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Establishment of endosymbiosis: The case of cnidarians and *Symbiodinium*

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ABSTRACT

The symbiosis between cnidarians and *Symbiodinium* algae (dinoflagellates) is the keystone responsible for the formation of the huge and important structures that are coral reefs. Today many environmental and/or anthropogenic threats compromise this tight relationship and lead to more frequent events of drastic loss of *Symbiodinium* pigments and eventually of algae themselves from cnidarians, better known as cnidarian bleaching. While the mechanisms underlying the collapse of the algae–coral symbiosis are progressively getting unraveled, the understanding of the mechanisms involved in the de novo infection of bleached cnidarians by *Symbiodinium* remains elusive. In this review, we describe the various steps needed to establish a stable symbiotic relationship between *Symbiodinium* and cnidarians. We review the mechanisms implicated in host–symbiont recognition and in symbiosome formation and persistence, with a special emphasis on the role played by lectins and Rab proteins. A better understanding of these molecular mechanisms may contribute to the development of strategies to promote post-bleaching recovery of corals.
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1. Introduction

The foundation of coral reefs, one of the most diverse and productive marine ecosystems, relies on the mutualistic relationship between invertebrate hosts (scleractininan corals) and their photosynthetic dinoflagellates of the genus *Symbiodinium* (commonly referred to as zooxanthellae; Fig. 1). In the cnidarian-*Symbiodinium* associations, photosynthetic algae are located in host-derived vacuoles (symbiosomes) within gastrodermal cells of the host (Wakefield and Kempf, 2001). Once in symbiosis, the symbiont provides up to 95% of its photosynthetic products (glycerol, glucose, amino acids or lipids) to the host (Muscatine, 1990), thus contributing massively to its energy demands (Gattuso et al., 1999). A significant part of the photosynthetically fixed carbon is translocated through processes controlled by host release factors (HRFs) which are yet to be clearly identified (Yellowlees et al., 2008). High O_2 concentration due to *Symbiodinium* photosynthesis also helps maintaining the high ATP level needed for the calcification process (see Jokiel, 2011). In return, the host ensures protection to the zooxanthellae and provides a source of inorganic nutrients (CO_2, NH_3 and PO_4^{3-}; see Yellowlees et al., 2008 for review). This mutual

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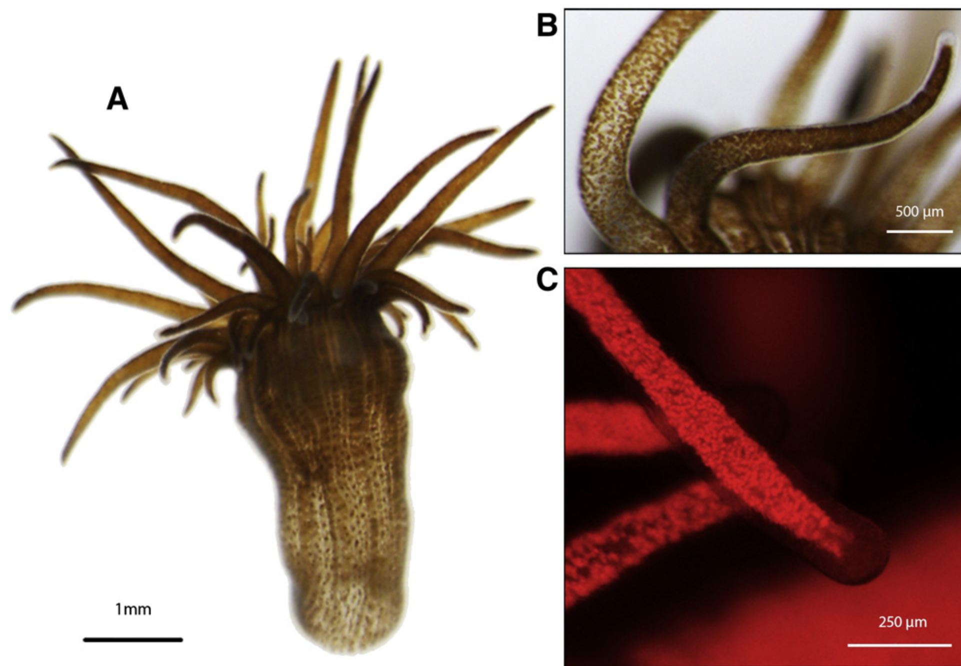

