp.				
Chromadoridae sp.			5.2	
A. galapagensis	-29.1	2.2	6.2	0.2

Blue Mats R743/ Village at Axial Volcano on the Juan de Fuca Ridge

Invertebrate	Mean δ13C	StDev	Mean δ15N	StDev
Amphiascus sp	-25.4	0.7	5.0	0.6
without gravid	-25.1	0.5	4.7	0.3
L.fucensis (juvenille)	-22.5	0.7	4.2	0.5
Folliculinopsis. sp	-32.5	0.1	5.5	0.1
A. galapagensis	-24	0.1	8.2	0.6

Blue Mats R854/ 2004/ Phoenix at ASHES Vent Field

Invertebrate	Mean δ13C	StDev	Mean δ15N	StDev
Amphiascus sp.	-26.4	0.7	4.7	0.1
w/t gravid	-27.7	0.1	4.6	0.1
Folliculinopsis sp.	-35.0		6.2	
D.globulus	-20.7	0.4	7.0	0.2
L.fucensis (juvenille)	-25.8	0.5	4.5	1.7
L.fucensis	-18.4	5.6	7.5	0.9
A. galapagensis	-25.7	3.5	4.9	1.5
Polynoid	-17.0	5.7	7.8	1.4
Tubeworm	-16.0	1.5	5.8	1.9
Pycnogonid	-22.3	1.8	7.0	1.8
Whelk	-21.1	2.3	5.8	1.1

Table 3. Fatty acid composition for *Folliculinopsis* sp, samples R682-0005 and R682-0006 from Clam Bed, Main Endeavor vent field, Juan de Fuca Ridge. SFA=saturated fatty acids, MUFA=monounsaturated fatty acids, PUFA=polyunsaturated fatty acids (3 or more double bonds), OBFA=odd and/or branched fatty acids, NMID=non-methylene-interrupted dienes, BACT=bacterial markers, i.e., ΣOBFA + ω4 dienes + 16:1ω7 + 18:1ω7.

Fatty acid	*Folliculinopsis* sp. 682-0005 (%)	*Folliculinopsis* sp. 682-0006 (%)
Saturates		
14:0	2.04	1.96
i-15:0	0.51	0.65
ai-15:0	0.5	1.3
15:0	0.72	0.79
i-16:0	0.26	0.25
16:0	11.36	15.31
i-17:0	0.38	0.36
17:0	0.72	0.61
18:0	2.74	2.91
20:0		0.25
Monounsaturates		
14:1	1.05	1.05
16:1ω7	30.47	29.5
18:1ω9 + ω13	3.39	5.06
18:1ω7	33.2	24.06
20:1ω13 + ω9	1.78	2.2
20:1ω7	2.46	2.49
Polyunsaturates		
20:4ω6		0.33
20:5ω3	1.05	0.69
Dienes		
18:2ω6	1.28	2.01
18:2ω4	4.17	5.6
20:2Δ5,11		*0.23*
20:2Δ5,13		*0.28*
20:2Δ13,16	*0.99*	*1.07*
22:2Δ7,15	*0.92*	*1.03*
SFA	19.23	24.39
MUFA	72.35	64.36
Dienes	7.37	10.22
PUFA	1.05	1.02
OBFA	3.09	3.96
NMID	1.91	2.61
Feeding indices		
PUFA/SFA	0.05	0.04
16:1ω7/16:0	2.68	1.93
18:1ω7/18:0	12.14	8.27
18:1ω9/ω7	0.1	0.21
ω4 dienes	4.17	5.6
BACT	70.94	63.12
BACT/PUFA	67.28	61.6
PUFA/BACT	0.01	0.02

3.5 DISCUSSION

On the periphery of the Juan de Fuca and Explorer Ridge vents sampled in this study (with the exception of Phoenix vent), the surface area occupied by blue mat ciliates (visible to the naked eye through observations of video recordings taken by the submersible) by far exceeds the area occupied by any other visible species and is indeed more impressive if the microscopic size of these ciliates is taken into account. The distribution of the folliculinid ciliates on diffuse flow basalt-hosted vents in the northeast Pacific resembles the Bathymodiolin mussel communities from the Galapagos Spreading Center as described by Johnson and colleagues (1994): shimmering hydrothermal effluents escaping from cracks in the basalt are closely surrounded by erect vestimentiferan worms whose bright red plumes are visible. Immediately surrounding the vestimentiferan tube worms are stacked limpets and white bacterial mats. The blue mats, similarly to the bathymodiolin mussels, circumscribe the stacked limpets, bacterial mats and tube worms. Phoenix vent was the only sulfide edifice site sampled in this study. Here, blue mats formed patches on the sulfide mineral edifice.

