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Understanding the symbiosis between the giant tubeworm *Riftia pachyptila* and chemoautotrophic sulfur-oxidizing bacteria

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Understanding the symbiosis between the giant tubeworm *Riftia pachyptila* and chemoautotrophic sulfur-oxidizing bacteria

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May 20, 2013

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Riftia pachyptila* is a deep sea tube worm that is mainly found along the East Pacific Rise and the Galapagos Rift in the Eastern Pacific. Here it inhabits deep sea hydrothermal vents, sea floor geysers harvesting high temperatures, low pH, high pressure and strong chemical fumes. Despite these conditions *R. pachyptila* thrives, with growth rates exceeding those of other tubeworm species. Lacking a mouth and a gut, *R. pachyptila* is unable to feed, and depends on its endosymbiotic sulfur-oxidizing bacterium Candidatus *Endoriftia Persephone* for organic carbon supply. The discovery of this symbiosis in 1981 opened doors for researchers and greatly contributed to understanding the endosymbiosis between chemosynthetic bacteria and vent dwelling macro-organisms. Although attempts in culturing Candidatus *E. Persephone* outside of its host have not yet succeeded, a large part of its genome and protein map has been analyzed and provides a better insight in the lifestyle, metabolic pathways and diversity of this endosymbiont.

1. Introduction

1.1 *Discovery of hydrothermal vents*

During the South Tow expedition in 1972 Klitgord and Mudie (1974) carried out the fist deep tow observations of the Galápagos Rift. A year later, Sclater and Klitgord (1973) proposed the presence of active convective cirulation of seawater through newly formed oceanic crust along this rift. The following year, Williams *et al.* (1974) measured the
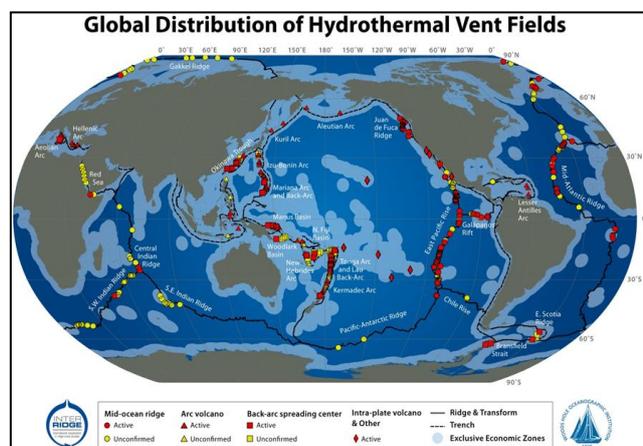

Fig. 1: Global distribution of hydrothermal vent fields. Red indicates active vents. Yellow indicates unconfirmed activity. (source: http://www.pmel.noaa.gov)

bottom water temperature and heat flow along the same traject. This expedition led to the discovery of vulcanic activity at the bottom of the ocean: the hydrothermal vent. The actual discovery was made by Corliss *et al.* in 1977, where visual observations and water samples were obtained along the Galápagos Rift during an expedition with the first manned deep-sea submersible *Alvin* (Corliss *et al.* 1979). With this expedition Corliss and his colleagues not only managed to prove the existence of the vents, but in addition determined the heat and seawater budgets of the hydrothermal systems and characerised the chemical composition of the vent fluid. To their surprise, they found that this newly discovered phenomena was not only an ocean vulcano, but in fact a thriving ecosystem packed with yet undiscovered organisms. This discovery opened a whole new world for scientific research and is considered as one of the most important discoveries of marine science (Yong *et al.* 2012).

1.2 Hydrothermal vents

Hydrothermal vents are deep sea vulcanos that occur particularly along the central rift valleys of the East Pacific. These vents can be found at depths of 2000-3000 meters and temperatures of the outflowing sea water can reach up to 350 ℃. The fumaroles (vulcanic smoke holes) contain a rich cloud of minerals such as sulphides, methane, manganese and many other trace metals (Kaiser *et al.* 2005). Not all hydrothermal vents are located in the deep sea: in California vents have been discovered only 20 meters under the ocean surface. These shallow vents will not be discussed in this review.

Hydrothermal vents are frequently perturbed by volcanic eruptions. These eruptions have tremendous impact on the surrounding area and can destroy the existing ecosystem. The eruptions together with the emerging diffuse flows do not only demolish, but also create a brand new colonisation ground for invertebrates, the first organsism to colonize the newly established vent (Gardebrecht *et al.* 2012). This colonisation is not a walk in the park, as the variable temperature and chemical regimes around the vents require spesialisation (Robidart *et al.* 2008). Moreover, no light can penetrate deep enough to reach such depths making photosynthesis impossible. These invertebrates rely entirely on bacterial chemosynthesis for energy.

1.3 Colonization of the hydrothermal vents

The development of hydrothermal vent fauna is influenced by many factors due to the wide range of conditions that vary in time and space (Tunnicliffe *et al.* 1997). The authors studied the colonisation of hydrothermal vents on the Juan de Fuca Ridge, discovered that the animal recruitment was greatest at the sites where bacterial activity was highest. They also suggested that H_2S erupting from the vents is an important factor and perhaps even a locator clue for nearby pelagic larvae, as the macrofaunal colonisation started as the sulphide increased. Deposit feeders also colonise quickly, as they find a great food source in the highly active microbial community near the vent (Tunnicliffe *et al.* 1997).

It is difficult to tell if the recruiting larvae originate from a local site or a remote vent site, as these larvae are small and difficult to track (Mullineaux *et al.* 2010). Larvae distribution is even harder to track due to little genetic differentiations between vent sites that are tens to even a hundred kilometers apart (Craddock *et al.* 1997; Jollivet *et al.* 1999; Won *et al.* 2003), suggesting that the colonizing larvae are supplied in a well-mixed pool (Vrijenhoek 1997).

Which species become pioneers depends on the larvae availability shortly after the eruption, influenced by time-variant transport processes (Adams and Mullineaux 2008) or spawning cycles (Tyler and Young 1999).

Pioneer colonists seem to travel long distances, as Mullineaux *et al.* (2010) found species which originated from sites far away from the investigated vent habitat at the East Pacific Rise. At least one of the prominent pioneer species, the marine gastropod mollusk *Ctenopelta porifera*, arrived from vents where the only known populations are located, possibly more than 300 km away.

Fig. 2: Dr. Meredith L. Jones holding a preserved Riftia pachyptila. Photo by Harold E. Dougherty; Image from Smithsonian Institution Archives (source: http://www.mnh.si.edu)

Species composition within a vent habitat change over time, as the chemical composition of hydrothermal fluids can change after eruption (Nees *et al.* 2008). An example is the tubeworm *Riftia pachyptila*, the invertebrate discussed in this review. This invertebrate prefers oxygen rich and sulfur poor water and only colonizes vent sites with favorable conditions (Shank *et al.* 1998, Mullineaux *et al.* 2012). Once activity diminishes and fluid fluxes and physicochemical properties change *R. pachyptila* slowly replaces the pioneer tubeworm species *Tevnia jerichonana* which prefers high sulfide and low oxygen conditions characteristic for more active vents (Nees et al. 2009).

