

## **Molecular cross-talk between sponge host and microbial associate partner**

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### **Abstract**

Marine organisms especially those that live sessile, as sponges, are well known to have specific relationships with a great variety of microorganisms including bacteria and fungi. As most simple metazoan phylum, the Porifera, which emerged first during the transition from the non-Metazoa to the Metazoa from the common ancestor, comprise wide arrays of recognition molecules, both for Gram-negative bacteria and for Gram-positive bacteria as well as for fungi. They react specifically with effector molecules to inactivate or to kill the attacking microorganisms. The elicitation and the subsequent effector reactions of the sponges towards these microbes are outlined. However, besides of the elimination of bacteria and fungi, some of those taxa are kept as symbionts of the sponges, allowing them, for example, to accumulate the essential element manganese or to synthesize carotenoids. The sponges produce low-molecular-weight bioactive compounds, secondary metabolites, to eliminate the microorganisms. In addition, they are armed with cationic antimicrobial peptides allowing them to defend against invasive microorganisms and, in parallel, to kill or repel also metazoan invaders. The broad range of chemically and functionally different compounds qualifies the Porifera as the most important animal phylum to be exploited as a source for the isolation of new potential drugs. First molecular biological strategies have been outlined to obtain those compounds in a sustainable way, by producing them recombinantly.

**Keywords:** Sponges, secondary metabolites, symbiosis, host defense systems, bacteria, fungi, immune reactions.

## Introduction

During the last two decades the phylogenetically oldest metazoan phylum, the Porifera (sponges), have gained special interest. Through the introduction of molecular biological techniques solid evidence has been elaborated which revealed that this phylum provides a cornucopia of new information which allows a grasping for the understanding of the dynamics of evolutionary processes that have occurred during the Earth period of Ediacara, the time prior to the Cambrian Explosion which can be dated back approximately 540 million years ago (Wang et al., 2010). Importantly, the species of this phylum are rich and valuable sources for bioprospecting, a discipline that promotes the translation of life-science discoveries to practical products or processes targeted for the benefit of the human society.

The elucidation of the evolutionary origin of metazoans in general and of sponges in particular was problematic since the beginning of modern biology in the 18<sup>th</sup> century. First Oken (Oken, 1805) suggested that Metazoa originated from an "Urschleim" which is composed of Infusoria; unicellular organisms are created by spontaneous generation. Then the "Gastraea"-theory was formulated by Haeckel 1874 (1874) who postulated that the Monera were the result of a spontaneous generation from which the Amoeba derived and aggregated to Moraea which subsequently developed to Blastaea and finally to the pelagic Gastraea. With respect to Porifera, James-Clark (1867) proposed the "Choanoflagellate theory" which assumes that flagellated Protozoa aggregate to a colonial sponge. Even about 10 years ago it was still outlined that sponges evolve from biofilms, accumulations composed of bacteria, choanoflagellates and their secreted mucus (Reitner et al., 1999). This view is still cited in modern textbooks, e.g. in Pechenik (2000), in which sponges are assumed to be composed of functionally independent cells.

The introduction of molecular cloning of genes, coding for "informative" proteins increased in a rapid, self-accelerating manner our knowledge on the evolution of the multicellular ancestor to the Porifera. This approach also facilitated the subdivision of this phylum into the three classes, the Hexactinellida, the Demospongiae and the Calcarea (Schütze et al., 1999). The first report in line with this new strategy was on the cloning of a galectin from the demosponge *Geodia cydonium* (Pfeifer et al., 1993; Hirabayashi and Kasai, 1993). This protein is known to be crucially important for cell-cell interactions (Müller et al., 1997); the deduced sponge protein showed high sequence similarity to the corresponding galectins of other metazoan taxa and – what was decisive – no relationship to proteins from other kingdoms. A number of subsequent publications (see: Müller, 1998a and 1998b) on the phylogeny of further functional proteins from sponges clearly demonstrated that the Porifera have to be grouped together with the other metazoan phyla into one monophyletic unit (Müller et al., 1994). The hypothetical ancestor of all metazoan phyla was finally named Urmetazoa (Müller, 2001; Müller et al., 2001a).

Based on calculations described earlier (Schütze et al., 1999) it was outlined that the transition to multicellularity must have occurred about 1,000 Mya. Later in evolution, 700 Mya, the green algae evolved, while the first sponge fossils have been dated back at least to 580 Mya. Hence, sponges lived more than 30 to 50 million years before the Cambrian Explosion, the time of main divergence of metazoan phyla. This conclusion was supported by calculations based on the extent of aa substitutions of two galectins from *G. cydonium*. It was found that these molecules diverged from the galectin isolated from the nematode *Caenorhabditis elegans*, approximately 800 Mya (Hirabayashi and Kasai, 1993; Pfeifer et al., 1993). Furthermore, data especially from studies with a series of Ser/Thr kinases suggested that the Calcarea might be a sister group to higher metazoan phyla (Müller et al., 1998).

### **Structural characteristics of the sponge Bauplan**

Sponges, as the oldest still extant metazoan phylum, are characterized by a simple Bauplan. They are filter-feeding organisms that are usually sessile. Their body is composed of an epithelial layer which surrounds a mesogleal compartment, the mesohyl; this is reticulated in a highly organized manner by a canal system. The cells of the sponges are highly orderly arranged within the body and interact in a tuned manner, providing the animals with the feature of “labor division” not only to build organ (-like) assemblies but also an already complex structural Bauplan.

*Molecules involved in cell-cell interaction:* Sponges have become a classical model for basic studies to understand metazoan cell-cell adhesion. It was Wilson (1907) who introduced this system to experimental biology which then became a traditional model to study both cell-cell- and cell-matrix adhesion (reviewed in: Müller, 1982). With the isolation of a galectin sequence (Pfeifer et al., 1993) as the first cell-cell adhesion molecule, and with an integrin sequence as the first cell-matrix adhesion receptor in the demosponge *G. cydonium* (Pancer et al., 1997; Wimmer et al., 1999), it became overt that sponges contain molecules highly related to those also known to promote adhesion in Protostomia and Deuterostomia.

*Molecules involved in cell-substrate interaction:* The dominant molecule present in the extracellular matrix of sponges which functions as a cell-matrix adhesion molecule is collagen. The corresponding gene was cloned both from the freshwater sponge *Ephydatia muelleri* (Exposito and Garrone, 1990) as well as from the demosponge *Suberites domuncula* (Schröder et al., 2000). Cell surface-spanning receptors, in particular the single- [1-TMR] as well as the seven-pass transmembrane receptor [7-TMR] proteins, serve as receivers for extracellular signaling molecules. The first 1-TMR detected in a sponge was the receptor tyrosine kinase (reviewed in: Müller and Schäcke, 1996); this molecule represents an autapomorphic character for Metazoa. As a first 7-TMR the metabotropic glutamate/GABA-like receptor was identified and characterized in *G. cydonium* (Perovic et al., 1999). This receptor was found to respond to extracellular agonists as well

as antagonists known from metabotropic glutamate/GABA-like receptors present on nerve cells from mammals (Perovic et al., 1999; Kaupmann et al., 1997).

*Molecules involved in morphogenesis:* In the extracellular space of sponges morphogens have been identified, e.g. the endothelial-monocyte-activating polypeptide (Pahler et al., 1998) or myotrophin (Schröder et al., 2000). Both are very likely involved in the organization/differentiation of cells within the sponge body.

*Transcription factors:* During development of animals, a set of genes, most of them transcription factors that are responsible for cell fate and pattern determination are expressed. Among them are the T-box, Forkhead and Homeobox gene families. From the T-box family, *Brachyury* is involved in the formation and differentiation of the third germ layer, the mesoderm, in triploblastic animals. Two T-box genes have been isolated from the sponge *S. domuncula*; a *Brachyury* gene and a homologue of the Tbx2-3-4-5 genes from chordates, which, interestingly, are involved in the formation of limbs (Adell et al., 2003a). Functional studies demonstrated that the expression of the *Brachyury* gene is upregulated in differentiating sponge cells during formation of canal-like structures (Adell et al., 2003a). Based on this observation we postulated that already in sponges a primordial axis formation is genetically fixed. This assumption was subsequently confirmed by the isolation and phylogenetic characterization of five members of the forkhead gene family from the sponge *S. domuncula* (Adell et al., 2002; Adell et al., 2004). Forkhead proteins form a subfamily within the large group of helix-turn-helix proteins. They are responsible for a wide range of functions and play key roles in early developmental processes, during organogenesis and also for the function of the major organs and tissues in the adult animals.

*Genes in S. domuncula indicative for Wnt signaling:* The Wnt signaling pathway controls a cell communication system which regulates cell-fate decisions, tissue polarity and morphogenesis. The Frizzled protein is the membrane receptor for the Wnt secreted glycoproteins. Through the canonical Wnt signaling pathway, the activated Frizzled binds to Dishevelled (Dsh), which leads to the stabilization and accumulation of  $\beta$ -catenin in the nucleus, where it activates the TCF/LEF transcription factor. All those molecules could be isolated from *S. domuncula*, e.g. the Frizzled receptor (Adell et al., 2003b).

*Molecules present in tight junctions:* The screening for a gene encoding a tight junction scaffold protein from a sponge, at first from *S. domuncula*, was also successful. The membrane-associated guanylate kinase with inverted arrangement (MAGI) could be isolated (Adell et al., 2003a). Moreover, the existence of one tetraspan receptor, tetraspanin, in *S. domuncula* has been reported. The tetraspanins belong to a group of hydrophobic proteins comprising four transmembrane domains with a series of conserved amino acid (aa) residues in the extracellular loops.