Bates (2008) shows that recruit-sized and juvenile *L. fucensis* settle in peripheral vent habitats and eventually move inward towards active flow where stacked adult limpets are most prolific. Since juvenile *L .fucensis* are present in all blue mat samples and since there is a continuity of habitat between the blue mats and other habitats, it is reasonable to suggest that these limpets may use the folliculinid colonies as a nursery. They may thus pass through their juvenile phase in the blue mats and eventually migrate along a hydrothermal gradient towards more intensive hydrothermal flow.

Faunal composition

Macrofaunal species such as *Amphisamytha galapagensis*, *Lepetodrilus fucensis* and *Depressigyra globulus* are not exclusively associated with our collected blue mat samples but rather are a recurrent component of hydrothermal vent fauna in the northeast Pacific. On the other hand, meiofaunal species such as the numerically dominant harpacticoid copepod *Amphiascus* sp. and the nematodes *Chromadoridae* sp. *Geomonhystera* sp. have not previously been reported from the Juan de Fuca or Explorer Ridges and are found in almost

all of the blue mat samples collected for this study. Harpacticoid copepods have often been found at vents (Huys & Conroy-Dalton 1997; Lee & Huys 2000; Willen 2003; Gomez & Boyko 2006; Gollner et al. 2006) though *Amphiascus* sp. has previously only been reported from shallow (90m) sub-polar Mid Atlantic Ridge vents (Fricke et al. 1989).

Aggregations of vestimentiferan tube worms (*Riftia pachyptila*) from the East Pacific Rise (EPR) host permanent benthic communities a third of whose total species richness is comprised of meiobenthic fauna (Gollner et al. 2007). Nematodes and copepods were the two most abundant meiobenthic taxa within both mussel beds and vestimentiferan bushes on the EPR (Gollner et al. 2007, Zekeley 2006). Faunal abundance in colonies of blue mats sampled from the Explorer and the Juan de Fuca Ridges was dominated by meiobenthic species. Since population densities increase with decreasing body size (Peters 1983, Reiss & Schmid-Araya 2008), the numerical dominance of meiobenthic species in blue mat is not surprising. However, there was also a greater number of meiobenthic species compared to macrobenthic species in all samples but those at Phoenix vent. Macrofauna such as adult limpets, pycnogonids and polynoid polychaetes found at Phoenix but not at other blue mat sites could be transients from elsewhere on the sulfide edifice.

Species composition of meiofauna at vents differs from adjacent non-vent soft bottom areas of the deep sea (Dinet et al. 1988; Tsurumi et al. 2003). Dinet et al. (1988) found that overall meiofauna abundance and species diversity did not vary greatly between samples collected from vents on the EPR and the Explorer Ridge. Species composition within vent fields however, does differ. For example, the dominant copepod species found in habitats close to vents on the Juan de Fuca Ridge is *Stygiopontius quadrispinosus* whereas *Aphotopontius forcipatus* was the most abundant copepod at new vents and *Benthoxynus spiculifer* at old vents (all three are siphonostomatoid copepod species) (Tsurumi et al. 2003). In this study we found a distinct meiobenthic community within blue mats with species not recorded from adjacent high flow vent sites. Further, meiobenthic species found within detrital matter from the base of the vents differ from those associated with megafauna such as the polchaete *Alvinella pompejana*, the vesicomyid clam *Calyptogena magnifica* clams and the giant tube worm *Riftia* pachyptila (Dinet et al. 1988). Small-scale spatial variability in food sources caused by variations in hydrothermal discharge can shape vent food webs and influence the spatial distribution of the meiobenthos (Limén et al. 2007). At the 21°N vent

field on the EPR, nematode species composition differed between hydrothermal vents as well as from surrounding oxic environments, cold seeps, and subsurface anoxic sediments (Vanreusel et al. 1997).

Food web structure

The blue mat ciliates may serve as a food source for other species. Folliculinids have been found in digestive tracts of galatheid crabs (Small & Gross 1985). Based on similar isotopic signatures, as well as observations of mouthparts and gut content, Bergquist et al. (2007) suggest that the gastropod *Clypeosectus curvus* associated with tube worm bushes on the Juan de Fuca Ridge is a specialized consumer of folliculinid ciliates. This gastropod was not found within the five blue mat samples analyzed in this study and our stable isotopic analyses do not reveal any potentially exclusive consumer of vent folliculinids within the blue mat assemblages. However, our comparison of average stable carbon and nitrogen values does show that metazoans associated with the blue mat are more depleted in carbon than they are outside of the blue mat areas. Thus, these metazoans appear to be deriving at least part of their nutrition from organic matter produced by the folliculinids (either through direct predation on the ciliates or through feeding on detrital mat material). This is supported by our microscopic observations of bluish pigmentation in the tissues of all sampled mat invertebrates. The blue mat fauna may also feed on allochtonous organic matter, of hydrothermal (chemosynthetic) and photosynthetic origin that accumulates within the matrix of the mats.