1.4 *The discovery of Riftia pachyptila*

The vestimentiferan *R. pachyptila* was discovered by Dr. Meredith L. Jones (fig. 2) after studying the samples supplied by Corliss and Ballard during their expedition in 1977 with *Alvin*. It is the first of the hydrothermal vent tube worms to be discovered. This unusually long marine tubeworm was classified as a new species, and with this discovery a whole new field within deep sea research born. The new discovery was accompanied with a series of questions. Where did the worm originate from? How can it survive the harsh and fluctuating environment of the hydrothermal vents? On what does the worm feed? Why is this specimen so much bigger than its fellow marine worms? From the day it was discovered, *R. pachyptila* quickly became the poster child for annelid hydrothermal vent research, and is still the subject of many deep sea studies to answer all questions about this unusual and highly adapted invertebrate.

2.   The giant tubeworm *Riftia pachyptila*

Riftia pachyptila (fig. 3) inhabits hydrothermal vent sites along the East Pacific Rise and the Galapagos Rift in the Eastern Pacific and is one of the most prominent members of the hydrothermal vent fauna (Desbruyères and Segonzac 1997). It is typically found clustered around diffuse or low flow vents (Steward and Cavanaugh, 2005) which form a mix between cold (~1.8℃), oxygen rich (110 μM) bottom water and the hot (~400℃) and acidic (pH ~3 to 6) vent fluid (Steward and Cavanaugh, 2005). The

Fig. 3: *Riftia pachyptila* (source: Stewart and Cavanaugh (2005) and http://www.marine-conservation.org.uk). The yellow tubes on the background belong to the other giant tubeworm species, *Tevnia jerichonana.*

result of this water mix is a fluctuating oxygen-rich habitat with temperatures between 1.8 and 40℃, a pH around 6 and low concentrations of reduced vent chemicals (Fisher 1995; van Dover 2000) like sulfur (Nees *et al.* 2009). This diffuse flow region where the worm lives at the base of the vent is a highly variable physicochemical region (Johnson et al 1988 a,b) and substrate availability is unpredictable. Species inhabiting these vents need to be highly resilient for the always changing environment.

2.1   Anatomy

The tubeworm *R. pachyptila* is dark-red with a white anterior and is covered with a white tube composed of chitin and scleroproteins, insoluble proteins which can be found in skeletal and connective tissue. The tube can grow up to three meters in length and protects the long soft body of the worm from the environment (Steward and Cavanaugh 2005). The body of the adult worm is divided in four main regions (fig. 4), each with its own function: the *branchial plume*, the *vestimentum*, the *trunk* and the *trophosome* (Stewart and Cavanaugh 2005). The branchial plume is a gill like organ that lies at the anterior of the worm and is in direct contact with the surrounding water. Metabolites (e.g. sulfide, oxygen, carbon dioxide and inorganic nitrogen) are taken up by the worm via the branchial plume, and waste products like ammonia and excessive protons from bacterial sulfide oxidation can be released into the water (Girguis *et al.* 2002; Stewart and Cavanaugh 2005).

Below the plume lies the vestimentum, which is a circular muscle that houses the heart and brain of the worm. The glands that produce chitin and scleroproteins for tube composition are also located in the vestimentum. This muscle also enables the worm to move within its tube, so that the worm can

withdraw when being predated by crabs or fishes, or extend to access sulfide rich vent fluids and oxygen rich bottom water through vertical migration (Stewart and Cavanaugh 2005). The trunk (not shown in fig. 4) secures the attachment of the worm's posterior to its tube.

Surprisingly, the adult *Riftia* worm has no mouth or gut and is not able to feed. This lacking digestive system puzzled scientists: how does the worm acquire organic carbon for nutrition and growth? The answer came in 1981, where scientists revealed the mystery behind the feeding strategy of the *Riftia* worm. The worm is supplied with organic carbon by sulfur-oxidizing bacteria. These bacteria that live inside the trophosome: an inner sack inside the host worm, specifically designed to house bacterial symbionts (Cavanaugh *et al.* 1981; Felbeck 1981; Jones 1981). Within the trophosome, the symbionts are further encapsulated within a host-derived membrane bound vacuole (Cavanaugh 1983, 1994; Fisher 1990). The trophosome is located in the coelom of the tube (Chaston and Goodrich-Blair 2010) and is organized into lobules that are each composed of a thick tissue of host cells: the bacteriocytes. These bacteriocytes take up 50-70% of the trophosome. Inside these
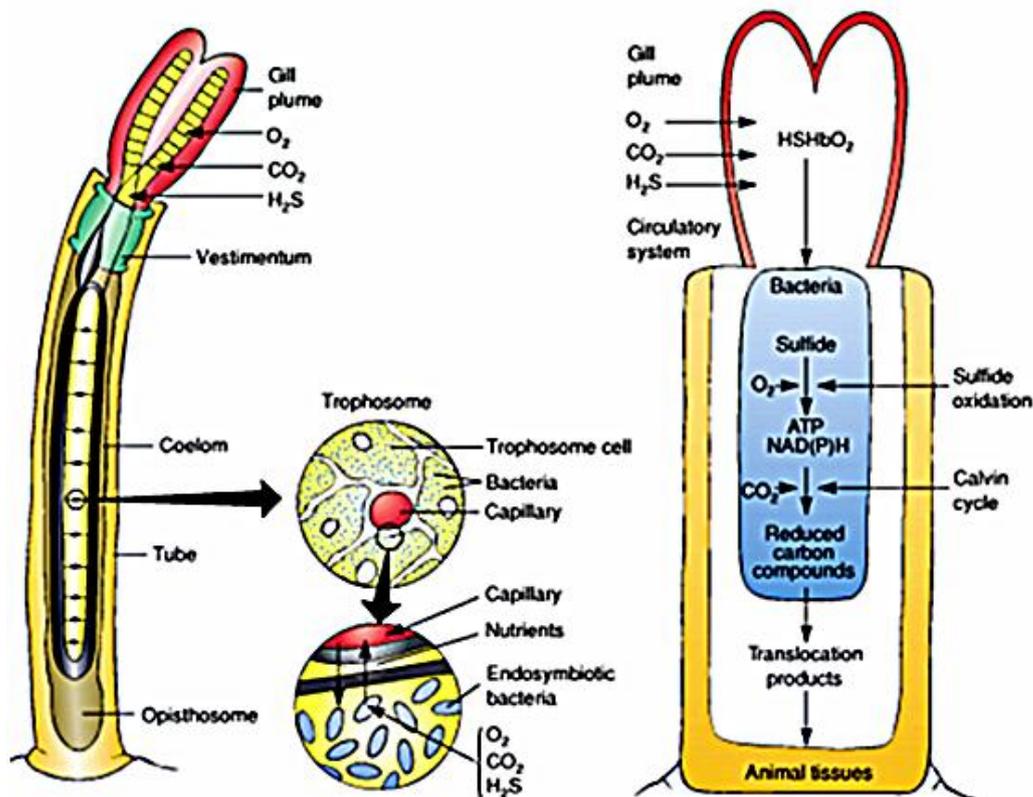
Fig. 4: *Riftia pachyptila anatomy (modified figure from cms.daegu.ac.kr)*

bacteriocytes, the symbiont accounts for approximately 25% of the total volume of the trophosome (Jones and Gardiner 1988; Bright and Sorgo 2003) and can reach cell densities of on average 10^9 cells per gram of fresh trophosome (Hand 1987). These bacteria are embedded in their own individual vacuoles, known as the symbiosomes (not shown in fig. 4), which are surrounded by the symbiosome membrane of the host (Cavanaugh *et al.* 1981) On the outside, the bacteriocyte tissue is surrounded by

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additional blood vessels and epithelial tissue that face out into the coelum, connecting the bacteriocyte tissue with the worm tissue (Chaston and Goodrich-Blair 2010).

