Anaerobic pathways in the Porifera: Strombine dehydrogenase, an opine dehydrogenase, from the sponge *Suberites domuncula*

Dissertation
zur Erlangung des Grades
Doktor der Naturwissenschaften

Am Fachbereich Biologie
der Johannes Gutenberg-Universität
in Mainz

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geb. in Zagreb (Croatia)

Mainz, 2007

Dekan:

1. Berichterstatter:

2. Berichterstatter:

Tag der mündlichen Prüfung:

Mojoj Baki i Čiki

1 Introduction

1.1 Sponges (Porifera)

1.1.1 The origins

Million years ago a group of single-celled "creatures" joined forces to form the first ever animal body, or "metazoan". Evidences for this event could be found in the first fossils that might represent a series of early "experiments" in multicellularity. For example, Doushantuo Formation in southern China that contains fossils of dividing embryos (Xiao et al. 1998) or a mishmash of strange animal-like creatures known as the Ediacaran fauna (such as *Dickinsonia, Spriggina, Parvancorina, Tribrachidium*). Aside from them, most known animal phyla make more or less simultaneous appearance during the Cambrian period (Cambrian explosion). Despite the progress that has been achieved in the last years by molecular studies, the origin of Metazoa is to some extent still enigmatic. The evolution of the Metazoa from unicellular/colonial organisms occurred some 1,300–600 million years (Myr) ago in the pre-Ediacaran period (Conway-Morris 1998). Morphological contributions to understanding of the transitional stages to the Metazoa suggest a colonial origin of Metazoa (see Dewel 2000). This view implies that, based on the Beklemishev's cycles of duplication and individuation (Beklemishev 1969), after duplication of an individual and the formation of a colony, this entity has to undergo individuation again. It has been pointed out that two such cycles were necessary in early evolution for the emergence of Metazoa: first the transition to multicellular organisms, with the sponge grade of organization and second the change to the modularized ancestor of the Bilateria (Dewel 2000). Recent phylogenies, based on rDNA, have suggested that the Metazoa are polyphyletic. The proposal suggested that the Porifera/Cnidaria evolved separately from the Triploblasts; both having originated independently through aggregation of protists belonging to two different lineages (Christen et al. 1991). This view is based on the early idea that sponges (Porifera) are grouped with the Protozoa (Spencer 1864). Later, ontogenetic evidence provided the basis for sponges to be considered as metazoans (Haeckel 1896). However, until recently it was generally accepted that the choanoflagellates were the sister group to Metazoa (see Nielsen 2001).

Even though sponge choanocytes are similar to choanoflagellates and are composed of a single flagellum, surrounded by a microvillar collar, the long-standing view of a homology of these types of flagella/cilia (Kent 1881; Lackey 1959) could not be substantiated (Karprov and Efremova 1994). Based on the study of protein molecules, Fungi are regarded as the nearest neighbor kingdom of the Metazoa (Schütze et al. 1999; Baldauf et al. 2000). The ‘‘traditional’’ animal phylogeny (Fig.1A) found in major zoology textbooks (Barnes 1985) is following the work of Hyman (1940). It is a good illustration of the long prevailing notion that animal evolution went from simple to complex through gradual steps, with extant animals actually representing grades of intermediate complexity supposed to have been those of their ancestors.

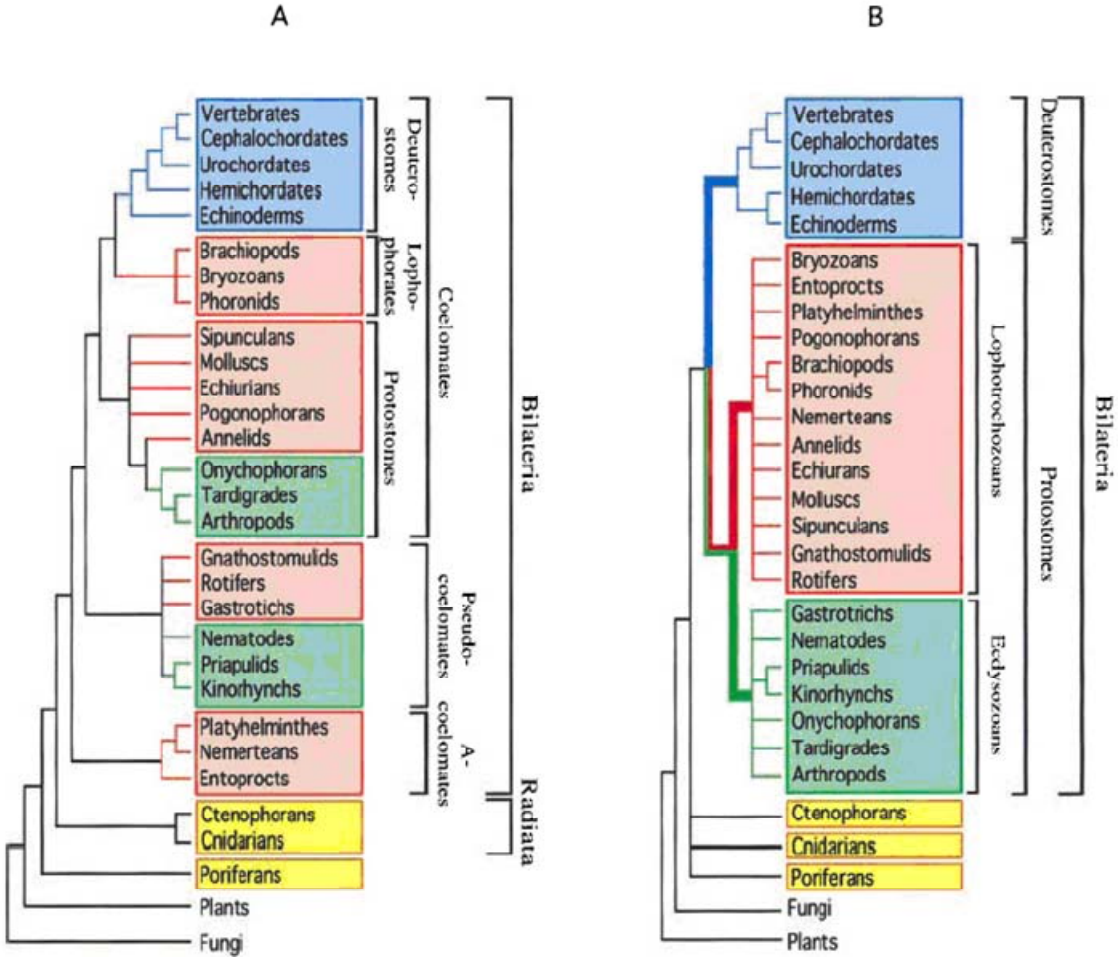

Fig.1. *Metazoan phylogenies: (A) The traditional phylogeny based on morphology and embryology, adapted from Hyman (1940). (B) The new molecule-based phylogeny. A conservative approach was taken into account (taken from Adoutte et al. 2000). Reorganization is showed in colored rectangles.*

New animal phylogeny proposed by Adoutte et al. (2000) is exclusively based on rRNA (Fig.1B). New animal phylogeny leaves us with no evolutionary ‘‘intermediates’’ and suggest a paraphyletic emergence of sponges at the base of metazoan tree, followed by a monophyletic Ctenophora and a possibly paraphyletic Cnidaria.

Regardless of evolutionary theorizing about Metazoan origin, today it is widely accepted that all metazoan phyla are of monophyletic origin (see Borchiellini et al. 2001; Müller 1995) with the hypothetical common metazoan ancestor, named Urmetazoa (Müller 2001). Sponges (Porifera) as the phylogenetically oldest metazoan phylum still extant today share the closest relationship to the Urmetazoa. Fossil sponges are among the oldest known animal fossils, dating from the Late Precambrian (Venedian) (Orlov 1971; Valentine 1994). A fossil record of the first sponge denoted as *Paleophragmodicty* was discovered near Australia in 1996. Marine species of order Triaxonida are dating from Proterozoik (10^9 years ago). The number of described fossil genera exceeds 900 (Hooper and Van Soest 2003). The sponges, as the most basal group in the animal phylogeny, laid the foundation for all animal life to follow. They emerged approximately 600–800 million years ago as calculated on basis on amino acid exchanges (Müller et al. 1994; Müller 2001) and since then their overall Bauplan (bluprint) have not changed.

Sponges are predominantly marine, with the notable exception of the family Spongillidae, an extant group of fresh-water demosponges whose fossil record begins in the Cretaceous. So far an estimated 15000 species have been known, and 8000 species well described (Hooper and Van Soest 2002). Generally, they are sessile, though it has been shown that some are able to move slowly (up to 4 mm per day) within aquaria. Their habitats range from the inter-tidal zone to depths of 8500 meter or deeper. Sponges are worldwide distributed, from waters of the polar to the tropical regions. They produce the highest and the most diverse quantity of secondary metabolites, many of which have been allocated for defense against predation in some fashion (Pawlik et al. 1995). Sponges are frequently exposed to intense predation, tissue infection by other microorganisms, and competition for space in tropical reefs. These marine invertebrates have a high nutritional value (protein source) and from prior studies seem to be prey for only selected groups of marine animals, such as turtles, a few fish species, nudibranchs, sea urchins and sea stars (Torres 2002).

Usage of sponges in commercial sources is dating back from Aristotle time. They are the richest source of bioactive compounds among the metazoan organisms (Faulkner 2000; Sarma 1993). Modern sponges promise to offer critical insights into the earliest events in animal genome evolution. Lately, the pharmacological properties of the secondary metabolites from sponges, have gained importance with regard to biotechnology and their potential application in human therapy (Bongiorni and Pietra 1996; Proksch et al. 2002).

1.1.2 Morphology

Within a sponge there is an intriguing body structure. They have the simplest type of metazoan organization – a cellular level of organization. While the sponge functions as a unified organism, each cell is a division of its own mini-organism. Essentially, a sponge is a combination of billions of cells that function together. Within the body of the sponge, several processes occur which are vital to its welfare. Most importantly, sponges conduct the processes of respiration, digestion, protection, development and reproduction.

The Latin name of the phylum (*porus* "pore" and *ferre* "to bear") derives from the numerous pores found on the body surface. These are respectively the entrances and exits for a complex system of channels and chambers through which the sponge pumps a current of water. It is amazing that sponges have the capacity to pump several tons of water per kilogram of body weight per day in order to obtain the required nutrition (Bergquist 1978). The general body plan consists of two cell layers surrounding a spongocoel. The body wall is perforated by many pores and channels through which water enters the animal, passing into the spongocoel and exiting through a large opening, the osculum. Water movement is driven by the beating of flagellae, which are located on specialized cells called choanocytes (collar cells). Particle uptake by sponges is highly efficient but largely unselective (Pile et al. 1996; Reiswig 1971; Ribes et al. 1999). It occurs at least at three functional sites: (*i*) large particles (> 50 μm) are taken up at the sponge surface by epithelial pinacocytes, (*ii*) smaller particles (< 50 μm) capable of entering the ostia are taken up by pinacocytes lining the progressively narrower canal walls and (*iii*) even smaller particles (< 5 μm) are trapped in the choanocyte chambers (Turon 1997). After particle capture by pinacocytes and choanocytes, food particles are

passed to the mesohyl were they are phagocytosed and digestion occurs within individual archaeocytes (Fig.2). Sponges of the family Cladorhizidae make an exception due to the fact that they are carnivorous. They typically feed by capturing and digesting whole animals (usually crustaceans) with their spicules and digestion takes place extracellularly (Vancelet and Boury-Esnault 1995).

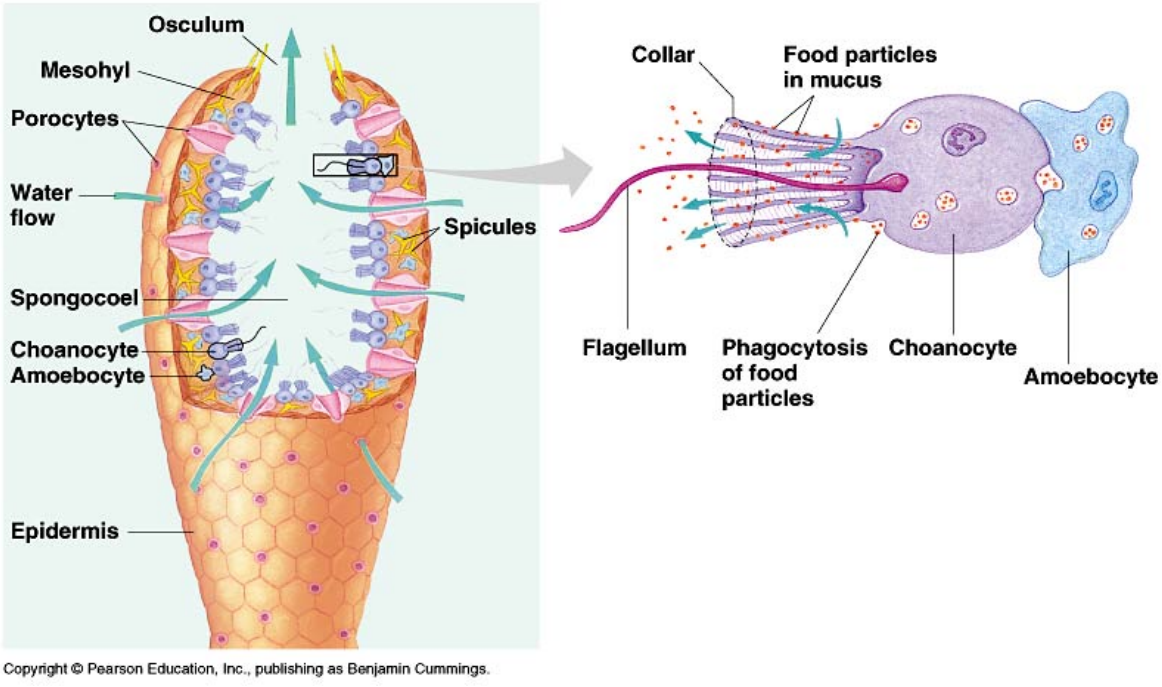

Fig.2. *Sponge anatomy with enlarged schematic picture of the choanocyte and particle uptake (taken from http://io.uwinnipeg.ca/~simmons/Chap3298/sld013.htm)*

1.1.3 Reproduction

Sponges can reproduce asexually when they are fragmented by water disturbances or by predator actions and pieces of the sponge grow into new individuals (external buds) (Wullf 1991). Some species also form internal buds, called gemmules, which can survive extremely unfavorable conditions that cause the rest of the sponge to die. Most poriferans that reproduce by sexual means are hermaphroditic and produce eggs and sperm at different times. Sexual reproduction takes place in the mesohyl. Male gametes are released into the water by a sponge and taken into the pore systems of its neighbors in the

same way as food particles. Spermatozoa are "captured" by collar cells, which then lose their collars and transform into specialized, amoeba-like cells that carry the spermatozoa to the eggs. Sponges may be either dioecious or monoecious, depending on the species. In most sponges for which developmental patterns are known the fertilized egg develops into a blastula, which is released into the water. Some sponges release their larvae immediately, while others retain them for some time. The larvae may settle directly and develop into juvenile sponges or could be planktonic for a while before they settle.

1.1.4 Classification

Sponges have three different types of body plans, although these morphologies do not define taxonomic groups. Asconoid sponges have the simplest type of organization. They are small and tube shaped sponges with internal part called the spongocoel that contains the collar cells. Syconoid sponges tend to be larger than asconoids and have a tubular body with a single osculum. The syconoid body wall is thicker and the pores that penetrate it are longer, forming a system of simple canals. These canals are lined by collar cells, the flagellae which move water from the outside, into the spongocoel and out through osculum. The third category of body organization is leuconoid. The sponges with this type of the skeleton are the largest and most complex. These sponges are made up of masses of tissue penetrated by numerous canals. Channals lead to numerous small chambers lined with flagellated cells. Water moves through the canals, into these chambers and out via a central canal and osculum. Leuconoid sponges are the best adapted to increase of sponge size.

According to the chemical composition of their internal skeleton, living poriferans have been classified into tree classes: Hexactinellida (glass sponges), Calcarea (calcareous sponges) and Demospongia. Hexactinellida with skeletons composed of silicon dioxide have two subclasses: Amphidiscophora with order Amphidiscosida and Hexasterophora with two orders (Hexactinosida and Lyssacinosida). Calcarea are radially symmetrical with skeletons made of calcium carbonate. They are considered to be the most primitive group divided in two subclasses: Calcaronea with two orders (Leucosoleniida and Lithonida) and Calcinea with order Lithonida.

Sponges in the class Demospongiae contain the largest, commonest and most widely distributed species. The skeleton is made of siliceus spicules of silica, whose number of rays vary from one to four, but these may be entirely replaced in some species by spongin fibers or a mixture of spongin and spicules. The class Demospongiae includes tree subclasses: Ceractinomorpha (with 8 orders: Agelasida, Criptocoeliidae, Dendroceratida, Dyctioceratida, Halichondria, Haplosclerida, Poecilosclerida and Verongida), Homoscleromorpha (with order Homosclerophida) and Tetractinomorpha (with 4 orders: Lithisida, Astrophorida, Hadromerida and Spirophorida). In the order Hadromerida there are ten families including Suberitidae with 8 genuses including Suberites with up today 6 identified species: *S. domuncula, S. ficus, S. fuscus, S. massa, S. suberia* and *S. virgultosa*. *Suberites domuncula* has two subspecies. *S. domuncula domuncula* (Olivi 1792) and *S. domuncula latus* (Lambe 1893). Two fossil groups are often included - the Stromatoporoidea and the Archaeocyatha. The former are most likely Porifera (possibly Sclerospongiae or Demospongiae), while the precise relationships of the latter remain controversial.

1.1.5 Symbiosis

Genus *Suberites* is well known as hermit crab sponges. Probably the best known of these is Mediterranean sponge *Suberites domuncula* (*domunculus* = "little house") shown in the Fig.3. Hermit crab sponges typically grow on snail shells, although some can grow on other substrates such as other mollusk shells or wharf pilings. These sponges are compact and slightly compressible and have a smooth waxy-like texture. They are usually colorful, occurring in shades of green, brown, orange or blue. These sponges occur in association with hermit crabs and therefore can live in habitats with sand or mud bottoms, where other sponges would not be able to survive because of the lack of a hard substrate and the risk of burial by sediments. Sponge benefits from this symbiosis by being moved from place to place by the hermit crab. Only certain hermit crab species, like *Pagurus impressus, Paguristes hummi, Paguristes oculatus* or *Dardanus arrosor,* use sponge as shelters.

Fig.3. *Marine sponge, Suberites domuncula, with a crab Paguristes sp. Picture was taken in aquarium, Mainz.*

As filter feeders sponges are capable of processing large volumes of seawater with microorganisms as the main sponge diet. Whereas most captured microorganisms serve as food particles and are phagocytotically digested, some are retained and form specific associations with sponges. These sponge–microorganism associations become a common phenomenon in nature. In the sponge "microcosmos" we can find unicellular algae (Vacelet and Donadey 1977), cyanobacteria (Thacker and Starnes 2003), dinoflagellates (Garson et al. 1998), zooxanthellae (Sara and Liaci, 1964), zoochlorellae (Gilbert and Allen 1973; Williamson 1979) and members of the domain Archaea (Preston et al. 1996; Fuerst et al. 1999). Discovery of bacteria in sponges (Vacelet 1975), that can attribute as much as 40-60% of the total biomass (Wilkinson 1978), opened a new field of scientific interest. Sponge-associated microbial consortia represent a huge variety from the poorly characterized phyla (Acidobacteria, Chloroflexi, Actinobacteria, the Alpha-, Gamma- and Delta-proteobacteria) to recently discovered candidate phylum Poribacteria (Fiesler 2005; Fiesler et al. 2004).

Bacteria's in sponges are located in the interior of the mesohyl tissue (Hentschel et al. 2003) where some of them are encapsulated in specific cells (Böhm et al. 2001) or on the surface of the sponge host. There are two pathways whereby a developing sponge may acquire bacterial symbionts. The first is by selective absorption of specific bacteria from the large diversity of bacteria in the surrounding water column that passes through the sponge during filter feeding. The second is by vertical transmission of symbionts through the gametes of the sponge by inclusion of the bacteria in the oocytes or larvae (Usher et al. 2001; Usher et al. 2005; Anakina and Drozdov 2001; Ereskovsky 2005). A major unresolved issue regarding sponge/bacterial symbiosis is the nature of the relationship between the bacteria and the sponge. It can be assumed that symbiotic bacteria coevolved for an unknown period together with the host, allowing it to interact with the "symbiont" in the complete range of relationship ranging from commensalism to mutualism or parasitism. Molecular biological techniques have allowed first insights into the functional symbiotic relationship between bacteria and their host. These symbiotic relationships are involved in nutrient acquisition (Wilkinson and Garrone 1980), supply of photosynthate from photoautotrophic symbionts to the sponge (Steindler et al. 2002; Wilkinson 1983), stabilization of the sponge skeleton (Rützler 1985), metabolic waste processing (Beer and Ilan 1998) and production of secondary metabolites (Schmidt et al. 2000). Sponges are a well-established source of important natural products and some of these may be produced by the bacterial symbionts (Haygood et al. 1999; Hill 2004). Bacteria in the sponges are the biosynthetic origins of highly diverse secondary metabolites that provide chemical defense against predation, microbial attachment and fouling by other invertebrates for the sponges (Kubanek et al. 2002). In addition, sponges serve as a shelter for bacteria against grazers or the substrate for bacteria to attach so that bacteria can proliferate more rapidly. Furthermore, sponge metabolites can serve as a consistent nutrient supply (Imhoff and Stöhr 2003). This close contact between sponges and bacteria and so far discover mutual influences gives optimal conditions to study potential horizontal gene transfer (HGT). In the process of breakdown and digestion of the bacteria some of the bacterial DNA is taken up by the sponges and incorporated into their genomes. Throughout the decades mutualism and reticulation supplemented by HGT are processes that are the most important in species evolution (Margulis and Sagan 2002; Kropotkin 1902). The role of

horizontal gene transfer in speciation, adaptation and evolution of life on earth has been studied intensively (Brown 2003). There has been a growing body of evidence for gene transfers among species (Nelson et el. 1999; Koonin 2001; Gogarten et al. 2002) and transfers from organelles to nuclei (Martin et al. 1998; Huang et al. 2003; Bergthorsson et al. 2003). Individual examples include transfer from Bacteria to Fungi (Garcia-Vallve et al. 2000) or Ciliates (Devillard et al. 1999) in the rumen. The transfer of 16 bacterial genes to Nematodes (Scholl et al. 2003) and of 96 such genes to *Entamoeba histolytica* (Loftus et al. 2005) are the only examples where HGT from Bacteria to Eukaryotes has been investigated on a large-scale. We have examples that conjugation from bacteria to eukaryotes still occurs nowadays under laboratory conditions (conjugative transfer to yeast, filamentous fungi and plants) as well as in natural environments (T-DNA transfer to plants) (de la Cruz and Davies 2000). The Ti plasmids from *Agrobacterium*, the nodulation plasmids from *Rhizobium* and the virulence plasmids from *Yersinia*, *Shigella* and *Escherichia coli* are examples of plasmids that, in essence, create new bacterial species when they are acquired.

1.2 Anaerobic pathways

Sponges as aerobic organisms depend on oxygen supply for their metabolism and for morphogenetic events (Perović et al. 2003). Oxygen saturation in sponge tissue depends of flow regime, culturing conditions and their pumping activity (Gatti et al. 2002). When oxygen flux is reduced below some critical level in most animals, full or partial anaerobic metabolism begins to occur (Gnaiger 1991). Historically, the first anaerobic process observed by man was the fermentation of sugar to alcohol by the action of various yeasts. This process has been exploited by commercial and amateur interests throughout most of man's recorded history. The other anaerobic process of historical note is the production of lactate associated with muscular activity in vertebrates. This process has received considerable attention from biologists; hence the degradation of glycogen via the Embden-Meyerhof pathway to lactate (i.e. anaerobic glycolysis) is recognized as an important metabolic route for providing energy in many species, especially vertebrates (Withers 1992). In the last years the number of species, which do not adhere to the

"lactate standard", has grown considerably. A number of marine invertebrates (cephalopods, some bivalves, sipunculids, polychaetes, anemones and sponges) capable of withstanding prolonged periods of environmental hypoxia or anoxia do in fact possess other anaerobic pathways which are alternatives to lactate formation (Fig.4). Phosphoenolpyruvate (PEP) is a branch point for the four main degradation pathways as follows: (1) glucose-lactate pathway (end-product: lactate); (2) glucose-opine pathway (end-product: opines); (3) glucose-succinate pathway (end products: succinate); and (4) aspartate-succinate pathway (end products: succinate).