There is evidence that some members of the blue mat fauna feed at different trophic levels. Based on gut content analyses, polynoid polychaetes appear to prey upon juvenile limpets at Phoenix vent. For the polychaete *Amphisamytha galapagensis,* mean $\delta^{13}C$ values for specimens from a single sample varied the most between samples and sites, suggesting that this potential mobile predator has a more generalized opportunistic diet. Adult *Lepetodrilus fucensis* limpets at Phoenix vent have less of a depleted blue mat-influenced signature than juveniles from the same site and may also be opportunistic feeders. This weaker isotopic signature could support the idea that adult limpets are transient in the mat.

Stable isotopic signatures also indicate at least two trophic levels within the meiobenthic assemblage: the nematode Chromadoridae sp. could be feeding on detritus including decaying bodyparts of, for example, the abundant copepod *Amphiascus* sp. (unlikely to catch live copepods). Abundant *Amphiascus* sp. in blue mats are likely to generate a detrital pool composed of decaying body parts of copepods which could serve as a food source to detritus/omnivorous invertebrates. Siphonostomatoid copepods at vents have previously been suggested to be consumed along with other particulate debris, in this case by paralvinellid worms (Limén et al. 2008). Another meiobenthic species potentially feeding on the numerically dominant *Amphiascus* sp. is the ostracod, *Euphilomedes climax*. At Juan de Fuca Ridge vents, *E. climax* has shown to be enriched in δ^{15} N (Limén et al. 2007) indicating a predatory feeding mode. Most members of its family (Philomedidae) are detritus feeders but examination of *E. climax* gut contents in other habitats has shown that it may also ingest small invertebrates such as copepods (Kornicker 1991). Finally, it is possible that metazoans associated with the blue mat assemblages derive nutrition from the episymbiotic microorganisms hosted by the folliculinid ciliates. A recent study (Chapter 2) reported the presence of epsilon and alpha proteobacteria on the surface of the ciliate lorica. Using16SrRNA sequence analysis and confirmed by fluorescent in situ hybridization (FISH), the authors found that the epsilon proteobacteria were closely related to known sulfur-oxidizing *Rimicaris exoculata* ectosymbionts (Chapter 2). Based on this and on our lipid data, carbon fixed through the oxidation of reduced sulfur species appear to lie at the base of the blue mat food webs.

3.6 CONCLUSION

Several lines of evidence suggest that the blue mats host a recurrent and interacting community of invertebrates. The three major macrofaunal species, known from other vent habitats, were present at all locations. At least three previously unreported meiofaunal species were found in our mat samples, one of which (*Amphiascus* sp.), was the most abundant metazoan in all collections. Stable isotope and gut content analysis and microscopic examination of faunal tissues indicate that the macro-invertebrates were at least partially feeding on the blue mat ciliates. Lipid analysis of mat material and a previous ultrastructure

study suggest that sulphide-oxidizing autotrophic symbionts are the primary source of carbon for the mats and their associated food webs. These mats occupy significant areas of substratum at peripheral and low flow hydrothermal areas of the northeast Pacific. This provides supplementary habitat (and food) for vent macro-invertebrates, and may represent the only habitat for three meiofaunal species.

3.7 ACKNOWLEDGMENTS

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CONCLUSION

Chemoautotrophic symbiosis

Symbioses are commonly occurring interactions between tightly associated organisms in almost all know ecosystems. The advent of molecular biology and its sophisticated and powerful experimental tools has reinvigorated symbiosis research, a field previously hampered by difficulties associated with the cultivation of symbiotic partners. Another major breakthrough in the field was the discovery that deep-sea hydrothermal vent ecosystems are dominated by symbiotic associations between chemoautotrophic bacteria and endemic metazoans. The finding that *Riftia pachyptila*, a gutless pogonophora tube worm, derives its nutrition from chemolithoautotrauphic (sulfur-oxidizing) bacteria (Cavanaugh et al. 1981, Feldbeck 1981) led scientists to investigate and subsequently unearth a previously unsuspected diversity of chemosynthetic symbioses ubiquitously distributed in both terrestrial and aquatic ecosystems (Dubilier et al 2008). Metazoans from seven phyla including vesicomyid bivalves (Mollusca), siboglinid polychaetes (Annelida), siphonolaimid nematodes (Nematoda) and alvinocarid shrimps (Arthropoda) are now known to host chemoautotrophic symbionts such as sulfur or methane oxidizers (Dubilier 2008). Bathymodiolin mussels are globally distributed at hydrothermal vents and cold seeps and, in certain species, simultaneously host both thiotrophs and methanotrophs (DeChaine and Cavanaugh 2005, Distel et al. 1995, Dubilier et al 1999).