Color variations can be seen between trophosomes, which are the result of different elemental sulfur concentrations within the periplasm of the symbionts. This elemental sulfur is stored when the symbionts are supplied with plenty of H_2S (Wilmot and Vetter 1990) and give the trophosome a light green, almost yellow colour. When H_2S is limiting the trophosome turns dark green or black (Pflugfelder *et al.* 2005).

2.2   The juvenile worm

The *R. pachyptila* worms are not born without a mouth and gut; juvenile specimen still have the ability to feed and do not carry a trophosome or bacteriocytes inside their coelom (Chaston and Goodrich-Blair 2010). The symbionts that are required for carbon generation during their adulthood are collected during this life stage, and were at first suggested to enter the juvenile worm by feeding on free living bacteria (environmental transmission, Gros *et al.* 1998). This hypothesis was ruled out when no evidence for actively feeding on free-living bacteria was discovered: the symbiont phylotype was never present in the digestive system of the juvenile worm, and the midgut tissues did not show any signs of production or involvement with symbionts (Nussbaumer *et al.* 2006).

Nussbaumer *et al.* (2006) and other scientists discovered that the free-living bacteria accumulate within a secreted mucous layer on the outside surface of the worm and actively invade the epithelial tissue. It is suggested that this mucus is derived from the chitin-secreting pyroform glands (Gaill *et al.* 1992; Shillito *et al.* 1995; Chamoy *et al.* 2001). From there the symbionts migrate towards the developing cells that will later form the trophosome (Nussbaumer *et al.* 2006). Not being sessile enables the juvenile worms to travel away from their parents to new places before they settle and develop their symbiotic organs (Shank *et al.* 1998; Marsh *et al.* 2001).

2.3   Discovery of the symbiosis between Riftia and bacterium

The symbiosis between *R. pachyptila* and an intracellular sulfur-oxidizing bacterium was the first association between a marine invertebrate and a chemoautotroph to be described (Cavanaugh *et al.* 1981; Felbeck 1981). Although scientists first thought these chemoautotrophic bacteria were filtered from the sea water only to support the worm's life (Lonsdale 1977; Corliss *et al.* 1979), others soon discovered that suspension feeding was impossible and that the *Riftia* tubeworms were completely dependent on their symbionts for organic carbon (Cavanaugh *et al.* 1981; Felbeck 1981; Jones 1981; Stewart and Cavanaugh 2005). Initial evidence for a chemoautotrophic symbiosis in *R. pachyptila* came from microscopic and biochemical analyses showing Gram negative bacteria packed within the trophosome, (Cavanaugh *et al.* 1981) that were later taxonomically classified as Gammaproteobacteria (Distel *et al.* 1988).

2.4   A mutualistic relationship

Riftia pachyptila endosymbionts are thioautotrophs, bacteria that oxidize reduced sulfur compounds to synthesize ATP for use in autotrophic carbon fixation via the Calvin cycle (Stewart and Cavanaugh 2005). To complete their chemoautotrophic process, these bacteria need access to reduced sulfur (HS^-), oxygen (O_2) and carbon dioxide (CO_2). As the symbionts live inside their host and are not in direct contact with their environment, they are dependent on their host for supplying the desired substrates (Childress and Fisher 1992). This co-dependence is a mutualistic symbiosis, where both organisms rely on each other for supply of the substrates they need for their independent existence.

The high bacterial activity and the constant supply of plenty of carbon give *R. pachyptila* one of the fastest growth rates known in marine invertebrates (Lutz *et al.* 1994). The symbionts in their turn enjoy the luxury of high nutrient concentrations inside the trophosome (Childress and Fisher 1992) which allow them to practice a higher metabolic activity than their free-living fellow species (Markert *et al.* 2007).

This symbiosis is not exclusive for the *R. pachyptila* tubeworm, but has been found in several other invertebrates (Annelida, Arthropoda, Echinodermata, Mollusca, Nematoda and Porifera) living near hydrothermal vents (Cavanaugh *et al.* 2006; Dubilier *et al.* 2008). The described form of symbiosis is quite common in these environments, as chemical energy sources needed for bacterial nutrition are not easily harvested by the bacteria. Chemoautotrophy requires both a source and a sink of electrons. The source of electrons (electron donor) consist of reduced sulfur or methane compounds, while O_2 functions as the sink of electrons (electron acceptor) These donors and acceptors do not co-occur in the same microenvironment since reduced sulfur or methane would oxidize spontaneously in the presence of oxygen (Zhang & Millero, 1993) and are found only in anoxic or microoxic zones (e.g. sediments) (Chaston and Goodrich-Blair 2010). The worm can provide both compounds, creating an microenvironment inside its body preferred by chemoautotrophs.

2.5   Research on the Riftia symbiont

When the symbiosis between *R. pachyptila* and sulfur-oxidizing bacteria was first discovered it raised a lot of questions. What mechanisms are used for symbiont acquisition? What are the spatio-temporal dynamics and processes of symbiont growth and metabolism? How do symbiont populations disperse and what is their genetic structure? (Sterwart and Cavanaugh 2005). Acquisition of symbionts has been discovered (Gaill et al 1992; Shillito *et al.* 1995; Chamoy *et al.* 2001; Nussbaumer *et al.* 2006), and processes of symbiont growth and metabolism have been researched through enzyme analysis, predicting the presence of active enzymes in the symbionts (Cavanaugh *et al.* 2006). More recently, genomic and proteomic approaches have been utilized to further explore this symbiosis (Woyke *et al.* 2006; Kuwahara *et al.* 2007; Markert *et al.* 2007; Sanchez *et al.* 2007; Robidart *et al.* 2007). Some questions remain due the inability to culture the bacteria outside their host (Stewart and Cavanaugh 2005).

3. The symbiosis between *Riftia pachyptila* and chemoautotrophic bacteria

3.1 *Supplying the host with organic carbon: first model of symbioses*

Stewart *et al.* (2005) proposed the first model of metabolism for the symbiosis between *R. pachyptila* and a chemosynthetic sulfur-oxidizing bacterium (fig. 5). This model explains all metabolic steps performed by the endosymbiont to supply the host with the desired end product: organic carbon.

Bisulfide (HS^-) and O_2 simultaneously bind to the worms hemoglobin and travel through the veins surrounding the bacteriocytes where they are collected by the symbiont for sulfide oxidation. HS^- is first oxidized to elemental sulfur (S) or directly to sulfite (SO_3^{2-}). Elemental sulfur is also oxidized to sulfite and all sulfite is further oxidized to sulfate (SO_4^{2-}). This last oxidation step is generated by the adenosine 5'-phosphosulfate (APS) pathway through APS reductase, an enzyme that catalyzes the two-electron reduction of APS to sulfite and AMP (Brychkova *et al.* 2012). This process is catalyzed by the enzyme ATP sulfurylase, and one ATP is yielded through substrate level phosphorylation (the formation of high-energy phosphate bonds by phosphorylation of ADP to ATP).

From here, the electrons that are liberated during sulfur oxidation pass through an electron transport system where O_2 is consumed and ATP + NADPH is produced.

The end product SO_4^{2-}, which is an undesired waste product, is released in the bacteriocyte tissue where it travels back through the worm blood to the branchial plume and is released into the seawater. The generated ATP travels to the Calvin Benson cycle (fig. 6), where ATP and NADPH provide the energy to produce organic carbon from CO_2 via the enzyme RubisCO. The converted CO_2 creates symbiont biomass. The host collects organic carbon through direct translocation of less complex nutritive compounds like amino acids and through digestion of its symbionts.