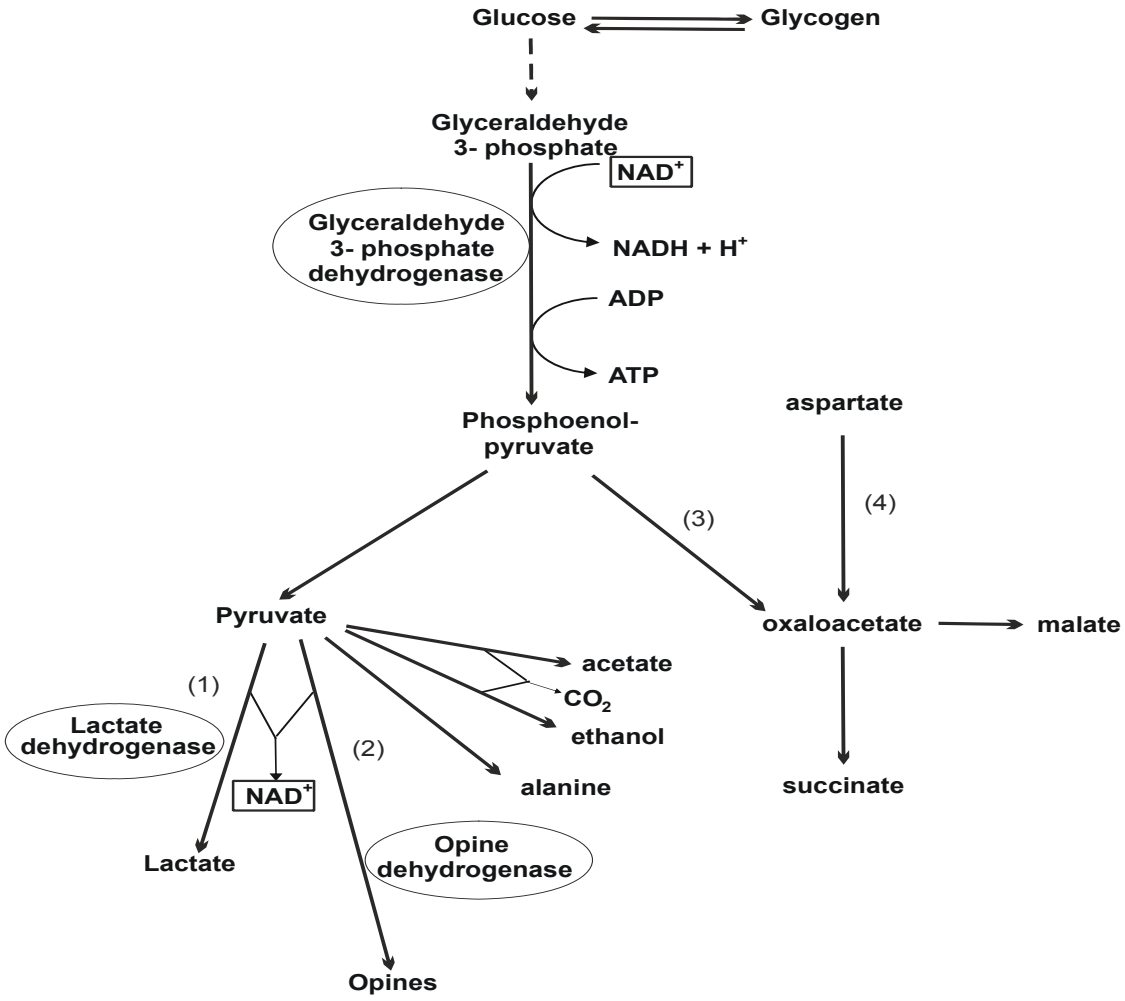

Fig.4. *The four main anaerobic pathways indicated by number in parentheses according to (Hochachka and Somero 1984; Livingstone 1983).*

In the glucose-opine pathway and glucose-succinate pathway lactate dehydrogenase (LDH) and other pyruvate reductases (i.e. opine dehydrogenases) ensures continuous flux of glycolysis and a constant supply of ATP by maintaining the NADH/NAD^+ ratio during exercise and hypoxia, as well as in the subsequent recovery period (Gäde and Grieshaber 1986). Although, the energy can be produced faster in these two pathways energy production is more efficient in glucose-succinate pathway and aspartate-succinate pathway where malate dehydrogenase, succinate dehydrogenase, ketoglutarate dehydrogenase and succinic thiokinase have functions similar to LDH and pyruvate reductases. These pathways accumulate succinate and can yield as almost twice much energy as the first two pathways.

1.2.1 Evolution of the anaerobic pathways

Any of the anaerobic reactions are suitable candidates for the production of anaerobic energy and it is likely that many were present at a very early stage of life what is indicated by their wide distribution in modern-day prokaryotes and eukaryotes. A number of observations support the proposal for the early appearance of amino acid based pathways due to the fact that amino acids were prominent components of the primeaval soup (Oro 1976). It has been suggested that one of the earliest systems for the generation of ATP by oxidoreduction reactions may have involved amino acids as electron-donors and electron-acceptors, the *Clos*tridium- type bacteria being modern-day examples of organisms using such a system (Clarke and Elsden 1980). A reasonable conclusion therefore is that amino acid pathways were prominent among the original anaerobic pathways. From these early beginnings, the aspartate-succinate and opine pathways may have evolved. These pathways are having in common a requirement for carbohydrate and amino acids. The ancestral opine pathways may have involved many amino acids but eventually the octopine pathway was selected in at least the Mollusca as being particularly useful. The glucose-succinate pathway may have arisen either from the aspartate-succinate pathway or directly from other sources.

A hypothetical scheme (Fig.5) is concluded on the basis of phylogenetic distribution of the pathways and describes the evolution of functional, integrated pathways and not of individual anaerobic reactions (Livingstone 1983; Livingstone et al. 1983).

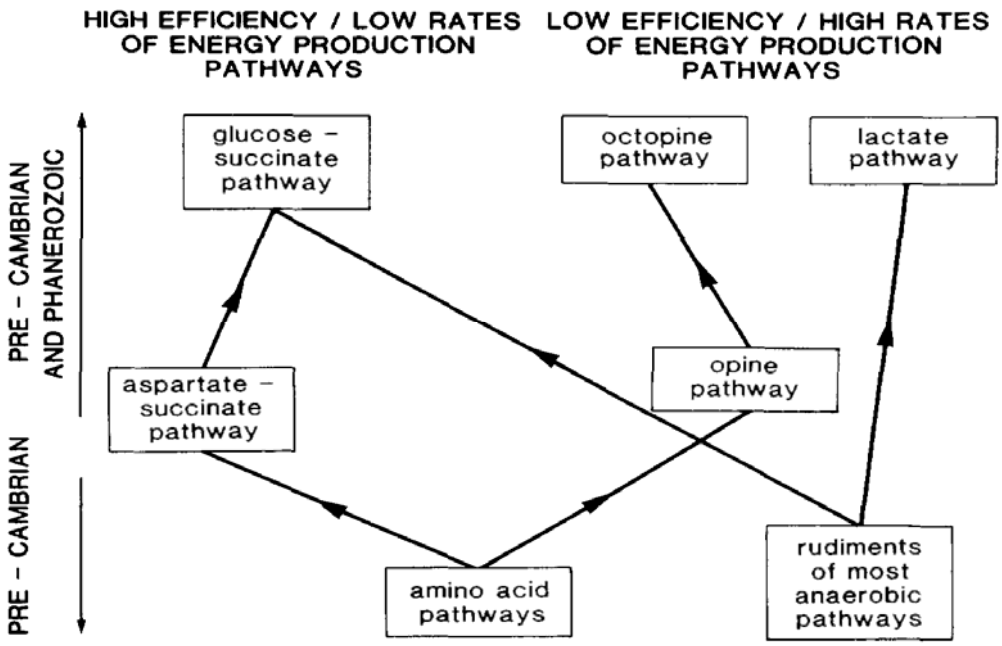

Fig.5. *Hypothetical evolutionary scheme of the anaerobic pathways (taken from Livingstone 1991).*

The lactate pathway could have originated independently of the opine pathways or possibly from them. A major advantage for the preferred selection of lactate formation in some phyla could result from the facts that lactate can easily diffuse from tissue into body fluids. Thereby the continuous reduction of pyruvate is facilitated since the reaction product is eliminated. The lactate pathway is evolutionarily the most advanced due to the presence in most, if not all, phyla. It is the sole major pathway in the Arthropoda, Echinodermata and Chordata. Opine pathways are less advanced, being present in marine species of the lower (Porifera, Cnidaria, Nemertina) and middle (Mollusca, Annelida, Brachiopoda, Sipuncula) phyla (de Zwaan et al. 1982). Important difference between the octopine and lactate pathways is that the anaerobic end product of the former is less acidic (Zammitt 1978). Opine (imino acid) will not change the intracellular pH as much as lactate would and therefore the intracellular osmotic pressure would be kept constant. Whereas potential selective advantages of glucose-opine pathways, particularly in amino acid rich cells such as those of marine invertebrates, are appreciated (Hochachka 1985; Hochachka and Somero 1984) their origin has remained obscure. This is because phylogenetically ancestral organisms do not form imino acid end products and so the

genes for these dehydrogenases seem to have arrived from nowhere in invertebrate evolution. They constitute an example par excellence of a true genetic innovation. A clue to the origin of for example octopine pathway comes from studies of this system in crown galls, the only other place it is known to occur. Hochachka (1988) has suggested that octopine dehydrogenase in mollusks might come from bacterial transfection, but if this is so then given the wide phylogenetic distribution of the pathway (Nemertina, Sipuncula) and/or ODH activity it must have been a general phenomenon and occurred early in the evolution of the Animalia. A prominent role was assigned to the opine pathways in the rise of metazoans in providing energy for the prolonged burrowing of infaunal worms of the Precambrian era (Livingstone 1983). This is still a function of the octopine pathway although it is likely that the present-day alanopine and strombine pathways have other uses such as survival under anoxia (de Zwaan and Zurburg 1981) and osmoregulation. Correlations between the size of free amino acid pools and the presence of opine pathways are seen in modern day species (Sato et al. 1987) and presumably this must have been a factor in their selection. Multiple opine pathways increase the capacity of the system (Gäde and Grieshaber 1986). Comparing opine production throughout the animal kingdom two observations can be made: (1) the variability in opine production corresponds to the variability in the amino acid contents and (2) in those species that are able to produce several opines using the glycolytic pathway during burst muscular activity as well as during ambient lack of oxygen, the pattern of end products produced is different in each anaerobic condition. Concerning environmental anaerobiosis it is obvious that the amino acid present in the highest tissue concentration forms the amino acid moiety of the opine which is predominantly accumulated.

1.2.2 Opines

Opines (imino acids) are end products of anaerobic glycolysis in invertebrates, biosynthesized by enzymes – opine dehydrogenases. The name, opine comes from octopine, the very first opine from octopus muscle, discovered by Morizawa in 1927.

There are at least 30 different opines described so far that chemically fall into two major structural classes:

1. Secondary amine derivatives formed by condensation of an amino acid with a:

(A) keto acid

NOPALINE family/ alpha-ketoglutarate
nopaline, nopalinic acid, leucinopine, glutaminopine, succinamopine

OCTOPINE family/ pyruvate
octopine, octopinic acid, lysopine, histopine, tauropine

(B) sugar

MANNITYL family/mannose
mannopine, mannopinic acid, agropine, agropinic acid

2. Agrocinopines form a small, separate class of opines. Chemically they are sugar-phosphodiesters (for example, agrocinopine A: phosphodiester of sucrose and L-arabinose).

Opines (acetopine and nopaline) can be a product of arginine metabolism in normal callus and plant tissue. Saccharopine is an intermediate in the metabolism of amino acid lysine and occurs in fungi, higher plants and mammals, including man. The poisonous mushroom *Clitocybe acromelalga* is a source of four opines: valinopine, epileucinopine, isoleucinopine and phenylalaninopine.

Opine dehydrogenases (OpDHs) are monomeric pyruvate oxidoreductases that catalyze the NADH-dependent reductive condensation of pyruvate and amino acid by the following reaction:

$$\text{pyruvate} + \text{amino acid} + \text{NADH} + \text{H}^+ \xrightarrow{\text{OpDH}} \text{opine} + \text{H2O} + \text{NAD}^+$$

To date 6 opine dehydrogenase activities have been detected in some marine invertebrates. Besides pyruvate and the coenzyme NADH, different opine dehydrogenases use the following amino acids as a secondary substrate:

- **octopine dehydrogenase, OcDH** (D-octopine: NAD oxidoreductase; EC 1.5.1.11; L-arginine),
- **alanopine dehydrogenase, AlaDH** (meso-alanopine: NAD oxidoreductase, EC 1.5.1.17; L-alanine),
- **strombine dehydrogenase, StDH** (D-strombine: NAD oxidoreductase, EC 1.5.1.22; glycine),
- **tauropine dehydrogenase, TaDH** (tauropine: NAD oxidoreductase, EC 1.5.1.23; taurine),
- **β -alanopine dehydrogenase, AlaDH** (β -alanopine: NAD oxidoreductase, EC 1.5.1.26; β -alanine),
- and **lysopine dehydrogenase, LyDH** (lysine: NAD oxidoreductase, EC 1.5.1.16; lysine).

Studies indicate that OpDHs have many common properties such as molecular mass, single polypeptide structure, reaction type, co-enzyme specificity and pH optima. Some opine dehydrogenases have high specificity for pyruvate and the respective amino acid, whereas others are characterized by relatively low specificity for their substrates (Sato et al. 1993; Manchenko et al. 1998). All known OpDHs from marine invertebrates are monomeric proteins of approximately 37 to 47 kDa and have similar catalytic properties.

Despite many similarities, recent studies, are suggesting that there are at least two types of OpDH in marine invertebrates (Kanno et al. 2005): (*i*) a mollusk/ annelid-type, which includes all of the previously described OcDHs and TaDHs from the mollusks and the annelid and (*ii*) a sponge-type which belongs to the OCD/mu-crystallin family. Sponge OpDH showed no significant amino acid sequence similarity with the previously described OpDHs. Instead the enzyme showed homology with proteins belonging to the OCD/mu-crystallin family which includes ornithine cyclodeaminases: lysine cyclodeaminase from *Streptomyces hygroscopicus*, AlaDH from *Archaeoglobus fulgidus* and NADP-regulated thyroid hormone-binding protein in human (Vié et al. 1997). Ornithine cyclodeaminase (OCD) is an unusual enzyme that converts L-ornithine directly into L-proline and releases ammonia. It has limited distribution among the specialized-niche Eubacteria and Archaea (Soto et al. 1994). OCD is a one of the enzymes that participate in the octopine or nopaline catabolism pathway in Agrobacteria that infect plants by tumor inducing plasmid-mediated gene transfer (Sans et al. 1987). In other bacteria, OCD is thought to be one of enzymes in the arginase pathway, a pathway of arginine catabolism (Cunin et al. 1986). At first glance, OCD and sponge OpDHs seem similar in several aspects: both are opine-relating enzymes, both catalyze imino acid (proline or opine) formation and both require NAD^+ as a co-substrate or as transient electron acceptor (Sans et al. 1988). It seems difficult to regard sponge OpDHs as homologs of the known OpDHs if the phylogenetic distance between sponge and mollusks/annelids is taken into account, suggesting that sponge OpDHs constitutes an independent enzyme class. In addition, it has been proposed that TaDH and StDH were an ancient OpDHs and most likely the ancestors of other opine dehydrogenases (Kanno et al. 2005). Due to the fact that glycine and taurine are often the largest free amino acid pools found in species this could have given the opine pathways an early advantage in functional anaerobiosis or even allowed them to have a role in anoxia survival (Livingstone 1991; Konosu 1971)

2 AIM OF THE STUDY

The aim of this study was to establish the presence and the physiological role of opine production pathway(s) in the sponge *Suberites domuncula.* Characterization of the OpDH gene was done by application of molecular biology techniques. Moreover, genetic variations of the gene were observed. Although, OpDH showed high sequence homology with bacterial OCD primary structure analysis supported assumption that the OpDH is integrated in the sponge genome. In addition, tertiary and quaternary structure was analyzed. Applying the enzymatic assays, characterization of the protein was done. Production of the recombinant protein and antibodies ensured localization of the OpDH *in situ* with immunohistological analysis. Following previous studies that imply involvement of the OpDH in anaerobic pathway, expression studies in the controlled conditions were done as well. Sponges, as phylogenetically the lowest marine animal group in which OpDHs activity has been demonstrated, are especially interesting in terms of evolutionary analysis. Due to the many specific properties that sponge OpDH showed special focus was set on the mutual influences between bacteria and sponge with emphasis on potential horizontal gene transfer.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

Agar	Roth, Karlsruhe
Agarose	Roth, Karlsruhe
Albumin Fraktion V	Roth, Karlsruhe
Ampicillin-Sodiumsalt	Sigma, Taufkirchen
Blocking reagent	Roche, Mannheim
5-Bromo-4-chloro-3-indolyl-b-D-galactopyranoside	Roth, Karlsruhe
5-Brom-4-chlor-3-indolylphosphat	Roth, Karlsruhe
Bromphenolblue	Serva, Heidelberg
CDP-Star™ ready to use	Roche, Mannheim
Chloroform	Roth, Karlsruhe
DigEasyHyb	Roche, Mannheim
dNTP-Mix	Peqlab Biotechnologie GmbH, Erlangen
Ethanol	Roth, Karlsruhe
Ethidium bromide	Boehringer, Mannheim
Formaldehyde	Roth, Karlsruhe
Formamide	Roth, Karlsruhe
Gelatine	Serva, Heidelberg
Gelcode blue stain reagent	Pierce, Bonn
Glycerol	Sigma, Taufkirchen
Glycine	Roth, Karlsruhe
Hydrochloric acid	Roth, Karlsruhe
Isopropanol	Merck, Darmstadt
Luria Bretani (LB) Broth	Roth, Karlsruhe
Magnesiumsulfat	Merck, Darmstadt
Maltose	Merck, Darmstadt
2-Mercaptoethanol	Sigma, Taufkirchen
NZCYM broth	Roth, Karlsruhe
Proteinase-Inhibitor-Cocktail,Complete-Mini	Roche, Mannheim
RNase Away	Roth, Karlsruhe
Roti-Quant R250	Roth, Karlsruhe
Sequagel complete	BIOZYM, Hess.Oldendorf
Sequagel XR	BIOZYM, Hess.Oldendorf
Sodiumdihydrogenphosphat-2-hydrate	Roth, Karlsruhe
Sodiumdodecylsulfat (SDS)	Roth, Karlsruhe
Technovit 8100	Heraeus Kulzer, Wehrheim
Triton X-100 (Octylphenolpoly(ethylenglycolether)$_n$	Roth, Karlsruhe
TRIzol®	Invitrogen, Karlsruhe
Piperazine-1,4-bis(2-ethanesulphonic acid) (PIPES)	Sigma, Taufkirchen

Tween 20 (Poly(oxyethylen)$_n$-sorbitan-monolaurat)	Roth, Karlsruhe
Yeastextract	Roth, Karlsruhe

3.1.2 Equipment

Centrifuge:	
Sorvall RC 5B	DuPont, Bad Nauheim
Eppendorf centrifuge 5402	Eppendorf, Hamburg
Heraeus Biofuge fresco	Kendro,Hanau
Electrophoresis apparatus	Bio-Rad, München
Heatblock Thermostat 5320	Eppendorf, Hamburg
Heatplate	IKA Labortechnik, Staufen
Laminar Flow	Sterilbank Slee, Mainz
Li-Cor Sequencer 4200	MWG-Biotech, Ebersberg
Light microscope	Olympus AHBT3, Hamburg
Mini Protean II	Bio-Rad, München
Pipettes (2, 10, 20, 100, 200, 1000 µl)	Gilson, Frankreich
pH-Meter Typ CG 840	Schott, Mainz
Spectrophotometer:	
UV/Visible GelDoc 2K System PC	Bio-Rad, München
Pharmacia LKB-Ultrospec III	Pharmacia Co., USA
Spectorphotometer SmartSpec Plus	Bio-Rad, München
Spectorphotometer Titertek Multiskan Plus	Bartolomey Labortechnik, Rheinbach
Sunrise microplate reader	TECAN, Switzerland
Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cel	Bio-Rad, München
Thermocycler:	
Bio-Rad i-cycler	Bio-Rad, München
Thermomixer comfort	Eppendorf, Hamburg
UV-Crosslinker Stratalinker 1800	Stratagene, La Jolla; USA
Vortexer	Labotech, Wiesbaden
Waterbath	Köttermann, Hängingen

3.1.3 Enzymes and Antibodies

Anti-(DIG) Digoxigenin-AP, Fab fragments	Roche, Mannheim
Anti-His (C-terminal)-IgG (mouse)	Invitrogen, Groningen; NL
Anti- *rabbit* IgG (Fab specific)–Alkaline Phosphatase antibody produced in goat)	Sigma, Taufkirchen
Cy3-conjugated goat anti-*rabbit*	Dianova , Hamburg
Expand DNA-polymerase mix	Roche, Ingelheim
Jump start, Accutaq	Sigma, Taufkirchen
KOD XL	Novagene, Darmstadt
Pwo-DNA-Polymerase, SAWADY	Peqlab Biotechnologie GmbH, Erlangen
Goat Anti - *rabbit* IgG-peroxidase	Dianova , Hamburg
Proteinase K	Qiagen, Hilden
Restorase	Sigma, Taufkirchen
SuperScript™III (Reverse Transkriptase)	Invitrogen, Karlsruhe
Taq-DNA-Polymerase, SAWADY	Peqlab Biotechnologie GmbH, Erlangen
Restriction enzymes:	
BamH I	Boehringer, Mannheim
Sal I	Boehringer, Mannheim
SacI	Boehringer, Mannheim
Eco RI	Sigma, Taufkirchen
DNA-ase	Ambion, Darmstadt
RNA-ase	Stratagene, NL-Amsterdam

3.1.4 Markers

Lambda DNA/Eco130I (StyI)	Fermentas, St. Leon-Roth
DNA-Ladder GeneRuler	MBI Fermentas, St. Leon-Rot
DNA-Ladder 1 kb	New England Biolab, Frankfurt am Main
DNA-Marker pUC 19/MspI	Roth, Karlsruhe
Mass ruller™ DNA ladder	Fermantas, St. Leon-Roth
2-log DNA ladder	New England Biolab, Frankfurt am Main
PeqGOLD Protein Marker I	PeqLab, Erlangen
Precision Plus Protein Standards, Dual Color	Biorad, München
RNA*later*	Ambion, Bad Soden

3.1.5 Kits

Ni-NTA Agarose	Qiagen, Hilden
Ni-NTA Spin Kit	Qiagen, Hilden
NucleoSpin® Extract II	Macherey-Nagel, Düren
NucleoSpin® Plasmid	Macherey-Nagel, Düren
PCR DIG Labeling MixPLUS	Roche, Mannheim
pGEM®-T Vector System	Promega, Madison, USA
QIAprep Spin Miniprep Kit	Qiagen, Hilden
QuantiTect Reverse Transkription Kit	Qiagen, Hilden
Sequagel Complete	National Diagnostics, Atlanta, USA
Rapid Excision Kit	Stratagene, Heidelberg
Thermo Sequenase Kit	Amersham-Pharmacia, Freiburg
Blood & Cell Culture DNA kit	Qiagen, Hilden
Lambda Isolation kit	Qiagen, Hilden
pTrcHis TOPO® TA Expression Kit	Invitrogen, Karlsruhe
pCR®II TOPO TA Cloning®Kit	Invitrogen, Karlsruhe

3.1.6 Vectors

Lambda Fix II	Stratagene, Heidelberg
pCR®II TOPO TA Clonig®Kit	Invitrogen, Karlsruhe
pGEM-T	Promega, Madison, USA
pTrcHis2 TOPO® TA Expression Kit	Invitrogen, Karlsruhe
pTriplEx2	Clontech, Heidelberg

3.1.7 Primers

Vector specific primers

M13-20	5’ GTA AAA CGA CGG CCA GT 3’	T_m = 59 °C
pTrcF	5’ GAG GTA TAT ATT AAT GTA TCG 3’	T_m = 54 °C
pTrcR	5’ GAT TTA ATC TGT ATC AGG 3’	T_m = 48 °C
SP6	5’ TAG GTG ACA CTA TAG AAT ACT CA 3’	T_m = 55 °C
SP6 800	5’ CTATTTAGGTGACACTATAG 3’	T_m = 51 °C
T3	5’ CGG AAT TAA CCC TCA CTA AAG 3’	T_m = 60 °C
T7	5’ GTA ATA CGA CTC ACT ATA GGG C 3’	T_m = 64 °C
T3 not	5’ CTCGCGGCCGCGAGCTCAATTAACCCTCACTAAGGG 3’	T_m > 75 °C
T7 not	5’ CTCGCGGCCGCGAGCTCTAATACGACTCACTATAGGGC 3’	T_m > 75 °C
T7 800	5’ GTAATACGACTCACTATAGG 3’	T_m = 53 °C

Gene specific primers

OrniF	5’ GCTCTACAAGATGTCCAAGAAG 3’	T_m =58,4°C
OrniR	5’ GTTAGACGGGTGGCATAGG 3’	T_m =58,8°C

Semiquantitative RT-PCR primers

MSN_QF	5'-CTGGACATTGCTGGATTC -3'	T_m =53,7°C
MSN_QR	5'-TCCTTTCTGCCTTGTACT -3'	T_m =54,5°C
TaDH_RT_F	5′ -TAGAGTTTGAGACTGCCAG- 3′	T_m =54,5°C
TaDH_RT_R	5′ -CTTCACCCACTCATCAC- 3′	T_m =52,8°C
Tub_RT_F	5′ -TTTATGACATCTGCTTCCGC- 3′	T_m =55,3°C
Tub_RT_R	5′ -TGACCAGGGAAACGGAG- 3′	T_m =55,2°C

Expression primers

SdOCDexF	5' -TCCAAGAAGCTTACTTTCC- 3'	T_m =52,4°C
SdOCDexR	5' -GACGGGTGGCATAGGTATAGG- 3'	T_m =61,8°C

Primers for polymorphism

Sd_polyF*	5' -TCAACAGTACGGAGACAGG- 3'; 5′ IRD 800	T_m = 56,7°C
Sd_polyR	5 '-GAATGGCAGTGATGTATGTC- 3'	T_m = 55,3°C

Degenerative primers

OrniDR	5' -ATCGGCAA(CT)GGCGCGCA(AG)AGC- 3'	T_m = 67,7°C
OrniDF	5' -(CT)TC(GT)GT(CT)TTGCC(AG)GGGCA(AG)TC- 3'	T_m = 62,8°C

Primers for cathepsin

SdCatLC2F	5’ CCAAAATGAAGGTGCTCATTC 3’	T_m = 55,9°C
SdCatLC2R	5’ GCATTAGACTCTAAACCA 3’	T_m = 52,4°C

3.1.8 Bacterial strains

E. coli XL1-*Blue MRF'* Genotype: RecA1 endA1 gyrA96 thi-1 hsdR17 supE44 [F' proAB lacIqZΔM15 Tn10 (Tetr)] relA1 lac	Stratagene, Heidelberg
NovaBlue Genotype: *endA1 hsdR17*(rK12$^-$mK12+) *supE44 thi-1 recA1gyrA96relA1lac*F'[*proA+B+laclqZΔM15*:: Tn*10* (TcR)]	Merck, Darmstadt
TOP10 Genotype:F-*mcr*AΔ(*mrr-hsd*RMS-*mcr*BC)Φ80*lac*Z ΔM15Δ*lac*X74*rec*A1*deo*R*ara*D139Δ(*araleu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen, Karlsruhe
LE 392 Genotype:*hsd*R514($r_K^-m_K^+$)*sup*E44 *sup*F58*lac*Y *gal*K2 *gal*T22 *met*B1 *trp*55 *mcr*A	Promega, Madison, USA

3.1.9 Culture medium

LB-Medium

10 g/l Trypton
5 g/l Yeastextract
5 g/l NaCl
x ml NaOH (pH 7.0)

2 x TY-Medium

16 g/l Trypton
10 g/l Yeastextract
5 g/l NaCl
x ml NaOH (pH 7.0)

LB-Agar 1.5%

10 g/l Trypton
5 g/l Yeastextract
5 g/l NaCl
15 g/l Agar
x ml NaOH (pH 7.0)

LB-Top-Agar 0.7%

10 g/l Trypton
5 g/l Yeastextract
5 g/l NaCl
7 g/l Agar or Agarose
x ml NaOH (pH 7.0)

NZY-Broth

10 g/l Trypton
5 g/l Yeastextract
5 g/l NaCl
2 g/l $MgSO_4$ x 7 H2O
x ml NaOH (pH 7.5)

NZY-Agar

10 g/l Trypton
5 g/l Yeastextract
5 g/l NaCl
2 g/l $MgSO_4$ x 7 H2O
15 g/l Agar
x ml NaOH (pH 7.5)

NZY-Top-Agar

10 g/l Trypton
5 g/l Yeastextract
5 g/l NaCl
2 g/l $MgSO_4$ x 7 H2O
7,5 g/l Agar
x ml NaOH (pH 7.5)

3.1.10 Computer programs

BLAST	http://genome.eerie.fr./blast/blast-query.html (Altschul et al. 1990)
FASTA	http://www.ebi.ac.uk/htbin/fasta.py?request (Pearson and Lipman 1988)
CLUSTAL V1.81	EMBL, Freeware: ftp.ebi.ac.uk (Thompson et al. 1994)
DNAStar V6	DNAStar Inc.
e-Seq V2.0 DNA	LI-COR, MWG Ebersberg
ExPASy ProtParam tool	http://www.expasy.org/tools/protparam.html
GENEDOC V2.6.002	www.cris.com (Nicholas and Nicholas 1997)
Genescan	Intron/Exon Splice Sites http://genes.mit.edu/GENSCAN.html
Pfam	Protein families database of alignments and HMMs: http://sanger.ac.uk/cgi-bin/pfam/
Prosite	http://www.ebi.ac.uk/searches/prosite.input.html (Bairoch 1988)
Mega 4	Tamura et al. 2007

3.2 Experimental animals

Live specimens of *Suberites domuncula* Olivi (Porifera, Demospongiae, Hadromerida) were collected by Scuba diving near Rovinj (North Adriatic, Croatia) from depths between 15 and 35 m. In this study either fresh collected sponges that were immediately frozen in liquid nitrogen were used or the sponges were brought to Mainz (Germany) and kept for at least five months in aquaria at 16°C, under controlled aeration before use in the experiments (LePennec et al. 2003).