### **Spicule network in sponges: A unique skeleton allowing axis formation of the individuals**

In living organisms four major groups of biominerals exist; (1) iron compounds, which are restricted primarily to Prokaryota; (2) calcium phosphates, found in Metazoa; (3) calcium carbonates, used by Prokaryota, Protozoa, Plantae, Fungi, and Metazoa; and (4) silica (opal) present in sponges and diatoms (Müller, 2005). The formation of the skeleton is a multifaceted process and could be explained in an exemplary way for Porifera. Even though these animals comprise the simplest Bauplan, the formation of their structure from biominerals is already highly complex. The underlying biological and molecular biological mechanisms have been worked out to some extent (reviewed in: Müller et al., 2009c). Silica is the major constituent of sponge spicules in the classes Demospongiae and Hexactinellida. The spicules of these sponges are composed of hydrated, amorphous, noncrystalline silica. The secretion of spicules occurs in Demospongiae in specialized cells, the sclerocytes. There, silica is deposited around an organic filament. The synthesis of spicules is a rapid process; e.g. the 100- $\mu\text{m}$  long megascleres can be formed within 40 h, as has been shown for *Ephydatia fluviatilis*. Studies revealed that the dominant protein that triggers the formation of monomeric to polymeric silica is an enzyme which was termed silicatein (Morse, 1999). The formation of the siliceous spicules in sponges is genetically controlled. This process also initiates the morphogenesis phase. A major step forward to elucidating the mechanism of spicule formation on a molecular level was the finding that the “axial organic filament” of the siliceous spicules is in reality the enzyme, silicatein, which mediates the apposition of amorphous silica and hence spicule formation. The skeletal framework of sponges is highly ordered, as analyzed for the demosponge *Lubomirskia baicalensis* (Müller et al., 2006a) and the hexactinellids *Euplectella aspergillum* and *Monorhaphis chuni* (reviewed in: Wang et al., 2009). Growth of those sponge species proceeds in a radiate accretive manner, reflecting that highly ordered growth zones are delimited by growth lines. These growth centers are constructed by an ordered arrangement of the spicules within the body.

### **Symbiotic relationship between sponges and microorganism**

One major reason why the sponges were so successful during their long evolutionary history is the molecular toolkit they developed to survive adverse and extreme climatic conditions (Müller et al., 2007c). They developed effective “resistance” molecules, suitable for the survival of “snowball earth” episodes. Sponges possess and express genes that allow them (1) to resist against adverse temperatures, and (2) to survive food restriction, and (3) protect them against ultraviolet radiation (reviewed in: Wang et al., 2010). (ad 1) Sponges have cryoprotective proteins, e.g. galactose-specific lectins, which stabilize the cell membrane against freezing and drying. Furthermore, the siliceous sponges comprise  $\beta/\gamma$ -crystallins, proteins that contain only small amounts of water and hence remain functionally active during adverse temperatures. (ad 2) Food deprivation and restriction conditions surely existed, at least initially, during the change in living conditions from an open marine environment to an ice-covered ocean. This critical situation has been compensated by

the establishment of a symbiotic relationship with microorganisms (Thakur and Müller, 2005c). The siliceous sponges possess a complex array of molecules that allow them to interact with Gram-positive (Thakur et al., 2005a; Thakur et al., 2005b; Thakur and Müller, 2005c) and Gram-negative bacteria (Wiens et al., 2005) as well as with fungi (Perović-Ottstadt et al., 2004a). The resulting eukaryotic–prokaryotic “labor division” provided the sponges with a flexible and rapid adaptation network towards changing environmental climate conditions. As an example, the endemic freshwater sponges in Lake Baikal can cope with the freezing season during which the Lake is covered by a meter-thick ice layer (Müller et al., 2006b). The cellular and molecular basis for this survival strategy is the symbiotic relationship between these sponges and specific single-celled algae. The algae autotrophically produce organic carbon molecules, primarily glycerol.

The sponge-microbial association has been a research topic for long time (Vacelet, 1975). During the feeding process, bacteria from the surrounding seawater are continuously swirled in by the sponge-driven currents and most of these bacteria are retained in the sponge body. Interestingly, bacterial density in sponges is attributed to the temporal variations in the surrounding environment and also linked to the irrigation system of the sponge (Imhoff and Stöhr, 2003). Apart from this, it has been proved that some bacteria permanently reside in the sponge mesohyle, pointing to a close interaction between the host and the associated bacteria (Webster and Hill, 2001; Friedrich et al., 2001; Böhm et al., 2001). It has been estimated that in some sponge species, as much as 40% of animal biomass must be attributed to the bacteria, which exceeds the concentration of bacteria in seawater by two to four orders of magnitude (Friedrich et al., 2001).

The first experimental data, gathered by application of molecular biological techniques came from studies with the sponge *Halichondria panicea* (Althoff et al., 1998; Müller, 1999). *H. panicea* is a marine sponge which abundantly occurs in the Adriatic Sea, North Sea, and Baltic Sea (Fig. 1a). Applying the technique of polymerase chain reaction (PCR) analysis of selected 16S rRNA gene segments, the microbial population had been analyzed. DNA was isolated from sponge material that had been sampled near Rovinj (Adriatic Sea), as well as near Helgoland (North Sea) and Kiel (Baltic Sea). The data gathered indicated that in all samples bacteria belonging to the genus *Rhodobacter* (Proteobacteria, subdivision a) were dominant, suggesting that these bacteria live in a symbiotic relationship with that sponge. The 16S rDNA sequences were aligned with similar sequences from *Roseobacter algocolus* and *Rhodobacter veldkampii* (Fig. 1b). A phylogenetic tree was constructed that revealed that the bacterial sequences obtained from *H. panicea* from the North Sea [HpaNS1-3], the Baltic Sea [HpaBS] and from the Adriatic Sea [HpaAS1 and HpaAS2] were not identical to those of *R. algocolus* and *R. veldkampii*. Importantly, the tree revealed that the bacterial sequences from the North Sea and the Baltic Sea clustered together, while those from the northern Adriatic Sea were distantly related. The *R. veldkampii* sequence shared more similarity with the two branches of the *H. panicea* bacterial sequences than did the *R. algocolus* sequence. From these data we

concluded that the marine sponge *H. panicea* harbors a dominant “very likely symbiotic” bacterial species belonging to the genus *Roseobacter*.

### **Bioprospecting**

The field of bioprospecting of Porifera may be of tremendous potential benefit for humans from the applied point of view. Taking into account that the chemical diversity of natural bioactive compounds is much higher than that of compounds obtained by standard combinatorial chemistry, and that natural compounds display an impressive selectivity, the high value of the secondary metabolites from natural resources in general and from sponges in particular can only be roughly imagined (Wessjohann, 2000). It is conceivable that, especially in preliminary screening programs aimed to discover new lead molecules directed against newly discovered targets, the natural compounds are superior to those generated by combinatorial chemistry (Harvey, 2000). The reason for the high selectivity and potency of the natural secondary metabolites is explainable by virtue of the evolutionary sharpening of the compounds. During the long evolutionary history of the Porifera, during the 800 Myr, the optimal specificity of the bioactive compounds against a particular organismic disorder could be shaped. On this background the highly diverse chemical compounds from sponges which are likewise provided with a diverse bioactivity are extremely valuable. This exiting research field was pioneered by Bergmann (Bergmann and Feeney, 1951) and Scheuer (1990) and culminated with the discovery of arabinofuranosyl cytidine [ara-C], a highly potent anti-leukemic agent (Fig. 2b; Zahn et al., 1972; Müller et al., 1972) that had been isolated from the sponge *Cryptotethya crypta*. A related species, *Tethya lyncurium* (Fig. 2a; *T. aurantium*), is likewise rich in bioactive compounds, among which a complement-related 21 kDa pore-forming peptide should be highlighted (Mangel et al., 1992). This protein, also called *Tethya* hemolysin, forms stable transmembrane pores with an effective diameter of about 2-3 nm in membranes of erythrocytes. Treatment of erythrocytes with *Tethya* hemolysin caused a rapid efflux of intracellular  $K^+$  and ATP, and simultaneously a rapid influx of extracellularly added  $Ca^{++}$  and sucrose. In negative-staining electron microscopy, target erythrocyte membranes that had been exposed to purified *Tethya* hemolysin displayed ultrastructural lesions but without visible pores (Fig. 2c). Bio-inspired by ara-C, the chemical synthesis of 9- $\beta$ -D-arabinofuranosyl-adenine [ara-A], a potent anti-herpes simplex virus drug (Müller et al., 1977) was performed. As an example, in Japan it is one of the most applied ointments against this disease [Arasena-A® (Mochida Pharmaceutical Co; Tokyo)]. Ara-A had been identified as a highly potent competitive inhibitor for the herpes simplex virus specific DNA polymerase, an enzyme first isolated by Müller et al. (1973).

The diversity of secondary metabolites produced in sponges has been highlighted in a large number of reviews (e.g. Faulkner, 1995; Sarma et al., 1993). They range from derivatives of amino acids and nucleosides to macrolides, porphyrins, terpenoids to aliphatic cyclic peroxides and sterols. This diversity reflects the efficient mechanisms of combinatorial biochemistry which the

animals have acquired during their evolutionary history. The question arises if the sponges as the host of associated/symbiotic bacteria are the real producers or whether the microorganisms which they harbor produce these substances. Recent data strongly favor the view that the microorganisms are the main producers of the natural products which are subsequently stored and accumulated in the sponge as chemical defense, although sponge metabolites can also be produced by specific sponge cells. As an example the phosphatase inhibitor okadaic acid can be cited (Tachibana et al., 1981). This compound was first isolated from the sponge *Halichondria okadai* and later found to be produced by the free-living microalgae *Prorocentrum lima* and perhaps even by bacteria which are associated with them (Murakami et al., 1982).

Very likely produced by the sponge host are the sesquiterpenoid quinones and hydroquinones, e.g. avarone and avarol (Fig. 2e). These compounds are produced in the demosponge *Dysidea avara* (Fig. 2d; Müller et al., 1985). Furthermore, those compounds, the avarol/avarone redox couple, display potent antileukemic activity *in vitro* and also *in vivo*, with a therapeutic index of 11.7 for avarone and 4.5 for avarol (Müller et al., 1985b). Lastly, the compounds inhibit selectively human immunodeficiency virus [HIV] in H9 lymphocytic cells (Sarin et al., 1987). Light and transmission electron microscopic studies revealed that avarol is probably stored only in spherular cells of the sponge (Müller et al., 1986). There, it had been found to be compartmented in intracellular cytoplasmic vesicles in a paracrystalline form; by that it protects the host cells against its inhibitory effect. This finding had been confirmed (Uriz et al., 1996). Based on these data it has been proposed that avarol is involved in regulating the bacteria with which the sponge is symbiotically associated; the antibacterial activity of avarol has also been described (Seibert et al., 1985).