The digestion of the symbionts was confirmed by Bright *et al.* (2000) whom found that the symbionts were consistently being consumed near the periphery of the trophosome lobule, which shows similarities with previous reports about symbiont digestion by the *Riftia* host (Bosch and Grassé 1984a; Hand 1987; Gardiner and Jones 1993). Experiments including ^{14}C-bicarbonate radiotracer fixation support these observations. Juvenile *Riftia* specimens were collected and incubated in pressure vessels filled with sea water containing labeled carbon ($NaH^{14}CO_3$) for 15 minutes, 1 hour and 3 hours (Bright *et al.* 2000) The appearance of the radioactive label in the host tissue was directly associated with the loss of this label in the trophosome during the incubation period showed that symbiont digestion also plays a role in host nutrition (Bright *et al.* 2000). Furthermore, Bright *et al.* (2000) Discovered the radiotracer in symbiont-free tissue after only 15 minutes of incubation and concluded that the endosymbionts release a significant amount of carbon directly after fixation.

Another clue towards this symbiont digestion is the relatively high lysozyme activity within the trophosome (Boetius and Felbeck 1995), an enzyme that can lyse bacterial cell walls (Tompkins *et al.*

1991).How many symbionts are lysed by the host is dependent on the growth dynamics of the symbiont population (Stewart and Cavanaugh 2005), and relies on the cell division rate in the center of the trophosome (see *Lifestyle of the endosymbiont*).

Next to HS^-, O_2 and CO_2, NO_3^- is also provided by the host for its bacterial symbionts. The transport mechanism is still unknown. This system could be simple diffusion into the host like proposed for the bivalve *Solemya,* which also carries chemoautotrophic symbionts. NO_3^- is the dominant nitrogen source for both organisms, and is reduced to nitrite (NO_2^-) by the symbionts via an assimilatory nitrate reductase (an enzyme involved in the reduction of nitrate to nitrite). NO_2^- is again reduced through an uncharacterized pathway and ammonia (NH_3) is yielded for host and symbiont biosynthesis.
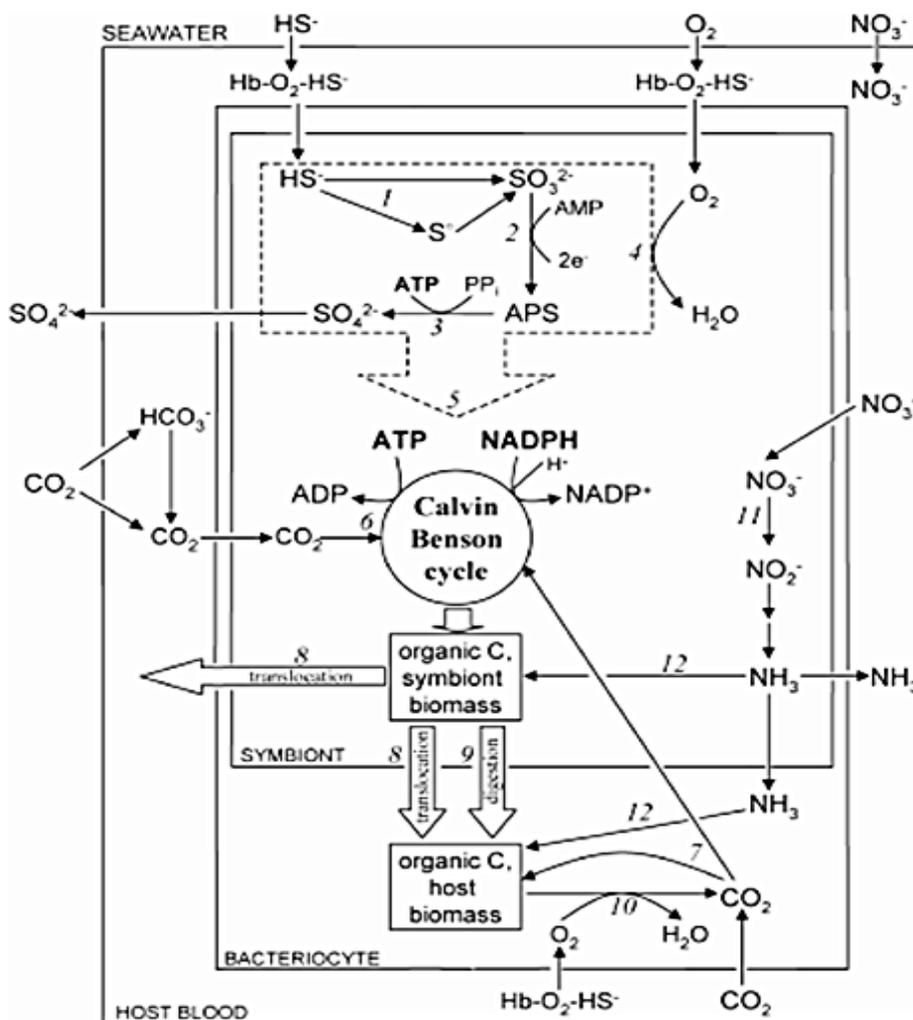

Fig. 5: First model of the sulfur-oxidizing Riftia endosymbiont (Stewart et al. 2005, modified by Stewart and Cavanaugh 2006)

The model proposed by Stewart *et al.* (2005) was investigated further by Markert *et al.* (2007) through functional genomics, to analyze the proteome and gain information on the physiology of the

uncultured symbionts to describe gene and protein functions and interactions through the dynamic aspects of the genome. Markert *et al.* (2007) were able to map out the major pathways existing in these symbionts and included the sulfide oxidation pathway and the reverse tricarboxylic acid (TCA) cycle. Also, they investigated the symbionts response to oxidative stress.

3.2 The sulfide-oxidation pathway unraveled through metagenomics

During their research, Markert *et al.* (2007) identified the three key-enzymes (DsrA, AprA/AprB, SopT) that play an important role in the sulfur cycle. Although these enzymes were originally described as part of the sulfate-reducing pathway, these enzymes can also function in the reversible mode and serve in sulfide oxidation (Markert *et al.* 2007).