Fig. 1. Illustration of the distribution of symbiotic algae in a sea anemone *Aiptasia pallida*. A. Overall view of *A. pallida* shows the general distribution of zooxanthellae. B. Closer view of some tentacles revealing the patchy distribution of brown zooxanthellae within the gastrodermis. C. The lack of chlorophyll autofluorescence in the ectodermis of a tentacle tip confirms the gastrodermal distribution of zooxanthellae.

relationship continuously adjusts to subtle changes in the environment to optimize the survival of the holobiont (entity including the host, the symbionts and the whole associated microbial fauna). In the current context of global changes threatening symbiotic cnidarians, especially reef building corals, it is critical to understand the mechanisms controlling the symbiosis in order to develop new management strategies to help preserve or improve the health of coral reefs. In this review, we describe the mechanisms involved in the various steps needed to establish a stable symbiotic relationship between *Symbiodinium* and cnidarians.

1.1. The diversity of the symbiosis

Although zooxanthellae were once all considered as members of a single pandemic species, *Symbiodinium microadriaticum* Freudenthal (1962), recent molecular and genetic analyses, based upon variation of nuclear ribosomal DNA (18s, ITS and 28s rDNA) and chloroplast 23s rDNA, have revealed that the genus *Symbiodinium* is divided into nine large clades (A to I), each comprising multiple strains or species (Coffroth and Santos, 2005; Pochon and Gates, 2010). Moreover, the ability for *Symbiodinium* to establish symbioses is not restricted to reef-building corals but also involves a variety of other cnidarians (octocorals, sea anemones and jellyfish) and some representatives of the Platyhelminthes, Mollusca, Porifera, Foraminifera and ciliates (see Stat et al., 2006 for review).

1.2. From the free-living state to the symbiosis

Under normal conditions, *Symbiodinium* densities in symbiotic corals reach a steady state wherein neither partner outgrows the other (Muscatine et al., 1989). The maintenance of this dynamic equilibrium suggests the existence of intrinsic and environmental factors that can potentially regulate algal density pre- or post-mitotically (Hoegh-Guldberg and Smith, 1989; Muscatine and Pool, 1979). This regulation involves a variety of mechanisms, like the limitation of algal nutrient supply (e.g. Falkowski et al., 1993), the digestion of algae (Jones and Yellowlees, 1997; Muscatine and Pool, 1979; Titlyanov et al., 1996), the expulsion of excess or dividing *Symbiodinium* (Baghdasarian and Muscatine, 2000; Hoegh-Guldberg and Smith, 1989; Jones and Yellowlees, 1997), the accommodation of excess algae by division of host cells (Titlyanov et al., 1996) and possibly the production of growth inhibiting factors (Smith and Muscatine, 1999). This fine regulation of algal density also occurs on daily (Fitt, 2000; Hoegh-Guldberg et al., 1987; Jones and Yellowlees, 1997; Stimson and Kinzie, 1991) and yearly bases showing, for example, seasonal variations (Brown et al., 1999; Fagoonee et al., 1999; Fitt et al., 2000; Stimson, 1997). Expelled algae can then be recruited by gastrodermal cells or released to the ambient environment. Symbiotic algae that are released every day to the external environment, either through density regulation mechanisms (Hoegh-Guldberg et al., 1987; Stimson and Kinzie, 1991) or through survival of corallivorous species digestion (Bachman and Muller-Parker, 2007; Muller-Parker, 1984), may contribute to the maintenance of a free-living *Symbiodinium* population. Indeed, several studies report the presence of free-living *Symbiodinium* in the water column, within sediments (Adams et al., 2009; Carlos et al., 1999; Coffroth et al., 2006; Gou et al., 2003; Hirose et al., 2008a; Littman et al., 2008; Manning and Gates, 2008; Pochon et al., 2010) and in association with macroalgae beds in the vicinity of coral reefs (Porto et al., 2008). Nevertheless, the density distribution of free-living *Symbiodinium* seems to be highly heterogeneous between and within reefs (Littman et al., 2008).