4 Methods

4.1 DNA extraction from tissue

Genomic DNA was isolated with Blood and Cell Culture DNA kit (Qiagen, Hilden, Germany) from dissociated tissue. Approximately 200 mg of tissue was cut into fine pieces and incubated in 30 ml of calcium magnesium-free sea water (CMFSW), supplemented with 2.5 mM EDTA, for one hour at room temperature with gentle shaking. After this treatment tube was left for 5 min for sedimentation of spicules and non dissociated pieces. The cells were collected from the supernatant by centrifugation at 1000 rpm for 10 min. Genomic DNA was isolated from those cells following QIAGEN recommendations. After lysis and digestion with protease K cleared lysate was loaded onto a pre-equilibrated QIAGEN Genomic-tip by gravity flow. The salt and pH conditions of the lysate and the superior selectivity of the QIAGEN Resin ensure that only DNA binds, while degraded RNA, cellular proteins and metabolites are not retained and appear in the flow-through fraction. The QIAGEN Genomic-tip is then washed with a medium-salt buffer that completely removes any remaining contaminants, such as traces of RNA and protein (e.g. RNase A) without affecting the binding of the DNA. This buffer also disrupts nonspecific interactions and allows removal of nucleic acid-binding proteins without the use of phenol. The low concentration of ethanol in the wash buffer eliminates nonspecific hydrophobic interactions, further enhancing the purity of the bound DNA. The DNA is then efficiently eluted from the QIAGEN Genomic-tip with a high-salt buffer.

CMFSW (calcium magnesium-free sea water):

- 400 mM NaCl
- 7 mM Na_2SO_4
- 10 mM KCl
- 10 mM HEPES
- 2.5 mM Na-EDTA

4.2 Phenol-chloroform DNA extraction

The standard and preferred way to remove proteins from nucleic acid solutions is by extraction with neutralized phenol or phenol/chloroform. Generally, samples are extracted by addition of 0.5 volume phenol to the sample, followed by vigorous mixing for a few seconds to form an emulsion. Subsequent centrifugation is recovering the aqueous (top) phase, containing the nucleic acid, which is transferred to a clean tube. Residual phenol is removed by extraction with an equal volume of chloroform. Following centrifugation is separating the phases. The chloroform (upper phase) is discarded and the nucleic acid is precipitated by adding 0.3 M sodium acetate and 0.8 volume of isopropanol. After additional centrifugation pellet is washed with 2 volumes of ethanol in order to remove residual salts. Replacing isopropanol with the more volatile ethanol is making the DNA easier to redissolve. Pellet is dissolve in apropriate buffer.

4.3 DNA precipitation

DNA was precipitated using ethanol. Concentration of monovalent cations in the sample was adjusted by adding 0.3 M sodium acetate to the aqueous phase. After addition of 2 volumes of absolute ethanol the solution was incubated at -70 °C for 1 hour. Precipitated DNA was recovered by centrifugation at maximum speed for 10- 30 min. For large DNA fragments the centrifugation times were reduced to 5-15 min to facilitate subsequent redissolving. The supernatant was carefully discarded and the resulting pellet was rinsed with ice-cold 70% ethanol to remove residual salts. Evaporation off residual ethanol was done for 5-10 min and DNA was resuspended in appropriate amounts of ddH_2O or TE buffer.

4.4 Restriction digestion of DNA

The digestion of DNA was performed in the final volume of 20 µl with recommended reaction buffer and temperature for 1-2 h according to the instruction from the company. Double digestions were carried out simultaneously in a buffer compatible with both

enzymes. If only specific bands or restriction products were required, DNA was separated on agarose gel and isolation of the desired bands was done by excision and subsequent isolation as described in 3.11.

4.5 RNA isolation

RNA was extracted with TRIzol (GibcoBRL) from 0.1-0.2 g tissue as recommended by the manufacturer (Invitrogen). TRIZOL is a ready-to-use reagent for the isolation of total RNA from cells and tissues. The reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, is an improvement to the single-step RNA isolation method developed by Chomczynski and Sacchi (1987). During sample homogenization or lysis, TRIZOL Reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. Addition of chloroform followed by centrifugation separates the solution into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase. After transfer of the aqueous phase, the RNA is recovered by precipitation with isopropyl alcohol. For semiquantitative RT-PCR purified RNA sample is briefly incubated in “gDNA Wipeout buffer” (Qiagen), at 42°C to effectively remove contaminating genomic DNA. The integrity of the RNA was assessed by denaturing agarose gel electrophoresis, followed by ethidium bromide staining and the quantity was determined by UV absorption at 260 nm.

4.5.1 RNAse inactivation using DEPC (diethylpyrocarbonate)

DEPC was added to solutions (1ml per 1L of solution) to inactivate RNAse. After overnight incubation, solutions were autoclaved to inactivate the DEPC. Bottles were opened only under the hood.

4.6 Southern Blotting

Southern Blotting is a method for localization of a particular DNA sequence within a complex mixture. For example, Southern Blotting could be used to locate a particular gene within an entire genome. It can be used as well for determination of the molecular

weight of a restriction fragment or for measuring relative amounts in different samples. For Southern Blotting 5 µg of genomic DNA was digested with EcoRI and/or BamHI restriction endonucleases and electrophoresed in 1%/TAE agarose gel. The gel was then blotted onto a Hybond N+ membrane (Amersham). Transfer is accomplished by the flow of buffer (capillary forces) through wick, membrane and adsorbent paper layers. After transfer, the DNA is permanently attached to the membrane by UV cross-linking. Prehybridization is done in 30-50 mL DIG EasyHyb solution (Roche) in glass tubes, for 30-40 min. Before hybridization the probe is denaturated for 5 min on 95°C. Next, the membrane with attached DNA is hybridized with DIG-labeled probe (3.18.3) at a final concentration of 10-20 ng/ml in DIG EasyHyb solution (Roche), depending on the further detection method. Hybridization was done at 42°C overnight in Rotary Hybridization oven. If probe molecules are complementary to molecules bound on the membrane, double-stranded DNAs will form. After the hybridization filters were washed in 2x SSC–0.1% sodium dodecyl sulfate (SDS) for 20 min at room temperature, followed by 1x SSC–0.1% SDS for 20 min at room temperature, and then 0.2x SSC–0.1% SDS for 15-30 min at 65-68°C. In washing steps poorly bound probe molecules are removed. After blocking for 30-60 min antibodies are added. Antibody dilution depends on the further selected detection method. Therfore, if chemiluminiscent method will be applied for detection of the desired probe 1:10000 dilution is used. For colorimetric method 1: 5000 dilution is used. Subsequent washing in P1 buffer and equilibration in P3 buffer are done. For chemiluminiscent method CDP-star reagent is used (20-30 drops on 100 cm^2), membrane is placed between the plastic folie in development box and exposed to X –rays film for 5-15 min. For colorimetric method development is done with NBT/BCIP reagents (in 10 ml of 1x P3 buffer 45 µl NBT and 35 µl BCIP was added).
Buffers and solutions used:

20x SSC:

- 3 M NaCl
- 300 mM Sodiumcitrat
- pH 7.0

Washing buffer (P1):

- 100 mM Maleic acid
- 150 mM NaCl
- pH 7.5

Detection buffer (P3):

- 100 mM Tris-HCl, pH 9.5
- 100 mM NaCl

NBT (nitobluetetrazolium) solution:

- 75 mg/ml NBT in 70 % N,N-Dimethylformamid (v/v)

BCIP (5-bromo-4-chloro-3-indolyl phosphate toluidinium salt) solution:

- 50 mg/ml BCIP in 100 % N,N-Dimethylformamid (v/v)

SDS stock solution:

- 10 g SDS (sodium dodecyl sulfate) in 100 ml dH_2O

4.7 Screening of the *S. domuncula* genomic library

Genomic DNA library was screened by plaque hybridization. The bacterial *E. coli* strains used for plating out genomic libraries were LE392 and KW 529. The size of a library of completely random fragments of genomic DNA that is necessary to ensure representation of a particular sequence of interest is dictated by the size of the cloned fragments and the size of the genome. The likelihood that the sequence of interest is present in such a random library can be estimated by simple statistic based on the Poisson distribution (Clarke and Carbon 1976), following the formula:

$$N = \ln (1 - P) / \ln [1 - (I / G)]$$

N : number of independent clones that must be screend to isolate a particular sequence
P : probability of isolating a fragment of interest (here 99 % = 0.99).
I : size of the average cloned fragment, in base pairs
G : size of the target genome, in base pairs

Bacteria were streak out on a LB plate, single colony was picked up and inoculated in LB medium supplemented with 0.2% maltose (induces higher expression of the lambda receptor). After spinning down, the cells were resuspended in 20-25 ml 10 mM $MgSO_4$. Checking the titer of the library is used to isolate pure populations of phage from a single plaque and provide the titer of the phage stock. Depending of the titer of the library (10^8 - 10^9 pfu/ml) serial dilutions of a phage lysate were made. The library stock solution (10 µl) was transferred to 990 µl SM solution. This "10^7" stock solution was diluted 1/100 (10^5 mix), then 1/100 dilution of the 10^5 mix (10^3 mix), and a 1/100 dilution of the 10^3 mix (10^1 mix) was done. In separate tubes, aliquots (2 µl) of each dilution are mixed with *E. coli* (100 µl). Phages are allowed to adsorb to the cells and the cell/phage mixture is then heated to 37°C, causing the phage to inject their DNA into the cells. Top agar is added to each tube, and the mixture is poured onto rich plates, which are incubated at 37°C until plaques appear. Each plaque contains phages derived from a single infecting phage. The plaques are counted and the titer of the library is calculated.

Buffers and solutions used are described in 3.6. Additional ones are listed below:

Denaturation solution:

- 500 mM NaOH
- 1.5 M NaCl

Neutralization solution:

- 400 mM Tris-HCl
- 1.5 M NaCl
- pH 7.4

SM (suspension medium):

- 0.1 M NaCl
- 80 mM $MgSO_4$
- 50 mM Tris-HCl, pH 8.0
- 0.1 % Gelatine (w/v)

4.7.1 Plating out the library and transferring the lambda phage to nylon membranes

The optimal library dilution was chosen for further plating out the library onto 245 x 245 mm plates. According to the scaling factor (F factor) amount of required bacteria is calculated. After incubation of the cell/phage mixture for 30 min at 37°C mix is transferred to 7 ml top agarose, poured over the lambda plate and incubated at 37°C. Hybond N+ membrane (Amersham) were placed on top of the top agarose for about 1-2 min. Using a needle, filled with india ink, the filter was marked. The back side of the lambda plate was marked with a marker as well. This is necessary for later aligning the filters against the plates. Using flat forceps the filter is lifted off the plate, making sure the top agarose stays on the plate. The lambda phage is then transferred to the filter. When the filter is dried it is soaked for 2-5 min in denaturation solution, then for 5 min in neutralization solution. After washing the filters two times in 2X SSC, UV fixation is required for 1-2 min. Pre-hybridization, hybridization and washing of the filters was done as described in 3.6. Finally, the location of the probe is detected by colorimetic method using NBT/BCIP detection reagents. The lambda plate and the filter are aligned according to the marks made before. A positive clone is picked with a pasteur pipette, placed in 1 ml of SM solution with a drop of chloroform that keeps bacteria from growing and put on 4°C overnight. The second screening is done similar to the one described above. Isolated phage was diluted 1/100, mixed with 100 µl of bacterial cells, incubated for 15 min at 37°C. The mixture was added with 3 ml top agarose. This time for plating 82 mm plates were used. The secondary plates are now ready for lifts, hybridization, washing and detection of the signals as described. Third screening was done due to the fact that clean plaque pure phage from secondary plates was not obtained. But in this last screening it was plated less then 100 pfu on the plates. The procedure is the same as described. To make sure that a plaque pure clone is isolated a last check ("plaque pure screen") was done. Plating out the plaque pure clone and re-screen it. Then from this plate with all the positive signals a clone is picked for further analysis-lambda DNA isolation. Three lambda plaques were selected and purified to individual clones. Phage DNA was isolated by Qiagen lambda kit, analyzed by restriction (EcoRI, BamHI

and SacI) and blot hybridization to ensure that the clones represent the same fragment of the *S. domuncula* genome. One lambda clone was sequenced.

4.7.2 Lambda DNA extraction

Extraction of lambda DNA was done with lambda isolation kit (Qiagen). The QIAGEN lambda isolation kit can be used to isolate DNA from:

1. Liquid cultures — in which bacteriophages are propagated in bacteria grown in liquid medium or
2. Plate lysates — in which bacteriophages are propagated in bacteria grown in soft agarose.

If lambda phage is propagated in liquid cultures, after achievement of fully lysed culture (contains considerable amount of bacterial debris) chloroform is added (2% v/v) and incubated at 37°C for a further 15 min to enhance lysis efficiency. In order to remove bacterial debris solution is centrifuged at >10000 x g for 10 min. Supernatant is retained for further isolation.

If DNA is isolated from plate lysates plates are overlaid with 5 ml SM (Suspension Medium) and incubated with shaking at room temperature for several hours. Liquid is transferred to a clean tube. Additional washing of plates with 1 ml SM was done and the washings were added to the tube. After addition of chloroform (2 % v/v), centrifugation at 10000 x *g* for 10 min was done in order to remove residual agarose. Supernatant is used for further isolation. In order to digest bacterial RNA and chromosomal DNA supernatant was incubated with appropriate buffer supplied with RNase A and DNase I for 30 min on 37°C. The hole procedure is based on an optimized PEG precipitation step, followed by anion-exchange chromatography on a QIAGEN-tip. Lambda DNA is bound to QIAGEN Anion-Exchange Resin under appropriate low-salt and pH conditions. Impurities such as proteins, carbohydrates and metabolites are removed by a medium-salt wash. Pure lambda DNA is then eluted in high-salt buffer and subsequently concentrated and desalted by isopropanol precipitation. The isolated DNA is free of protein and RNA and can be used for all downstream applications, including automated or manual sequencing, PCR or other enzymatic reactions and in vitro packaging.

4.8 Isolation of plasmid DNA

To obtain high pure plasmid DNA from bacterial cultures NucleoSpin Plus kits were used. The procedures were followed as outlined in the manufacturer's protocol. In order to obtain small amount plasmid DNA, LB medium (4 ml) supplemented with an appropriate antibiotic and containing *E.coli* was cultured overnight. After lysing the bacterial pellet in alkaline/SDS buffer, precipitating the genomic DNA and clearing the supernatant by a quick centrifugation step, the lysate is transferred into the NucleoSpin Plus spin column, which contains a silica membrane filter. The chaotropic salt in the loading buffer leads to a reversible disruption of the hydrate shell of the plasmid DNA, which allows it to be adsorbed onto the membrane. After removing the contaminants (e.g. protein and RNA) with the washing buffer, the plasmid DNA is eluted in Tris buffer (pH 8.0–8.5). Purified plasmid can be used for restriction analysis, PCR, sequencing and other molecular biology applications.

4.9 Agarose gel electrophoresis

To separate and visualize DNA/RNA samples, standard gel electrophoresis was used (Sambrook et al. 1989). Separation of molecules by size is achieved by moving negatively charged nucleic acid molecules through an agarose matrix with an electric field (electrophoresis). Factors affecting migration in the agarose gels are length and conformation of the DNA molecule, agarose concentration, voltage and buffer composition. Gel electrophoresis can be used for the separation of DNA fragments from 50 base pairs (bp) up to 20000 bp. Routinely gels containing between 0.8% to 1.5% agarose were used. Gels were run at 40-80V. Before electrophoresis, DNA samples were mixed with loading dye which enables visualization and sedimentation of the sample in the gel well. Negatively charged indicators keep track of the position of the DNA. Xylene cyanol and bromophenol blue are usually used. They run at about 5000 bp and 300 bp respectively, but the precise position varies with percentage of the gel. The electrophoresis buffer was 1X TBE or 1X TAE. Molecular weight markers were used for checking the size of the DNA fragments. The most common dye used in agarose gel

electrophoresis, for visualization of DNA, is ethidium bromide (EtBr). It fluoresces under UV light when intercalated into DNA (or RNA). By staining agarose gel with EtBr and visualizing it with UV light, distinct bands of DNA become visible. The electrophoresis results were documented with digital camera.

10x TAE-Elektrophorsese buffer:

- 97.6 g Tris-Base
- 20 ml Glacial Acetic acid
- 5.84 g EDTA
- 2 l ddH_2O

10x TBE-Elektrophorsese buffer:

- 121 g Tris-HCl, pH 8.3
- 61.8 g Boric acid
- 7.4 g EDTA
- 1 l ddH_2O

4.10 Isolation of DNA from agarose gel

DNA was run on agarose gel. Using a sterile scalpel blade, a gel slice containing the DNA band was carefully excised under UV illuminator. The DNA was purified with NucleoSpin® Extract II (Macherey-Nagel, Düren) kit. Colums provided by the manufacturer bind DNA, in the presence of chaotropic salts, to a silica membrane. Contaminations like salts and soluble macromolecular components are removed by a simple washing step with ethanolic buffer. Pure DNA is finally eluted under low ionic strength conditions with slightly alkaline buffer (5 mM Tris-Cl, pH 8.5).

4.11 Polyacrylamide gel

Polyacrylamide gel consists of polymerized acrylamide with the addition of urea, a molecule that binds with hydrogen bonds to DNA/RNA bases and denatures the double

strands into single strands. Acrylamide forms a linear polymer (polyacrylamide) that can be cross-linked with N,N'-methylene bisacrylamide and forms a gel matrix of controlled pore size. Acrylamide stock solutions are typically found with varying ratios of acrylamide/bis-acrylamide to create pores of different size. In this study ratio of acrylamide/bis-bcrylamide 29:1 ratio was used (40% solution containing 38.67% (w/v) acrylamide and 1.33% (w/v) bis-acrylamide). Polymerization of the gel is catalyzed by free radicals, generated by agents such as ammonium persulfate in the presence of N,N,N',N'-tetramethylethylenediamine (TEMED). Polyacrylamide Gel is used in electrophoresis of biomolecules, such as proteins or DNA fragments. Traditional DNA sequencing techniques such as Maxam-Gilbert or Sanger methods used polyacrylamide gels to separate DNA fragments differing by a single base-pair in length so the sequence could be read.

4.12 Photometric measurements

Concentration and purity of dsDNA in solution was assessed by measuring the absorption values at 260 nm and 280 nm using disposable plastic cuvettes and a spectrophotometer. DNA with 260/280 quotient of 1.8-2.0 was regarded as high pure. Alternatively the concentration of PCR products and plasmid DNA solutions was estimated comparing the gel band intensity of different DNA dilutions (1:1 and 1:10) with the band intensity of known DNA ladder mixtures.

4.13 Cloning

4.13.1 A-Tailing of DNA for TA ligation and cloning

Thermostable DNA polymerases with proofreading (3´-->5´ exonuclease) activity, such as Pfu DNA polymerase, Pwo DNA polymerase, KOD DNA polymerase and enzyme mix from Eppicentre generate 95% blunt-ended fragments during PCR amplification. For efficient cloning PCR products needs to be 3' adenylated and purified. Therefore PCR fragments generated using these polymerases have to be modified with dATP using Taq

DNA polymerase before ligation into the vectors. This A-tailing can also be used to subclone DNA fragments generated by restriction enzymes which produce blunt ends. Linearized vectors like pCR®-TOPO® or pGEM-T, used in this study, contain single 3´ terminal thymidines (T's) at each end which complement the A overhang added by the polymerase. Thus vector can be easily ligated with the PCR product.

4.13.2 TOPO TA cloning

The pCR®-TOPO® vectors are provided linearized with 3´-T overhangs and topoisomerase I from *Vaccinia* virus wich functions both as a restriction enzyme and as a ligase. Its biological role is to cleave and rejoin DNA during replication. Topoisomerase I bind to duplex DNA at specific sites and cleave the phosphodiester backbone after 5′-CCCTT in one strand (Shuman 1991). The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3′ phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5′ hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase (Shuman 1994). To harness the religating activity of topoisomerase, TOPO® vectors are provided linearized with topoisomerase I covalently bound to each 3´ phosphate. This enables the vectors to readily ligate DNA sequences with compatible ends. In only 5 minutes, at room temperature, the ligation is complete and ready for transformation into *E. coli.*

4.13.3 pGEM-T cloning

Vector and inserts were prepared according to standard protocols provided by manufacturer (Promega). Usually, 50 ng of linearised and dephosphorylated vector was ligated to a 5-7 molar excess of insert DNA. For calculation of the appropriate amount of PCR product (insert) necessary for successful ligation, the following equation was used:

$$\frac{\text{ng of vector x kb size of insert}}{\text{kb size of vector}} \text{ x insert:vector molar ratio } = \text{ng of insert}$$

Vector and insert DNA were mixed in a 10 µl volume with 1 µl of ligase buffer and 1 µl of T4 DNA ligase. Samples were incubated over night at 4°C and 1-5 µl of the ligation mixture was transformed into competent cells.

4.14 Transformation of competent cells

Bacterial transformation is the process by which bacterial cells take up naked DNA molecules. If the foreign DNA has an origin of replication recognized by the host cell DNA polymerases, the bacteria will replicate the foreign DNA along with their own. When transformation is coupled with antibiotic selection techniques, bacteria can be induced to uptake certain DNA molecules and those bacteria can be selected for that incorporation. Bacteria which are able to uptake DNA are called "competent" and are made so by treatment with calcium chloride in the early log phase of their growth. This treatment produces competent cells which can uptake DNA after a heat shock step (at 42°C for 30 sec). However, due to the low number of bacterial cells which contain the plasmid and the potential for the plasmid not to propagate itself in all daughter cells, it is necessary to select for bacterial cells which contain the plasmid. This is commonly performed with antibiotic selection. *E. coli* strains used in this study (TOP 10 and Novablue) are sensitive to common antibiotics such as ampicillin. Plasmids used for the cloning and manipulation of DNA have been engineered to harbor the genes for antibiotic resistance. Thus, if the bacterial transformation is plated onto media containing ampicillin, only bacteria which possess the plasmid DNA will have the ability to metabolize ampicillin and form colonies. In this way, bacterial cells containing plasmid DNA are selected. Selection for transformants was done on LB/ampicillin/IPTG/X-Gal plates. Successful cloning of insert in the vector interrupts the coding sequence of β-galactosidase. Therefore recombinant clones can be identified by color screening on indicator plates. When IPTG and X-Gal are included in plasmid DNA transformation, blue colonies represent bacteria harboring non-recombinant vector DNA since the lacZ gene region is intact. IPTG induces production of the functional galactosidase which cleaves X-Gal and results in a blue colored metobolite. It follows that colorless colonies

contain recombinant DNA since a nonfunctional galactosidase is induced by IPTG which is unable to cleave the X-Gal.