The identification of these three enzymes proved the sulfur oxidation pathway suggested by Stewart *et al.* in 2005. These enzymes were suggested to play an important role in the symbiont sulfide oxidation because they constituted for more than 12% of the total cytosolic (located in cytoplasm) symbiont proteome in a pH range of 4-7: around the same pH value of the *R. pachyptila* habitat which fluctuates around 6 (Stewart and Cavanaugh 2006).
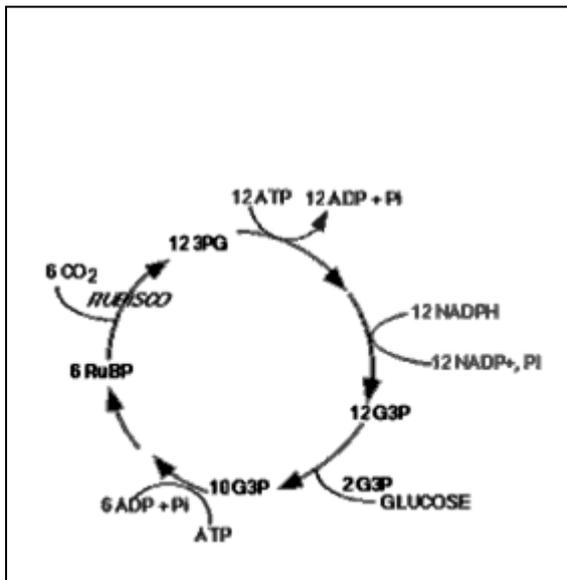
Fig. 6: *The Calvin Benson cycle, where CO_2 is fixated through RuBisCO into organic carbon. (source: http://www.biology.arizona.edu)*
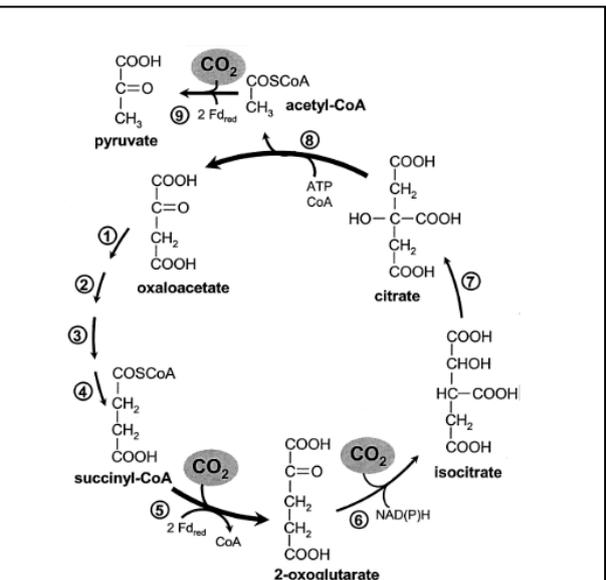
Fig. 7: *The reverse TCA cycle, where CO_2 is fixated through the enzymes ATP citrate lyase, 2-oxoglutarate:ferredoxin oxidoreductase, and fumarate reductase for the production of carbon molecules. (source: http://basic-microbiology.blogspot.nl)*

3.3   The importance of the Calvin-Benson cycle and the reverse tricarboxylic acid cycle

Later research showed that the Calvin-Benson cycle was less important than first suggested by Stewart *et al.* (2005) and Markert *et al.* (2007). Other enzymes were detected (Markert *et al.* 2007) which were involved in the energy-generating tricarboxylic acid (TCA) cycle. Moreover, the enzymes could run the TCA cycle in the reductive direction, the reductive or reverse TCA (rTCA) cycle (fig. 7) (Markert *et al.* 2007). This cycle represents an alternative CO_2 fixation mechanism that requires less energy than the Calvin-Benson cycle for each three carbon units formed. Considering the great abundance of the key enzymes found (KorB, Por1, Por2, PorA/PorG, SdhA/SdhC, CoA, ScsB), it is not only possible that the reductive TCA cycle exists, but also that it might play a very important role in the carbon metabolism of the *Riftia* symbiont (Markert *et al.* 2007). These enzymes showed higher activity than the Calvin-Benson cycle related RuBisCO enzyme, which constituted of only ~1% of the total protein map (Markert *et al.* 2007). In bacteria that mainly use the Calvin Cycle for CO_2 fixation, this protein is usually accounts for 4-50% of the total soluble protein (Tabita 1988). The enzymes found by Markert *et al.* (2007) indicate that there is a possible co-existence of both pathways in the *Riftia* symbiont.

Markert *et al.* (2007) also found evidence that the endosymbionts can adjust their enzyme production in different environmental conditions. Markert and colleagues compared bacterial protein patterns from naturally occurring sulfur-rich and from sulfur depleted trophosome tissues and found that the high sulfide symbionts showed higher peptide masses for enzymes involved in sulfide oxidation and for RuBisCO than those at low sulfur conditions. Several enzymes involved in the rTCA cycle also showed different peptide masses for the two conditions. Symbionts from the dark (sulfur depleted) trophosome showed elevated rTCA cycle enzymes compared to those from the light green (sulfur rich) trophosome. This suggested that the symbiont might be capable of switching between the two carbon fixation cycles when sulfur levels are low: from the high energy demanding Calvin-Benson cycle to the lower demanding rTCA cycle.

The probably important role of the rTCA cycle was also found by Robidart *et al.* (2008) and Gardebrecht *et al.* (2012). Both research teams found high activity in all enzymes involved in the rTCA cycle, confirming the findings by Markert *et al.* in 2007. Although it was first thought that the enzymes involved in the symbionts TCA cycle could catalyze in both the oxidative and reductive direction (Markert *et al.* 2007), new data showed that these reactions were carried out by separate sets of enzymes (Gardebrecht *et al.* 2012).

Robidart *et al.* (2008) presented the endosymbiont with its name: Candidatus *Endoriftia Persephone.* This name was chosen to reflect the dual nature of its life stages: inside the "internal oasis", nourished by the tubeworm (endo-riftia) and outside in the harsh environment of the hydrothermal vent (Robidart *et al.* 2008). The name *Persephone*, the Greek goddess of harvest and fertility, reflects the organic carbon providing function of the endosymbiont.

3.4   Organic nitrogen and nitrate respiration

Organic nitrogen, which is provided by Candidatus *Endoriftia Persephone* through nitrate assimilation, is a necessary substrate required for biosynthesis in *R. pachyptila* (Girguis *et al.* 2000). If organic nitrogen acquisition by *R. pachyptila* is completely mediated through the endosymbionts is not sure. The enzyme glutamate dehydrogenase, one of the primary enzymes that mediate the assimilation of ammonia into amino acids, was found to be highly active in the symbiont-free branchial plume tissue (Minic *et al.* 2001). This indicates that *R. pachyptila* filters ammonia directly from the surrounding water and might be independent or less dependent on the symbiont for organic nitrogen.

Besides being used for organic nitrogen production, Candidatus *E. Persephone* has been proposed to be capable of using nitrate as an alternative electron acceptor (instead of O_2) (Hentschel and Felbeck 1993; Pospesel *et al.* 1998; Gardebrecht *et al.* 2012). Whether the symbiont is capable of nitrate respiration through denitrification (reducing nitrate to N_2 gas) is not sure, as the gene coding for the enzyme, a nitrous oxide reductase, was not found by Robidart *et al.* (2008). However, all enzymes needed for nitrate respiration were discovered by Gardebrecht *et al.* (2012), suggesting that Candidatus *E. Persephone* can use nitrate for respiratory purposes. Still, further research is necessary to determine if the symbionts are able to gain energy from denitrification, and to clarify the details of this process (Gardebrecht *et al.* 2012).

4.   Physiology of the symbiont: before and after infecting the host

4.1   Physiology of the free-living pre-symbiont

Before the free-living bacteria become endosymbionts, they are suggested to live as heterotrophs (fig. 8) (Cary *et al.*, 1993; Feldman *et al.*, 1997; Millikan *et al.*, 1999; Nussbaumer *et al.*, 2006; Stewart and Cavanaugh, 2006). The idea of a free-living pre-symbiotic lifestyle and mixotrophic abilities was born when all the genes coding for all the enzymes of the Krebs Cycle were identified (Robidart *et al.* in 2008). The Krebs Cycle is the basis of energy metabolism of numerous cell types, principally found in aerobes (Gest 1981). Together with this discovery, identification of genes coding for fructose degradation and glycolysis highly suggest that the symbiont is indeed a heterotrophic microorganism in its free-living life stage.