A further potent cytostatic agent is aeroplysinin-1 and the structurally related dienone (Fig. 2h) (Fattorusso et al., 1970; Koulman et al., 1996) which are both synthesized by the demosponge *Aplysina aerophoba* (Fig. 2g). The bromotyrosine derivatives are likely to be produced and stored in different compartments of the sponge or sponge cells. Experimental evidence has been presented that these compounds are enzymatically cleaved from precursor compounds after the sponge is damaged, for example, by predators (Ebel et al., 1997). The enzymatically formed products [aeroplysinin-1 and dienone] have been found to be more toxic than the parent compounds, suggesting that these compounds are involved in the defense of this sponge species. An accumulation of aeroplysinin-1 is seen at the surface of the sponge, while the dienone is predominantly found in the more central region of the tube-like sponge (Fig. 2i); Kreuter et al., 1990.

In the previous paragraph the antiviral activity of 9- $\beta$ -D-arabinofuranosyl-adenine was addressed. Building on this finding we asked where the (potential) virus particles are located within the sponge mesohyl. By using the freshwater sponge *Lubomirskia baicalensis* and applying the technique of PCR one transposon [Baikalum-1] has been detected in this species. Baikalum-1



embraces a continuous open reading frame, putatively coding for a polyprotein that consists of nucleocapsid, protease, reverse transcriptase, RNase H, and integrase, all proteins/enzymes characteristic of retrotransposons (Wiens et al., 2009); Fig. 2k. Baikalum-1 was discovered in all freshwater sponge species endemic to Lake Baikal, as well as in cosmopolitan sponge species that inhabit a Lake Baikal-feeding rivulet. Retrotransposons are highly related to retroviruses. Both have a similar structural organization, and nucleotide sequences of the two are similar; the most important difference between retrotransposons and retroviruses is the existence of an envelope gene in the genome of the retroviruses. Such a virus has been identified and completely cloned from the sponge *S. domuncula* (Fig. 2j). Using the reverse transcriptase gene segment as a probe the location of the *S. domuncula* retrovirus could be determined in the surface layers that surround the aquiferous canal system (Fig. 2l). Until now it is not yet clear which function could be attributed to the virus in *S. domuncula*.

### **Bacterium-sponge relationship**

The biology of bacterium-sponge relationship has elicited considerable interest among researchers, as marine sponges have been considered as a rich reservoir of bioactive compounds. The role of sponge associated microorganisms in the synthesis of compounds of biological interest is the subject of scientific debate, as in some cases, since these bacteria are reported to produce bioactive metabolites (e.g. Imhoff and Stöhr, 2003). The role of sponge associated bacteria in the host eubiotic defense has also been highlighted in our studies (Thakur et al., 2003). However, there are some conflicts about the origin of compounds in sponges; i.e., surfactin like depsipeptides were originally isolated from a marine sponge. Later it could be shown that the bacterium, *Bacillus pumilus*, which is associated with the sponge, is the producer of these compounds (Kalinovskaya et al., 1995). Similar was the case with okadaic acid, a cytotoxic compound, which is now commercially available for biochemical research. Recently, our group has isolated a compound, 2-methylthio-1,4-naphthoquinone, from an unknown bacterium, associated with marine sponge *D. avara*, which showed strong anti-angiogenic and antimicrobial properties (Müller et al., 2003) These observations highlight the importance of sponge-bacteria association not only for biodiversity research but also for biotechnology.

Like bacteria, sponges also exhibit an association with marine fungi which produce bioactive substances. In the course of those studies, Sorbicilactone A, a novel-type alkaloid was reported from a sponge (*Ircinia fasciculata*) associated fungi, *Penicillium chrysogenum*. This compound showed promising activities in several mammalian and viral systems, qualifying it for therapeutic human trials (Bringmann et al., 2003).

Polyketide biosynthetic genes from bacteria and fungi have been cloned, sequenced and expressed in heterologous hosts. Polyketide synthases (PKSs) are a class of enzymes that are

involved in the biosynthesis of secondary metabolites such as erythromycin, rapamycin, tetracycline, lovastatin and resveratrol (Gokhale and Tuteja, 2001). Some sponge associated bacteria with antimicrobial assets are also detected to have polyketide synthases gene clusters and our group is presently working in this area.

### **Potential functional cooperation of manganese-oxidizing bacteria and sponges**

Recently, we could demonstrate for the first time that a Mn(II)-oxidizing bacterium, a *Bacillus* strain [termed BAC-SubDo-03] coexists with *S. domuncula* (Wang et al., 2011). The bacterial strain has been isolated and cultivated. The functional analyses revealed that Mn(II) stimulated bacterial growth and induced sporulation. Furthermore, it was demonstrated that these bacteria immobilized manganese on their cell surface (Fig. 3b). Co-incubation studies of BAC-SubDo-03 with 100  $\mu$ M MnCl<sub>2</sub> and >1  $\mu$ M of CuCl<sub>2</sub> showed an increase in their Mn(II)-oxidizing capacity. In order to prove that a multicopper oxidase-like enzyme(s) [Fig. 3a; MCO] exists in the cell wall of the *S. domuncula*-associated BAC-SubDo-03 *Bacillus* strain the gene encoding this enzyme was cloned (*mnxG-SubDo-03*). Sequence alignment of the deduced MCO protein (MnxG-SubDo-03) revealed that the sponge bacterium clustered together with known Mn(II)-oxidizing bacteria. Based on these findings, we assume that BAC-SubDo-03 might serve as a Mn reserve in the sponge providing the animal with the capacity to detoxify Mn in the environment.

It appears that Mn(II)-oxidizing bacteria are essential for the maintenance of the physiological Mn concentrations in the sponge (Wang et al., 2011). Since only minute levels of Mn usually exist in the surrounding seawater a substantial accumulation of Mn on the surface of the bacteria is proposed. It can be postulated that, if required, the release of bacterial-precipitated Mn(IV) suffices the physiological need for Mn. According to the reaction mechanisms recently described for the MCO from *Escherichia coli*, and based on the experimental proven four-electron reduction of dioxygen by the MCO, a reduction and, in turn, a dissolution of Mn(IV) to Mn(II) may be postulated (Fig. 3c). Furthermore, we postulated that the presence of BAC-SubDo-3 bacteria is required as a protection against higher, toxic concentrations of Mn; after oxidation of Mn(II) to Mn(IV) the ion becomes insoluble (Bargar et al., 2000).

### **Sponge-associated bacteria as producers of carotinoids**

Retinoic acid is one central signaling molecule involved in Bauplan formation of vertebrates (Marlétaz et al., 2006). In our previous studies we could identify the first retinoid X receptor (RXR) in the sponge *S. domuncula* (Fig. 4a; Wiens et al., 2003a). First functional investigations with primmorphs, the three-dimensional cell aggregates (Fig. 4b), revealed that retinoic acid controls the expression of a LIM homeodomain protein, a transcription factor whose expression parallels the formation of the aquiferous canals in sponges. From studies with vertebrates it is established that retinoic acid operates both on molecular level and on cellular level during axial patterning (Durst

et al., 1997), especially during early embryogenesis. Finally, earlier studies showing that retinoic acid has a stimulatory effect on spicule formation in growing cell aggregates (Imsiecke et al., 1995). In view of these preparative results we determined the spectrum of retinoids in *S. domuncula* and found that  $\beta$ -carotene comprises among those compounds the highest percentage (Müller et al., 2011c). The analytical data revealed that the highest levels exist in red specimens with 137 nmoles of  $\beta$ -carotene per g of dry tissue, while significantly lower levels are found in magenta or whitish colored specimens. The level of  $\beta$ -carotene in those animals is strongly correlated with the length of cultivation in aquaria after sampling of the specimens from the sea. Already after 6 days in the tank the concentration of  $\beta$ -carotene dropped significantly, if compared with specimens that were analyzed immediately after sampling. Experiments with primmorphs kept under controlled conditions in the presence of antibiotics showed that these aggregates almost completely lost their  $\beta$ -carotene after 28 days of incubation. From these data we conclude that the level of  $\beta$ -carotene in tissue/primmorphs is correlated with the bacterial load. In *S. domuncula* the bacteria are encapsulated into bacteriocytes (Böhm et al., 2001); Fig. 4c. An experimental confirmation of this assumption was obtained by a semi-quantitative PCR analysis. Thus, the data strongly suggest that bacteria produce retinoid precursors such as beta-carotene and store them in cells that surround the spicules, the chromocytes. This  $\beta$ -carotene compound undergoes cleavage to *all*-E-retinal via the  $\beta,\beta$ -carotene-15,15'-dioxygenase (Fig. 4e). Parallel to this pathway *all*-E-retinal is reversibly oxidized to retinaldehyde formed via the enzyme short-chain dehydrogenase/reductase [SDR]. In turn *all*-E-retinal is enzymatically oxidized to retinoic acid via the SDR. The underlying genes, encoding these enzymes, have been identified and cloned from *S. domuncula* (Müller et al., 2011c).

Surprising was the finding that depending on the time of maintenance of the sponges in aquaria the size of the silintaphin-2 protein (Wiens et al., 2011i) is truncated to a 13 kDa protein. Subsequent protein sequencing revealed that the *N*-terminus of the short silintaphin-2,  $\Delta$ silintaphin-2, comprises an Asp moiety. Further analysis revealed that this processing of silintaphin-2 is mediated by the protease bone morphogenetic protein-1 [BMP-1] under cleavage at the dipeptide Ala↓Asp site. During this process silintaphin-2 becomes activated and functions as a  $\text{Ca}^{2+}$  ion delivering protein;  $\text{Ca}^{2+}$  is required for the formation of the silicatein/galectin complex that allows the silicatein to deposit poly(silicate) around the growing spicules (Schröder et al., 2006d).