Nevertheless, the symbiotic relationship is highly sensitive to environmental or anthropogenic disturbances and may be disrupted, thus leading to a phenomenon, commonly referred to as coral bleaching, in which *Symbiodinium* densities are drastically reduced (Brown, 1997; Douglas, 2003; Glynn, 1993). Debate still rages on whether expelled *Symbiodinium* following a stressful event remains viable and is able to contribute to the recovery of bleached corals. Some studies reported that a majority of expelled symbionts remained healthy (Bhagooli and Hidaka, 2004; Ralph et al., 2001, 2005; Sandeman, 2006), others claimed that *Symbiodinium* survival did not last (Hill and Ralph, 2007; Perez et al., 2001; Steen and Muscatine, 1987; Strychar et al., 2004). For instance, Hill and Ralph (2007) reported that *Symbiodinium* cells released in the water column after a thermal stress had a drastically reduced photosynthetic activity after 5 days, suggesting that their survival could be compromised. Therefore their contribution to the free-living stocks may then be limited.

Free-living *Symbiodinium* may constitute a reservoir of various clades from which juvenile and adult corals can select, to establish the symbiosis in normal conditions or after bleaching. Dinoflagellate symbionts may be acquired by vertical transmission, where *Symbiodinium* are present in the egg or brooded planula larvae prior to release from the maternal colony. Alternatively, aposymbiotic coral larvae recruit symbionts from the environment (around 80% of scleractinian corals; Fabricius et al., 2004; Rodriguez-Lanetty et al., 2006; Schwarz et al., 1999; Szmant, 1986).

During this so-called horizontal transmission many studies showed that larvae acquire their symbionts during a nutritional process (Harii et al., 2009; Hirose et al., 2008b; Rodriguez-Lanetty et al., 2004; Rodriguez-Lanetty et al., 2006; Schwarz et al., 1999, 2002; Weis et al., 2001). Although symbiont infection through feeding is probably the major acquisition mechanism, it could also be facilitated by active mechanisms deployed by *Symbiodinium*. Indeed, during their life cycle, *Symbiodinium* cells interchange between a vegetative cyst, which is the dominant form when endosymbiotic, and a motile zoospore comprise thecal plates and a transverse and a longitudinal flagellum (Freudenthal, 1962). The motility of flagellated *Symbiodinium* is regulated in a diel-cycle and cells are active under illumination (Crafts and Tuliszewski, 1995; Fitt and Trench, 1983b; Fitt et al., 1981; Lerch and Cook, 1984; Yacobovitch et al., 2004). During that period, *Symbiodinium* seems able to swim in an oriented manner, thus showing phototaxis (Hollingsworth et al., 2005) and/or chemotaxis (Fitt, 1984; Pasternak et al., 2004). However, a potential host-seeking behavior remains limited by the flow velocity and the proximity of a host (Pasternak et al., 2006).

2. Establishment of the symbiosis

2.1. Making contact

In order to establish the symbiosis, both partners first have to recognize each other. Recognition mechanisms could be similar to the winnowing mechanism, which consists of a multi-step process involving both partners, that was first described by Nyholm and McFall-Ngai (2004) for the symbiotic relationship between the bobtail squid, *Euprymna scolopes*, and its bioluminescent bacterium, *Vibrio fischeri*. These mechanisms necessarily take place before, during, and after contact with *Symbiodinium* and its internalization, leading ultimately to the formation of a stable mutualistic relationship. Recognition mechanisms between host cells and algae seem to be necessary, at least at some point, during vertical or horizontal transmission. Indeed, while it is quite obvious for horizontal transmission, Marlow and Martindale (2007) showed that recognition events could also be involved during vertical transmission in *Pocillopora meandrina*.