In addition Robidart *et al.* (2007) revealed the
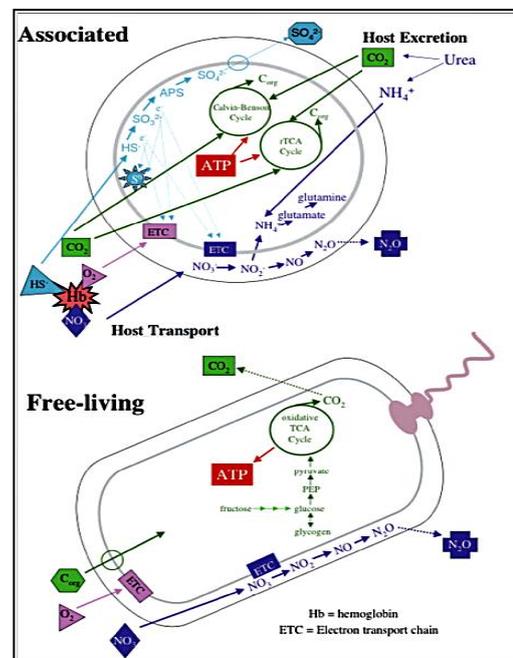
Fig. 8: *the two life stages of the Riftia symbiont as suggested by Robidart et al (2008). Picture modified from Robidart et al.*

presence of regulatory systems in the genome that respond to carbon compounds. They discovered the presence of a large number of genes involved in chemotaxis, which aides the free-living bacteria to find the substrates they need. The bacteria also revealed the ability to **1)** respond to external carbon sources via catabolite regulation [a regulation in which only the favored carbon/energy source is used and the cell is usually prevented from producing enzymes which catabolize other carbon compounds (Aharonowitz and Demain 1978)], **2)** the phosphotransferase system [the system involved in both the transport and phosphorylation of a large number of carbohydrates in the regulation of a number of metabolic pathways, including chemotaxis (Postma *et al.* 1993)]. Furthermore, the endosymbiont has the genes that are necessary for a functional flagellum. All these findings suggest that the free-living bacteria is able to actively "hunt" for substrates to feed on, with help from chemotaxis and a flagellum.

4.2   Invading the host

It is suggested that the free-living bacteria attach themselves to the surface of the juvenile worm with the help of fimbriae [adhesion organelles expressed by many Gram-negative bacteria (Connell *et al.* 1996)], pili [organelles that support a flagella-independent form of bacterial translocation over moist surfaces (Mattick 2002)], or even with a flagellum (Gardebrecht *et al.* 2012). The protein involved in this attachment could be an adhesive like protein, a hyaline [a proteins involved in cell adhesion (http://www.bio.davidson.edu)] or a fibronectin type III domain protein [fibronectin mediates a wide variety of cellular interactions with the extracellular matrix (ECM) and plays important roles in cell adhesion, migration, growth and differentiation (Mosher, 1989; Carsons, 1989; Hynes, 1990; Yamada and Clark, 1996)]. All the genes encoding these proteins were discovered in the *Riftia* symbiont metagenome (Gardebrecht *et al.* 2012). A cell wall-associated biofilm protein might also be involved in bacterial attachment, which is located on the mucus layer that coats the tubeworm larvae (Nussbaumer *et al.* 2006).

The symbionts have been found to colonize the host tissue in very low numbers. Nussbaumer *et al.* (2006) found fewer than 20 bacterial cells in the invading tissue during the early stages of invasion, and predicted that even fewer cells actually initiate the colonization within the trophosome. Sequencing the highly variable internal transcribed spacers (ITS) revealed that three different individual tubeworms from the same location were predominantly colonized by the same bacteria (Nussbaumer *et al.* 2006). These ITS regions are regions of non functional RNA, which are not translated into proteins, but have a critical role in the development of functional rRNA and are used as signature regions for molecular assays (Iwen *et al.* 2002).

In *Oligobranchiae mashikoi,* another marine annelid, the worms collected from a single site together contained a total of seven distinct bacterial phylotypes, but only a single phylotype dominated the symbiont population in each worm separately (Kubota *et al.* 2007). This supports a model in which one or a few individual bacterial cells initiate colonization of a single *R. pachyptila* worm (Kubota *et al.* 2007). Due to the relatively simple microbial communities, together with host diversity, general

experimental tractability and tendency, invertebrates function as perfect models for research on bacterial symbiosis (Chaston and Goodrich-Blair 2010). The low diversity simplifies the task of investigating complex molecular and cellular interactions between the symbiont and the host (McFall-Ngai, 2002; Ruby 2008).

4.3   Inside the host: different morphotypes of the endosymbiont

The endosymbionts inside the trophosome are present in two different morphotypes depending on their location within the trophosome lobule (Bosch and Grassé 1984a,b; Gardiner and Jones 1993; Bright *et al.* 2000). The bacteria inhabiting the bacteriocytes located in the central zone of the lobule are rod shaped with little variation in size, while the bacteria in the bacteriocytes nearer the periphery of the trophosome are mostly coccoids which vary in size from 1.6 to 10.7 µm in diameter (Bright *et al.* 2000). The rod-shaped symbionts inside the center of the trophosome are actively dividing, while the coccoids from the periphery show various stages of autolysis [self-digestion of cells by enzymes (Kavanaugh 2005)] and digestion by the host (Bosch and Grassé 1984a; Gardiner and Jones 1993; Bright and Sorgo 2003).

The different life stages are thought to have different contributions towards CO_2 fixation. Symbiont fixation rates are almost equivalent in both the center and the periphery of the trophosome, which suggests that the substrates are equally divided and do not offer locations for high or low chemosynthetic activity (Bright *et al.* 2000). It appears that the rod-shaped symbionts inhabiting the central zone fix carbon to support cell division (Bosch and Grassé 1984a; Gardiner and Jones 1993; Bright and Sorgo 2003) while the coccoid cells from the peripheral zone fixate carbon to support an increase in per cell biomass. Indeed a great margin in cell size was observed in the periphery cells (Bright *et al.* 2000).

Why there are different morphotypes within the trophosome is not sure, but it could be caused by a difference in biochemical gradients which impact symbiont metabolism, morphology and growth (Stewart and Cavanaugh 2005). It is also plausible that changed cell morphology is a response to a different in life stages. The endosymbionts might switch their morphology during or after migration towards the periphery, where they undergo lysis and degradation by direct ingestion of the host (Bosch and Grassé 1984a,b Hand 1987; Bright *et al.* 2000). Support for this cell cycle hypothesis was given by Bright and Sorgo in 2003, who discovered that division by the rod shaped bacteria inhabiting the central zone, was in balance with lysis of the cocci at the periphery of the trophosome. He also found intermediate stages (between rod shaped and coccoid) within the trophosome, supporting this hypothesis.

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4.4   Effect of low energy conditions: mixotrophy and carbon storage

When Candidatus *E. Persephone* is subjected to long lasting or severe low energy conditions, they might switch from being autotrophs to practicing a heterortrophic lifestyle (Markert *et al.* 2007). This was suggested because glycogen was found as a carbon storage compound (Sorgo *et al.* 2002). The lack of sulfur compounds for energy conversion might be compensated for by burning carbon reserves (glycogen) through glycolysis and the oxidative TCA cycle. Glycogen is an important substrate for glycolysis in pro- and eukaryotes and therefore as an important energy source when nutrients are low (Sorgo *et al.* 2002). This suggests once more that the symbiont might be able to use the TCA cycle in the oxidative and reductive direction when environmental conditions change.

A gene discovered by Gardebrecht *et al.* in 2012 suggested another method of energy storage, this time in the form of an enzyme generating cyanophycin. This enzyme is a storage amino acid biopolymer containing both nitrogen and carbon (Ziegler *et al.* 1998; Krehenbrink *et al.* 2002). These different types of storage once again indicate the great flexibility of Candidatus *E. Persephone* towards environmental changes in an environment that is everything but stable (Markert *et al.* 2007).