One aliquot (50 µl) of the competent bacteria were thaw on ice. Ligated DNA was added and carefully mixed without pipetting. Cells were kept on ice for 30 min and subsequently heat shocked for 30 sec in the water bath at 42°C. After the heat shock, cells were immediately transferred to ice and kept there to recover for 2 min. 250 µl of preheated LB or SOC medium was added to the cells and it was incubated at 37°C for 1 h, to allow the bacterial to recover and to express the antibiotic resistance marker encoded by the plasmid. Finally 50-200 µl of the cells were plated on a LB/ampicillin/IPTG/X-Gal plates and incubated over night at 37°C. Single bacteria colonies were picked with sterilized toothpicks and put in a bacterial culture-tube containing LB-medium supplied with ampicilin for overnight incubation. The plasmid DNA was finally isolated after overnight culture. Positive clones containing inserts of the indicated length are identified by colony PCR and inserts of 3 clones are verified by sequencing using vector specific primers.

Solutions used are:

X-Gal-stock solution:

- 50 % X-Gal (w/v) in N,N'-Dimethylformamid

LB/Amp/IPTG/X-Gal-plates (per plate):

- 20 ml LB
- 1.5 % Agar (w/v)
- 0.01 % Ampicillin (w/v)
- 0.5 mM IPTG
- 0.02 % X-Gal (v/v)

LB-Agar is autoclaved. After cooling down to 50°C, the components listed above are added, stirred and poured into the Petri dish.

4.15 Optic density (OD) of bacterial cultures

Bacterial growth was measured with a spectrophotometer at a wavelength of 600 nm in 1 ml disposable plastic cuvettes. Cultures with an OD_{600} of 0.5-0.7 were considered optimal as they are in the logarithmic growth phase.

4.16 Generation of primers

Primers were ordered from MWG-Biotech, Germany (http://www.mwgbiotech. com). They were designed manually and extra checked for hairpins and dimer formation using Lasergene 6 software. When making gene specific primers the following rules were applied:

- varying length of 16- 23 nucleotides
- G+C content of about 40-60 %
- adapted annealing temperature of both, upstream and downstream, primer
- no self-compatibility
- no palindrome sequence
- no possibility to build primer-dimers or any secondary structures

At least one G or C nucleotide was at the 3´end of the primer to ensure a tight binding of the oligo at this crucial point. Working aliquots with 10 pmol/µl were used for PCR. Sequencing primers were labeled (IRD 800 or IRD 700) and working aliquots of 2 pmol/µl were used.

Degenerative primers were designed on the basis of conserved regions of the ornithine cyclodeaminase (OCD) from the *Brucella suis* (accession number Q8FVE4, α-proteobacteria), *Pseudomonas putida* (Q88H32, γ-proteobacteria), *Ralstonia solancearum* (Q8XSP9, β-proteobacteria) and *Methanosaeta thermophila* (YP843143, Archaea) with amino acids positions chosen to minimize the degeneracy. Working aliquots with 50 pmol/µl were prepared and 1 µl was used for PCR

Reverse primer used for expression of SUBDO_TaDH was design to remove the native stop codon in the gene of interest and preserve the reading frame through the C-terminal tag in order to include the *c-myc* epitope and polyhistidine region. The forward primer was designed to ensure that ORF is in frame with the initiation codon.

4.17 PCR (Polymerase Chain Reaction)

There are three major steps in a PCR. During the **denaturation** step the double strand melts and open to single stranded DNA. At the **annealing** step primers are jiggling around, caused by the Brownian motion. Ionic bonds are constantly formed and broken between the single stranded primer and the single stranded template. The more stable bonds last a little bit longer (primers that fit exactly) and on that little piece of double stranded DNA (template and primer) the polymerase can attach and starts copying the template. Once there are a few bases built in, the ionic bond is so strong between the template and the primer that it does not break anymore. At the **extension** step primers that are on positions with no exact match, get loose again (because of the higher temperature) and don't give an extension of the fragment. The primers, that have a few bases built in, already have a stronger ionic attraction to the template than the forces breaking these attractions. The bases (complementary to the template) are coupled to the primer on the 3' side (the polymerase adds dNTP's from 5' to 3', reading the template from 3' to 5' side; bases are added complementary to the template). For PCR reactions the following parameters were used and optimized for the specific need and template/primer combination. Exemplary parameters are given below:

Cycle 1:	1-3 min	95°C	Initial denaturation
Cycle 2-30:	30 sec	95°C	Denaturation
	30 sec	T_m	Primer-Annealing
	15 sec-15 min (depending on the product size)	70°C-72°C	Elongation
Cycle 31:	5 sec-10 min	70-72°C	Final elongation

The annealing temperatures varied depending on the melting temperature (T_m) of the primers. Several formulas are used to calculate melting temperature. The fastest and the most common one established by Thein et al. (1986) is:

$$T_m = 2(A + T) + 4(G + C)$$

Annealing temperatures were determined using software and the information's supplied by the oligonucleotide manufacturers. In many cases optimal annealing conditions had to be adjusted using touch down PCR, keeping all parameters constant, just altering the annealing temperatures. All PCRs were performed using Taq polymerase (Peqlab). If maximum proofreading was required, KOD DNA polymerase was used. If proofreading and high fidelity was required, because for example the amplification with KOD failed (e.g. difficult templates or low DNA concentrations), the Expand High Fidelity PCR System (Roche) was used. It contains a mixture of Taq and proofreading polymerases achieving high yield and high fidelity. The kits were used following the manufacturers recommendations.
Standard conditions for 50 µl reaction were as follows:

10x PCR buffer	5µl
$MgCl_2$	2.5 mM
dNTPs	10 nmol
Forward primer	10 pmol
Reverse primer	10 pmol
Taq-Polymerase	1 U
DNA	1 ng
Aq. *bidest*.	up to a volume of 50 µl

4.17.1 Long and accurate (LA) PCR

For amplification of larger DNA fragments, LA PCR with AccuTaq LA DNA polymerase was applied. This enzyme mix combines a highly processive polymerase with

a second polymerase that exhibits a 3'→5' exonucleolytic activity. By using the proofreading polymerase to repair terminal mis-incorporations this blend increases the length of amplification products. This repair allows the polymerase to resume elongating the growing DNA strand. Effective denaturation is accomplished by the use of higher temperatures for shorter periods of time or by the use of co-solvents, such as dimethyl sulfoxide (DMSO). Addition of DMSO in the reaction at a final concentration of 1-4% is increasing yield and improving reliability of the system. Extension times are long and extended for each additional cycle.

4.17.2 Touch down PCR

Touchdown PCR is modification of conventional PCR that results in a reduction of nonspecific amplification (Don et al. 1991). Touchdown PCR uses a cycling program with varying annealing temperatures. The annealing temperature in the initial cycle is 5–10°C above the Tm of the primers. In subsequent cycles, the annealing temperature is decreased in steps of 1–2°C/cycle until a temperature is reached that is equal or 2–5°C below of the T_m of the primers. The primer will anneal at the highest temperature which is least-permissive of nonspecific that it is able to tolerate. Thus, the first sequence amplified is the one between the regions of greatest primer specificity; it is most likely that this is the sequence of interest. These fragments will be further amplified during subsequent rounds at lower temperatures and will swamp out the nonspecific sequences to which the primers will bind at lower temperatures. This method was used in a case where estimated annealing temperatures of the primers were of a great difference (more than 5°C).

4.17.3 DIG labeling PCR

The DIG System is an effective system for the labeling and detection of DNA, RNA, and oligonucleotides. The DNA probes were generated by PCR using the PCR DIG labeling kit from Roche. A standard PCR was done substituting dNTPs with the PCR-DIG labeling mix containing digoxigenen-11-dUTP (lithium salt). Extra $MgSO_4$ was added to

final concentration of 1 mM. DNA probes obtained in this way must be denatured by boiling in a waterbath. The labeled probes were cleaned with purification kit and analyzed on an agarose gel. DIG-labeled DNA probes can be used for all types of filter hybridization, for single copy gene detection in total genomic DNA and for *in situ* hybridization.

Exemplary parameters are given below:

Cycle 1:	2 min	95°C	Initial denaturation
Cycle 2-6:	30 sec	95°C	Denaturation
	30 sec	52°C	Primer-Annealing
	45 sec-1.5 min (depending on the product size)	72°C	Elongation
Cycle 7-36:	20 sec	95°C	Denaturation
	20 sec	58°C	Primer-Annealing
	45 sec-1.5 min (depending on the product size)	72°C	Elongation

Standard conditions for 50 µl reaction were as follows:

10x PCR buffer/$MgCl_2$	5 µl
DIG-Mix	2.5 µl
dNTPs	0.2 mM
Forward primer	10 pmol
Reverse primer	10 pmol
Taq-Polymerase	1 U
DNA	0.1-1 ng
Aq. *bidest*.	up to a volume of 50 µl

4.17.4 Checking PCR

Checking PCR is performed in order to confirm success of the ligation and presence of specific inserts in a DNA construct. Transformed colonies (3.15) were picked, from LB-plate, using a sterile pipette tip or tooth pick and incubated in 100 µl LB medium. After 30 min of incubation at 37°C, 1 µl of bacterial suspension was taken as a template for standard PCR reaction (3.18) in a final volume of 10 µl. Forward and reverse vector specific primers were used. PCR products were analyzed on the agarose gel and checked for the correct insert size.

4.17.5 Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was performed on total RNA from three different sets of *S. domuncula*, each containing three animals. One set of the animals was taken directly from the sea and immediately frozen in liquid nitrogen. The second set were animals kept for over two months in aquarium under controlled conditions. The third set were aquarium animals exposed for three days to constant elevated aeration [the 50-ml incubations cambers were flowed through by one bubble of air per sec to reach maximal oxygen saturation]. Under these conditions no adverse effect on the integrity of the tissue was observed.

Total RNA was extracted from all samples as described (3.5) and incubated in gDNA wipeout buffer (Qiagen) at 42°C for 2 min to effectively remove any genomic DNA contamination. First-strand cDNA was synthesized from 1 µg total RNA with optimized blend of oligo-dT and random primers using Quantitec reverse transcriptase (Qiagen) as recommended in QuantiTect RT handbook. Reverse transcription was performed at 42°C for 15 min. Incubation at 95°C for 3 min inactivated Quantiscript reverse transcriptase. Obtained cDNA was then used as a template for semi-quantitative RT-PCR. To obtain meaningful results, all PCR products were determined for a linear range of cycles, in a period of the PCR in which the amplification efficiency is at its maximum and remains constant over a number of cycles. In order to obtain the linear range, the PCR mixture was split into 7 aliquots and was subjected to PCR. Aliquots were then removed every 5

cycles (starting with cycle 10 and ending with cycle 40). RNA samples that were not reverse transcribed verified that no products were amplified from contaminating genomic DNA. Products (10 µl of each) were visualized by ethidium bromide staining in 1.5 % agarose gels and scanned using the GS-525 Molecular Imager (Bio-Rad, Hercules, CA, USA).

4.18 DNA Sequencing

The cycling parameters used for sequencing were as follows:

Cycle 1:	2 min	95°C
Cycle 2-30:	20 sec	95°C
	20 sec	54°C
	45 sec (depending on the product size)	70°C
Cycle 31-45:	30 sec	95°C
	30 sec	72°C

In this study classical chain-termination or Sanger method was used. This method requires a single-stranded DNA template (1.5 µg), a DNA primer (2-4 pmol/µl), DNA polymerase, fluorescently labeled nucleotides and modified nucleotides that terminate DNA strand elongation. The DNA sample is divided into four separate sequencing reactions, containing the four standard deoxynucleotides (dATP, dGTP, dCTP and dTTP) and the DNA polymerase. To each reaction only one of the four dideoxynucleotides is added. These dideoxynucleotides are the chain-terminating nucleotides, lacking a 3'-OH group required for the formation of a phosphodiester bond between two nucleotides during DNA strand elongation. Incorporation of a dideoxynucleotide into the nascent (elongating) DNA strand therefore terminates DNA strand extension, resulting in various DNA fragments of varying length. The dideoxynucleotides are added at lower concentration than the standard deoxynucleotides to allow strand elongation sufficient for sequence analysis. To the PCR tubes, with newly synthesized and labeled DNA

fragments, 4 µl of formamide + bromphenol blue is added. Samples are heat denatured at 80-85°C for 4 min and size separated (with a resolution of just one nucleotide) by gel electrophoresis on a polyacrylamide gel. Each of the four DNA synthesis reactions is run in one of four individual lanes (G, A, T, C). The DNA bands are then visualized on gel image and automatically red with eSeq computer program. Obtained sequences were analyzed and verified using DNAStar (as described in 3.20).

In order to analyze allelic variation of OpDH gene (genotyping), genomic DNA from 19 specimens of *S. domuncula* was extracted as described before (3.1). An internal fragment (453 bp) of the OpDH gene spanning intron2 was amplified using 5´-labeled forward primer (IRD 800) Sd_polyF* and the reverse primer Sd_polyR. Standard PCR conditions were used (3.18)**.** Prior to further analysis, the PCR products were visualized by electrophoresis in a 1.5% agarose gel stained with ethidium bromide and photographed under UV transillumination. According to the amplification rate, dilutions of the samples were made in loading buffer (formamide–dye solution) and denatured at 95°C for 4 min, then loaded onto a denaturing polyacrylamide sequencing gel. The sequencing ladder of the same fragment was used as a size marker. The genotyping results obtained by this method were confirmed by cloning and sequencing representative PCR products of each allelic variation.

4.19 Sequence analysis

Computer analyses and comparisons of DNA sequences were performed using Lasergene processing software (DNASTAR, Inc., Madison, Wis., USA). The BLAST network service (http://www.ncbi.nlm.nih.gov/) was used for sequence homology searches. Multiple alignments were performed with CLUSTAL W Ver. 1.6 (Thompson et al. 1994). Phylogenetic tree was constructed with Mega 4 on the basis of amino acid (aa) sequence alignments by the neighbour-joining method, as implemented in the ‘Neighbor’ program from the PHYLIP package (Felsenstein 1993). The graphic presentations of the alignments were prepared with GeneDoc (Nicholas and Nicholas 1997). Sequences of the PCR products obtained with degenerate primers were assembled and deduced amino

acids were analyzed with the basic local alignment search tool (BLAST) on the National Center for Biotechnology Information (NCBI) website.

4.20 Isolation of total protein extract

Tissue samples were mechanically homogenized in lysis-buffer (1x TBS [Tris-buffered saline], pH 7.5, 1 mM EDTA, 1% Nonidet-P40, 10 mM NaF, protease inhibitor cocktail (Roche, Mannheim; Germany) [1 tablet/10 ml]), shaken for 1 h at room temperature, centrifuged and the supernatants were taken for further analysis. Protein concentration was measured with Bradford and quality/purity estimated with SDS-PAGE.

4.21 SDS-polyacrylamid gel electrophoresis (SDS-PAGE)

SDS-PAGE has a number of uses, which include the establishment of protein size, protein identification, determination of sample purity, identification of disulfide bonds, quantification of proteins and blotting applications. SDS, sodium dodecyl (lauryl) sulphate, is an anionic detergent that binds quantitatively to proteins, giving them linearity and uniform negative charge, so that they can be separated solely on the basis of their size. The SDS has a high negative charge, which overwhelms any charge of the proteins giving them an approximately equal negative charge. The number of SDS molecules that bind to a protein is proportional to the number of amino acids in the protein. SDS also disrupts the forces that contribute to protein folding (tertiary structure), ensuring that the protein is not only uniformly negatively charged, but then linear. SDS is present in the loading dye, the gel and the running buffer. All proteins migrate to the anode. Furthermore, β-mercaptoethanol is also present in the loading dye, so that the tertiary structure of the proteins is destroyed by cleavage of disulfide bonds, thus breaking the only known covalent bond between the polypeptide chains. The following denaturation at 95°C for 5 min is responsible for an unfolded state and an elongated conformation of the proteins. The migration distance during the electrophoresis is then linearly dependent on the logarithm of the molecular size (Weber and Osborn 1969).

Through using protein of known molecular mass, it was possible to elevate the molecule mass of the polypeptide chains.

In a discontinuous system, used in this study, a non-restrictive large pore gel (stacking gel) is layered on top of a separating gel (resolving gel). Each gel is made with a different buffer and the tank buffers are different from the gel buffers. In the presence of the radical former ammonium persulfate (APS) and the catalyst tetramethylendiamine (TEMED), acrylamide can polymerize into long chains which are linked to form a net-like porous gel structure by N,N'-methylenbisacrylamide. The size of the pores of the matrix is determined by the concentration of acrylamide and N,N'-methylenbisacrylamide so that the range of separation can be selected as required. For performing PAGE, vertical electrophoresis tanks from Mini Protean II from Bio-Rad were used (minigels: 7 cm x̧ 8 cm x̧ 0.75 or 1 mm). The gels were used directly or stored under humid conditions at 4°C until use. Separation of the samples was done in 10% resolving gels, overlaid with a 5 % stacking gel (Tab.1). PAGE was carried out under denaturing conditions, as well as nondenaturing (native) and seminative conditions, following the procedure of Laemmli (1970).

	Resolving gel (10%)	**Stacking gel (5%)**
40% acrilamid /bis stock	2.5 ml	0.63 ml
1.5 M Tris HCl (pH 8.8)	2.5 ml	-
1.5 M Tris HCl (pH 6.8)	-	0.63 ml
10% SDS	0.1 ml	0.05 ml
glycerol	0.02 ml	-
Aq. bidest.	4.88 ml	3.58 ml
APS 10% (w/v)	0.1 ml	0.1 ml
TEMED	0.01 ml	0.01 ml

Tab.1: *Pipetting scheme for 2 minigels.*

4.21.1 Native/Seminative PAGE

While the separation of proteins during an SDS-PAGE exclusively occurs due to their molecular size, during a native PAGE, untreated proteins are separated electrophoretically due to their molecular size combined with their net charge and conformation (tertiary structure). In Tab.2 solutions used in different SDS systems are listed. Note that the buffers, used for native/seminative PAGE, contained neither or less SDS and β- mercaptoethanol and that the sample denaturation step at 95°C was omitted.

	SDS-PAGE	Native PAGE	Seminative PAGE
Resolving buffer	1.5 M Tris/HCl pH 8.8 0.1% SDS	1.5 M Tris/HCl pH 8.8 -	1.5 M Tris/HCl pH 8.8 0.1% SDS
Stacking gel buffer	1.0 M Tris/HCl 0.1% SDS pH 6.8	1.0 M Tris/HCl - pH 6.8	1.0 M Tris/HCl 0.01% SDS pH 6.8
Running buffer	25 mM Tris, pH 8.3 192 mM glycine 20% MeOH 0.1% SDS	25 mM Tris, pH 8.3 192 mM glycine -	25 mM Tris, pH 8.3 192 mM glycine 20% MeOH 0.1% SDS
Loading dye (4 x)	0.5 M Tris pH 6.8 40% Glycerol 8% SDS 20% β-mercaptoEtOH Bromophenol blue	0.5 M Tris pH 6.8 40% Glycerol - - Bromophenol blue	0.5 M Tris pH 6.8 40% Glycerol - - Bromophenol blue

Tab.2: *Solutions used depending on the system: System specific solutions for SDS, native and seminative PAGE.*

4.21.2 Coomassie staining with GelCode® Blue stain reagent

GelCode® Blue Stain Reagent (Pierce) utilizes the colloidal properties of Coomassie® G-250 for protein staining on polyacrylamide gels. After electrophoresis, gel was washed twice with distilled water with gentle shaking for 15 min. For an 8 x 10 cm mini gel 20 ml of GelCode® Blue Stain Reagent was used. Stain intensity reaches a maximum within approximately 1 h. Gels may be stained overnight without increased background. After staining, gels were washed with distilied water (1-2 h) for optimal results. This step enhances stain sensitivity as weak protein bands continue to develop.

4.21.3 Measuring protein concentration (Bradford)

The bradford dye-binding assay is a colorimetric assay for measuring total protein concentration (Bradford 1976). It involves the binding of Coomassie Brilliant blue to protein. Roti quant 5x (BioRad) is diluted with water in ratio 4:1. For each sample (25 µl) 1 ml of this dilution is added and incubated at RT for 5 min. Samples are placed in 96-well plate in duplicates (100 µl per well) and set on the reader. Absorbance is measured at a wavelength of 580 nm. Samples to be measured are diluted appropriately and if the concentration of the protein is unknown several samples of various dilutions are made.

4.21.3.1 Protein Standards

Protein standards are prepared in the same buffer as the samples to be assayed. A convenient standard curve is made using bovine serum albumin (BSA) with concentrations of 0, 62.5, 125, 250, 500 and 1000 µg/mL for the standard assay. Using protein standards a graph of absorbance at 580 nm vs. [Protein] is prepared. The Bradford assay gives a hyperbolic plot for absorbance versus protein concentration, but within a range of relatively low protein concentrations, the hyperbolic curve can be approximated reasonably well by a straight line. The results are used to graph the standard curve from which unknown protein concentration is determined.

4.22 Western Blot

For the identification/quantification of opine dehydrogenase in sponge tissue, total protein extracts were prepared as described (3.21). Prior loading the samples protein was mixed with 4x loading buffer and heated at 99°C for 5 min. The samples and prestained molecular marker were loaded on the 10% SDS-PAGE. Separated proteins were transferred to PVDF membrane (Immobilon) by semi-dry blotting, using Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cel (Bio-Rad) at 40 mA in transfer buffer for 90 min. The membrane (Millipore, Schwalbach) was activated in methanol and wetted in blot buffer before transfer. Non-specific binding sites were blocked by 1% (w/v) blocking solution in TBST for 1 h at room temperature or at 4°C overnight. After, washing (2x 5 min) with TBST the blots were incubated with primary antibody ((PoAb-TaDH [1:25000 dilution]; or Anti-His_6-Peroxidase [1:3000]) with shaking for 1-1.5 h at room temperature. Unbound reagents were removed and background was reduced by five washing steps, 5 min each, in TBST with shaking. Subsequently, anti-rabbit IgG-horseradish peroxidase (HRP) conjugate (1:10000 dilution in blocking buffer) was added to detect bound proteins of interest. After 1 h of incubation at room temperature, the membranes were repeatedly washed three times with TBST and two times with TBS. After equilibration in P3 for 3 min the antibody-protein complexes are visualized with colorimetric substrates NBT/BCIP.

Blotting (transfer) buffer:

- 25 mM Tris
- 192 mM Glycin
- 20% Methanol (v/v)

Blocking solution:

- 1% Blocking-Reagent in TBS (v/v)

TBS:

- 10 mM Tris-HCl, pH 8,0
- 150 mM NaCl

TBST:

- 0.1% Tween 20 in TBS (v/v)

Antibody solution:

- 0.5% blocking solution in TBST (v/v)

4.23 Recombinant His-tag fusion protein

The cDNA encoding StDH was amplified by PCR from *S. domuncula* cDNA library, using the primers designed to amplify the complete open reading frame (ORF). Touch down PCR (3.18.2), with annealing temp. 57-55-53°C, was used to obtain 1.380 kb insert. The amplified DNA fragment was cloned into the *pTrcHis2-TOPO/lacZ* expression vector (TOPO TA Cloning kit, Invitrogen). Vector used in this study encodes a C-terminal peptide containing the *c-myc* epitope and a 6xHis tag for detection and purification of the recombinant protein. It is designed with the initiation ATG (located at bp 413-415) correctly spaced from the optimized ribosome binding site to ensure optimum translation. Two amino acids, encoded in the DNA between the initiation codon and the TOPO® Cloning site, plus additional amino acids at the C-terminal end and 6 His tags in the vector are increasing the size of recombinant protein for 3 to 4 kDa.
After successful ligation (3.14) and transformation (3.15), analysis of plasmids was performed with checking PCR (3.18.4) using forward primer specific for target insert and reverse vector specific primer. In addition, sequencing (3.19) of the selected clones was performed in order to select the clone in the correct orientation. Once the correct clone was identified, colony was purified and glycerol stock was done for long term storage (0.85 ml of culture was mixed with 0.15 ml of sterile glycerol and stored at -80°C).

4.23.1 Expression of His-tag fusion protein

To test the expression of the fusion construct, 10 ml of overnight culture (LB medium supplemented with 50 μg/ml of ampicillin) was inoculated with single recombinant *E. coli* colony and grown overnight at 37°C with shaking. The next day overnight culture was added to fresh LB medium (with 50 μg/ml ampicillin) at 1:50 ratio and grown at

37°C till the OD_{600} reached 0.4-0.6. Expression of fusion protein was then induced with isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 0.1 mM. Growth was continued for 5 or more hours at 37°C-25°C with shaking. Every hour, 1 ml of cells aliquot was removed centrifuged at maximum speed for 30 sec and the cell pellet was stored at -20°C for further analysis. When all the time points have been collected, each pellet was resuspended in 25 µl of 4x SDS-PAGE sample buffer and 5 µl of each sample was analyzed on an SDS-PAGE gel. Negative control (TOP10 cells only) was used to distinguish recombinant proteins from background proteins and positive control (TOP10 cells containing pTrcHis2-TOPO®/*lac*Z) to confirm that growth and induction was done properly. Optimal growth and expression conditions for the protein of interest were established with small-scale cultures before large-scale protein purification was attempted. By checking the 6xHis-tagged protein present at various times after induction in the soluble and insoluble fractions the optimal induction period and conditions were established. Large scale expression of fusion protein was obtained in 50 ml of LB (with 50 µg/ml ampicillin). For maximum expression of the protein induction with 0.1 mM IPTG was done and culture was grown for next 9 h at 25°C. Cells were harvested by centrifugation and stored at -80°C for further analysis/purification.