2.1.1. Lectin/glycan interactions

According to Weis (2008), among others, the onset of the infection by *Symbiodinium* involves the same mechanisms as those acting in the recognition of pathogenic organisms (Fig. 2). For many eukaryotes, the innate immune system implies the production of pattern recognition receptors (PRRs) able to recognize and bind to specific conserved components of microbe cell walls (carbohydrates, proteins, lipids; Kilpatrick, 2002). Among those PRRs, lectins (carbohydrate-binding proteins; Goldstein et al., 1980) are widely distributed in most classes of living organisms and are thought to play an important role in various symbiotic associations (e.g.: Bulgheresi et al., 2006; Hirsch, 1999; Muller et al., 1981). To date, lectins have been described in at least two cnidarian classes (Hydrozoa and Anthozoa) and have been reported in five scleractinians, including *Acropora millepora* (Kvennefors et al., 2008), *Montastrea faveolata*, *Oculina patagonica* (Hayes et al., 2010), *Pocillopora damicornis* (Vidal-Dupiol et al., 2009) and *Ctenactis echinata* (Jimbo et al., 2010). Several studies have thus examined the implication of lectin/glycan interactions in the recognition process between cnidarians and *Symbiodinium*.

Lin et al. (2000) identified ten different proteins on the cell surface of symbiotic dinoflagellates. Among those figured five glycoproteins with two kinds of terminal sugar residues: mannose–mannose and galactose-β(1–4)-N-acetylglycosamine. Moreover, cell-surface glycan profiles of cultured *Symbiodinium* seem to be stable throughout their life history, indicating that *Symbiodinium* may maximize the potential for host recognition by retaining recognition molecules throughout their vegetative growth (Logan et al., 2010). The importance of these membrane-bound glycoproteins during the infection of the sea anemone *Aiptasia pulchella* with *Symbiodinium* has been demonstrated by masking or removing cell surface glycans of the dinoflagellate. Indeed, *Symbiodinium* incubated with either trypsin, α-amylase, N-glycosidase, O-glycosidase or with different kinds of lectins, in order to remove or mask carbohydrate groups, showed a significant decline in the infection rate of an aposymbiotic host (Lin et al., 2000). Similarly, *Symbiodinium* treated to digest or mask α-mannose/α-glucose and α-galactose residues failed to efficiently infect larvae of the fungiid coral *Fungia scutaria* (Wood-Charlson et al., 2006).

These membrane-bound glycoproteins could play a role in *Symbiodinium* recognition by acting as "markers" that could be bound by various types of lectins. SLL-2 is one of these lectin-type proteins. This (N-glycosylated) galactose-binding lectin, isolated from the octocoral *Sinularia lochmodes*, is preferentially localized not only in nematocysts but also on the cell surface of *Symbiodinium*, whether they are present within a host cell or in the coelenteron (Jimbo et al., 2000). The most surprising propriety of SLL-2 is its ability to induce the switch of flagellated and motile *Symbiodinium* cells to a non-motile coccoid form still able to divide (Koike et al., 2004). Such physiological transformations may favor a condition more suited for the establishment of symbiosis. SLL-2 action on *Symbiodinium* seems to present more than a simple carbohydrate binding property as shown by the lack of algal modification in the presence of proteins showing similar properties as SSL-2. Koike et al. (2004) suggested that SLL-2 could be stored within nematocysts and released into the gastrodermal cavity in the presence of micro-algae. Response of these algae to SLL-2 could then act as a first screening process, modifying algal physiology to favor further interaction with the host or causing damages to micro-algae unsuitable for symbiosis. Recently, CecL discovered in *C. echinata* showed effects on cell transformation highly analogous to those of SLL-2. It seems that CecL lectin also has the ability to temporarily suppress the rate of cell division without affecting cell viability and thus, can regulate *Symbiodinium* density in the coral gastrodermis, where only a limited number of algae can be accommodated (Jimbo et al., 2010).