4.5   Oxidative stress

Candidatus *E. Persephone*, like other aerobic bacteria, experience the downside of oxygen use: reactive byproducts of oxygen, such as superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radicals (OH). These byproducts are generated continuously in their cells and causing oxidative stress, which occurs when the concentration of active oxygen exceeds the cell's defense capacity (Cabiscol *et al.* 2000).

The *R. pachyptila* host has a high oxygen buffering capacity in its hemoglobin (Arp and Childress 1983) and uses a part of the required O_2 for respiration. Still a lot of oxygen is passed on to the endosymbionts inside its trophosome. The endosymbionts do not tolerate high oxygen concentrations (Fisher *et al.* 1989), and needs some sort of protection against oxidative stress.

Candidatus *E. Persephone* do not have a catalase for cellular hydrogen peroxide (H_2O_2) protection (Blum and Fridovich 1984) and there was no evidence of the respective gene found during the first proteogenomic research carried out by Markert *et al.* in 2007. The protection against oxidative stress is thought to be given in the form of AhpC (alkyl hydroperoxide reductase), an enzyme which reduces organic hydroperoxides caused by H_2O_2 and is present in large amounts in the symbiont cytoplasmic (Markert *et al.* 2007). The symbionts might also enhance expression of thioredoxin reductases (TrxB) as an oxidative stress response (Gardebrecht *et al.* 2012). These are enzymes that have the ability to reduce oxidized thioredoxins, a group of small redox-active peptides (Mustacich and Powis 2000) which are shown to reduce oxygen stress in other bacteria (Helmann *et al.* 2003; Ballal and Manna 2010).

An addition to this genetic map was realized in a later proteogenomic research (Gardebrecht *et al.* 2012) and yet another gene for the protection against oxidative stress was discovered: the superoxide dismutase SodB. Superoxide dismutase (SOD), which catalyzes the dismutation of superoxide radicals, is an enzyme found in various bacterial species and forms the first line of defense against oxygen toxicity (Carlioz and Touati 1986). In this latest research by Gardebrecht *et al.* the gene encoding for a catalase that could reduce hydrogen peroxide was not found, again suggesting that Candidatus *E. Persephone* uses other pathways to protect itself from the oxygenic environment of its host.

5.   *R. pachyptila* and Candidatus *E. Persephone*: co-speciation?

5.1   *The environmental theory*

The symbiont acquisition model suggested by Nussbaumer *et al.* (2006), where free-living bacteria infect the host through the mucus layer on the outside surface of the worm, could possibly be generalized to all vestimentiferan worms (Vrijenhoek 2010). This means that the symbiont phylotyles are possibly more related to the local environment than to a particular host species (Vrijenhoek 2010). There is no evidence of co-speciation between vestimentiferans and their symbionts (Feldman *et al.* 1997; Nelson and Fisher 2000; McMullin *et al.* 2003) and their phylogenies show no similarity (Vrijenhoek 2010). Vrijenhoek and coworkers discovered that the symbionts are indeed associated with habitat type and also with biogeographical regions.

5.2   *Co-speciation?*

Studies in which 16S rRNA sequences were amplified show that there are two primary phylotypes (I and II) of symbionts found in the trophosomes of all vestimentiferan species (fig. 9) (Feldman *et al.* 1997; Di Meo *et al.* 2000; Nelson and Fisher 2000; McMullin *et al.* 2003; Vrijenhoek *et al.* 2007).

The degree of sequence divergence (4.3%, Vrijenhoek 2010) suggests that the two discovered phylotypes diverged more than 200 million years ago (Feldman *et al.* 1997) while the vestimentiferans are only about 60 million years old (Black *et al.* 1997). Another hint for a rather geographical relation instead of a direct host relationship is derived from the eastern Pacific seep worm *Escarpia spicata*, which mainly hosts phylotype I, while specimens of this species found near a hydrothermal vent in the Gulf of California host phylotype II (Di Meo *et al.* 2000). This finding agrees with the environment theory proposed by Vrijenhoek in 2010: vestimentiferan species are not bound to a particular symbiont, but can switch to other phylotypes when the larvae colonize this phylotypes habitat.

The proteogenomic research carried out by Gardebrecht *et al.* (2012) on endosymbionts of the two most abundant tubeworm species *R. pachyptila* and *Tevnia jerichonana* showed that these

endosymbionts were highly similar, again disproving the probable species specific nature of these bacteria.
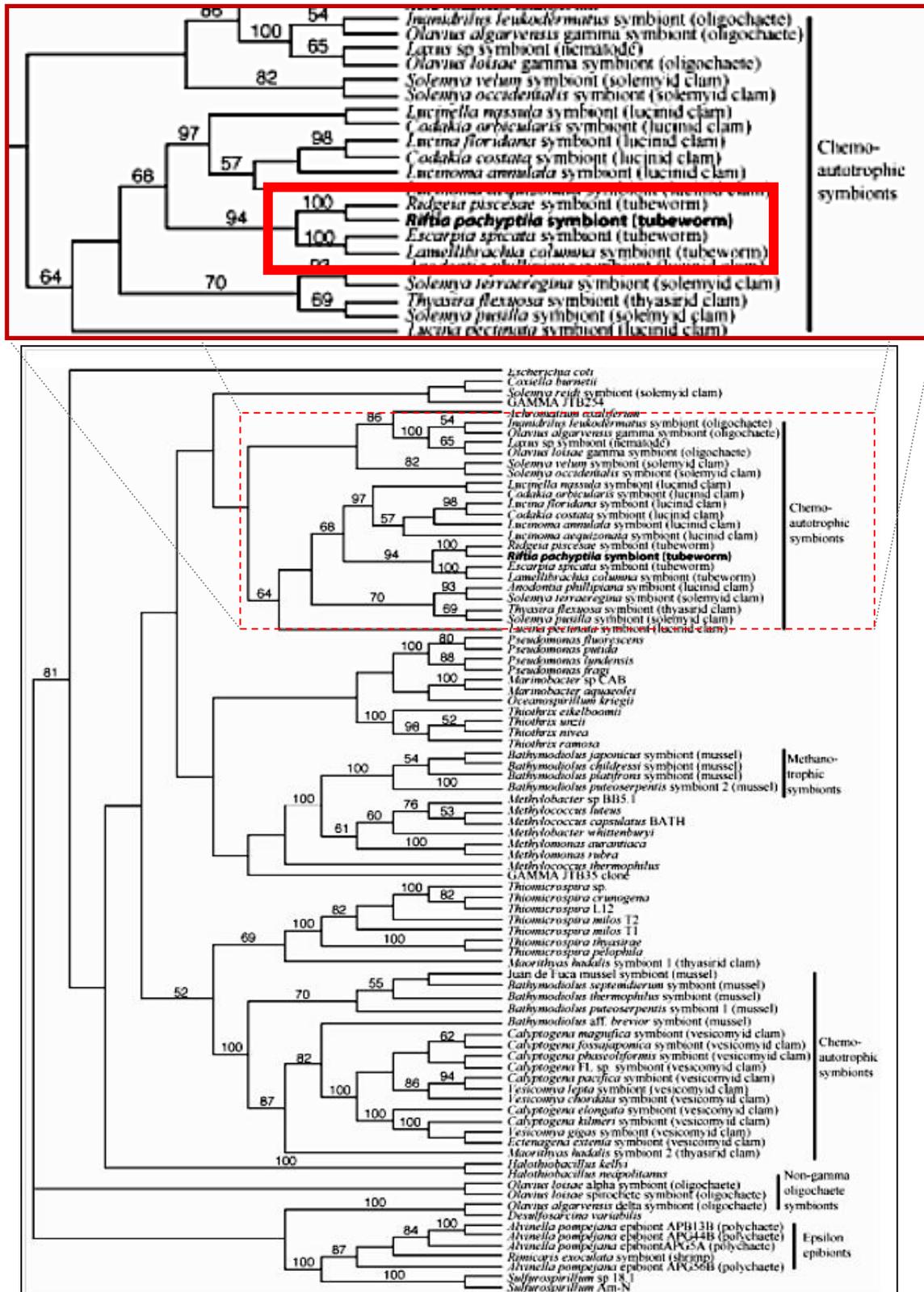