These data show that retinoids, most likely produced by sponge-associated bacteria, play an essential role surely not only in the intermediate metabolism, but also in the formation of the siliceous spicules (Fig. 4d). They underscore that the cascade-like interaction of the structural components that form the organic cylinder around the growing spicules, silicatein-galectin, is controlled by the mature,  $\text{Ca}^{2+}$ -binding silintaphin-2. This organic cylinder functions as a scaffold for the formation of the siliceous shell during growth of the spicule. The processing of silintaphin-2 is probably mediated by the protease BMP-1. One key signal for the assembly of the organic cylinder

around the growing spicule is retinoic acid which is produced in chromocytes from carotenoid precursors that originate from microorganisms. Hence, in the most ancient still existing metazoan phylum, retinoic acid plays a similar/the same signaling function as in higher vertebrates during skeleton formation (Campbell and Kaplan, 1992).

### **Defense by antimicrobial peptides**

It is well established, even though it came unanticipated, that sponges comprise besides the wide spectrum of directly acting, inhibitory secondary metabolites, a very efficient and effective immune system (Müller et al., 1999b). Most of the biological and molecular biological studies on immune responses in sponges have been performed with the demosponge *S. domuncula*. For example, fungi are recognized by specific D-glucan carbohydrate receptors (Perović-Ottstadt et al., 2004a) and subsequently eliminated by expression of a fibrinogen-like protein. Gram-positive bacteria are killed by an increased induction of lysozyme (Thakur et al., 2005b), resulting in lysis of the microorganisms and subsequent phagocytosis. Gram-negative bacteria are recognized by sponges through a lipopolysaccharide-interacting protein and eliminated by expression of perforins through activation of a MyD88-dependent signaling pathway (Wiens et al., 2005). It should be noted that most of the recognition and effector molecules display high sequence similarities to polypeptides identified in deuterostomians. However, sponges also produce molecules that are only found in some protostomian taxa, e.g. a GlcNAc/GalNAc-binding lectin (tachylectin; Schröder et al., 2003c) but not in deuterostomia.

Considering the large-scale and diverse number of noxious prokaryotic and eukaryotic organisms, it appeared to be compelling also to screen for antimicrobial peptides in sponges. Among those peptides, the cationic antimicrobial peptides that are produced in higher metazoans act as host defense against microorganisms (reviewed in: Zhua et al., 2005). Two of them, the defensins and the ASABF (*Ascaris suum* antibacterial factor) are grouped to those peptides (see: Nijnik and Hancock, 2009). Recently, by screening the *S. domuncula* database that comprises over 40,000 sequences (<https://octavia.vk.medizin.uni-mainz.de/login.cgi>), several ESTs (Expressed Sequence Tag) were identified that displayed defensin-like sequence similarity. More specific, the deduced protein shared highest relationship with the antimicrobial peptide, designated as ASABF. ASABF peptides were first identified in *Ascaris suum* and subsequently also in *Caenorhabditis elegans* (Kato et al., 2002). The ASABF peptides and the defensins are both cysteine-rich antimicrobial peptides. While the defensins are widespread both in protostomians and in deuterostomians (Selsted and Ouellette, 2005), the ASABF peptides are, among the triploblastic animals, restricted to the nematodes (Minaba et al., 2009). In contrast to the defensins which comprise two to three antiparallel  $\beta$ -sheets that are linked together by three to four intramolecular disulfide bridges, the characteristic ASABFs have eight cysteine residues that form four disulfide bridges (Kato, 2007).

Very recently, the first sponge ASABF has been identified on molecular level and the protein was prepared in a recombinant manner (Fig. 5a). The mature peptide from *S. domuncula*, comprising 64 aa (amino acid) residues, is cationic (isoelectric point >8), a characteristics that it shares with the other known ASABFs. The sponge molecule comprises the characteristic CS $\alpha$  $\beta$  (cysteine-stabilized  $\alpha$ -helix and  $\beta$ -sheet) structural motif (Fig. 5b). The recombinant sponge ASABF was found to be biologically active, as microbicidal (as expected) peptide which also possesses hemolytic activity. Furthermore, we elucidated for the first time that an ASABF peptide can also be toxic for metazoans. It was shown that the ASABF peptide is toxic for the marine gastropod/mollusc *Bittium* sp. which settles on apoptotic patches on the sponge surface (Schröder et al., 2002). The gastropod is attracted by the sponge after induction of the *3-hydroxyanthranilate 3,4-dioxygenase (HAD)* gene, through quinolinic acid, a metabolite of the kynurenine pathway. In parallel with the increase in the steady-state level of *HAD* the expression of a gene encoding a *caspase* is induced (Wiens et al., 2003b) that is known to be one key enzyme induced during apoptosis in *S. domuncula*. Both effector limbs, grazing of the gastropod and the caspase-dependent apoptosis pathway, lead to the removal of the apoptotic tissue. Temporarily delayed, expression of the *ASABF* gene encoding the functionally active peptide occurs, a process that results in the elimination of the gastropods. The data schematically summarized in Fig. 5c show that in the sponge system the evolutionary most ancient ASABF peptide is involved not only in the microbicidal defense but also in the elimination of eukaryotic invaders. Both functions contribute to the rich arsenal of the innate immunity in sponges, the most basal metazoan phylum.

### **Molecular recognition between sponges and their associated microorganisms**

Sponge-bacteria interactions are probably among the oldest host-bacteria interactions, dating back more than 500 million years in time (Wilkinson, 1984). Several studies have revealed that permanent associations exist between certain host sponges and specific microorganisms, however their interactions remained largely unknown (Althoff et al., 1998, Friedrich et al., 1999; Schmidt et al., 2000). Moreover, sponges may also succumb to microbial and fungal infections which result in the disintegration of the sponge fibers/tissue and ultimately lead to sponge death (Vacelet et al., 1994). A more detailed understanding of the molecular interaction between sponges and their associated microorganisms was achieved by application of the technique of molecular biology. This approach allowed a bottom-up understanding of the cause and effect reactions proceeding during the host [sponge] and associate microorganism interaction. Based both on the similarity of the motifs existing within a protein, deduced from a sponge nucleotide sequence, and the subsequent functional analyses and verifications of these molecules in the cellular systems [intact organism or in the primmorph model] an elucidation of the real cross-talk responsive and signaling transduction pathways became possible.

It is known that sponges possess molecules whose domains are similar to those functioning during the mammalian immune response (Müller et al., 1999b). As examples, elements of the innate immune system, e.g. scavenger receptor cysteine rich domains and macrophage derived cytokine-like molecules have been identified which are upregulated during auto- and allografting experiments. Furthermore, the (2'-5') oligoadenylate synthetase system might be mentioned that exists in sponges as well (Schröder et al., 2008). Precursors of the adaptive immune system have also been identified and were shown to be functional in sponges (Müller et al., 2004).

**INTERACTION WITH GRAM-NEGATIVE BACTERIA:** Like in mammals, also in sponges the lipopolysaccharide (LPS)-mediated pathway exists and functions after exposure to Gram-negative bacteria (Fig. 6). LPS, an endotoxin derived from the outer cell wall of Gram-negative bacteria, binds to the cell surface molecule CD14 (Ulevitch and Tobias, 1994). In mammalian system, this interaction is mediated by LPS-binding protein(s) (Scott et al., 2000). CD14, a plasma membrane linked molecule, presents LPS to a specific transducer resulting in an enhanced production of reactive oxygen metabolites and gene expression (reviewed in: Jiang et al., 2000). Downstream, serine-threonine directed MAP [mitogen-activating protein] kinases transmit the signals, elicited from the interaction of LPS with the surface molecules of the mammalian cells. The MAP kinases are grouped into three main families, the extracellular signal-regulated kinases [ERKs], the p38 kinases and the c-jun N-terminal kinases [JNKs] (Seeger and Krebs, 1995). Using *S. domuncula* as a model for demosponges we demonstrated (Böhm et al., 2001) that cells exposed to LPS, respond with the activation [phosphorylation] of the stress-activated MAP kinases p38 and JNK. Furthermore, molecular and functional studies disclosed that this sponge responds to Gram-negative bacteria with an interaction of LPS and distinct receptors, integrated into the cell-surface, the LPS-binding protein (Wiens et al., 2005).

The cDNA encoding the LPS-binding protein was isolated and the protein was expressed. The data suggested that binding of LPS to the LPS-binding protein results in dimerization of this molecule (Wiens et al., 2005); Fig. 6. Subsequently, this surface-associated protein interacts with MyD88, after exposure of the animals towards LPS. The sponge MyD88 is composed of two protein interaction domains, a TIR domain (present in Toll-like receptors and in MyD88) and a death domain (present in MyD88 and IRAK). Northern blot experiments and *in situ* hybridization studies showed that the level of LPS-binding protein does not change after LPS treatment, while MyD88 is strongly upregulated. As an executing molecule of this pathway (LPS-binding protein - MyD88) the macrophage expressed protein, a perforin-like molecule ( $M_r$  74171), was identified and its cDNA was isolated. The gene encoding the perforin-like protein is highly expressed after LPS treatment, especially at the surfaces of the animals. The recombinant protein comprises biological activity and eliminates Gram-negative bacteria (*E. coli* and BL21 [sponge-associated Gram-negative bacteria] were used). It is inactive against the Gram-positive bacterium *Staphylococcus aureus*. These data

indicate that *S. domuncula* is provided with an innate immune system against Gram-negative bacteria. The ligand LPS (a pathogen-associated molecular pattern) is recognized by the pattern-recognition receptor (LPS-binding protein) which interacts with MyD88. A signal transduction is established which results in an elevated expression of both MyD88 and the macrophage expressed protein, as executing proteins.

**INTERACTION WITH GRAM-POSITIVE BACTERIA:** Since their discovery in 1922 (Fleming and Allison, 1922) lysozymes have been known to be present in different tissue fluids of mammals where they act as bacteriolytic enzymes for Gram-positive bacteria. After identification of their amino acid sequence (Jollès et al., 1963) and their functional role in mammals, related enzymes were found also in plants (reviewed in: Jollès, 1996). The metazoan lysozymes are grouped into the class of c-type (chicken) lysozymes, i-type (invertebrate) lysozymes, and lactalbumin and display considerable overall sequence homology (Bachali et al., 2002). They catalyze the hydrolysis of the  $\beta$ -1,4-glycosidic linkage between N-acetyl muramic acid and N-acetylglucosamine of peptidoglycans (PPGs) of Gram-positive bacteria (reviewed in Jollès, 1996). Lysozymes are quite ineffective against Gram-negative bacteria (McKenzie and White, 1991). When PPGs or whole bacteria are taken up by metazoan cells via endocytosis they cause inflammation (reviewed in Ginsburg, 2002).