Another lectin has been identified in *A. millepora* (Kvennefors et al., 2008). Named millectin, this lectin possesses a Ca^{2+}-dependent carbohydrate-binding site that preferentially binds to mannose and similar sugars. According to immunohistochemical analysis, millectin is localized in nematocysts present in the epidermal tissue (Kvennefors et al., 2010). This protein is phylogenetically close to collectins, which play a key role in vertebrate innate and adaptive immune responses. Millectin expression is up-regulated in response to lipopolysaccharides and peptidoglycans. Moreover, it has the ability to bind to both gram+ and gram− bacteria (including *Vibrio coralliilyticus*, a coral pathogen) and to various clades of *Symbiodinium* (C1, C2 and A2) in vitro and in vivo (Kvennefors et al., 2008; Kvennefors et al., 2010). When bound to pathogens, millectin prevents their dispersion into the host and induces opsonization for phagocytosis and destruction. Finally, numerous millectin isoforms showing amino acid substitution sites in close proximity to the binding site have been identified. This vast diversity of millectins suggests a probable appearance of some recognition specificity and a role of millectins in the winnowing process (Kvennefors et al., 2008).

Recently, the sequence variation of a putative coral immunity gene, *tachylectin-2*, has been investigated in the coral *O. patagonica*

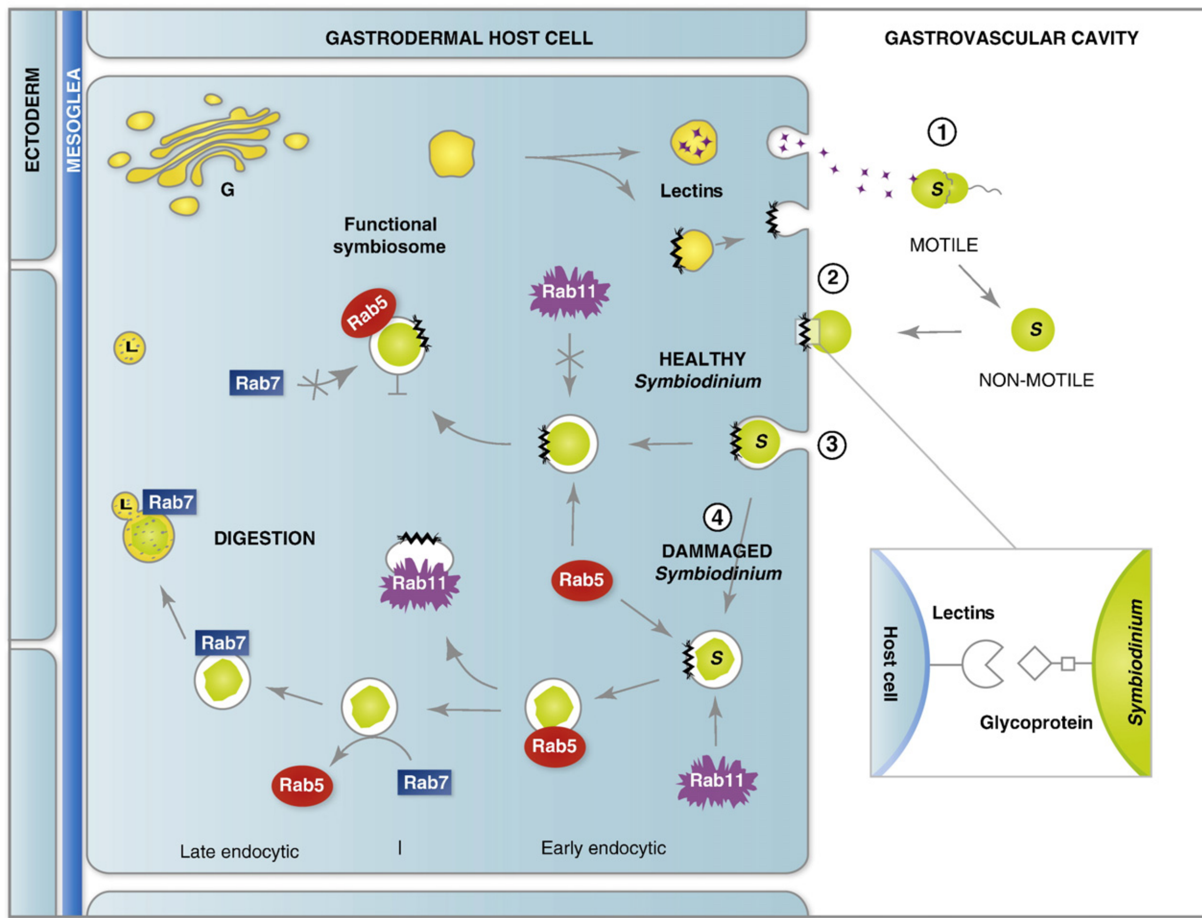

Fig. 2. Schematic illustration of *Symbiodinium* infection of a cnidarian host cell. Motile or non-motile (cyst) *Symbiodinium* enter the gastrodermal cavity of the host. (1) Lectins secreted by the host cell induce motile algae to progression to the cyst stage. (2) Contact and recognition are mediated by other lectins present on the host cell surface and glycoproteins on the surface of non-motile *Symbiodinium*. (3) *Symbiodinium* are phagocyted and directed to the early endocytic compartment. (4) Healthy *Symbiodinium* end in a functional symbiosome while damaged *Symbiodinium* are digested by fusion with lysosomes after transiting through late endocytic compartment. Rab5, Rab7 and Rab11 are respectively involved in the early endocytic compartment, late endocytic compartment and endosome recycling (see text for more information). G: Golgi apparatus, L: Lysosome, S: *Symbiodinium*.