Fig. 9: *Phylogeny showing the placement of the Riftia symbiont (in bold) relative to other chemosynthetic symbionts. The close relation shows the possibility of one symbiont for all vent tubeworms. Adapted from McKiness (2004) and Cavanaugh et al. (2005) (source: Stewart and Cavanaugh 2005).*

6.   Discussion

6.1   Culturing Candidatus E. Persephone and Riftia Pachyptila in the lab

Although *R. pachyptila* and Candidatus *E. Persephone* have been the subject of many studies since their discovery in 1981, there are still unanswered questions that need attention before this symbiosis can be fully understood. The metagenome of the *Riftia* symbiont is not closed, and there is still no 100% clarity about the possible differences between the symbionts (Gardebrecht *et al.* 2012).

Another question still remaining is the importance and possible switch between the Calvin-Benson cycle and the rTCA cycle. The comparative study carried out by Markert *et al.* (2007) showed a clear link between trophosome sulfur enrichment and the preferred CO_2 fixation cycle. If the endosymbionts are capable of switching according to the available sulfur or if the sulfur level determines symbiont evolution through an evolutionary bottleneck is not clear. Further research in which sulfur-depleted *R. pachyptila* worms are subjected to sufficient sulfur and sulfur-rich *R. pachyptila* to lowered sulfur levels is necessary to investigate if the switch is a short or long time (evolutionary) process. This involves in situ culturing of the *R. pachyptila* tubeworm (which acquires high-pressure incubations environment simulating conditions) or isolation and culturing of the endosymbiont. Since both methods have yet not been successful, investigating the possibilities would be an important and necessary step towards further *Riftia* symbiont exploration.

Culturing *R. pachyptila* in the laboratory could also give more information about symbiont acquisition in the juvenile stage. Although the mechanism of infection has been discovered, there are still questions remaining. What mechanisms are used by the bacteria to locate the host? Since the bacteria are thought to be heterotrophs during their free-living life stage, it is likely that the host secretes a certain compound that triggers the bacterial chemotaxis. This compound could be an organic carbon preferred by the required pre-symbiont or a certain chemical that attracts the bacteria towards the host.

The specificity of the endosymbiont per host suggests that the host can select which bacteria enter its tissue and which bacteria are blocked from entering. This could explain the massive apoptosis (regulated cell death) of host epidermis found by Nussbaumer *et al.* (2006) in freshly settled juvenile *Riftia* worms. The apoptosis could be caused by a natural reaction towards any bacterial invasion, or by selectively killing cells that contain the 'wrong' bacteria.

It is also possible that the bacteria select the host, and that symbiont selection occurs within the host through an evolutionary bottleneck. Since only a few bacteria infect the host (Nussbaumer *et al.* 2006) this bottleneck is very plausible and could be the reason why the bacteria are so alike. Findings by Robidart et al. (2008) confirm this selection, as they discovered that *Candidatus E. Persephone*

possesses defenses that help the bacteria to protect itself from the immune system of *R. pachyptila* during the earlier stages of infection.

6.2 The environmental theory and endosymbiont diversity

Gardebrecht *et al.* (2012) compared the endosymbionts of two different species of hydrothermal vent tubeworms (*R. pachyptila* and *T. jerichonana)* to find more answers about the genetic differences between the symbionts. Strangely, they found that the *Riftia* symbionts taken from two different specimens showed more resemblance to the *Tevnia* symbionts than to each other. This was explained through incompleteness of the genome due to differences in quality of the symbiotic DNA, but still there were little variations between the *Riftia* and *Tevnia* symbionts, even though they originated from very different conditions (e.g. low/high pH, low/high sulfur compound flow). This shows that, if the symbionts are indeed one and the same species, they show great flexibility towards the environment they originate from. This was also noticed by Robidart *et al.* (2008), whom suggested that these endosymbionts show an increased ability to sense their environment and respond when necessary. This finding disagrees with the environment theory, where it was suggested that the symbiont phylotypes are possibly more related to the local environment than to a particular host species (Vrijenhoek 2010). If this symbiont is indeed extremely flexible towards its environment, it would be unlikely to suggest that different symbiont phylotypes inhabit different geochemical locations. Only one phylotype (or perhaps a few, as found by Feldman *et al.* 1997; Di Meo *et al.* 2000; Nelson and Fisher 2000; McMullin *et al.* 2003 and Vrijenhoek *et al.* 2007) could suffice since bacteria easily evolve and adapt to a certain environment.

Despite the yet little acquired information about the free-living life stage it can be suggesting that environmental flexibility occurs in this life stage as well. Even more, if the free-living symbiont is heterotrophic, it would be less sensitive to mild changes in the chemical environment and will mostly be dependent on the flow of organic carbon in its habitat.

The endosymbiont associated with *R. pachyptila* (phylotype II) has been found to settle in biofilms on basaltic surfaces (volcanic rocks) and has been filtered from the seawater sampled up to 100 m from an eastern Pacific vent, which suggests that these organisms form potentially large environmental pool from which they can infect juvenile tubeworms (Harmer *et al.* 2008). The hydrothermal vent tubeworms *R. pachyptila, T. jerichonana* and *Oasisia alvinae*, which all live in the same area (eastern Pacific) and were sampled from the same vent site, seemed to share the same symbiont phylotype (Feldman *et al.* 1997; McMullin *et al.* 2003) and might comply with the environment infection model (Vrijenhoek 2010) which states that tubeworm species that settle together in the same habitat should be infected by the same local strains of the symbiont (Vrijenhoek 2010). Again this finding is unreliable due to conservative 16S rRNA sequences and differences could be missed, again putting a doubt on the environment theory.

If the symbiont indeed knows a heterotrophic free-living life stage, culturing might be possible by forcing the bacteria back to this stage by, for instance, providing them with sufficient organic carbon. If the host provides something yet undiscovered, culturing the bacteria outside of the host would be impossible until the missing link is found. This would suggest that certain traits are lost once the bacteria live inside the tube worm, and that the bacteria die once taken out of the trophosome. This also suggests that the bacteria need an exit strategy for when the host dies (Vrijenhoek 2010). If there is no strategy and certain traits are lost during their endosymbiotic life stage, the bacteria would die as well. If the traits can be re-activated, the symbiont could leave its host and can become a heterotroph once more. Vrijenhoek (2010) suggested that the symbionts could leave the worm through the worms respiratory structures, excretory pores or even with the gametes through the gonopores in cases of emergency, like damage to the host tissue or sulfide starvation. The symbiont could rely on its reserves during the transition to heterotrophy, although in case of sulfide starvation due to a diminishing vent flow these reserves might have already been used prior to leaving the host. If this transition is possible is not known and further research could not only clarify the faith of the symbionts once their host is enabled to further nurture its "garden", but could also create the desired cultivation possibilities for this yet uncultured and mysterious micro-organism.

7. References

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