PPGs were used as a probe for Gram-positive bacteria to clarify the response of *S. domuncula* on molecular level (Thakur et al., 2005b). The gene expression studies revealed that the sponge reacts to this polymer with an increased expression of both the *lysozyme* and the *AdaPTin* [adaptor-related protein complex 1 molecule] gene *AP-1*. AP-1 together with the other members of the adaptor protein [AP] complexes, AP-2, AP-3 and AP-4, are the key molecules required for vesicle formation and synaptic vesicle recycling (Glyvuk et al., 2010), processes that drive the process of endocytosis. Whole bacteria or their outer PPGs are known to be endocytosed (Ginsburg, 2002), a process that is followed by an activation of an overall cell metabolism, including induction of inflammation and the synthesis of a series of hydrolytic enzymes. Among those are the lysozymes that degrade PPG (reviewed in Jollès, 1996). In *S. domuncula* the expression of the *lysozyme* gene is upregulated. The deduced protein shares sequence similarity to both the i-type and c-type lysozymes as well as to the lactalbumins. This observation provided the first clue that in *S. domuncula* bacterial cell-wall proteins elicit the same response and increase the lytic (lysozyme) activity, as in invertebrates and vertebrates. In *S. domuncula* the *lysozyme* gene is expressed especially in cells that are embedded in the mesohyl compartment which is lined by a surface layer of pinacoderm cells. The mesohyl in sponges is very bulky and composed of lectins, collagen, and glycoproteins (Müller, 1982). There, the 25- $\mu$ m large cells, intensively stained with hematoxylin/eosine, were identified as those that strongly express the lysozyme gene. Based on earlier cytological and functional observations these 25- $\mu$ m large cells are the gray cells (Boury-Esnault and Doumenc, 1979). These cells have been described as eosinophiles displaying

phagocytic activity (Sarà, 1955). In the mesohyl compartment especially the symbiotic bacteria occur in clusters in specialized cells, the bacteriocytes (Fig. 4c) (Lévi and Porte, 1962). This localization has also been described for *S. domuncula* (Böhm et al., 2001). Striking and impressive was the finding that antibodies against the sponge lysozyme stain bacteria very brightly and (almost) none of the other cells that are scattered within the mesohyl. The antibodies failed to recognize those bacteria that are present in clusters within bacteriocytes. This result suggests that only the extracellularly present bacteria are digested by lysozyme while the intracellular microorganisms are protected against the lytic enzyme. From a previous study it can be assumed that the bacteria in the bacteriocytes are symbiotic (Böhm et al., 2001). In consequence, the elimination process of the Gram-positive bacteria, present in a scattered arrangement in the extracellular space of the mesohyl, can be divided into an activation phase of gray cells and an effector phase.

Even though since Metchnikoff (1892) it has been suggested that sponges phagocytose/endocytose bacteria this process has not been demonstrated on molecular level. In eukaryotic cells clathrin-coated vesicles mediate endocytosis of surface receptors and also extracellular bacteria (Le Borgne and Hoflack, 1998). The formation of these vesicles is initiated by the assembly of the adaptor proteins (AdaPTins) that form complexes on the target membranes. The AdaPTins are highly conserved proteins in the metazoans (Boehm and Bonifacino, 2001). In our studies we selected the AdaPTin protein AP-1, the small sigma 2 subunit protein, for the isolation of the corresponding gene. As expected, this sponge AP1 molecule was again found as the ancestral molecule of this group of adaptor proteins for Metazoa. AP-1 molecules function together with clathrin and are involved in the arrangement of the trans-Golgi network and/or the endosomes (Boehm and Bonifacino, 2001).

In conclusion, these studies indicate that in sponges Gram-positive bacteria elicit an immune response, which is triggered by the cell-wall PPG. As known from the (1→3)-β-D-glucan responsive pathway in *S. domuncula* (Perović-Ottstadt et al., 2004a), the activation phase of the cell is paralleled by the upregulation of the expression of membrane-associated molecule(s), here AdaPTin, which proceeds prior to the synthesis of the effector molecule, lysozyme (Fig. 7).

**INTERACTION WITH FUNGI:** Next to the bacteria, the molecular interaction of sponge cells with fungi was studied using *S. domuncula* (Perović-Ottstadt et al., 2004a). For this study the model glucan polymers, the (1→3)-β-D-glucans, which can be isolated from cell walls of plants, but also fungi (Riggi and Luzio, 1961) were used; e.g. curdlan, a linear polysaccharide from *Alcaligenes faecalis* or laminarin from the alga *Laminaria digitata*. Our group identified that sponges (here *S. domuncula*) indeed react to exposure with (1→3)-β-D-glucan. We found that curdlan caused no effect on the phosphorylation of the MAPKs by *S. domuncula*. Subsequently, we determined whether an effect on the tyrosine kinase pathway is seen in response to curdlan. By applying antibodies against tyrosine



kinase, such a phosphorylation process could be demonstrated, an effect which could be inhibited by the tyrosine kinase inhibitor, Aeropylsinin (Fig. 8). To progress, the respective (1→3)-β-D-glucan-binding protein was identified. Such a molecule had previously been isolated and cloned from a number of protostomians (Lee et al., 2000). Subsequently we continued our search for other potential binding proteins that might be involved in the recognition of the D-glucan. This screening resulted in the identification of fibrinogen and of the epidermal growth factor, two molecules that cause an engulfment of foreign organisms and an increased proliferative response of the sponge cells.

These data provide experimental evidence that *S. domuncula* has the toolkit to react and in turn to eliminate fungi that might attack them.

### **The future: evochemistry**

Thanks to the progress initiated by the pressure of the society for a sustainable use of natural resources for human benefit, the exploitation of natural biodiversity became possible through the application of the techniques of molecular biology and modern cell biology. It appears to be enigmatic why Porifera and also other phylogenetically old taxa, all of them being sessile filter feeders, have been so successful during the long evolutionary period of 800 Myr. Answers have been given in the last years. As summarized (Breter et al., 2003) the expected number of genes in the sponge genome might be >100,000. This number is comparatively large; the human genome contains ≈35,000 genes; *Caenorhabditis elegans* (nematode [worm]) ≈19,000, the insect *Drosophila melanogaster* ≈14,000 and the yeast *Saccharomyces cerevisiae* 6,000 genes. Two major conclusions must be drawn: FIRSTLY, during the transition from Yeast to the Urmetazoa a period of dramatic gene duplication must have occurred and SECONDLY, during the period of evolution from the Urmetazoa to the Protostomia [*C. elegans* and *D. melanogaster*] and to the Deuterostomia [*Homo sapiens*] a reduction of the number of genes must have taken place. In parallel with the metazoan *evolution by loss of genes [domains and families]* a second evolutionary progress, which occurred after the Urmetazoa, must be postulated for the metazoan phyla, an *evolution by diversification of gene families*. The hypothetical Urmetazoa has been proposed as the last common ancestor for all Metazoa.

The finding that the number of sponge genes is higher than that in the “crown” phyla also allows an explanation why the diversity of secondary metabolites in sponges and other filter feeders is comparably so high. These animals had – in order to survive environmental threads – to form bioactive compounds from the “regular” metabolites, the secondary metabolites. During their long evolutionary history the biological/defensive/pharmacological effectiveness of these compounds were trimmed for higher potency and selectivity (evochemistry). In consequence, the richness of the

sessile filter feeders in bioactive compounds is due to their growth and nutrition characteristics and their comparably large genetic repertoire.

## **Conclusion**

During the last 15 years multifold evidence has been presented that the sponges are provided with genes and in turn also deduced proteins highly related to those of higher phyla. This finding provides a major clue also for future secondary metabolite screening programs. It underscores that the bioactive secondary metabolites are targeted not only against sponge molecules but also – perhaps with a different binding affinity – to related mammalian/human regulatory and structural proteinaceous polymers. Especially the recognition molecules of sponges towards their microorganism share not only structural but also functional similarities to the crown metazoan phyla. Consequently, it is a fortune that, according to the fossil records, the phylogenetic oldest metazoan phylum, the Porifera did not become extinct during the last 800 Myr. By analyzing their genetic diversity it became obvious that all Metazoa originated from one common ancestor, the Urmetazoa. With respect to the functional aspect it could be demonstrated that the degree of complexity of the Porifera is beyond any expectations. It becomes now increasingly obvious that they are provided with most of the metabolic pathways and genetic elements required for constructing a metazoan Bauplan (Müller et al., 2004). Finally it should be stressed that the potential commercial value of sponge biodiversity is of an unpredictable scale. A new application field which very likely will have a considerable impact in biotechnology is the question of the synthesis of bioactive, low-molecular weight compounds in a recombinant manner. To reach this goal both the gene cluster required for the synthesis of these compounds from the sponge host and the associated symbiotic bacteria have to be isolated and expressed in a heterologous system.