4.23.2 Determination of target protein solubility

For best purification strategy, it is important to determine whether the protein is soluble in the cytoplasm or located in cytoplasmic inclusion bodies. If the protein forms inclusion bodies it must be solubilised with strong denaturants, such as guanidine hydrochloride (GuHCl) or urea, prior to purification. Purification under denaturing conditions ensures that all tagged proteins in the cell are solubilised and can be purified. In addition, the tag is fully exposed under denaturing conditions, which leads to more efficient purification. If purification under native conditions is preferred or necessary, the tagged protein must be soluble. Purification under non-denaturing conditions can result in reduced yields if the tagged protein is only partially exposed due to native protein folding. However, even when most of the recombinant protein is present in inclusion bodies, there may be some soluble protein, which can be purified in its native form. Protocol from the Qiagen

handbook ("The Qiaexpressionist™") was used. Harvested cells obtained in 3.24.1 are resuspended in 5 ml of lysis buffer for native purification. The samples are frozen in liquid nitrogen and thawed in cold water. After sonification 6x for 10 sec each at 200 - 300 W centrifugation at 10000 x g at 4°C for 20-30 min was done. Supernatant with a soluble protein is separated from the pellet (insoluble protein). The pellet is resuspended in 5 ml of lysis buffer. Both fractions were analyzed by SDS-PAGE (3.22) to settle on the location of the recombinant protein. Afterwards the appropriate purification was done depending on the future analysis.

4.23.3 Purification of 6xHis-tagged fusion proteins using Ni-NTA spin columns

The presence of the C-terminal 6xHis-tag in *pTrcHis2-TOPO/lacZ* allows affinity purification of recombinant fusion protein using a nickel-chelating resin (Ni-NTA, Qiagen), where the Ni-NTA silica combines Ni-NTA with a macroporous silica support material, optimized to suppress non-specific hydrophobic interactions. The procedure described in the Qiagen handbook ("The Qiaexpressionist™") was followed. Nitrilotriacetic acid (NTA) is a tetradentate chelator. NTA occupies four of the six ligand binding sites in the coordination sphere of the nickel ion, leaving two sites free to interact with the 6xHis- tag. The QIA*express*® System is based on the selectivity and affinity of nickelnitrilotriacetic acid (Ni-NTA) metal-affinity chromatography matrices for biomolecules which have been tagged with 6 consecutive histidine residues (6x His-tag). This purification method can be performed under native and denaturing conditions. A primary consideration for recombinant protein expression and purification is the experimental purpose for which the protein will be utilized. For biochemical and structural studies it is often important to optimize conditions for the expression of soluble, functionally active protein, whereas for antigen production, the protein can be expressed either in native or denatured form. In this study protein was purified under native conditions for use in enzymatic assays and under denaturating conditions for antibody production.

Under native conditions the frozen cell pellet was thawed on ice for 15 min and resuspended in lysis buffer at 4 ml per gram wet weight. Sonication step was done to

clear the lysate and to cleave the DNA. The lysate was centrifuged at 10000 x g for 20 - 30 min at 4°C to pellet the cellular debris and the supernatant was transferred to a fresh tube. The cleared lysate (supernatant) containing the 6xHis-tagged protein was loaded onto the equilibrated Ni-NTA spin column and then washed twice with 600 µl wash buffer. The elution of the protein was done with 2x 200 µl elution buffer.
Buffers used for purification under native conditions are:

Lysis buffer:

- 50 mM NaH2PO4
- 300 mM NaCl
- 10 mM Imidazole
- Adjust pH to 8.0 using NaOH

Washing buffer:

- 50 mM NaH2PO4
- 300 mM NaCl
- 20 mM Imidazole
- Adjust pH to 8.0 using NaOH

Elution buffer:

-50 mM NaH2PO4
- 300 mM NaCl
- 250 mM Imidazole
- Adjust pH to 8.0 using NaOH

For protein purification under denaturing conditions, the cells were thawed for 15 min, resuspended in 1 ml lysis buffer and incubated with agitation for 1 h at room temperature. The lysate was centrifuged at 10000 x g for 20-30 min at 4°C to pellet the cellular debris and the supernatant was collected. The cleared lysate (supernatant) containing the 6xHis-tagged protein was loaded onto the pre-equilibrated Ni-NTA spin column. The column was then washed twice with 600 µl wash buffer and the elution of the protein was done 2x 200 µl with elution buffer.

Buffers used for purification under denaturating conditions are:

Lysis buffer:

- 100 mM NaH_2PO_4
- 10 mM Tris/Cl
- 8 M Urea
- Adjust pH to 8.0 using NaOH

Washing buffer:

- 100 mM NaH_2PO_4
- 10 mM Tris/Cl
- 8 M Urea
- Adjust pH to 6.3 using HCl

Elution buffer:

- 100 mM NaH_2PO_4
- 10 mM Tris/Cl
- 8 M Urea
- Adjust pH to 4.5 using HCl

4.24 Antibody production

Polyclonal antibodies (PoAb) were raised against recombinant fusion protein by immunization in female rabbits (White New Zealand) as described (Schutze et al. 2001). PoAb are derived from different B-cell lines. They are a mixture of immunoglobulin molecules secreted against a specific antigen, each recognizing a different epitope. Injection of antigen (900 µg/ml) into the rabbit induces the B-lymphocytes to produce IgG immunoglobulins specific for the antigen. This IgG is purified from the rabbit's serum. The antibodies (1.5 mg) were adsorbed with 0.1 mg of sponge recombinant protein (antigen) in phosphate-buffered-saline (PBS) for 30 min at 4ºC prior to use. After three boosts the serum was collected and screened in a conventional ELISA assay as well as by Western Blotting.

4.25 Immunohistochemistry

Immunohistochemistry (IHC) refers to the process of localizing proteins in cells of a tissue section exploiting the principle of antibodies binding specifically to antigens in biological tissues. Immunohistochemical staining is widely used in clinical diagnostics and in laboratory research for understanding the distribution and localization of proteins in different parts of a tissue. Tissue was fixed in 2% paraformaldehyde in phosphate buffer pH 7.4 for 3-4 h. After overnight washing in PBS buffer (pH 7.4) containing 6.8% sucrose at 4°C the tissue is dehydrated in 100% acetone for 60 min at 4°C. During the first 5 min acetone should be renewed a few times until the solution remains clear. The infiltration of the samples was done using Technovit 8100 (Heraeus Kulzer) according to the instructions of the manufacturer. The infiltration solution consists of Technovit 8100 (100 ml base-liquid) and 1 bag hardener I (0.6 g). The tissue to be embedded is immersed in the solution (15 ml infiltration solution and 0.5 ml hardener) and agitated for 5 min. The well mixed embedding solution is poured into the embedding mould and the tissue specimen is properly placed. Right afterwards, the mould is sealed hermetically with the cover foil and placed on crushed ice at 4°C. Fixing of embedded specimens is done in histobloc using Technovit (mixed in a ratio of 2 parts by volume powder to 1 part by volume liquid) that is poured into the recess in the back of the histobloc up to a level of about 2 mm above the base of the Histobloc. After 5-10 min the specimen can be removed from the mould and are ready for cutting. Sections of 3 µm thickness were prepared through the use of a rotary microtome and slices were mounted on silane coated slides to avoid adhesion-problems. The prepared sections were kept for 30 min in 1% Saponin in PBS (to open the pores). Washing 3x 15 min in PBS was done prior blocking with 0.3% bovine serum albumin for 30 min. The samples were incubated with PoAb (1:25000 dilution) overnight at 4ºC in the humid chamber. After washing 3x 30 min in PBS, the slides were incubated with Cy3-conjugated goat anti-rabbit IgG (diluted 1:800 in PBS-T supplemented with 3% (w/v) BSA and 15 mM sodium azide) for 90 min in the dark on RT. After additional washing 3x 30 min in PBS, staining with DAPI (4'-6-Diamidino-2-phenylindole, 2 µM in 0.3% BSA) for 10 min was done in order to highlight the nuclei. After washing and mounting with the gel/mount hardened (Natutec) nail

polish was put around the cover slip to avoid the preparate dries out irregularly and the sections were inspected by immunofluorescence with an Olympus AHBT3 light microscope. Pre-immune serum was used as a negative control.

DAPI is known to form fluorescent complexes with natural double-stranded DNA, showing fluorescence specificity for AT, AU and IC clusters. Because of this property DAPI is a useful tool in various cytochemical investigations. When DAPI binds to DNA its fluorescence is strongly enhanced, what has been interpreted in terms of a highly energetic and intercalative type of interaction. For fluorescence microscopy DAPI is excited with ultraviolet light. When bound to double-stranded DNA its absorption maximum is at 358 nm and its emission maximum is at 461 nm.

Fixation buffer:

- 3.7% (v/v) formaldehyde
- PBS

Blocking buffer:

- 4% (w/v) BSA
- 0.1% (v/v) Triton-x 100
- PBS

Mounting media/Anti-fading agent, pH 8.0

- 10% (w/v) Elvanol
- 3.3% (v/v) Glycerine
- 0.1% (w/v) Phenylenediamine
- PBS

4.26 ELISA (Enzyme Linked Immunosorbent Assay)

The Enzyme-Linked ImmunoSorbent Assay (ELISA) is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample. ELISA may be run in a qualitative or quantitative format. Qualitative results provide a

simple positive or negative result for a sample. The standard deviation is often used to distinguish positive and negative samples. In quantitative ELISA, the optical density or fluorescent units of the sample is interpolated into a standard curve, which is typically a serial dilutions of the target. The antigen was applied on the microtiter plate at a concentration of 5-10 µg/ml PBS (50-100 µl per well) for 1-3 h at RT or overnight at 4°C. Non-specific adsorption in this step is immobilizing antigen to the surface of the plate. After washing for 3x with PBST (0.05%)- 200 µl per wash, blocking buffer 3% BSA in PBS (150 µl per well) was added for 2-3 h at RT, or overnight at 4°C. Solution of non-interacting protein such as BSA is blocking non-specific adsorption of other proteins to the plate. Washing with PBS-T (0.05%)- 200 µl per wash was done before incubation with primary antibody (100-150 µl) for 1 h at RT. This antibody will only bind to immobilized antigen on the well surface, not to other serum proteins or the blocking proteins. Polyclonal antibodies used in this study were diluted in 0.3% BSA in PBS. The plate is washed 3x with PBST (0.05%), to remove any unbound detection antibody. After this wash, only the antibody-antigen complexes remain attached to the well. Goat Anti – Rabbit IgG-peroxidase (secondary antibody) diluted 1:5000 in 0.3% BSA is added to the wells. Incubation for 30 min at RT allows binding to any remaining detection antibodies. These secondary antibodies are conjugated to the substrate-specific enzyme. Additional washing steps are removing excess of unbound enzyme-antibody conjugates. Substrate (TMB/HRP) was applied for 2-5 min which allows conversion by the enzyme to elicit chromogenic or fluorogenic signal. After addition of 10-25 µl per well 1 M sulfuric acid the reaction was stopped (yellow color) and optical absorbance was read at 450 nm. As a negative control preimmune serum at the same dilutions was used.

PBS-Puffer (pH 7.2):

- 137 mM NaCl
- 2.7 mM KCl
- 9.6 mM Na_2HPO_4
- 1.8 mM KH_2PO_4

Carbonate puffer:

- 0.1 M $NaHCO_3$

Substrate solution (TMB):

- 1.25 mM Tetramethylbenzidin
- 2.2 mM H_2O_2

4.27 Enzyme assays

Opine dehydrogeanse activity and characterization was determined using spectrophotometer (equipped with a graphical pen recorder and a thermostated cell holder) at 340 nm by the initial rate of substrate dependent NADH/NAD^+ formation according to the method of Kanno et al. (1997). Difference in the ultraviolet absorption spectra between the oxidized and reduced forms of the coenzyme (NADH) makes it simple to measure the conversion of one to another in enzyme assays (Fig.6).

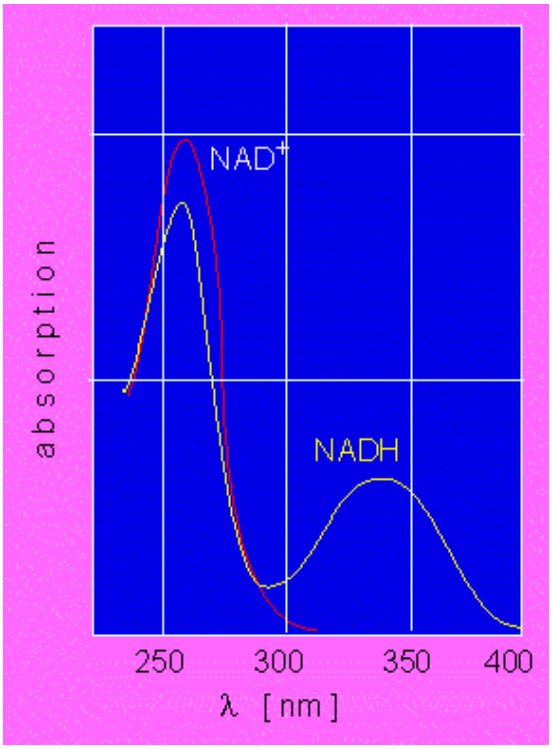

Fig.6. *Optical Test According to O. Warburg. The reduced state of NAD (yellow curve) is characterized by absorption at 340 nm. The oxidized state (red curve) displays no absorption at this wave length. In a given probe, the optical density (ordinate) is accordingly directly proportional to the amount of reduced NAD. The abscissa shows wave lengths between 250 and 400 nm. Taken from: http://www.biologie.uni-hamburg.de/b-online/e19/19b.htm*

Measurements were done in 1 mL semimicro disposable cuvettes (Ratiolab GmbH) with purified recombinant protein, as well as with total protein extract from the sponge. The complete opine-biosynthetic reaction contained 100 mM of PIPES (Piperazine-N,N´-bis[2-ethanesulfonic acid]) pH 6.8, 100 mM of amino acid, 0.3 mM of Na-pyruvate, 0.3 mM of NADH and an aliquot of protein preparation in a final volume of 1 ml. The reaction mixture lacking substrate was preincubated at 30°C for 2 min, and the reaction was started by adding the substrate. The reaction was monitored for at least 3 min and reaction velocity was calculated from the changes in the slope of trends. After estimation of optimal conditions in terms of pH, different substrates (alanine, glycine, taurine and ornithine) and concentration of enzyme, kinetic analysis was done. One enzyme unit was defined as the amount of enzyme oxidizing or producing 1 μmole of NADH per min. Beer's law is used for units calculation in spectrophotometric based assay:

$$A = \varepsilon \, l \, C$$

Where A = absorbance ($M^{-1}cm^{-1}$), b = path length of the cell (1 cm), c =concentration of the absorbing species (M) and ε = the molar extinction coefficient. When assaying enzyme activity we use ΔA/min (change in absorbance per time). Due to the fact that NADH has an extinction coefficient of 6.2 x 10^3 $m^{-1}cm^{-1}$ following formula for specific activity (U/mg) could be applied:

$$\frac{\Delta A_{340}/\text{min} \times V_{\text{reaction}}}{\varepsilon / m^{-1}cm^{-1} \times 1\ \text{cm} \times V_{\text{enzyme}}} \times \text{Homogenization factor}$$

$$\text{Homogenization factor} = \frac{V_{\text{tot}}}{m_{\text{tissue}}}$$

4.27.1 Substrate Saturation of the Enzyme

Maximum rate of an enzyme mediated reaction was determined by increasing the substrate concentration ([S]) until a constant rate of product formation is achieved. This is the maximum velocity (V_{max}) of the enzyme. In this state, enzyme active sites are saturated with substrate. The speed V means the number of reactions per second that are catalyzed by an enzyme. With increasing substrate concentration [S], the enzyme is asymptotically approaching its maximum speed V_{max}, but never actually reaching it. Therefore, no [S] for V_{max} can be given. Instead, the characteristic value for the enzyme is defined by the substrate concentration at its half-maximum speed ($V_{max}/2$). This K_M value is also called the Michaelis-Menten constant. K_M approximates the affinity of enzyme for the substrate. A small K_M indicates high affinity and a substrate with a smaller K_M will approach V_{max} more quickly.

The Michaelis-Menten equation describes the relationship between the rates of substrate conversion by an enzyme to the concentration of the substrate, with following formula:

$$v_0 = \frac{V_{max}\ [S]}{K_M + [S]}$$

This equation defines the shape of a square hyperbola, which is the shape shown for the plot of V_o vs. [S]. The constants (V_{max} and K_M) can be obtained from a set of experimental data where V_o is measured at different [S]. This is most easily observed by plotting initial velocity versus substrate concentration (Fig.7).

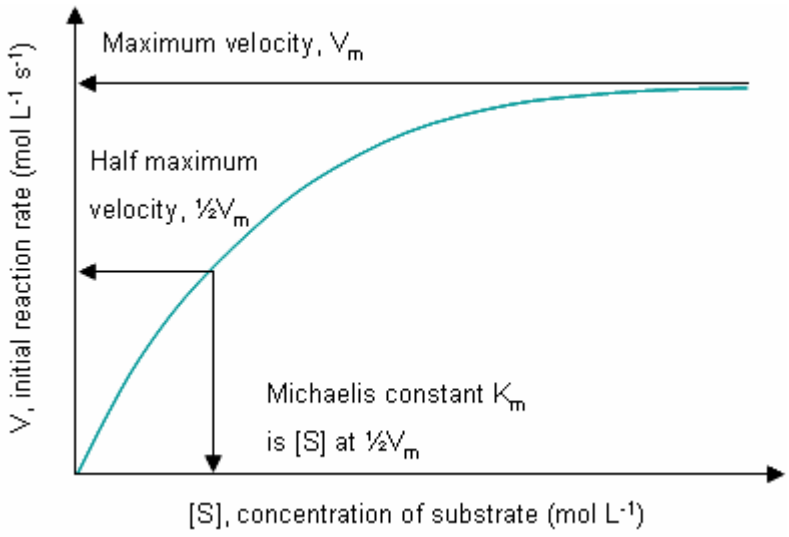

Fig.7. *Graphical display of Michaelis-Menten kinetics.*

Kinetic data were analyzed by Lineweaver-Burk plot, which is a linear transformation of the Michaelis-Menton equation generated by taking the reciprocal of both sides of the equation:

$$1/V_o = 1/V_{max} + ((K_m/V_{max}) \times (1/[S]))$$

Homogenization buffer recipe:

- 50 mM TRA (3- ethanolamine hydroclorid)
- 1 mM EDTA/DTT
- pH 7.3

4.28 Protein modeling

The overlay and the initial structure alignment of the sequences were done using the Swiss PDB viewer, version 3.7 (Guex and Peitsch 1997). The sequence overlay was submitted to the SwissProt modeling server to optimize the structure. The resulting model was checked for major deviations from the template and for unrealistic conformations using the same software.

5 RESULTS

The main goal of this doctoral thesis was to establish the presence and investigate the physiological role of opine pathway(s) in the sponge *S. domucula.* The first step was finding the transcript in *S. domuncula* cDNA library (Pfeifer et al. 1993).

5.1 Cloning of the cDNA encoding the putative tauropine dehydrogenase (TaDH)

The complete sponge putative tauropine dehydrogenase *SD_TaDH* cDNA was isolated and completed from the *S. domuncula* cDNA library, as described under "Materials and Methods". The sequence (Acc. No. AM712888) is 1304 nucleotides (nts) long (excluding the PolyA tail) and comprises one ORF of 1015 nts which ranges from $nt_{85\text{-}1099(stop)}$. The deduced polypeptide, the putative TaDH termed SUBDO_TaDH comprises 337 amino acids (aa) (Fig.8), with a calculated size of 36923 Da and a theoretical pI of 5.44. Motif Scan database search (PF02423.6; http://myhits.isb-sib.ch/cgi-bin/motif_scan) reveals one prominent domain, the ornithine cyclodeaminase/mu-crystallin family region, which spans in the *S. domuncula* sequence aa_{40} to aa_{318} with an "expect value" (Coligan et al. 2000) of $1.1e^{-78}$.