A type of symbiosis thus far overlooked in hydrothermal vent research is that between ciliate hosts and prokaryotes. This oversight is surprising not only because ciliates are 'pre-adapted' to be suitable hosts for symbionts (Fokin 2004) but because ciliates (referred to as 'blue mats') have been known to cover extensive areas surrounding northeast Pacific hydrothermal vents for over twenty years (Tunnicliffe et al. 1985). In hydrothermal vent ecosystems, all eukaryotic fauna that physically dominate considerable space (ex. tube worm bushes, limpet beds and mussel beds) host bacterial symbionts. Ciliates from other

environments host both heterotrophic and chemoautotrophic microorganisms though the latter include just two known examples (the sand-dwelling ciliates *Kentrophoros* spp. and the sessile colonial ciliate *Zoothamnium niveum*). Ciliates from anaerobic environments such as the rumen, anoxic freshwater environments, sewage sludge, sapropel and anoxic sands are known to harbor archaeal (methanogenic) symbionts (Fenchel & Finlay 1991, Görtz 2006).

Advantages to the ciliate host of having symbionts includes using bacteria as source of nutrition, as a chemical defense against potential predators repelled by a 'shell' of (ectosymbiotic) bacteria, as a source of reduced organic carbon or as a means to reduce toxicity in the immediate surroundings of the ciliates (Rosati 2002; Kicklighter *et al.* 2004).

In the first two chapters of this dissertation, the possible protozoan-prokaryote symbiosis at hydrothermal vents was explored for the first time. The primary aim of the first chapter was to use scanning electron microscopy (SEM) and transmission electron microscopy (TEM) to determine whether or not the blue mat folliculinids host symbiotic bacteria. The possibilities of both external and internal symbioses were investigated. We also used TEM to characterize the macronucleus ultrastructure in order to identify organisms to the genus level (Fauré-Fremiet 1936). Symbioses are a common feature at hydrothermal vents and also in ciliated protozoa. Relatively few folliculinids have been studied using electron microscopy (Mulish *et al.* 1993) and, to our knowledge, no other folliculinid ciliate is reported to host symbiotic bacteria. This first study resulted in several key observations. First, while bacteria appear to foul the blue mat lorica surface, regular colonization of microorganisms between rows of somatic cilia and especially on the peristomal lobes may indicate an ectosymbiosis. Second, within the *Folliculinopsis* sp. cytoplasm, we identified a regular distribution of coccoid bacteria adjacent to the cortex, an area devoid of lytic degradation of food bacteria, suggests an endosybiosis. Finally, electron dense, vacuole bound features characterized by stacked membranous structures were also found within the ciliate cytoplasm. Morphological examination alone cannot confirm whether these latter features are methanotrophs or pigment granules. Based on our observation of the beaded macronucleus we determined that the blue mat ciliates belong to the genus *Folliculinopsis* sp.. To our knowledge, this is the first report of a protozoan-bacterial symbiosis at vents as well as the first reported symbiosis in folliculinid ciliates.

The purpose of the second chapter was to identify the hydrothermal vent *Folliculinopsis* sp. ciliate symbionts and to determine their distribution within the ciliate cell. Our results suggested that *Folliculinopsis* sp. ciliates at northeast Pacific hydrothermal vents harbor multiple phylogenetically distinct symbionts located in different parts of their cell. FISH results confirmed the presence of archaea and bacteria known from our constructed phylogenetic (16S) clone libraries for *Folliculinopsis* sp.. The cytoplasm is largely occupied by archaea and relatively few (though phylogenetically distinct) bacteria. In contrast, a larger number of bacteria appear on the ciliate loricae and to the exterior of the cortex, areas entirely devoid of archaea. The symbiosis between folliculinids and their multiple symbionts is most likely flexible in nature allowing the protozoans to thrive adjacent to peripheral vent flow where conditions are highly variable.