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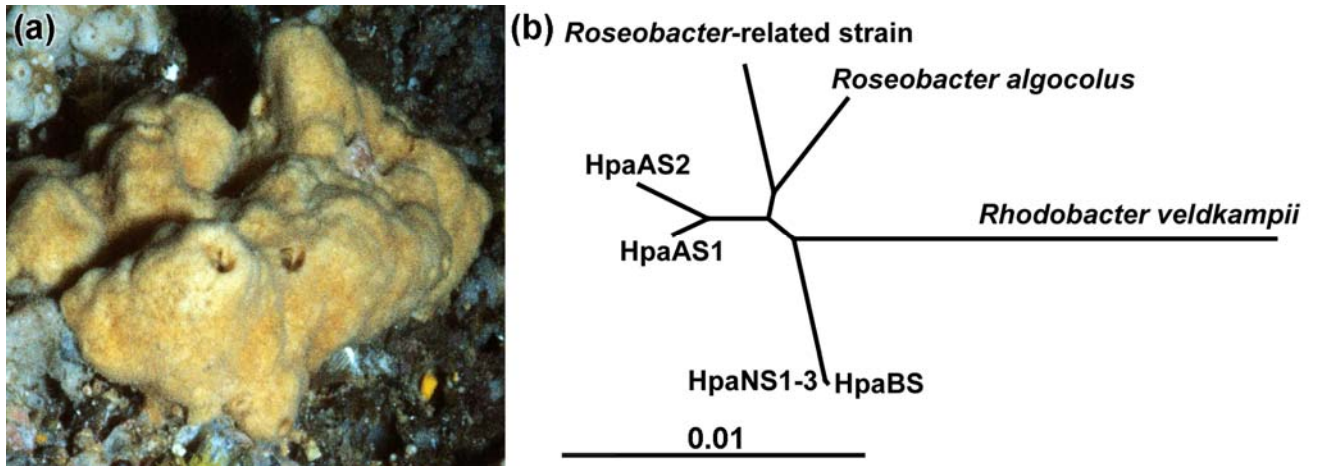
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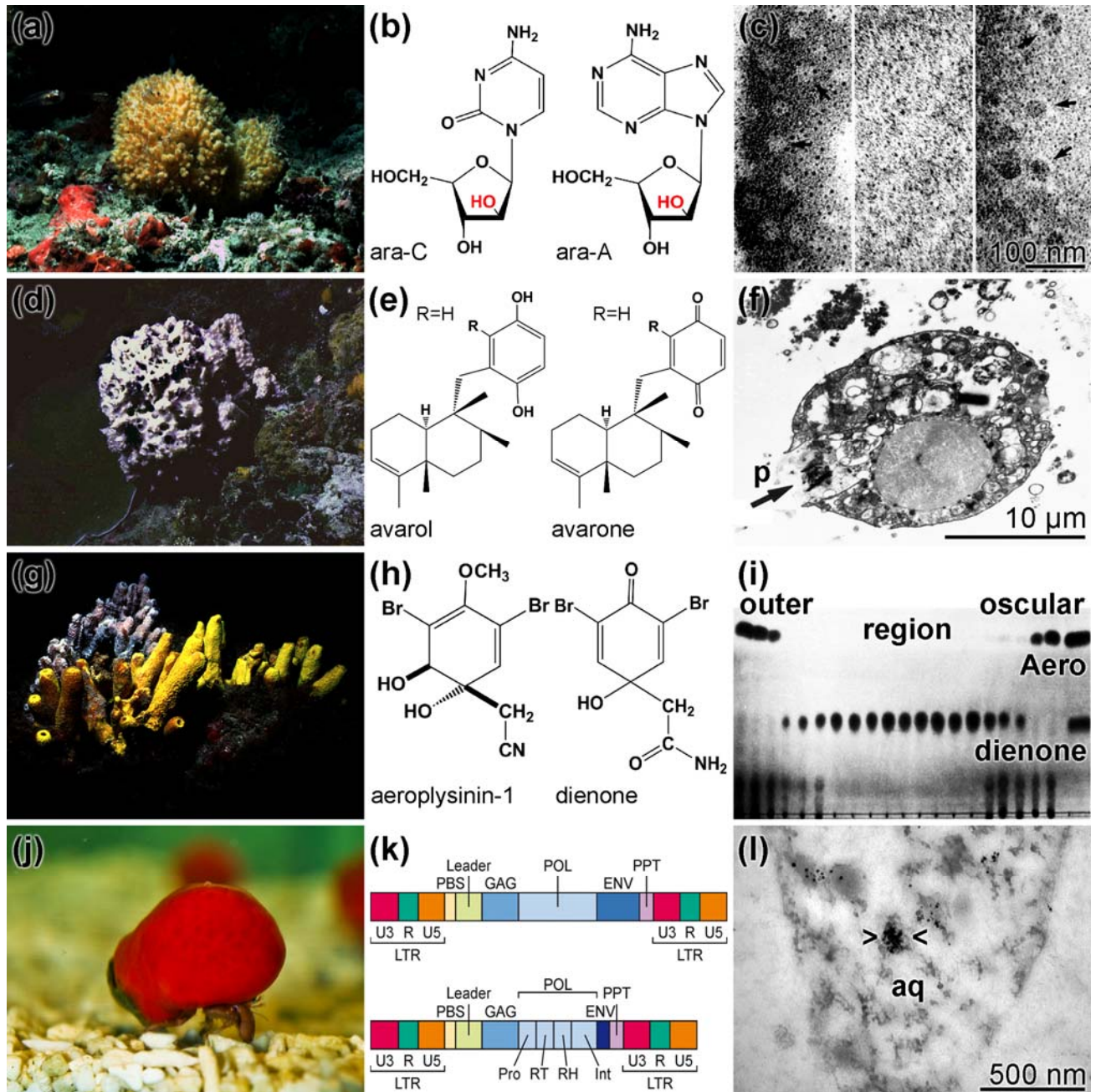


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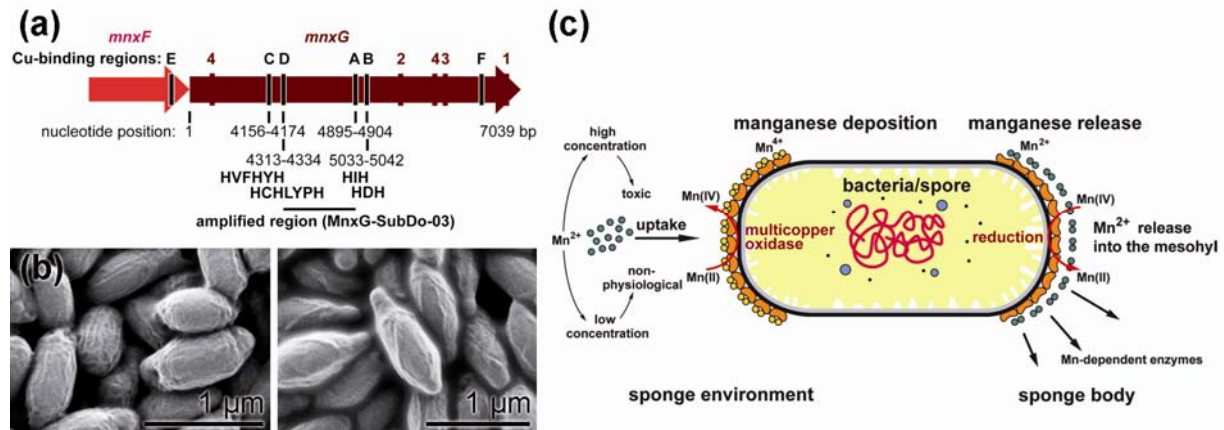


**Fig. 1.** (Potential) symbiotic [gram-negative] bacteria, colonizing the marine sponge *Halichondria panicea*. (a) Specimen collected from the Adriatic, close to the Rovinj area [Istria]. (b) Partial bacterial nucleotide sequences for the 16S rRNA segment of *Roseobacter algocolus* (*R. algicola*) and from *Rhodobacter veldkampii* were aligned with those sequences obtained from the microorganism of the *H. panicea*, collected from the North Sea [HpaNS1-3], the Baltic Sea [HpaBS] and from the Adriatic Sea [HpaAS1 and HpaAS2]. The phylogenetic tree (unrooted) was constructed showing the close relationship of the 16S rRNA sequences, especially those from specimens collected in the North Sea [HpaNS1-3] and those from the Baltic Sea [HpaBS]. The scale bar indicates a distance of 0.01 fixed mutations per nucleotide position.

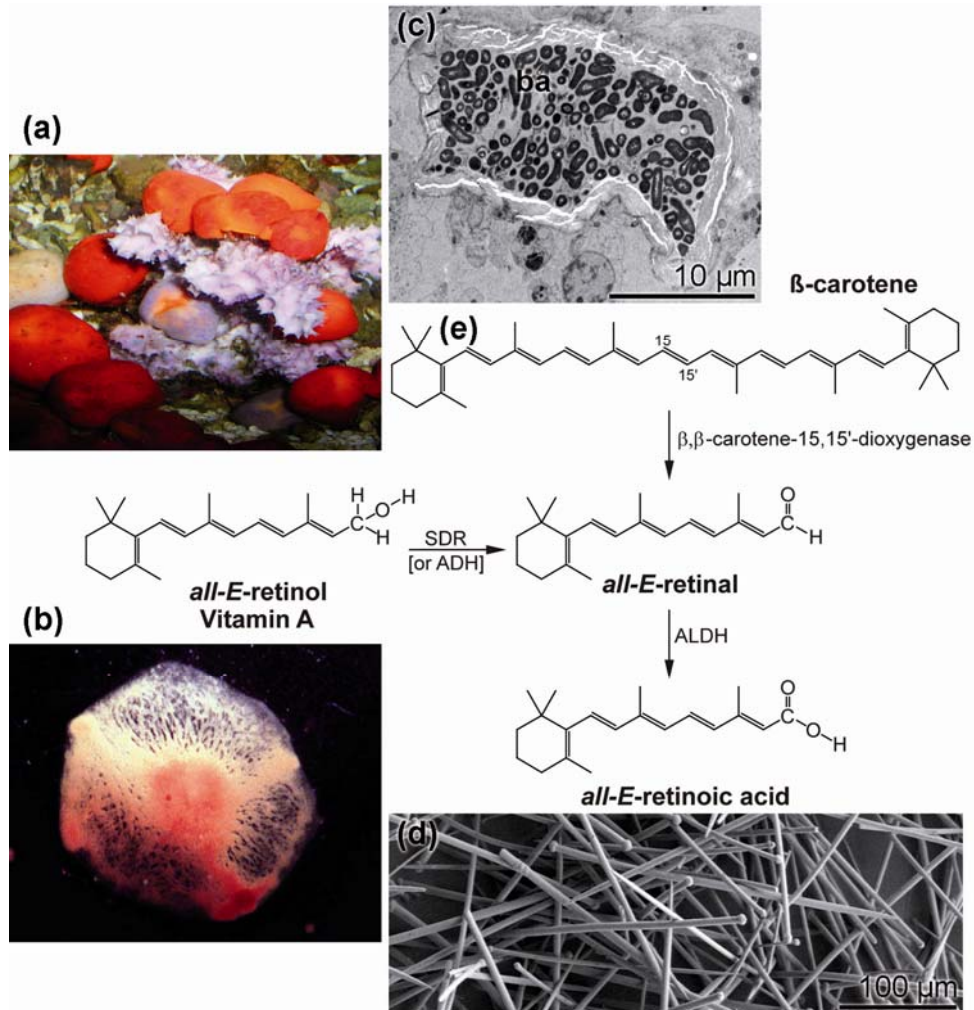


**Fig. 2.** Selected bioactive compounds, or targets, from sponges. **(a)** *Tethya lyncurium*, an abundantly occurring demosponge from the Northern Adriatic; size 3-5 cm. **(b)** Structures of arabinofuranosyl-cytidine [ara-C] and 9-β-D-arabinofuranosyl-adenine [ara-A], two bioactive nucleoside analogues from this species. **(c)** Negative-staining electron microscopy of membrane lesions induced by *Tethya* hemolysin. Membranes were isolated from sheep erythrocytes incubated for 1 h without or with 14 pg/ml *Tethya* hemolysin. (Left) Membrane fragment of erythrocyte lysed with *Tethya* hemolysin, displaying stain-free circular areas (arrows). (Middle) Control erythrocyte membranes prepared by hypotonic lysis. (Right) For comparison, negatively stained membrane