The purine A (adenine) of the ATG codon is designated as +1, with positive and negative integers proceeding 3' and 5', respectively. Vicinity of the start codon in the *S. domuncula* revealed that GCCA/GCC-*ATG*G established as the optimal context for initiation of translation in vertebrates is slightly modified (Kozak 1991). Although, at the position -3 is purine A, yet at the position +4 substitution of purine G into pyrimidine T is observed. The stop codon at position 1011 was followed by the polyadenylation signal (AATGAA) starting at position 1238. This alternative signal (Beaudoing et al. 2000), required for accurate and efficient cleavage and polyadenylation of pre-mRNAs in vivo, is not 100% homologous to eukaryotic consensus AATAAA (Zarkower et al. 1986). Targeting signal in the primary structure, indicating the potential signal transduction pathway, was not identified, which is consistent with the fact that opine dehydrogenases are not secreted, but are located in the cytoplasm (Kimura et al. 2005).

```
  AGATCTCGAACGAAGGCAAAGTCGATCTTCTCTCCTGGATCCTGGCATCACTGTTCAT      -58
CCTCTCTACTGAAAGCTGCTCTACAAGATGTCCAAGAAGCTTACTTTCCTCTATCTCTCT       33
                            M  S  K  K  L  T  F  L  Y  L  S        11

GAACCTGACGTCATCAAGACAGGTCTAACGCTCACAGAAACCATCGAGTTGTGTACGGAG       93
 E  P  D  V  I  K  T  G  L  T  L  T  E  T  I  E  L  C  T  E        31

AGTTTGAAGCAGCATGGCCTGAAAGAAGTCGAGAACCCCCCTAAACCAGGAGTACATCCT      153
 S  L  K  Q  H  G  L  K  E  V  E  N  P  P  K  P  G  V  H  P        51

AAAGAAGATTCCTTCATTAATGCCATGCCTGGATGGCTCAAGCAGAAGGGAGTGTGCGGT      213
 K  E  D  S  F  I  N  A  M  P  G  W  L  K  Q  K  G  V  C  G        71

ATAAAGTGGGTGTCTGGATTTCCAGAGAATGTCAAACAGAGGCTCCCCTCCATTGTGGGT      273
 I  K  W  V  S  G  F  P  E  N  V  K  Q  R  L  P  S  I  V  G        91

GTGATCATCCTCAACAGCACGGAGACAGGTTTCCCAACTGCTGTTATGGATGGGACATAC      333
 V  I  I  L  N  S  T  E  T  G  F  P  T  A  V  M  D  G  T  Y       111

ATCACTGCCATTCGTACTGCTGCTGTCTCGGGTGTCTCAGCCAAATACCTCGCTCGTAAG      393
 I  T  A  I  R  T  A  A  V  S  G  V  S  A  K  Y  L  A  R  K       131

GACTCTGAGGTGTTGGCCGTCATTGGCACAGGAGTACAGGGCAAGTACAACACTCTCTGC      453
 D  S  E  V  L  A  V  I  G  T  G  V  Q  G  K  Y  N  T  L  C       151

TTGACAACCGTCCTGCCTAGCATCAAGAAAGTAAAGATCTTTGACACATGGGCACCCAGT      513
 L  T  T  V  L  P  S  I  K  K  V  K  I  F  D  T  W  A  P  S       171

CTCCAGACTTTTCAAGATCAAATCCGTCCCCTCTTGCCAAACGTAGAGTTTGAGACTGCC      573
 L  Q  T  F  Q  D  Q  I  R  P  L  L  P  N  V  E  F  E  T  A       191

AGTAGCATGGAGGAAGCCATTCGTGACTCTGATGTGATTGTCGGCGCGACGGCTAAACTT      633
 S  S  M  E  E  A  I  R  D  S  D  V  I  V  G  A  T  A  K  L       211

ACTGAGACGGTCTACAGTGATGAGTGGGTGAAGCCAGGTGCTCTTGTTCTCCCTATTCAA      693
 T  E  T  V  Y  S  D  E  W  V  K  P  G  A  L  V  L  P  I  Q       231

GTGGGAGGATGGGATCCTGATGTTTTATCAAAGTTTGACAAAGTTGTGGCTGACGATTGG      753
 V  G  G  W  D  P  D  V  L  S  K  F  D  K  V  V  A  D  D  W       251

GCTCAACTCAAAGCACAACAAGACTTTAGTCGATACACTTCAAACTTCGACACACCGTAT      813
 A  Q  L  K  A  Q  Q  D  F  S  R  Y  T  S  N  F  D  T  P  Y       271

GCCGAGCTCGGCGAAATTGTCATGGGCAAAAAACCGGGACGTGAAAACGATCAAGAAAAG      873
 A  E  L  G  E  I  V  M  G  K  K  P  G  R  E  N  D  Q  E  K       291

ATCATCAATTTCAACAAGGGGTTAGCCGTTCACGATATGATCTGCGGTGGCCGTGTTCTT      933
 I  I  N  F  N  K  G  L  A  V  H  D  M  I  C  G  G  R  V  L       311

GAGAAGGCAAAGAAACAAGGATTAGGTGTAGAGTTAGAGCTGATGGACCTCGGCTCTCCT      993
 E  K  A  K  K  Q  G  L  G  V  E  L  E  L  M  D  L  G  S  P       331

ATACCTATGCCACCCGTCTAACTTCATGGATAAACTGTATGAGGATAGCCTGTATGGTAC     1053
 I  P  M  P  P  V  *                                              337

ATTTGCTTAGATACTTCTCTAGAGATAGGTGCAATCATGATGTTGTTGACTTGATTTGCA     1113
CACATGCTTAGCTAGTTTTATACGTACGTATAGTCATATAATTATAGTCATATATCAGTA     1173
TAGTGTAGTGTTGGGTTCTTTCAAAATGAATTTATAATTATGCATG(A)n               1219
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Fig.8. *The cDNA and deduced amino acid sequence of the putative SUBDO_TaDH. The first base of ATG initiation codon of the cDNA is numbered as +1. Deduced amino acid sequence is given below the nucleotide sequence. The nucleotides are in the 5′ to 3′ direction. Start and stop codon are marked in* ***bold****. Stop codon in aa sequence is marked with asterisk (*). 5′ and 3′ untranslated regions are in blue and green letters, respectively. PolyA signal at position -21 is marked in* ***bold green underlined*** *letters.* $(A)_n$ *indicates 18 adenine residues in poly A tail.*

5.2 Sequence analysis of *S. domuncula* TaDH-like protein

Fasta and BlastP searches with SUBDO_TaDH showed highest sequence homology with the *Halichondria japonica* tauropine dehydrogenase (Acc.No. BAD52445.1; Kanno et al. 2005), with a 68% identity and 86% similarity (Fig.9). Considerably lower is the relationship to the highest similar sequences from the following metazoans: the human mu-crystallin sequence (24%/42%, identity/similarity; Acc. No. U85772), the *Drosophila melanogaster* predicted protein CG4872-PA (16%/33%, identity/similarity, Acc. No. NP_573173), the hypothetical protein F54G2.1b from *Caenorhabditis elegans* (4%/10%, identity/similarity; Acc. No. NP_001024712), the predicted Caf130p sequence from *Saccharomyces cerevisiae* (3%/7%, identity/ similarity; Acc. No. NP_011650) and the ornithine cyclodeaminase/mu-crystallin family protein from *Arabidopsis thaliana* (20%/36%, identity/ similarity; Acc. No. NP_200093). In contrast, the similarity of the sponge TaDH-like protein to prokaryotic enzymes is relatively high, with ornithine cyclodeaminase (OCD) from *Rhizobium meliloti (Sinorhizobium meliloti)* [Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales] as the most prominent one (33%/53%, identity/ similarity; Acc. No. P33728) followed by the alanine dehydrogenase from *Methanococcoides burtonii* [Archaea; Euryarchaeota; Methanomicrobia; Methanosarcinales] (28%/51%, identity/similarity; Acc. No. YP_565945.1) as shown in Fig 9. Visual inspection of the aligned amino acid sequences revealed highly conserved residues. Among these, a GxGxxG/A motif (G139-T-G141-V-Q-G144 in the *S. domuncula* sequence), suggesting a NAD binding site, is located in the central part of the protein. No apparent homology to any other metazoan OpDHs except of demospongiae *Halichondria japonica,* suggested that OpDHs are not all homologues as it was believed. This study confirmed hypothesis that sponge opines constitutes an independent class of enzymes (Kanno et al. 2005).

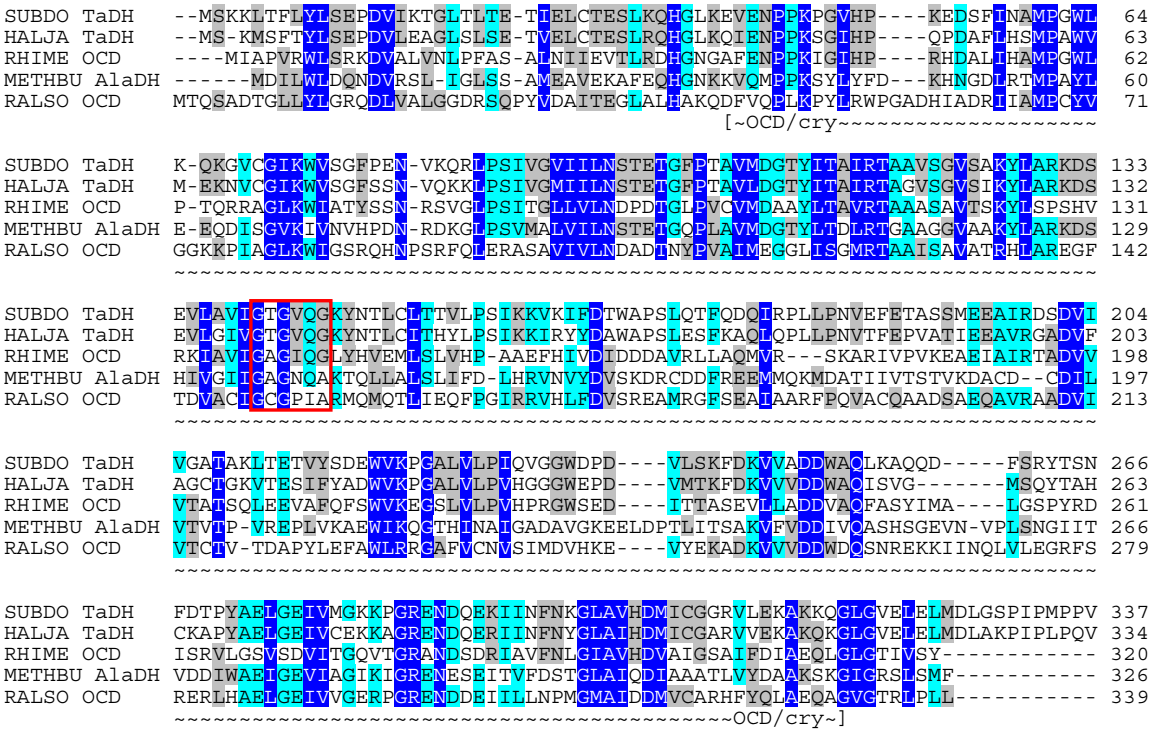

***Fig.9**. SUBDO_TaDH was compared with the TaDH from Halichondria japonica (HALJA_TaDH; BAD52445), ornithine cyclodeaminases (OCD) from Rhizobium meliloti (RHIME_OCD; X64613.1), Ralstonia solanacearum (RALSO_OCD; Q8XSP9) and alanine dehydrogenase from Methanococcoides burtonnii (METBU_AlaDH; YP_565945). Conserved amino acids in all sequences are highlighted in blue and those matching in four sequences are marked in turquoise. Residues marked with gray background are identical in two of the sequences. The GxGxxG/A motif known to be conserved among NAD(P)-dependent dehydrogenases is marked with red box. Ornithine cyclodeaminase/mu-crystallin family domain is indicated below the sequences. The full protein sequences have been used for all species.*

5.3 Ornithine dehydrogenases in sponge-associated bacteria

Considering the relatively high sequence homology of the SUBDO_TaDH with bacterial ornithine cyclodeaminases (OCDs) and the fact that the sponge OpDHs belongs to the same protein family (ornithine cyclodeaminase/mu-crystallin; Kanno et al. 2005) isolation, cloning and sequencing of bacterial OCDs from *S. domuncula* was performed. By application of degenerative primers, designed against bacterial OCDs eight different sequences were obtained which could be attributed to *S. domuncula*-associated bacteria. By means of BLAST search it was confirmed that amplified sequences termed

SUBDO_BAC1-8 (300 nts long) are indeed bacterial. Sequences were deposited in EMBL/GenBank under Acc. No.: AM712890, AM712891, AM712892, AM712894, AM712895, AM712896, AM712897 and AM712898. Surely this result does not give an answer if these bacteria are symbiotic/commensalic microorganisms or only accidentally present in the sponge prior to DNA extraction. Nevertheless, previous data demonstrate that *S. domuncula* harbors a series of bacteria, in special cells, bacteriocytes (Bohm et al. 2001) and on the surface of the marine sponge (Müller et al. 2004).

Obtained sequences, more precisely deduced polypeptide consisting of 100 amino acids, were included in the phylogenetic tree in order to show the relationship between OCD/mu-crystallin family protein and metazoan OpDHs. Fig.10A shows the alignment of the OCD fragment of one bacterial sequence (SUBDO_BAC2; deduced polypeptide) with the *S. domuncula* sequence (SUBDO_TaDH) as well as with the complete bacterial OCD from *Agrobacterium tumefaciens* (AGRTU_OCD; CAB44645.1). Due to the fact that bacterial sequences obtained in this study are relatively small and potentially not relevant for construction of the phylogenetic tree, additional tree was made using the whole protein sequences and without bacterial OCDs. No significant difference was observed. Alignments performed with CLUSTAL W Ver. 1.6 (Thompson et al. 1994) by the neighbour-joining method, included amino acid sequences from OCD/mu crystallin protein family which is highly diverse and includes proteins from Bacteria, Archea and Vertebrates. Opine dehydrogenases from marine invertebrates and OCDs from bacterial sequences obtained in this study, associated with *S. domuncula*, have been included in the comparison. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura et al. 2007). High sequence similarity of the two sponge OpDHs and bacterial OCDs (from *R. meliloti and R. solanacearum*) is reflected in the rooted phylogenetic tree. Although, bacterial sequences cluster in a separate branch they are still within the cluster of OCD/mu-crystallin protein family which is highly distinguished from marine invertebrates OpDHs which are forming a separate cluster what is pointed out with the long branches as seen on the Fig.10B.

A

```
SUBDO_TaDH   ---MSKKLTFLYLSEPDVIKTGLTLTETIELCTES----LKQHGLKEVENPPKPGVHPKEDSFINAMPGWLKQKGVCGIKWVSGFPENVK   83
AGRTU_OCD    MPALANLNIVPFISVENMMDLAVSTGIENFLVQLAGYIEEDFRRWESFDKIPRIASHSR-DGVIELMP--TSDGTLYGFKYVNGHPKNTK   87
SUBDO_BAC2   ------------------------------------------------------------------------------------------    -

SUBDO_TaDH   QRLPSIVGVIILNSTETGFPTAVMDGTYITAIRTAAVSGVSAKYLARKDSEVLAVLGTGVQGKYNTLCLTTVLPSLKKVKIFDTWAPSLQ   173
AGRTU_OCD    SGRQTVTAFGVLSDVDSGYPLLLSEMTILTALRTAATSAIAAKYLARKDSRTMALIGNGAQSEFQALAFKALIG-VDRIRLYDIDPEATA   176
SUBDO_BAC2   ----------------------------------------------------IGNGAQSEFQSLAMKALCG-LTSVRLYDKDPAATA    34

SUBDO_TaDH   TFQDQLRPLLPNVEFETASSMEEAIRDSDVIVGATA-KLTETVYSDEWVKPGALVLPIQVGGWDPDVLSKFDKVVADDWAQLKAQQDFSR   262
AGRTU_OCD    RCSRNLQRFG--FQIEACTSAEQAVEGADIITTATADKHNATILSDNMHGPGVHINGVGGDCPGKTEMHRDILLRSDIFVEFPPQTRIEG   264
SUBDO_BAC2   KCAANLAGSG--LTVVSGSSPEDAIEGAQLLTTGTADKQNAKILTDNMHGSGVHINAIGGDCPGKTE----------------------    99

SUBDO_TaDH   YTSN--FDTPYAELGEIVMGKKPGRENDQEKITNFNKGLAVHDMICGGRVLEKAKKQGLGVELELMDLG-SPIPMPPV-----------   337
AGRTU_OCD    EIQQLAPDHPVTELWRVMTGQDVGRKSDKQITLFDSVGFAIEDFSALRYVRDRVEGSSHSSPLDLLADPDEPRDLFGMLLRRQAFRRLGG   354
SUBDO_BAC2   ------------------------------------------------------------------------------------------    -
```

B

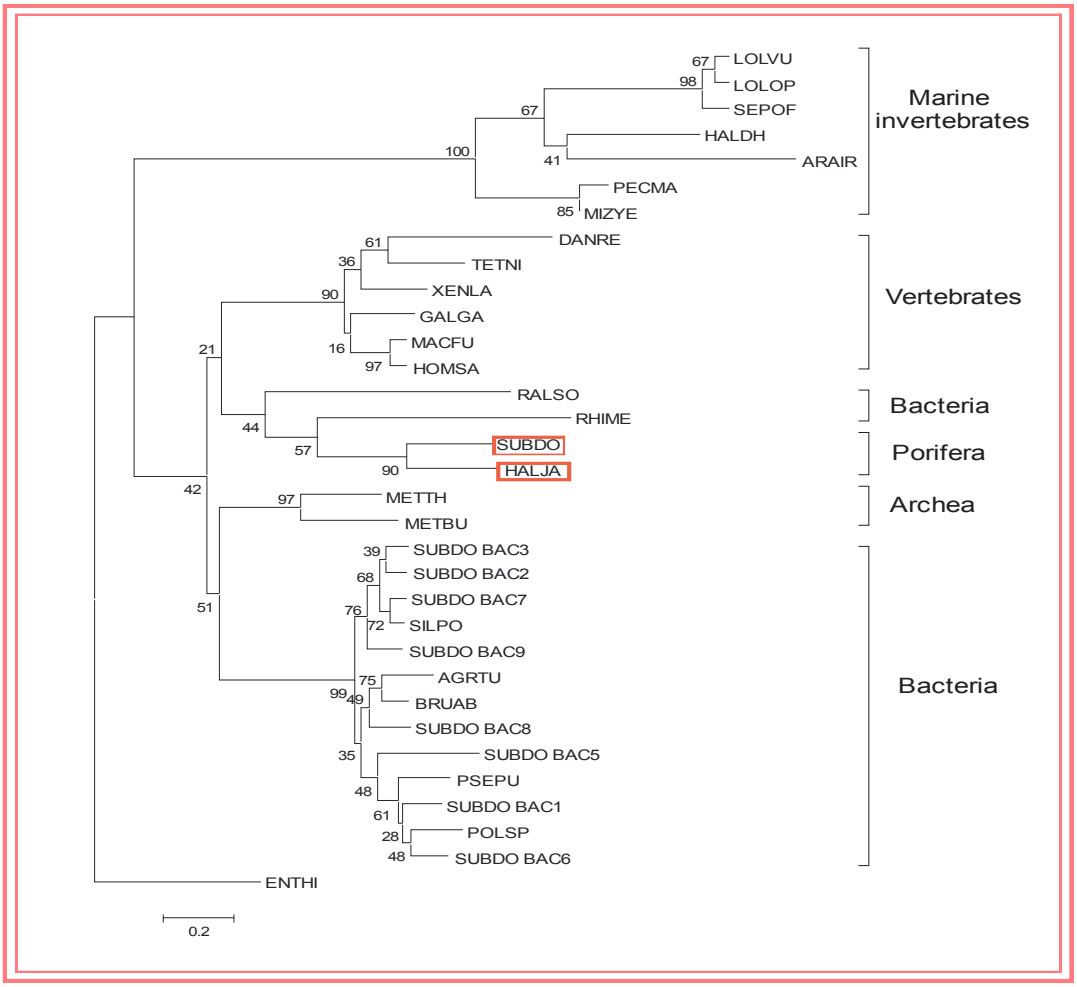

Fig.10. *Phylogenetic analysis of SUBDO_TaDH. (A) Alignment of the whole SUBDO_TaDH sequence with the complete sequence of the OCD from A. tumefaciens and the isolated OCD (fragment) from the sponge-associated bacterium (SUBDO_BAC2). (B) Rooted phylogenetic tree includes OpDHs from marine invertebrates and members of mu-crystallin protein family from Bacteria, Archea and Vertebrates. As outgroup OCD related sequence from Entamoeba histolytica (ENTHI) was used. The number of each of the paragraphs shows bootstrap support. Species and accession numbers in EMBL/Genbank/DDBJ databases are noted in Appendix1. Sponge sequences are marked by red box. The scale bar indicates an evolutionary distance of 0.2 amino-acid substitutions per position in the sequence.*

5.4 Cloning of the gene encoding the *S. domuncula* TaDH- like protein

Phylogenetic analysis of SUBDO_TaDH showed more similarities to the bacterial OCDs then to the same proteins from other marine invertebrates which fall in highly distinguish separate cluster. At this point it was not quite clear whether the TaDH-like gene is bacterial or poriferian. Therefore, Southern Blotting on the genomic DNA and subsequent screening of the genomic library was done. The cDNA clone (*SD_TaDH*) used as a template in PCR for generation of a gene fragment, comprised the whole open reading frame of the TaDH-like gene. The resulting PCR product was DIG-labeled (DIG_SDTaDH probe) as described under Materials and Methods and used as a hybridization probe to determine the presence of the TaDH-like gene in *S. domuncula* genomic DNA. Southern Blotting of the digested genomic DNA and subsequent hybridization with DIG_SDTaDH probe is shown in Fig.11.

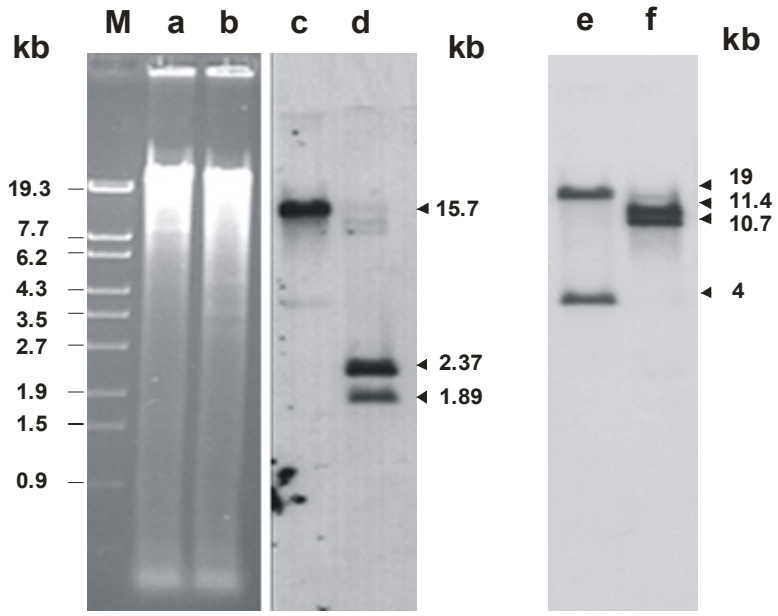

Fig.11. *Southern Blot analysis. S. domuncula genomic DNA was restricted with EcoRI (a) and BamHI (b) and size-separated by a 1% agarose gel. Digested DNA was hybridized with DIG_SDTaDH probe. The EcoRI digested DNA resulted in only one signal (c), while the BamHI digest gave two bands (d), suggesting the presence of a TaDH related gene in the S. domuncula. Size markers are given (M- λEco130I). The arrows indicate target fragments. As control, the restriction digestion of the gene for Cathepsin are shown as indicated (e, f).*

While digestion with EcoRI showed a single fragment 15.7 kb long, restriction with BamHI gave rise to intense bands of 2.37 and 1.89 kb, indicating that there are internal BamHI sites. Further analysis included screening of the genomic library done by plaque hybridization with the same probe used for Southern Blotting. The resulting three lambda (λ) clones were digested with the restriction endonucleases (EcoRI, BamHI, SacI) and further analyzed by Southern Blotting.

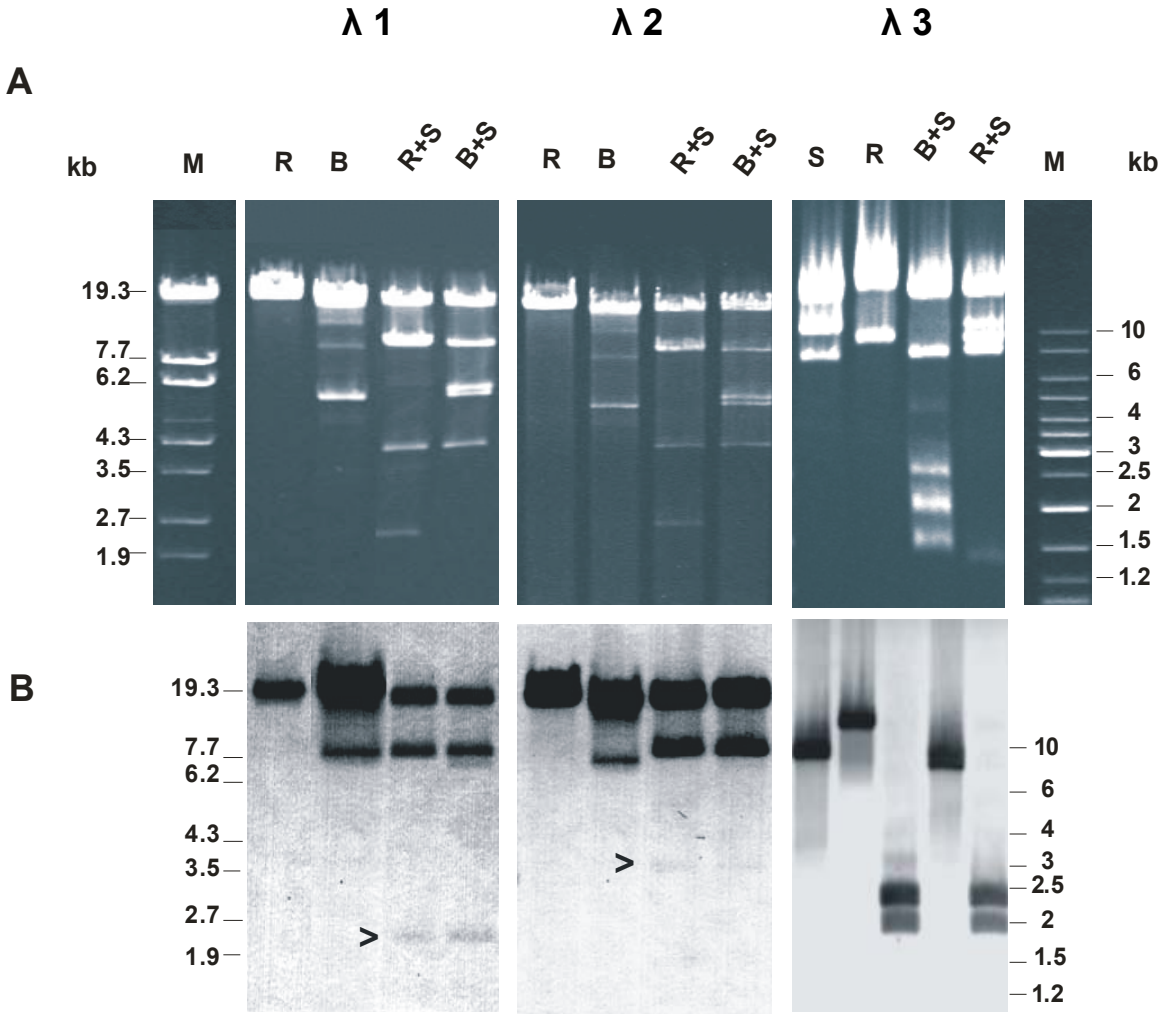

Fig.12. *(A) Restriction digestion combinations used for mapping three clones obtained after screening of the library: EcoRI (R); BamHI (B); SacI (S), EcoRI + SacI (R+S) and BamHI +SacI (B+S). Markers (M) that were used are: λEcoR130I (left side) and Gene ruller (right side). λ1-3 indicate three different lambda clones obtained after library screening. (B) The same λ clones as in A, but subjected to Southern hybridization with DIG_SDTaDH probe. Marked with arrows are four less visible bands (sizes 2 kb and 3.5 kb respectively), that corresponds to SacI restriction fragments.*

The results showed that there are at least one internal BamHI and SacI sites in all three clones (Fig.12). Appearance of one intense band of ~11 kb in λ3 indicated one EcoRI site in this clone but not in the gene of interest (Fig.12B). In λ1 and λ2 there is no EcoRI site which results in one prominent band of ~19 kb. Restriction with BamHI and subsequent Southern Blotting gave rise to the bands corresponding the size of 9 kb in λ 1 and λ 2 and 1.5-2 kb long fragment in λ 3, while restriction with SacI gave rise to bands 2 kb in λ 1, 3.5 kb in λ 2 and 10 kb long fragment in λ 3.

Lenhgt of the three λ clones and position of the TaDH-like gene within were estimated more precisely with serials of PCRs. Primer combinations used were either with vector specific primers (T3 and T7) in order to estimate the full size of the clone or gene specific primers (F or R) with vector specific primers (T3 or T7) in order to estimate the position and the orientation of the TaDH-like gene (Fig.13).

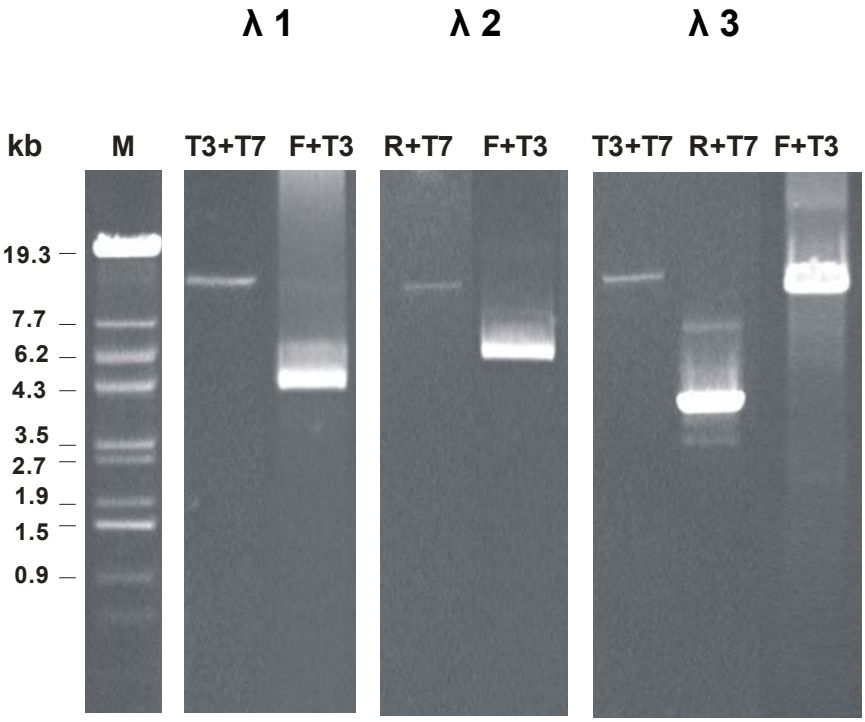

Fig.13. *Serials of PCRs were done on three λ clones (λ 1-3) in order to estimate the length of the clones and position of TaDH-like gene within. Different combinations of the primers were used: vector specific primers (T3 and T7) in order to estimate full size length of the clones and gene specific primers (F or R) with vector specific primers (T3 or T7) in order to estimate the position of the TaDH-like gene.*

These results showed that the sizes of the three λ clones are approximately 13.5, 15 and 13.5 kb respectively while the gene of interest is 3 kb long. Based on the results of restriction digestion, subsequent Southern Blotting and PCRs, mapping of the λ clones was done (Fig. 14).

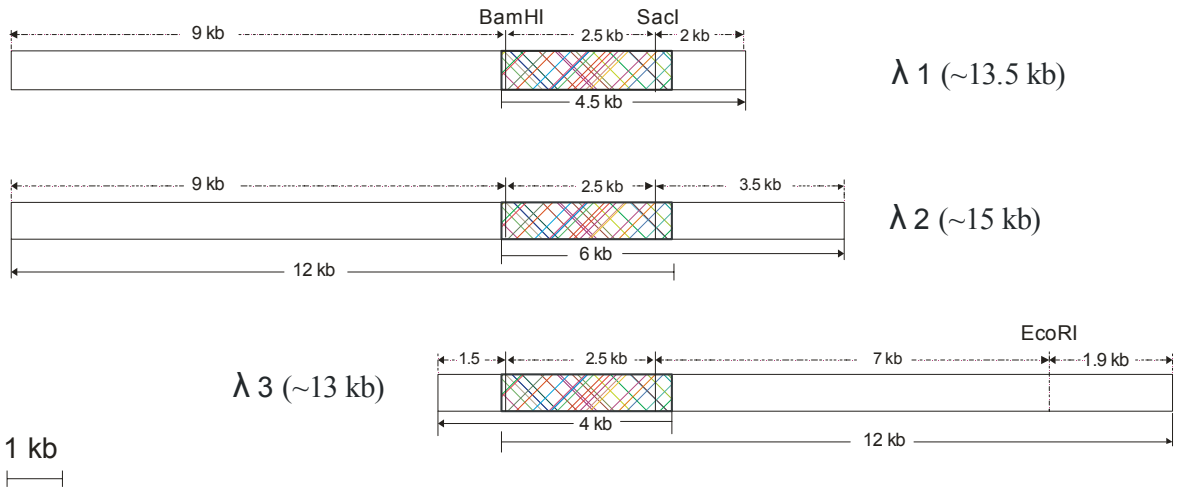

Fig.14. *Graphical display of tree λ clones based on the restriction digestion and PCRs results. Drawing made using CorelDRAW12 is to scale with respect to restriction fragment sizes and precise positioning of TaDH-like gene within the λ clones.*

Due to the best position of the TaDH-like gene, λ2 clone (~15 kb long) was sequenced to completion by primer walking method as described in "Materials and methods". Based on the obtained results, map of TaDH-like gene was constructed (Fig.15). The 2992-kb gene contains 3 exons (being 184, 118 and 712 bp long) and two introns (1229 and 390 bp long). The whole gene sequence is given in the Fig.16. These results confirmed that *SD_TaDH* gene is integrated in sponge genome.

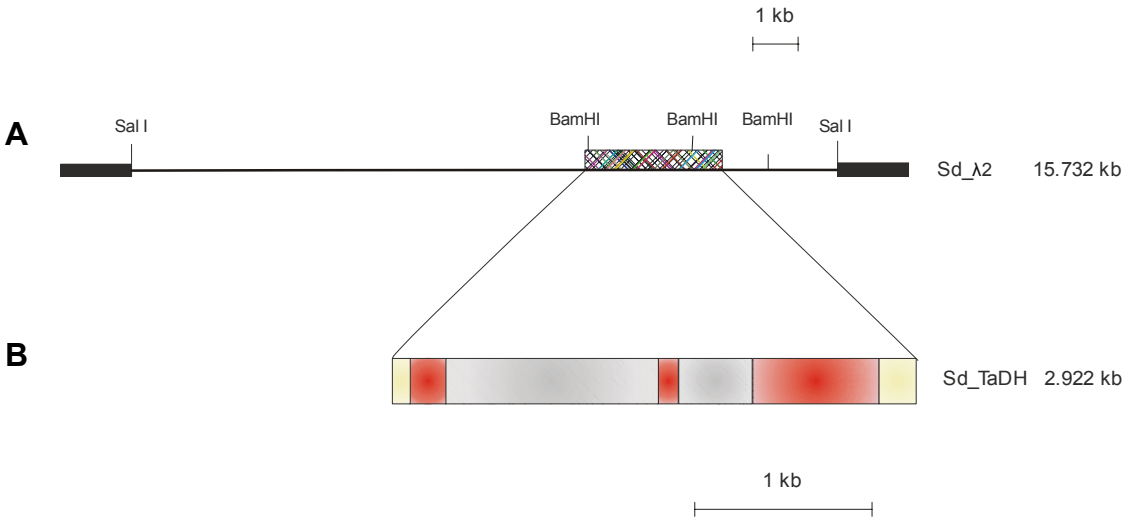

Fig.15. *S. domuncula TaDH-like gene. (A) Physical map of the 15.732 kb clone SD_TaDH-SD_λ2. The stripped boxed region indicates TaDH-like gene. (B) Physical map of the SD_TaDH gene (length: 2.992 kb). The* ***red*** *boxes represents the coding region (exons 1-3) and the* ***gray*** *boxes introns (introns 1-2), while 5' and 3' non coding regions are indicated with beige boxes.*

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  AGATCTCGAACGAAGGCAAAGTCGATCTTCTCTCCTGGATCCTGGCATCACTGTTCAT      -58
CCTCTCTACTGAAAGCTGCTCTACAAGATGTCCAAGAAGCTTACTTTCCTCTATCTCTCT      33
                            M  S  K  K  L  T  F  L  Y  L  S       11

GAACCTGACGTCATCAAGACAGGTCTAACGCTCACAGAAACCATCGAGTTGTGTACGGAG      93
 E  P  D  V  I  K  T  G  L  T  L  T  E  T  I  E  L  C  T  E       31

AGTTTGAAGCAGCATGGCCTGAAAGAAGTCGAGAACCCCCCTAAACCAGGAGTACATCCT     153
 S  L  K  Q  H  G  L  K  E  V  E  N  P  P  K  P  G  V  H  P       51

AAAGAAGATTCCTTCATTAATGCCATGCCTGGTAAGATGATTGTTTATGTATGCTCACTT     213
 K  E  D  S  F  I  N  A  M  P                                     61

TTTGTTTGCTTTTTAGAAACACCTCTACTTGTGACTACCTGTTCTCCACGACCACTTGTA     273
CAATGTATGTGCTTCCTCTCCTATATAATTGCAATGATCTTACACTATACACAGTACTGT     333
TGACCGCTCATCTTCTAATTGATATGTCCCTAGAAAAATAACTTACTCTGGGGCTTTTCA     393
TACCAGAAACAATCAATTGTACATGTACATATATAGTGCCTAATGAGCAAACACCTTGGT     453
GGCTCATACTTAGAAGGCTGCCTCAAACAAAAGTTCTAAGTATGATAGTCATTTGATTGC     513
GTTGGTTAGAGTACACTAATTTACTAGTTTTCATGTAGCAGGACTTCCCTATTGGAAGGT     573
ATAGAATGAGCCTTGGACCTTACTATACCTTGTCTGTCCACAGAACTGTTAGTGTATGTA     633
CGTAACTCTTTTTGTTTTGGGGTGAGGTCTTACTTTACCTAAAATGACACATTGTCCATA     693
AAACCTGCAATAGGCAATACTTCAAAATCTTAATCCCACCTTTACTCAACCTTGCCATGG     753
ACTCTTACAAATCTGTGTATACACTGTCTTTTAGAACTAGCTGCACTGACAATGTGTAGT     813
CAACCCCTACAGTACTAGATTCTCTACACCCATAGTCAACACCATCGCTCTGTTGCGTAA     873
CTGTCCAGCACAGTACACTTTATGAGTACCCAGGATGTAATAGACAATGACCTTTAGTCT     933
CCTTACCAAAATAGTGATGTTCACACAGAATTTTGTTGTTCATTATGCGCATTGTAATCT    1053
TTCATTTCTGTTCATATCCCTGCTTGTGGCGAGCATTGCATAGTTAACCTCCTCATACAT    1113
GTACAATGAAGTGTGTGTTTGACTTTTTATATACATGTATATATATACAAGTACTTAGGC    1173
TATTGGTAGTGTAAGTCCATCCATGCACATCTCCCATTGTGCTTCTTAATGCGAGTACAT    1233
TCATACTATACATAATAATACATAGGATGCTGTGGTTACACTGTGTGTACTCTTGTGCTA    1293
GTTTAAAAGTTGTCTCTTTTGCTGTTAAACATTGATACTCTTAACATGCATCAAGTTTTA    1353
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CACATGATTATAATAGTAATAAGCAGTGTATATTAATGACTCCATATTGTGTCCAAACA     1412
GGATGGCTCAAACAGAAGGGAGTGTGTGGTATAAAGTGGGTGTCTGGATTTCCAGAGAAT    1472
 G  W  L  K  Q  K  G  V  C  G  I  K  W  V  S  G  F  P  E  N      81

GTCAAACAGAGGCTCCCTTCCATTGTGGGTGTGATTATCCTCAACAGTACGGAGACAGGT    1532
 V  K  Q  R  L  P  S  I  V  G  V  I  I  L  N  S  T  E  T       100

CAGTTGTTTGACTGCAGAAATATTTTTATTGTGGTAAATCAAAGGTGACCTTTTAGCTGT    1592
ATAATTATAGTGTGGTTTCTATTTGAGTATTGTAGGTTCACATGTATCAGTTAGCAGACT    1652
ACTTGTTTAAGTGTGCTTCATCAGTTCAGACTCTTGGGGTCACCACTAATTAGATTTTGT    1712
TTATGCACAAATCAGACAAGAAAAATTTCAAGCAACCCCTTTAGAATGAGAAGTTCTACA    1772
AGAGTTCACCTTGCGTGTTGGTTCCAATCTTGGTTAAATTAGGAAAAGTCAACTCTCCTA    1832
GCCTTCTGCTGTCTTAAAATAGACCCCGATGCTTGGGAGGAAAACTGGCTACCAAAACAT    1892
ACTTGTCTATTTTCTTTTTACTGTTTAGGTTTCCCAACTGCTGTTATGGATGGGACATAC    1952
                           G  F  P  T  A  V  M  D  G  T  Y       111

ATCACTGCCATTCGTACTGCTGCTGTCTCAGGTGTCTCAGCCAAATACCTCGCTCGTAAG    2012
 I  T  A  I  R  T  A  A  V  S  G  V  S  A  K  Y  L  A  R  K      131

GACTCTGAGGTGTTGGCCGTCATTGGCACAGGAGTACAGGGCAAGTACAACACTCTCTGC    2072
 D  S  E  V  L  A  V  I  G  T  G  V  Q  G  K  Y  N  T  L  C      151

TTGACAACCGTCCTGCCTAGCATCAAGAAAGTAAAGATCTTTGACACATGGGCACCCAGT    2132
 L  T  T  V  L  P  S  I  K  K  V  K  I  F  D  T  W  A  P  S      171

CTCCAGACTTTTCAAGATCAAATCCGTCCCCTCTTGCCAAACGTAGAGTTTGAGACTGCC    2192
 L  Q  T  F  Q  D  Q  I  R  P  L  L  P  N  V  E  F  E  T  A      191
AGTAGCATGGAGGAAGCCATTCGTGACTCTGATGTGATTGTCGGCGCGACGGCTAAACTT    2252
 S  S  M  E  E  A  I  R  D  S  D  V  I  V  G  A  T  A  K  L      211

ACTGAGACGGTCTACAGTGATGAGTGGGTGAAGCCAGGTGCTCTTGTTCTCCCTATTCAA    2312
 T  E  T  V  Y  S  D  E  W  V  K  P  G  A  L  V  L  P  I  Q      231

GTGGGAGGATGGGATCCTGATGTTTTATCAAAGTTTGACAAAGTTGTGGCTGACGATTGG    2372
 V  G  G  W  D  P  D  V  L  S  K  F  D  K  V  V  A  D  D  W      251

GCTCAACTCAAAGCACAACAAGACTTTAGTCGATACACTTCAAACTTCGACACACCGTAT    2432
 A  Q  L  K  A  Q  Q  D  F  S  R  Y  T  S  N  F  D  T  P  Y      271

GCCGAGCTCGGCGAAATTGTCATGGGCAAAAAACCGGGACGTGAAAACGATCAAGAAAAG    2492
 A  E  L  G  E  I  V  M  G  K  K  P  G  R  E  N  D  Q  E  K      291

ATCATCAATTTCAACAAGGGGTTAGCCGTTCACGATATGATCTGCGGTGGCCGTGTTCTT    2552
 I  I  N  F  N  K  G  L  A  V  H  D  M  I  C  G  G  R  V  L      311

GAGAAGGCAAAGAAACAAGGATTAGGTGTAGAGTTAGAGCTGATGGACCTCGGCTCTCCT    2612
 E  K  A  K  K  Q  G  L  G  V  E  L  E  L  M  D  L  G  S  P      331

ATACCTATGCCACCCGTCTAACTTCATGGATAAACTGTATGAGGATAGCCTGTATGGTAC    2672
 I  P  M  P  P  V  *                                             337

ATTTGCTTAGATACTTCTCTAGAGATAGGTGCAATCATGATGTTGTTGACTTGATTTGCA    2732
CACATGCTTAGCTAGTTTTATACGTACGTATAGTCATATAATTATAGTCATATATCAGTA    2792
TAGTGTAGTGTTGGGTTCTTTCAAAATGAATTTATAATTATGCATG                  2838
```