(Hayes et al., 2010). Tachylectin-2 was originally isolated from the Japanese horseshoe crab (*Tachypleus tridentatus*) and has been demonstrated to possess anti-microbial activity (Okino et al., 1995). Nevertheless, a role for Tachylectin-2 in the symbiosis of coral species has not been confirmed yet.

Lectins also appear to play an important role under stress conditions. Indeed, the downregulation of the transcriptional expression levels of two C-type lectins has been observed in aposymbiotic larvae of *A. millepora* (Rodriguez-Lanetty et al., 2009) and nubbins of *P. damicornis* exposed to thermal stress (*PdC-lectin*; Vidal-Dupiol et al., 2009). Conversely, the same gene has been shown to be upregulated when exposed to the pathogen *V. coralliilyticus* under virulent conditions (Vidal-Dupiol et al., 2011). These contrasting results underline the complexity of these lectin/glycan interactions and the tenuous link between the coral physiological response and the establishment of the symbiosis.

2.1.2. Other proteins

Lectin-type proteins are not the only molecules involved in mutual recognition between host and symbiont; other proteins have also been reported to be over-expressed in the presence of symbionts. These include AtSym-02, a glycosylated membrane protein identified in *Acropora tenuis* (Yuyama et al., 2005), and Sym32 identified in *Anthopleura elegantissima* (Reynolds et al., 2000). AtSym-02 may belong to the fasciclin-I (*fasI*) gene family, also known to be involved in cell–cell recognition mechanisms (Yuyama et al., 2005). Sym32 can be found both in anemone protein homogenates and bound to membranes. Sym32 is also expressed more in anemones infected by *Symbiodinium* than in those hosting green algae (zoochlorellae), leading the authors to hypothesize that the symbiont has the ability to control the expression of some host genes (Reynolds et al., 2000).