Blue mat habitat

While one aspect of symbiosis involves the actual association between host and symbiont(s), in environments such as hydrothermal vents where symbiotic fauna occupy relatively large amounts of space, another aspect of symbiosis becomes important: symbiotic fauna create physical habitats for other organisms where biotic factors such as predation, competition, and recruitment are at play and where food sources may accumulate.

Sampling and manipulative experiments are logistically challenging at deep-sea hydrothermal vents, which makes tools such as stable isotope and lipid analyses particularly useful for studying dietary components and trophic interactions of vent organisms (Limén et al 2008). In addition to free-living and epibiotic microbial primary production available for direct grazing, food sources such as particulate organic matter (POM) and detritus are accessible to non-symbiont hosting fauna on both sulfide edifices and basalt hosted diffuse flow vents (Levesque et al. 2006, Limén 2007). Variations in flow regime and biological processes drive transitions between communities of organisms (Sarrazin et al. 1997). Faunal distribution and the relative abundance and use of food sources at vents vary on decimeter scales relative to hydrothermal discharge (Limén et al. 2007, Sarrazin et al. 1997). POM is more abundant and serves more as nutritional source further away from the primary source of venting (Levesque 2006) while bacterivory is most common adjacent to intense flow (Limén

et al. 2007). Structure-creating organisms such as Bathymodiolin mussels and vestimentiferan tube worms create habitats where detritus and POM can accumulate. The accumulation of potential food sources may, in part, explain why other species are a recurring component of these habitats.

Relatively little is known about communities associated with diffuse-flow vents characterized by lower temperatures and lower concentrations of reduced chemicals compared to sulfide edifice habitats (Bergquist 2007, Urcoyo 2003). Diffuse flow environments can be more stable and persistent than rapidly changing, unstable sulfide edifice environments (Bergquist et al. 2007). While *Folliculinopsis* sp. can form patches on sulfide edifices or individually attach to mobile invertebrates, we have mostly observed them in densely stacked colonial mats carpeting basalt-hosted diffuse flow environments. To our knowledge, no other colonial ciliate has ever been reported to blanket such extensive areas in the marine environment.

The third chapter of this dissertation aimed to describe the metazoan organisms living in association with the blue mats at NE Pacific vents, to determine whether or not there was a recurrent 'blue mat fauna' and to delineate their trophic relationships. For the latter, we undertook stable carbon and nitrogen isotope analyses of organisms found in blue mat samples, and used fatty acid biomarker analysis to identify the dietary components of the blue mat ciliates. Several lines of evidence suggest that the blue mats host a recurrent and interacting community of invertebrates. Three major macrofaunal species, known from other vent habitats, co-occurred at all locations (the gastropods *Lepetodrilus fucensis* and *Depressigyra globulus*, and the polychaete *Amphisamytha galapagensis*). Three previously unreported meiofaunal species were found in our mat samples, one of which (*Amphiascus* sp.), was the most abundant metazoan in all collections. Stable isotope and gut content analysis and microscopic examination of faunal tissues indicate that the macro-invertebrates were at least partially feeding on the blue mat ciliates. Lipid analysis of mat material suggest that sulfur-oxidizing autotrophic symbionts are the primary source of carbon for the mats and their associated food webs. These mats occupy significant areas of substratum at peripheral and low flow hydrothermal areas of the northeast Pacific. This provides supplementary habitat (and food) for vent macro-invertebrates, and may represent the only habitat for three

meiofaunal species. Based on our observations we suggest that juvenile *Lepetodrilus fucensis* limpets may use the folliculinid colonies as a nursery.

The findings reported in this dissertation speak to the importance of considering symbiosis not only as an interaction between hosts and symbionts but also in the larger context of community ecology. The blue mat symbiosis has a direct impact on hydrothermal vent fauna in the northeast Pacific because it permits the ciliates to colonize widespread areas of diffuse-flow venting consequently creating a habitat and contributing to local nutrition.

RESEARCH PERSPECTIVES

Results of this first comprehensive study of a protozoan-prokaryote symbiosis at northeast Pacific hydrothermal vents engender many new questions. Drawing from our conclusions I propose to speculate here on future research efforts that would expand from what we have learned.