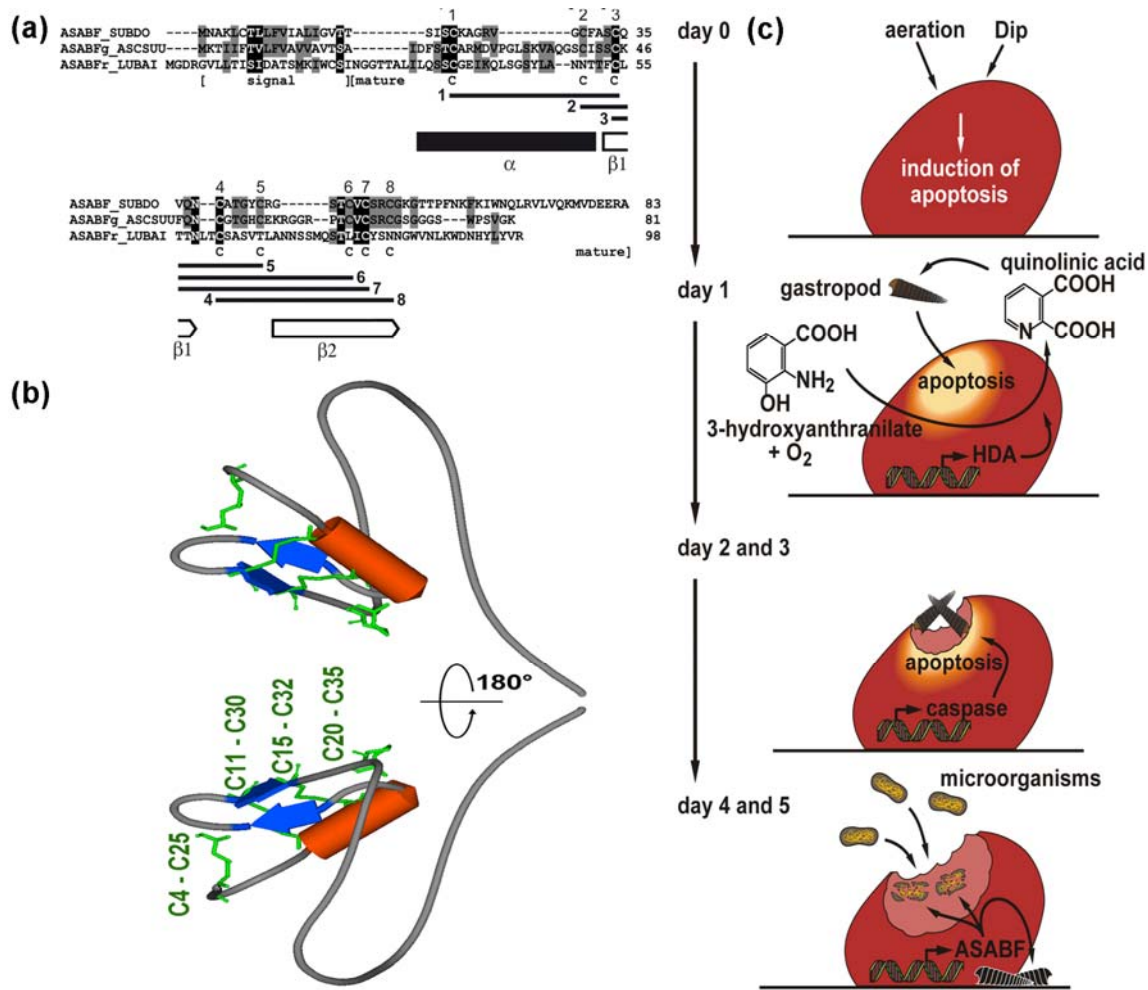
from erythrocytes lysed by streptolysin 0 is shown. The pores formed by this toxin have an approximate diameter of 30 nm. **(d)** *Dysidea avara*, a demosponge which forms the sesquiterpenoid quinones and hydroquinones avarone and avarol; size 5-20 cm. **(e)** Molecular structures of avarol (R = H) and 6'-hydroxy avarol (R = OH) and avarone (R = H). **(f)** Transmission electron micrograph of a spherular cell from *D. avara*. The vacuoles contain lamellar inclusions in which paracrystalline material (p) is packed. **(g)** *Aplysina aerophoba* (size of the gold-colored sponge  $\approx$ 15 cm; a purple-colored *Dysidea avara* specimen is seen in the background) the producer of the **(h)** cytostatic agents aeroplysinin-1 and the related dienone. **(i)** Regional distribution of the two compounds aeroplysinin-1 and the related dienone. In contrast to aeroplysinin-1 the dienone is accumulated at the surface regions of tube-like branch; both that one directed to the oscular, the water exhalant channel system, and that one directed to the outer region that is perforated by the inhalant pores. Extracts were prepared and separated on a TLC plate. The spots, representing aeroplysinin-1 [Aero] and the related dienone, are highlighted. **(j)** The demosponge *Suberites domuncula*; size of the globular sponge  $\approx$ 3.5 cm. **(k)** Genome organization of a retrotransposon (above) and of a retrovirus (below). The ends of the linear genome are framed by a long terminal repeat [LTR] that is built of U3, R and U5 regions. Between the LTR and the group-specific antigen [GAG] region the primer-binding site [PBS] is located. The region encoding the enzymes, the polymerase [POL], is composed of the protease [Pro], reverse transcriptase [RT], the RNase H [RH] and the integrase [Int]. In the genome of the retrovirus an envelope [ENV] gene exists. **(l)** Immuno-TEM [transmission electron microscope] image showing the localization of the retrovirus assemblies (> <) in *S. domuncula* in the cells layers, surrounding the aquiferous canals (aq). The reverse transcriptase gene segment was used as a probe (unpublished).



**Fig. 3.** Mn(II)-oxidizing *Bacillus* strain, co-existing with the demosponge *Suberites domuncula*. (a) Sequence structure of the *mnxG* gene (termed *mnxG-SubDo-03*), identified in the *Bacillus*-related strain, isolated from *S. domuncula*. The schematic representation of the *mnx* region with the central gene MnxG-SubDo-03 is attributed to the MCO [multicopper oxidase] enzyme. The locations of the putative Cu-binding regions within MnxG-SubDo-03, the regions E to F, have been indicated. The primers to identify the MCO from the sponge-associated bacteria (BAC-SubDo-03) span the regions C to B; the respective locations are indicated using the numbering of the gene isolated from the bacterial strain SG-1 (Dick et al., 2008). The consensus Cu-binding sequence motifs (C/D and A/B) found in all MCOs are marked. (b) In contrast to the controls (left) the bacteria that had incubated in Mn-containing K-medium become decorated with a manganese shell (right). (c) Schematic representation of the proposed role of the BAC-SubDo-03 *Bacillus* strain, associated with *S. domuncula*, as an agent in Mn storage. It is outlined that under high Mn concentrations the bacteria take up Mn(II) from the environment through the multicopper oxidase [MCO] and deposit the ions as insoluble Mn(IV) on their cell wall. If Mn exists in the environment only at low concentrations the MCO allows the enrichment of the element to physiological levels. Intracellularly, in the sponge body, Mn is solubilized by reduction from Mn(IV) to Mn(II) released from the cell wall and becomes available as co-factor in a series of essential enzymes involved in detoxification of reactive oxygen species (ROS).

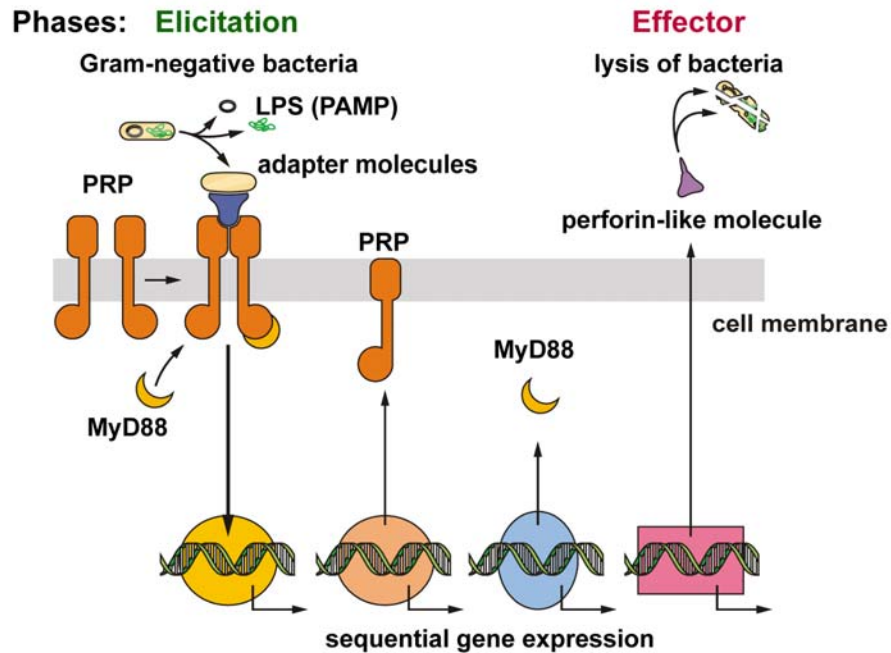


**Fig. 4.** Formation of retinoids/retinoic acid in *S. domuncula* (a). (b) Exposure of primmorphs to retinoic acid results in the formation of canal-like structures within these three-dimensional aggregates. (c) Section through a bacteriocyte, harboring bacteria (ba). (d) Pile of siliceous 100-200  $\mu\text{m}$  long spicules [tylostyles], isolated from *S. domuncula*.