2.2. Symbiosome formation

After the recognition step, symbionts are internalized by a phagocytic process conducted by host cells, probably through the formation of a lectin-binding complex. Then, a wide range of other cellular processes is necessary for the maturation of *Symbiodinium*-containing phagosomes into functional symbiosomes able to avoid fusion with the host endolysosomal system during endosymbiosis (Chen et al., 2003; Fitt and Trench, 1983a). Indeed, while normal phagosomes mature by fusing with lysosomes, early observations showed that healthy *Symbiodinium*-containing symbiosomes did not reach that stage (Fitt and Trench, 1983a), suggesting the existence of host and/or symbiont-specific molecules involved in maintaining the symbiosis once established.

Although the mechanisms of symbiosome membrane formation are still poorly understood, some evidence in *A. pulchella* indicates that ApARF1 and ApRab genes could be involved in this particular process. ApARF1 is homologous to ARF1, a member of a family that regulates intracellular vesicle transport and its gene expression appeared reduced in symbiotic anemones (Chen et al., 2004b).

However, further investigation is needed to clarify apARF1 function in the establishment of symbiosis. The role of ApRab seems more convincing. ApRab shows similarities with Rab family members, coding for small GTP binding proteins found in many vertebrates, where they assume the regulation of vesicular trafficking, membrane fusion and also the biogenesis and the function of membrane-bound organelles (Hong et al., 2009). To date, four of these ApRab proteins have been shown to play a role in the establishment of symbiosis (Fig. 2).

ApRab7 usually participates in late acidic endocytic and phagocytic pathways (Vitelli et al., 1997). However, in *A. pulchella*, ApRab7 is excluded from symbiosomes containing either resident or newly internalized *Symbiodinium*. ApRab7 can also be found on phagosomes containing heat-killed or PSII-impaired (DCMU-treated) *Symbiodinium*, where it probably promotes their maturation and fusion with lysosomes (Chen et al., 2003).

ApRab5 appears before ApRab7 and is present in the early endocytic and phagocytic compartments where it can promote their fusion (Barbieri et al., 1994). In *A. pulchella*, ApRab5 localizes on symbiosomes containing healthy algae and is absent from symbiosomes in which algae have been damaged by heat or DCMU treatment. These observations suggest that active retention of ApRab5 by *Symbiodinium* participates in their persistence in the host, possibly by preventing ApRab7 binding. Indeed, these two proteins never co-localize in *A. pulchella* vesicles (Chen et al., 2004a).

ApRab11 acts during endosome recycling processes, a necessary step towards maturation (Zerial and McBride, 2001). In *A. pulchella* ApRab11 can be located on phagosomes containing damaged symbionts but is absent from those containing healthy ones (Chen et al., 2005). By actively excluding ApRab11, the authors observed that *Symbiodinium* interferes with the vesicular recycling process and thus prevents the maturation of their symbiosome and its fusion with lysosomes.

ApRab3 is the last of the ApRab family members identified in cnidarians. It appears to be preferentially localized in the compartments of the biosynthetic pathway including both the Trans Golgi Network and a subpopulation of secretory vesicles. In *A. pulchella*, symbiosome formation seems to involve interactions with ApRab3-positive vesicles. However its function in the symbiosis establishment remains to be determined, as phagosomes containing either healthy or damaged *Symbiodinium* or even latex beads progressively accumulate ApRab3-specific labeling (Hong et al., 2009).