Perhaps one of the most intriguing and perplexing results stemming from my work is that *Folliculinopsis* sp. potentially harbors methanogenic endosymbionts. Not only are archaeal symbionts not present in any other hydrothermal vent or cold seep host but methanogens in particular are only known to be symbionts of anaerobic ciliates. Methane, like hydrogen sulfide, is present in vent effluent and available to free-living and symbiotic microbes as a carbon source and vents are aerobic environments. To provide further evidence for the presence of methanogen symbionts in *Folliculinopsis* sp. we could perform functional gene analysis of Methyl Coenzyme M Reductase A (mcrA) genes. Methyl coenzyme M reductase is a key gene of methanogenesis and a thus a 'diagnostic indicator of methanogenesis and methanogenic archaea' (Hallam et al. 2003).

Blue mat food web structure and faunal composition was studied from four hydrothermal vents within three vent fields while the identification of bacterial symbionts was performed on samples from only one vent. To detect whether there is spatial variation of folliculinid symbionts between vents, vent fields and even ridge systems, we could compare phylogenies of symbionts from several sites. While 16S rRNA gene sequence analysis provides information on prokaryotic phylogeny, 18S rRNA gene sequence comparisons

should also be used to determine whether several species of *Folliculinopsis* ciliates exist at northeast Pacific hydrothermal vents.

The study of the biogeography of folliculinid symbionts and ciliates could include cold seep sites from the Gulf of California where deep-sea folliculinids are also found (Lobban et al. 2009). Cold seeps are geologically diverse reducing environments that can be fuelled by a variety of organic hydrocarbon sources such as methane, petroleum, other hydrocarbon gases and gas hydrates (Levin 2005). Communities of organisms including symbiont bearing tube worms, mussels and clams are abundant in cold seep environments. Cold-seep megafaunal species are generally closely related to hydrothermal vent species (Sibuet and Olu 1998). Similarly to hydrothermal vents, cold-seep biota is largely dependant on the oxidation of reduced sulfur and methane by microorganisms for nutrition (Levin 2005). *Folliculinopsis* sp. archaeal sequences all fall within a clade of closely related environmental sequences from cold seeps. This makes the potential comparison of symbiont and host phylogeny between vent and seep microbes even more interesting.

APPENDIX A

TRAVEL LOG

During the course of my doctoral studies, I was fortunate enough to draw on the expertise of three different laboratories in order to complete the work for my dissertation. I received three bursaries (a European Union Marie Curie Early Stage Training Fellowship and two Québec Ministry of Education, Leisure and Sport's International mobility bursaries) to complete four internships in Europe:

2008 (01 – 06)	Max Planck Institute for Marine Microbiology, Bremen, Germany. Project title: *Identification and characterization of deep sea hydrothermal vent Folliculinopsis* sp. *ciliate symbionts using Methyl Coenzyme M Reductase A (mcrA) gene analyses.* Supervisor: Dr. Nicole Dubilier, Symbiosis Research Group, Department of Molecular Ecology.
2007 (02 – 05)	Max Planck Institute for Marine Microbiology, Bremen, Germany. Project title: *Molecular characterization of deep sea hydrothermal vent Folliculinopsis* sp. *ciliate symbionts using catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH).* Supervisor: Dr. Nicole Dubilier, Symbiosis Research Group, Department of Molecular Ecology.
2006 (09 – 12)	Max Planck Institute for Marine Microbiology, Bremen, Germany. Project title: *Identification and characterization of deep sea hydrothermal vent Folliculinopsis* sp. *ciliate symbionts using 16S rRNA phylogenetic analyses*. Supervisor: Dr. Nicole Dubilier, Symbiosis Research Group, Department of Molecular Ecology.
2005 (03 – 06)	Université Pierre et Marie Curie (Paris 06), UMR 7138 Systématique Adaptation Evolution, CNRS, Paris, France. Project title: *Electron microscopic characterization of the morphology and symbiosis in deep*

sea hydrothermal vent folliculinid ciliates. Supervisor: Dr. Françoise Gaill, Équipe Adaptation aux Milieux Extrêmes.

The purpose of this Travel log is to briefly document the units that I worked with and the basic techniques that I learned during these four international collaborative research internships.

In 2005, I gained experience in molecular and cellular biology during an internship with Dr. Françoise Gaill's "Adaptation aux Milieux Extrêmes" (AMEX) team of the "Systematique, Adaptation Evolution" research unit. This research unit brings together scientists from the "Université Pierre et Marie Curie" (UPMC), the "Musée National d'Histoire Naturelle", the "Institut de Recherche pour le Développement" (IRD) and the "Centre National de Recherche" (CNRS) in Paris, France. Dr. Gaill's AMEX group consists of highly specialized scientists that focus their research on living organisms from extreme environments such as hydrothermal vents. Here I received electron microscopy training: sample fixation and preparation, cutting ultrathin and thin sections and operation of both transmission and scanning electron microscopes.