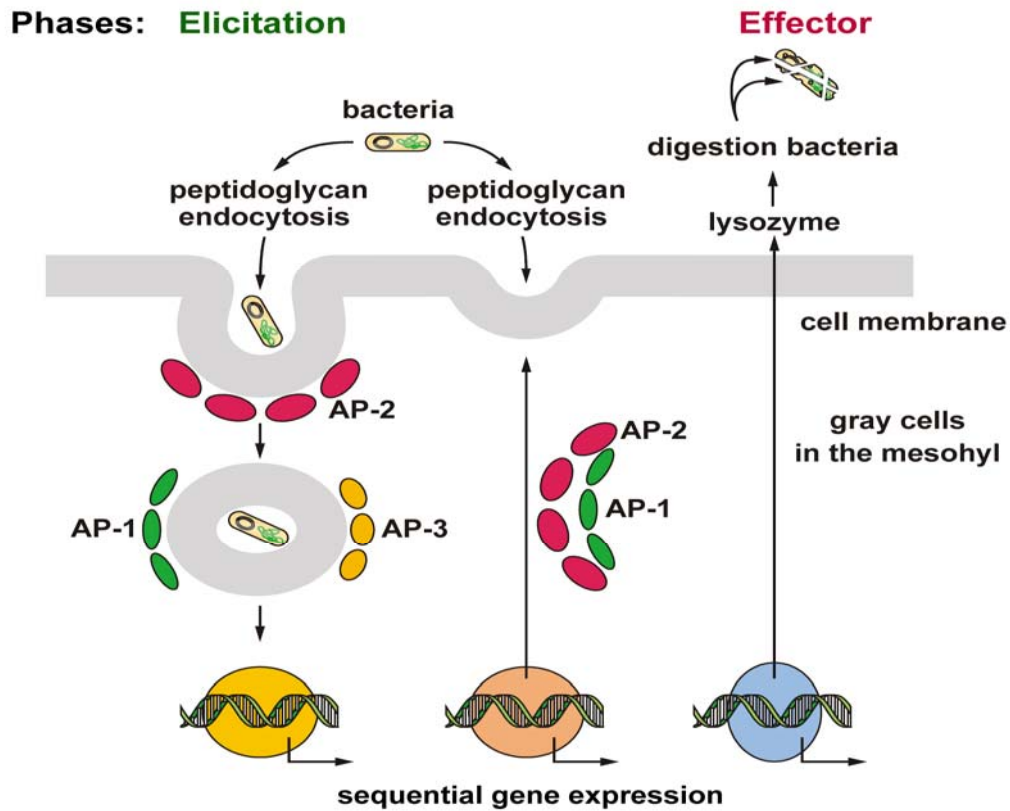


**Fig. 5.** The *S. domuncula* ASABF peptide (ASABF\_SUBDO; accession number HE573030). **(a)** The deduced sponge protein was aligned with the most related sequence from the database, the ASABF- $\gamma$  peptide from *A. suum* (ASABFg\_ASCSUU; BAC00498.1) and the distantly similar sequence, the ASABF-related peptide from the Baikalian sponge *L. baicalensis* (ASABFr\_LUBAI). Residues conserved (identical or similar with respect to their physico-chemical properties) in all sequences are shown in white on black; those which share similarity to at least two residues are in black on grey. The borders of the signal peptide and of the mature peptide are given. The 8 Cys moieties that are involved in disulfide bridge formation are numbered; the connectivity of the Cys are indicated. The  $\alpha$ -helix and the two  $\beta$ -strands are marked with a black solid bar and two open arrows, respectively. **(b)** Topology of the CS $\alpha\beta$  motif, existing within the ASABF peptide. The single  $\alpha$ -helix and two  $\beta$ -strands are indicated with cylinder and arrows, respectively. The green lines represent the disulfide bridges; the numbers of the cysteine moieties within the mature peptide are given (model). **(c)** Schematic outline of the elimination of apoptotic tissue from the sponge *S. domuncula*, after induction with the Fe<sup>++</sup> chelator Dip, or after lowering the aeration within the aquarium. Initially (day 0), the expression of the *HAD* gene is induced as a response to Dip. After initiation of Dip-induced apoptosis (day 1) the *caspace* gene is upregulated and, sequentially, the increased expression *ASABF* gene sets on. While quinolinic acid attracts the gastropod *Bittium* sp. (day 2 and 3), the terminally expressed ASABF causes, after removal of the apoptotic tissue, an repelling of the gastropods as well as a killing of the microorganisms (day 4 and 5).

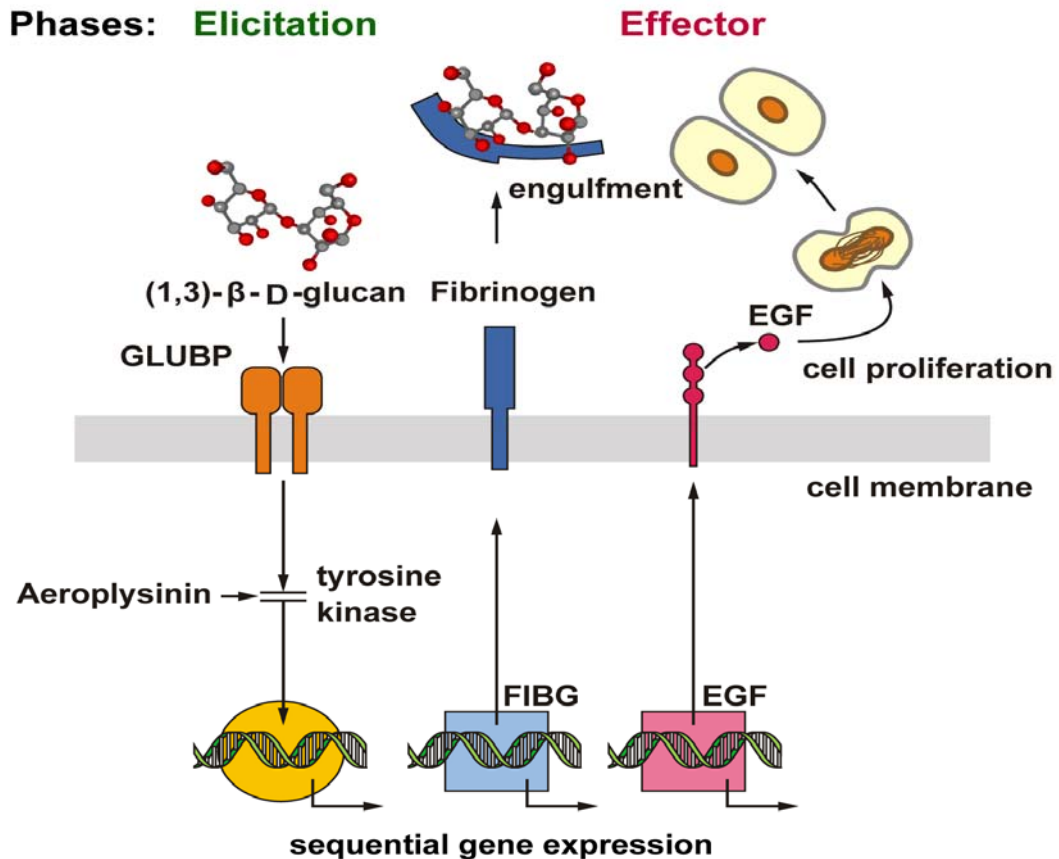




**Fig. 6.** Interaction of sponge cells [*S. domuncula*] with Gram-negative bacteria (scheme). It is outlined that bacterial LPS, acting as PAMP [pathogen-associated molecular pattern] molecules, react with PRP, an LPS-interacting protein, and causes its (homo)dimerization. After complex formation between PAMP (ligand) and PRP (receptor) a signal transduction cascade is initiated resulting in an integration of MyD88 into this complex. Downstream, intermediate members of the signaling pathway, which probably involves interleukin-1 receptor-associated kinases, TRAFs, NF- $\kappa$ B, and also the LPS-induced tumor necrosis factor- $\alpha$ , transduce an increased expression of the activation phase's key molecules, PRP and MyD88, and finally of the executing effector molecule, the perforin-like protein termed MPEG [macrophage-expressed protein]. This reaction chain most probable terminates in a lysis of the microorganism. After Wiens et al. (2005).



**Fig. 7.** Scheme describing the immune defense response of *S. domuncula* after exposure to Gram-positive bacteria. The microorganisms or PPG are taken up by endocytosis. This glycan causes during the elicitation phase an induction of gene expression for AdaPTin [in complex with AP-1 to AP-3] and lysozyme in the gray cells. During the effector phase lysozyme lyses the bacteria and/or their PPG, covering the cell surface.



**Fig. 8.** Proposed pathway by which sponges react to exposure to fungi. The model polymer curdian [(1→3)-β-D-glucan], decorating the surface of fungal cells, was used for elicitation. This polymer reacts to the corresponding cell-surface receptor of *S. domuncula*, the GLUBP [(1→3)-β-D-glucan-binding protein]. In turn signal molecules, among them tyrosine kinases are activated by increased phosphorylation, a process that can be inhibited by Aeroplysinin. During the effector phase the sponge cells express the genes encoding for FIBG [fibrinogen] and for EGF [epidermal growth factor], two molecules causing engulfment of curdian or increased proliferative response of the sponge cells.