2.3. Towards a functional symbiosome

The differentiation of phagosomes into functional symbiosomes obviously requires some particular cellular developments in order to maintain the endosymbiosis and to optimize mutual exchanges. Indeed, the analysis of symbiosome structure conducted on *Aiptasia pallida*, using transmission electron microscopy and immunological techniques, revealed a membrane complex composed of a single host-derived outer membrane and a multilayered inner membrane originating from the algal symbiont (Wakefield and Kempf, 2001; Wakefield et al., 2000). The presence of symbiont thecal vesicles in situ suggests that this multilayered membrane could be the result of a continuing delayed in situ ecdysis cycle (Wakefield et al., 2000). Whatever, the formation of this kind of structure requires some regulation by the cytoskeleton of the host cell and its symbionts, allowing normal host cell physiology (Peng et al., 2010). Moreover, proteomic analyses of isolated symbiosome membranes conducted on *A. pallida*, highlighted the presence of proteins highly similar to GPCR family proteins. These receptor-like proteins are distributed to symbiosome surface and should be involved in various cellular responses and modulation of host gene expression (Peng et al., 2010). The presence of ATP synthase complexes and ABC transporters on this structure demonstrates the pivotal process of molecular transport during the mutualistic association (Peng et al., 2010). Indeed, the symbiosis is considered to be optimally functional when a significant amount of photosynthate generated by *Symbiodinium* is transferred to the host cells. Conversely, the host cells provide symbionts with nutrients and inorganic carbon (see Yellowlees et al., 2008 for review). Finally, the presence of stress or chaperone proteins, like Hsp70 and alpha-B crystallin (Peng et al., 2010), is critical for preventing damage from oxidative stress and ultimately the induction of host cell apoptosis (see Weis, 2008 for review).

3. Ecology of the symbiosis

Larvae or juveniles of most coral species are able to initially associate with either homologous (same as adult colony) or heterologous clades of *Symbiodinium* (Abrego et al., 2009; Harii et al., 2009; Little et al., 2004; Schwarz et al., 1999). Such early association has also been reported in cultured gorgonians and sea anemones (Coffroth et al., 2001; Davy et al., 1997). However during the following months, the initial distribution of symbionts progressively adjusts to the most common distribution found in adjacent adult colonies (Abrego et al., 2009; Coffroth et al., 2001; Gomez-Cabrera et al., 2008; Little et al., 2004). This suggests the presence of specific mechanisms to favor the development of an optimal relationship between the coral and the symbiont.

The symbiosis between *Symbiodinium* and corals is in dynamic equilibrium and remains subjected to constant regulation in response to changes in environmental conditions. In *A. millepora* colonies, the predominance of *Symbiodinium* clade C shifted to clade D following a bleaching event (Jones et al., 2008; van Oppen et al., 2009). Although modifications to the relative densities of clades may impact the thermotolerance or the overall resistance of the coral to stress, acclimation and photobiological flexibility result from a complex integration of the physiological processes of both the host and symbiont. Fragile coral–algae interactions are exemplified by the reversion to the original clades some time after environmental perturbations returned to normal (Thornhill et al., 2006). In contrast to these flexible associations others have been reported to last even through bleaching events (van Oppen et al., 2009). These observations suggest that the mechanisms that regulate, select or maintain *Symbiodinium* clades in corals may vary according to the host and its physiological state.

4. Conclusions

In light of these studies, it seems that cnidarians initially capture their symbionts through nutritional mechanisms, detect and internalize them. The algae then disrupt the host cell machinery to avoid digestion and finally the transformation of the phagosome into a functional symbiosome occurs. An important step in the selection or winnowing mechanism seems to occur in response to binding with secreted and membrane-bound lectin-type glycoproteins. In addition unknown mechanisms also take place to favor particular clades among all successfully integrated *Symbiodinium*. Biomolecular technologies and approaches (transcriptomics, proteomics…) recently applied to the study of coral biology begin to provide additional data on the mechanisms of symbiosis. Those findings combined with holistic approaches of ecological systems will unravel the signals that regulate the density of *Symbiodinium*, the selection processes of clades, the response to stress and reveal a broader picture of the relationship between corals and *Symbiodinium*. This will hopefully also help managers to develop strategies to mitigate cnidarian bleaching and/or to promote post-bleaching re-establishment of symbiosis.

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