From 2006-2008 Dr. Nicole Dubilier's Symbiosis Group at the Max Planck Institute for Marine Microbiology (MPIMM) hosted three of my internships. The MPIMM is an internationally renowned research institution that hosts microbiologists, molecular biologists and biogeochemists that work together to understand basic principles of marine microbial ecology. Dr. Dubilier's Symbiosis Research Unit focuses on the distribution, diversity, phylogeny and metabolism of fauna from chemosynthetic environments. During my internships I received training in 16S rRNA gene sequence analysis, oligonucleotide and catalyzed reporter deposition fluorescence *in situ* hybridization (FISH and CARD-FISH) and phylogenetic tree reconstruction (including using BioEdit, Sequencher, Sequence Analysis Software and ARB). FISH training included learning to design specific oligonucleotide probes. While at the MPIMM I attended the lectures of an ARB software training program (February 26-29, 2008) and the International workshop on RNA technologies (April 7-0, 2008). During my last internship with Dr. Dubilier I aimed to characterize methyl coenzyme M reductase A (mcrA) and 18S rRNA genes in *Folliculinopsis* sp.. The results of these studies were not conclusive enough to include in my dissertation.

APPENDIX B

SUPPLEMENTARY RESULTS

Table 1. Oligonucleotide probes used on *Folliculinopsis* sp. thin sections not reported in Chapter 2.

Name	Target group	Probe sequence (5' to 3')	Position[a]	FA [%][b]	Litterature reference
ALF968	Alphaproteobacteria	GGT AAG GTT CTG CGC GTT	968 - 985	35	Neef (1997)
ARC94	Arcobacter	TGC GCC ACT TAG CTG ACA	94-111	35	Snaidr et al. (1997)
ARCH915MM	*Folliculinopsis* sp. archaeal symbiont	GTG CTC ACC CGC CAA TTC CT	915-934	0-35	This study
BET42a*	Betaproteobacteria	GCC TTC CCA CTT CGT TT	1027 - 1043	35	Manz et al. (1992)
CF319a	Bacteroidetes	TGG TCC GTG TCT CAG TAC	319 - 336	35	Manz et al. (1996)
CFB560	Bacteroidetes	WCC CTT TAA ACC CAR T	560 - 575	20	O'Sullivan et al. (2001)
CFB563	Most Flavobacteria	GGA CCC TTT AAA CCC AAT	563 - 580	20	Weller et al. (2000)
Cren554	Crenarchaea Group I	TTA GGC CCA ATA ATC MTC CT	554-573	0	Massana et al. (1997)
DBB660	Some Desulfobulbus	GAA TTC CAC TTT CCC CTC TG	660 - 679	60	Devereux et al. (1992)
DELTA495a*	Delta proteobacteria	AGT TAG CCG GTG CTT CCT	495 - 512	35	Loy et al. (2002)
DSB706	Most Desulfobulbaceae and Thermodesulforhabdus	ACC GGT ATT CCT CCC GAT	706 - 723	35	Loy et al. (2002)
DSS658	Desulfobacteraceae and other Bacteria	TCC ACT TCC CTC TCC CAT	658 - 675	60	Mussmann et al. (2005)
EPSY549	Epsilonproteobacteria	CAG TGA TTC CGA GTA ACG	549 - 566	55	Lin et al. (2006)
EPSY682	Epsilonproteobacteria	CGG ATT TTA CCC CTA CAC M	682-700	20	Moussard et al. (2006)
EUBI	Most bacteria	GCT GCC TCC CGT AGG AGT	338 - 355	35	Amann et al. (1990)
EUBII	Planctomycetales	GCA GCC ACC CGT AGG TGT	338 - 355	35	Daims et al. (1999)
EUK516	Eukarya	ACC AGA CTT GCC CTC C	502 - 517	35	Amann et al. (1990)
EURY806	Euryarchaea	CAC AGC GTT TAC ACC TAG	806-823	0	Teira et al. (2004)
Gam42a*	Gammaproteobacteria	GCC TTC CCA CAT CGT TT	1027-1043[c]	35	Manz et al. (1992)
HGC69a*	Actinobacteria	TAT AGT TAC CAC CGC CGT	1901 - 1918	30	
PLA46	Planctomycetales	GAC TTG CAT GCC TAA TCC	46 - 63	30	Neef et al. (1998)
PLA886*	Planctomycetales	GCC TTG CGA CCA TAC TCC	886 - 904	35	Neef et al. (1998)

a. Position in the 16S rRNA of *E. coli*.
b. Formamide concentrations used in CARD-FISH hybridization buffer in percentage (v/v)
c. Probe target position on E. coli 23S rRNA
* Includes an unlabeled competitor probe