Fig.16. *The TaDH-like gene. The first base of ATG initiation codon is numbered as +1. Deduced amino acid sequence is given below the nucleotide sequence. The nucleotides are in the 5′ to 3′ direction. Start and stop codon are marked with* ***bold*** *letters. Stop codon in aa sequence is marked with asterisk (*). 5′ and 3′ untraslated region are in blue and green letters respectively. Introns are marked with red letters. PolyA start signal in 3′ untraslated region is* ***underlined and bolded****.*

Unfortunately, in the whole almost 16 kb long lambda clone no other relevant genes were found. By usage of Softberry FGENESH gene prediction program four genes in + chain and one gene in – chain were predicted. BLAST search with deduced amino acid sequences showed really low homology either with predicted or unknown proteins (Fig.17).

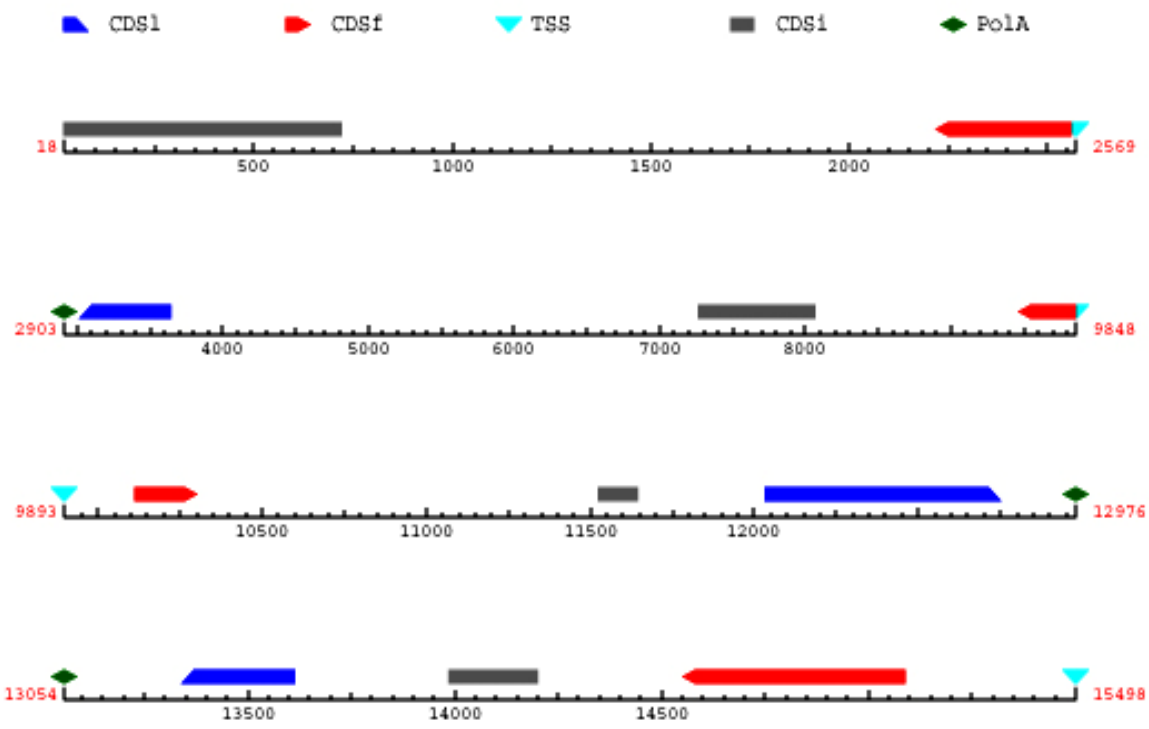


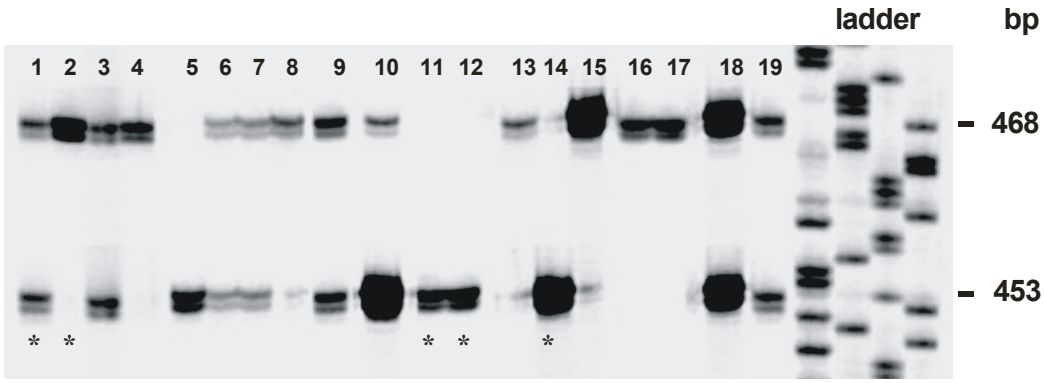
Fig.17. *Gene prediction output performed using Softberry FGENESH program (Salamov and Solovyev 2000). CDSf - First (Starting with Start codon;, CDSi - internal (internal exon; CDSl-last coding segment, ending with stop codon); TSS-Position of transcription start (TATA-box position and score); PolA-Polyadenilation signal.*

5.5 Allelic variations

Little is known about allelic variations in opine dehydrogenases protein family. Therefore, analysis of potential polymorphism was done on 19 specimens of *S. domuncula.* For this purpose, specific primers were applied on the intron2. This is the first report about intraspecies divergences, on the gene level, within OpDHs were a size polymorphism (453 bp and a 468 bp long) was found Fig.18. The polyacrylamide gel gave an optimal resolution for genotype analysis of 19 *S. domuncula* specimens (Fig.18A). According to the different sizes, samples migrated differently. Due to the fact that amplification was performed with non-proofreading DNA polymerases a single adenine nucleotide was added to the 3´-end of the PCR product (Clark 1988; Hu 1993) what gave rise to the double bands of each of the size classes. Confirmation of the allelic variations of the TaDH-like gene, observed on the polyacrilamid gel, was done with subsequent sequencing. Five representative individual DNA samples (lanes: 1, 2, 11, 12 and 14 on Fig.18A) were taken for further analysis. Cloning and sequencing was done on 3–5 clones from each sample. Sequence alignments confirmed genotype analysis from the vertical gel. As indicated in the Fig.18B, two alleles are distinguished by sixteen single nucleotide transitions or deletions and two seven-/nine nucleotide long deletions. Seven out of sixteen single nucleotide transitions could be attributed to the Taq polymerase base substitution error. This is the most familiar type of the mutation that results from the misincorporation of an incorrect dNTP during DNA synthesis. Even under high-fidelity reaction conditions, most of the Taq polymerase errors are the result of T·G mispairs that lead to A·T→G·C transitions (Tindall and Kunkel 1988; Eckert and Kunkel 1991).

Obtained results showed that each individual has one or two differently sized alleles being 453 and/or 468 bp long. Two alleles of the corresponding fragment (intron2) and 3 different genotypes were detected: two types of homozygotes depending on the size class either 468-bp or 453-bp and heterozygotes which include both size classes present as a double bands in one individual.

A



B

```
spec#2   TCAACAGTACGGAGACAGGTCAGTTGTTTGAGCGCAGAAATAATTT-ATTGTGGTAAATCAAAGGTT  65
spec#11  TCAACAGTACGGAGACAGGTCAGTTGTTTGACTGCAGAAATATTTTATTGTGGTAAATCAAAGGTG  67
         +++ Sd_polyF' ++++                *          #                    #

spec#2   ACCTTTTAGCTGTATAATTATAGTGTGGTTTCTATTTGAGTATTGTAGGTTCACTTGTATCAGTTAG 132
spec#11  ACCTTTTAGCTGTATAATTATAGTGTGGTCTCTATTTGAGTATTGTAGGTTCACATGTATCAGTTAG 134
                                     *                      #

spec#2   CAGACTACTTGTTTAAGTGTGCTTCATCTGTTCAGACTCTTGGGGTCACCACTAATTAGATTTTGTT 199
spec#11  CAGACTACTTGTTTAAGTGTGCTTCATCAGTTCAGACTCTTGGGGTCACCACTAATTAGATTTTGTT 201
                                    #

spec#2   TATGCACAAATCAGACAAGAAAAATTTCAAGCAACCCCTTCAGAATGAGAAGTTCTAAAAGAGTTTA 266
spec#11  TATGCACAAATCAGACAAGAAAAATTTCAAGCAACCCCTTTAGAATGAGAAGTTCTACAAGAGTTCA 268
                                              *                #       *

spec#2   CCTTGCGTGTTGGTTCCAATTTTGGTATTGAATTAAATTAGGAAAGGTCAACTCTCCTTGCCTTCTG 333
spec#11  CCTTGCGTGTTGGTTCCAATCTTGGT-------TAAATTAGGAAAAGTCAACTCTCCTAGCCTTCTG 328
                           *                      *                #

spec#2   CTGTCTTAAAATAGACCACGGTGCTTGGGAGGAAAACTGGCTACCAAAACATACTCGTCTACATTTT 400
spec#11  CTGTCTTAAAATAGACCCCGATGCTTGGGAGGAAAACTGGCTACCAAAACATACTTGTCTA------ 389
                         #  *                                    *

spec#2   CTATTTTCTTTTTACTGTTTAGGTTTCCCAACTGCTGTTATGGATGGGACATACATCACTGCCATTC 467
spec#11  ---TTTTCTTTTTACTGTTTAGGTTTCCCAACTGCTGTTATGGATGGGACATACATCACTGCCATTC 453
                                                    |++++ Sd_polyR +++++
```

Fig.18. *Polymorphism in the TaDH-like gene in intron2. (A) Samples from 19 individuals of S. domuncula TaDH-like gene resolved on vertical polyacrylamide gel (LiCor). DNA samples in lanes 1, 3, 6, 7, 9, 10, 18 and 19 [coming from different S. domuncula individuals] are heterozygotes; lanes 2, 4, 8, 13, 15, 16 and 17 homozygotes (size class: 468-bp), lanes 5, 11, 12 and 14 homozygotes (size class: 453-bp). The asterisk (*) indicates subsets of the original samples that were sequenced. (B) Alignment of the two different TaDH-like intron sequences (intron2) from S. domuncula #2 and #11, which are representative for the respective size-classes (468-bp and 453-bp). Deletions are indicated by dashes. Mononucleotide substitutions are shown with asterisk (*). Potential Taq polymerase errors (A→G/T→C) are marked with scale (#). Exon/intron junction is marked with the red box. Primers used for PCR reaction are marked below the sequence.*

5.6 Semi-quantitative RT-PCR analysis: effect of oxygen on TaDH-like gene expression

Previous studies indicated that opine dehydrogenases are employed during anaerobic glycolysis, when the oxygen level is low (Gade and Grieshaber 1986). Therefore, it was interesting to see if SUBDO_TaDH would be down-regulated on the transcriptional level in animals that were kept at more intense aeration (oxygen partial pressure). Semi quantitative RT-PCR method was used to estimate the variation in gene expression between samples from intact control animals and animals that had been exposed for three days to elevated oxygen levels. The expression of housekeeping genes, the myosin light chain (size of the PCR amplified product: 138 bp) and the β-tubulin (117 bp) were used as reference/control.