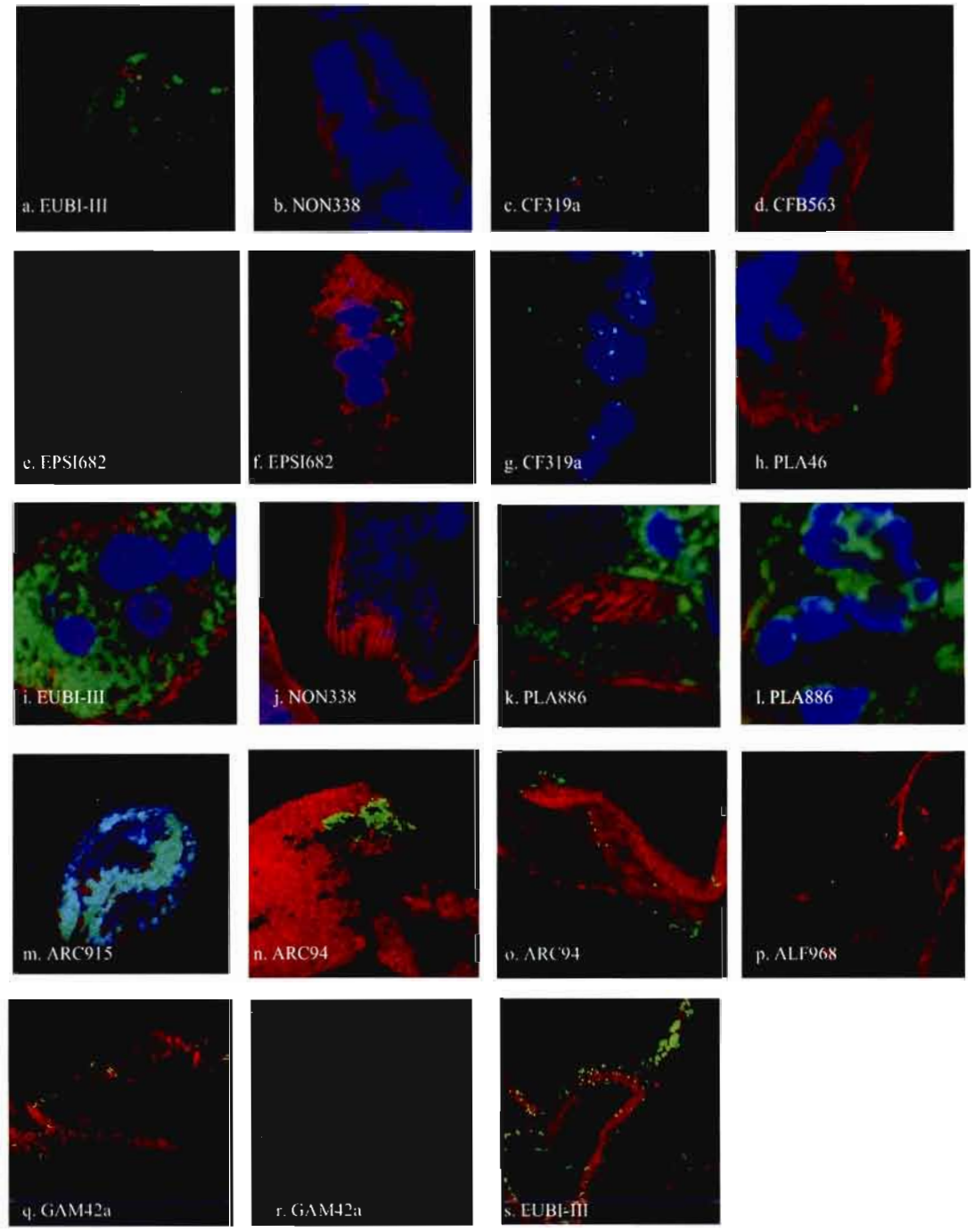

Figure 1. Fluorescence *in situ* hybridization (FISH) identification of microorganisms in *Folliculinopsis* sp. ciliates. Probe name indicated in each image. Images other than *a* and *m* are frontal sections of an individual ciliate along an anterior-posterior axis (A-P axis). Images *a* and *m* are transverse sections of a ciliate. Images *c*, *p*, *q* and *s* show the ciliate loricae. Ciliate autofluorescence color is red. Hybridized cells are green. DAPI staining is visible in purple. Magnification = 100x.

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