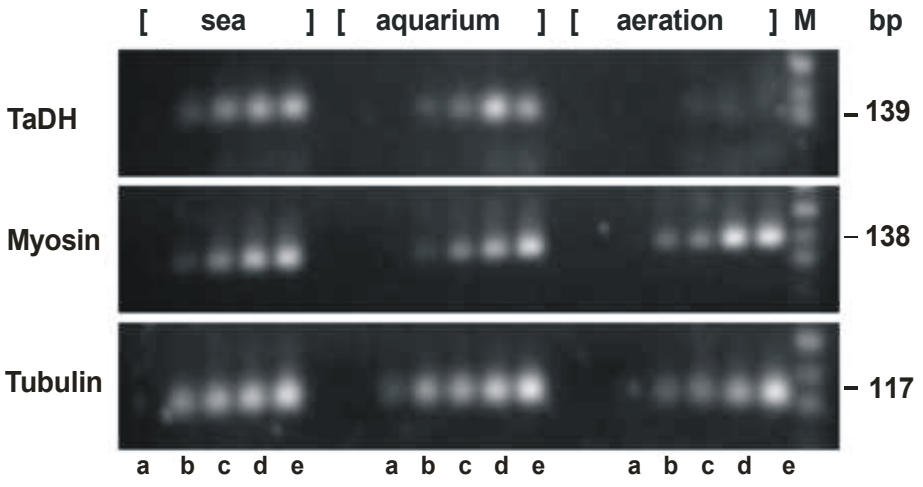


Fig.19. *Expression levels of TaDH-like gene in sponge determined by semi quantitative RT-PCR. Total RNA from 3 different samples of S. domuncula were subjected to semi-quantitative RT-PCR. The PCR products after amplification of the TaDH-like are given in the top picture gel (TaDH; size of the fragment 139 bp); in the middle gel the amplification signals for the gene encoding the myosin light chain (138 bp fragment) is shown; the bottom gel shows the PCR products for tubulin gene (117 bp fragment). The PCR products were applied to the gel after 20 (a), 25 (b), 30 (c), 35 (d) and 40 cycles (e). Tissue from specimens immediately taken from the sea (sea) or from the aquarium after 2 months in controlled conditions (aquarium) and from animals which had been kept under elevated aeration for 3 days (aeration) was analyzed.*

As shown in Fig.19 in animals exposed to elevated oxygen level [aeration] no or only minor expression of TaDH-like gene could be detected (139 bp fragment) in comparison with intact control animals, irrespective of whether the animal was maintained in the aquarium or was taken from the sea. Two control groups of animals show equally high expression of the TaDH-like gene.

5.7 Recombinant protein in *E. coli* cells

Production of recombinant protein was done in order to produce polyclonal antibodies required for further analysis like immunofluorescence analysis (localization of TaDH-like protein *in situ*) and for enzymatic assays. Therefore, TaDH-like gene from *S. domuncula* cDNA library was cloned into the *pTrcHis2-TOPO/lacZ* vector and expressed as a 6-His fusion protein in *E. coli* (TOP10) cells as described in Materials and Methods. Expression was monitored on the 10% SDS-PAGE by analysis of crude cell lysates taken every hour after induction with IPTG. The result is depicted in Fig.20A. One prominent band with a molecular mass of about 40 kDa was visible 1h after induction. This molecular mass of the recombinant protein confirms the expected size of 37 kDa (deduced from the cDNA) plus 3.4 kDa extra from the expression vector. Recombinant protein (r-TaDH) was purified twice on Ni-NTA columns (Fig.20B) under denaturating conditions. Approximately 900 μg/ml of r-TaDH that yielded a preparation with only a few minor impurities as determined by SDS-PAGE was obtained and used to prepare polyclonal antibodies (PoAb).

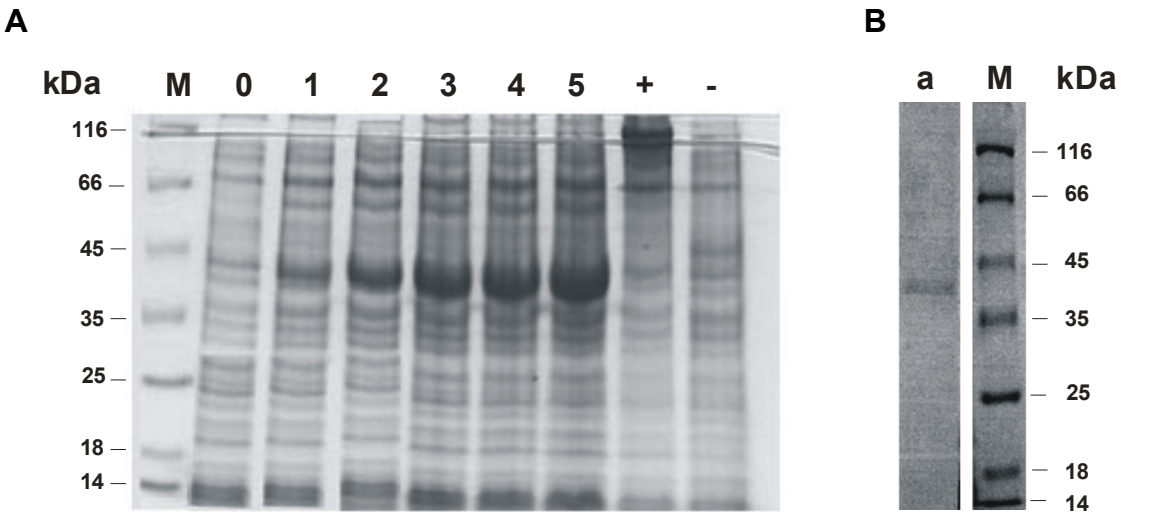


Fig.20. *(A) Expression of the TaDH-like protein in the E. coli cell extracts. Crude cell lysates were separated by 10% SDS-polyacrylamide gel electrophoresis and stained with Coomassie Brilliant Blue R-250.* ***M-*** *molecular mass standard;* ***Lane 0*** *– crude cell lysate taken at time zero, before induction;* ***Lanes 1–5*** *crude cell lysates containing TaDH-like protein after induction with IPTG, taken every hour;* ***Lane –,*** *E. coli TOP10 cell lysate (negative control);* ***Lane +****, E. coli TOP10 cell lysate with vector (positive control). (B) Protein purified twice with Ni-NTA column. The protein size of approximately 40 kDa indicated monomeric structure.*

Western Blot analysis with anti-his antibody was done in order to ensure that the correct fusion protein is isolated. Appearance of the band corresponding to the expected size is visible in Fig.21A. Native purifying conditions were applied for further usage of the fusion protein in enzymatic assays. Due to the fact that protein was less soluble when standard conditions recommended by manufacturer were applied, expression parameters were changed: temperature after induction was lowered to 25°C (instead of 37°C), so that the bacteria cells grew more slowly, IPTG concentration was lowered to 0.1 mM instead 1 mM and induction time was extended to 9 h. Purity of the protein was analyzed by 10% SDS-PAGE and additionally with Western Blot using anti-his antibody (Fig.21B). Approximately 80µg/mL of SUBDO_TaDH purified under native conditions was used for further analysis.

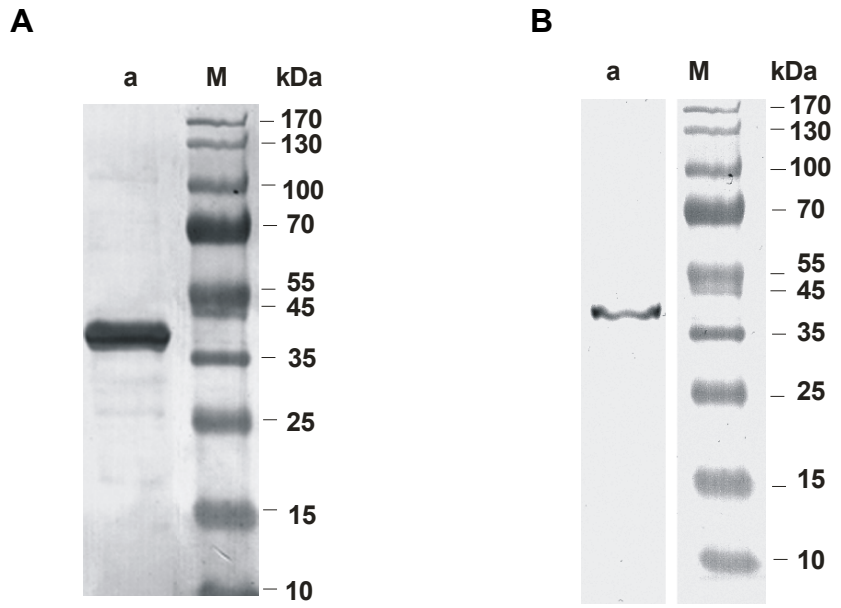


Fig.21. Western Blot *analysis of recombinant TaDH-like protein purified under (a) denaturating and (b) native conditions from Ni-NTA spin column. The blot was developed using anti-His antibodies and alkaline phosphatase conjugated secondary antibody. PeqGOLD protein-Marker IV (Prestained) was used as the molecular weight marker (M).*

5.8 Protein distribution in the sponge

After antibody production Western Blot analysis on total protein extraction was performed in order to confirm TaDH-like expression in *S. domuncula* tissue extracts. Sponge extract was subjected to SDS-PAGE and subsequently stained with Coomassie brilliant blue (Fig.22 lane a). For Western Blot analysis the size-separated proteins were transferred to a membrane which was then reacted with PoAb-TaDH. The 37 kDa band became brightly visible (Fig.22 lane b). This result is a proof that SUBDO_TaDH is present in the sponge *S. domuncula.*

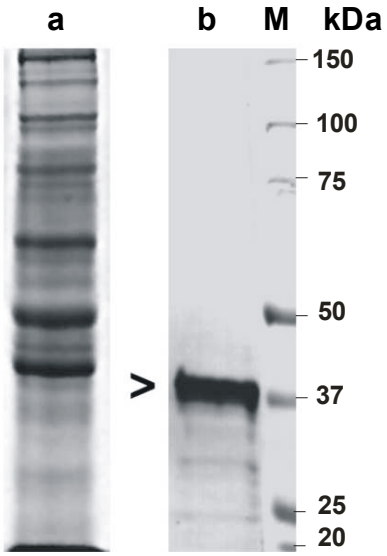


Fig.22. *Western Blot analysis of the SUBDO_TaDH in sponge tissue extracts. Total protein extract from S. domuncula was separated by 10% SDS-PAGE and stained with Coomassie Brilliant Blue R-250 (lane a). Protein size of 37 kDa confirms the expected molecular mass deduced from the cDNA (lane b). Precision Plus ProteinTM Standards (Dual Color) was used as molecular weight marker (M).*

Oxygen supply is crucial for sponge metabolism in general and for morphogenetic events (Perović et al. 2003). Concentration of oxygen in adult sponges is strongly dependent on the surrounding flow regime, molecular diffusion and sponge pumping activity as well as on culturing conditions. Distribution of TaDH-like protein in different parts of the sponge and potentially differences could be related with oxygen profiles within the sponge. Therefore, sponge was divided into two pieces upper and lower as pointed out in Fig.23.

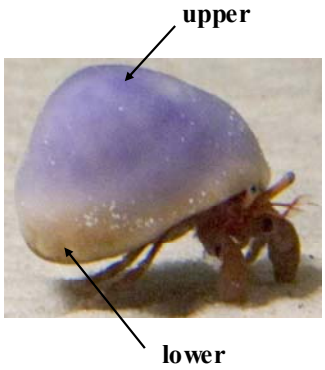


Fig.23. *Sponge, S. domuncula, with the marked upper and lower parts used for estimating protein distribution.*

Tissue from the upper piece of the animal was taken around osculum where utmost flow through of sea water occurs. After total protein extraction tissue was analyzed with Western Blot applying PoAb-TaDH. As seen on the Fig.24 there is no difference in TaDH-like expression levels in the sponge, implying equal distribution of oxygen. Surely this result does not give an answer on oxygen distribution in the sponge. Nevertheless the data demonstrate that *S. domuncula* TaDH-like protein is equally present in sponge tissue.

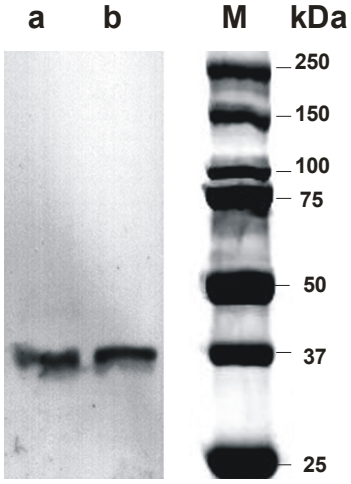


Fig.24. *Expression levels of TaDH-like in sponge. Upper part (lane a) and lower part (lane b). M-molecular mass marker Precision Plus ProteinTM Standards (Dual Color).*

5.9 Immunohistology analysis

Localization of the TaDH-like protein in the sponge tissue was done with immunohistology analyses. Slices from tissue were prepared as described under "Materials and methods". If tissue from animals living under ambient aeration was used for analysis the antibodies brightly stained almost all sponge cells (Fig. 25A). Especially strong are the reactions around the choanoflagellate chambers. In contrast, if antibodies which had before been adsorbed with recombinant SUBDO_TaDH were used, no distinct signals can be seen, even in regions were choanoflagellates chambers are present (Fig. 25B). From previous studies it is known that *S. domuncula* harbors some bacteria in special cells, bacteriocytes (Bohm et al. 2001), and these bacteria can be distinguished from others in the mesohyl which are scattered throughout the tissue (Thakur et al. 2005).

In order to clarify whether the sponge TaDH-like accumulates also around the bacteria, tissue from specimens which were kept for three days under elevated aeration was analyzed. No or almost no SUBDO_TaDH could be identified in sponge cells, with the exception of the bacteriocytes, which were brightly stained (Fig. 25D and G). In parallel, the sections were stained with DAPI (Fig. 25E and H); after this treatment the accumulation of the bacteria clusters in the bacteriocytes can be brightly seen. In a control experiment, pre-immune serum was used which did not give a signal (Fig. 25J) around the DAPI-stained bacteriocyte (Fig. 25K).

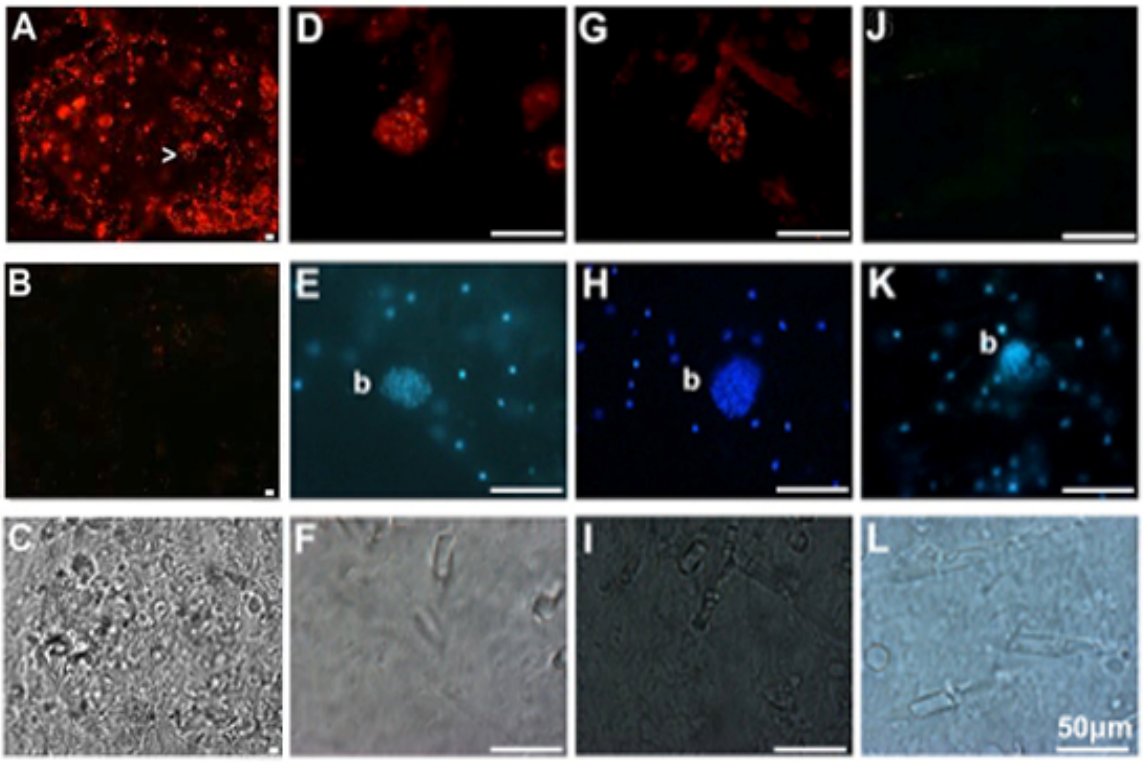


Fig.25. *Immunohistological identification of sponge TaDH-like in tissue from animals kept in the aquarium under optimal oxygen pressure. Immunofluorescence analysis of sections (A, D and G), which had been reacted with PoAb-TaDH antibodies. In A visible choanocyte clusters are marked (>). A parallel section was reacted with antibodies, which had been adsorbed with recombinant sponge TaDH (B). In (D and G) a bacteriocyte is brightly stained with PoAb-TaDH. The slides were additionally stained with DAPI (E and H) to identify both the sponge cell nuclei and the bacteriocyte (b). In addition, no signal is seen in the section reacted with pre-immune serum (J) and DAPI (K); there is no signal also around the bacteriocytes (b). Parallel sections (C, F, I and L) were inspected by light microscopy (Nomarsky images). Size bars (50 µm) are given.*

5.10 Enzyme assays

For many newly sequenced genes, probable cellular roles and functions can be assigned on the basis of homology to known sequences. That way, *S. domuncula* cDNA/gene was annotated as tauropine dehydrogenase due to the sequence homology with the same protein from *Halichondria japonica*. However, additional observation such as, protein structure, biochemical characterization and protein activity measurements are needed for linking functional and sequence data. Combining these sources of information is making identification of new proteins possible. Therefore, the enzyme activities of OpDHs in whole sponge body were measured by monitoring the rate of enzymatic conversion of NADH into NAD^+ (opine biosynthetic reaction) at 340 nm using spectrophotometer. Conditions used in the experiments were according to enzyme assays established by Kanno et al. (1997). One enzyme unit was defined as the amount of enzyme oxidizing or producing 1μmole of NADH per min. Measurements were done with the recombinant protein and crude sponge extracts. When recombinant protein was used in experiments no results were obtained. Maybe due the formation of inclusion bodies or extra amino acids added from the expression vector influenced the reaction. Nevertheless, activity of opine dehydrogenase in the crude extracts of the sponge *S. domuncula* was determined. Surprisingly, StDH (strombine dehydrogenase) activity was dominant in this sponge (79 mU/mg of tissue), while the activities of other OpDHs were not detected at all. Based on these results and according to the previous terminology the new opine dehydrogenase from sponge *S. domuncula* was named strombine dehydrogenase (StDH). The sponge StDH showed a specific requirement for NAD(H). This property is common to all OpDHs characterized so far (Gade and Grieshaber 1986). The enzyme was highly specific to glycine. No enzyme activity was observed when other amino acids (alanine, arginine and taurine) were used in the experiment. Due to the high sequence similarity with OCD, StDH was tested for possible OCD activity. The crude extracts were incubated with ornithine at 25°C and 37°C with standard conditions applied for previous experiments. However, no activity was detected what suggested that *S. domuncula* StDH is not an OCD.

The effect of pH on the activity of StDH was examined using five different pH values (6.2, 6.8, 7.0, 7.2 and 9.0). Other conditions were the same as for the standard assay. Sponge StDH showed maximal activity at pH 7.0 in the strombine-biosynthetic direction.

5.10.1 Apparent Michaelis-Menten Constant

Appling different substrate (Na-pyruvate) concentration in the enzymatic reaction (up to 5 mM) showed that strombine-biosynthetic reaction was markedly inhibited by the substrate (Fig.26). Optimum substrate concentration was 2.0-2.3 mM and the inhibitory concentration yielding the half-maximal reaction rate was 4.0 mM. On the other hand glycine and NADH exhibited no inhibitory effect on the reaction up to concentrations of 200 mM and 1 mM respectively. Apparent K_m value determined using constant co-substrate concentrations (100 mM glycine and 0.3 mM NADH at pH 7.0) was 0.38 mM for pyruvate (Fig.27) which corresponds to the values for StDHs from different organisms published so far, that are varying from 0.32-1.2 mM.

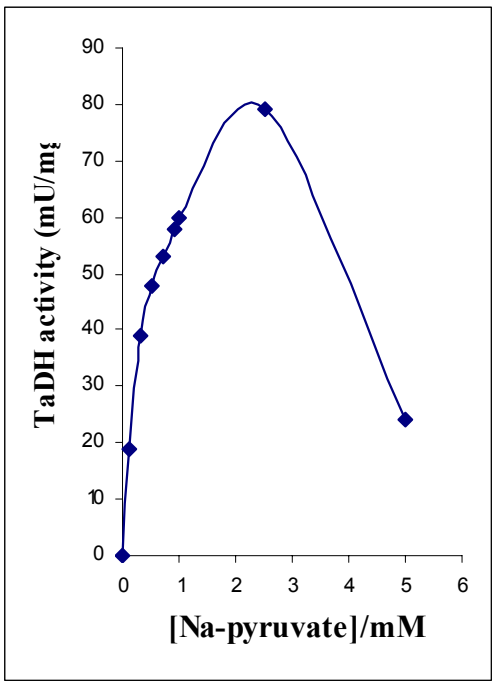


Fig.26. *Pyruvate saturation kinetics for the StDH in crude extracts. The enzyme activity was determined using standard conditions for the strombine-biosynthetic reaction, except that the concentrations of pyruvate were varied from 0 to 5 mM under fixed concentrations of co-substrates.*

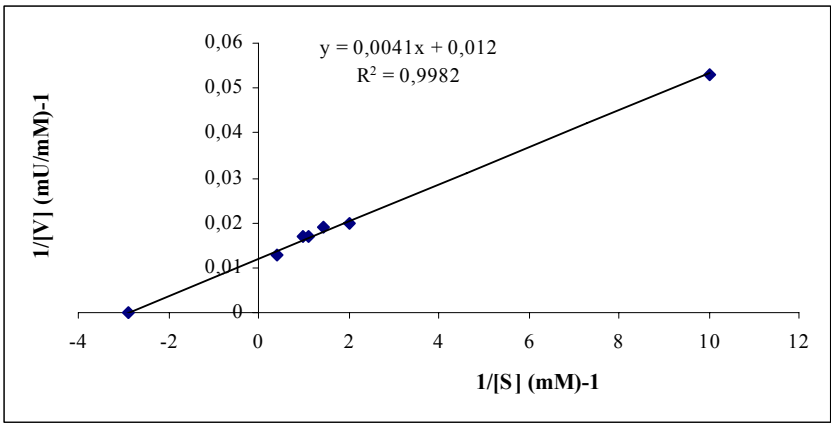


Fig.27. *Lineweaver-Burk plot showing the pattern of inhibition by pyruvate. Michaelis-Menten constant was calculated by linear regression plots.*

5.11 Tertiary and quaternary structure prediction

Previous studies indicated that all so far described opine dehydrogenases have monomeric structure (Kimura et al. 2005) with a notable exeption of alanine dehydrogenase from Archea *Archaeoglobus fugidus* AF_AlaDH (Gallagher et al. 2004). Taking into account specific properties of sponge StDH, described in this study, the question arose about tertiary and quaternary structure of StDH from *S. domuncula*. Therefore, total protein extract from the sponge was subjected to native and seminative PAGE and analyzed by Western Blotting with specific antibodies. When native PAGE was applied one band was visible really high on the gel. Due to the fact that with native gels determination of the size is not possible seminative PAGE was applied as well. In this case two bands become visible corresponding to a molecular size > 37 kDa and ~ 75 kDa indicating dimeric structure (Fig.28).

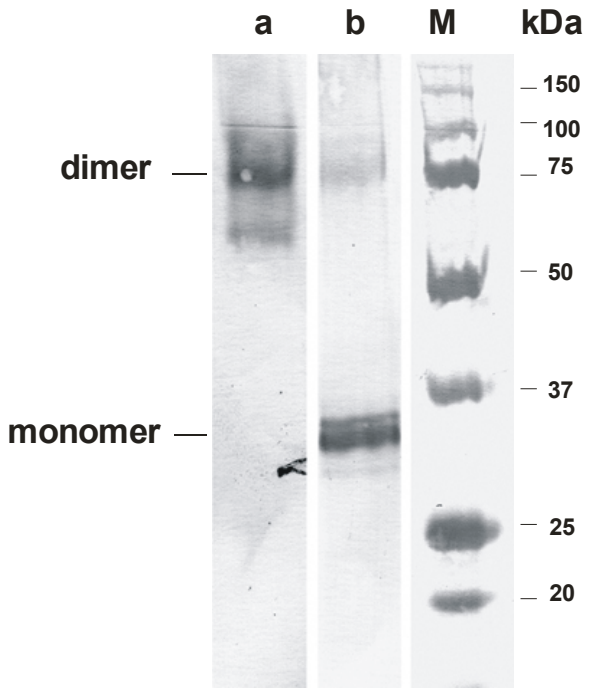


Fig.28. *Total protein extract from S. domuncula were analyzed by SDS-PAGE in native (a) and seminative (b) conditions. Gels were analyzed by Western Blotting, developed with PoAb_TadH antibody and peroxidase-coupled goat anti-rabbit Ig as a secondary reagent. Molecular size marker (in kDa) is indicated.*

This intriguing result was an encouragement for tertiary and quaternary structure prediction. Usage of SwissProt website alignment search tool showed OCD from *P. putida* (PDB ID: 1x7d, resolution 1.6 angstrom by x-ray crystallography) as a high level candidate for the modeling process. Following this interpretation and taking into account similarity in biological function, areas of sequence similarity and the results of fold-recognition methods, presumptive model of sponge strombine dehydrogenase was built (Fig.29). The superimposition and the initial alignment of the sequences were done using the Swiss PDB viewer, version 3.7 (Guex and Peitsch 1997). This preliminary model was submitted to the SwissProt modeling server in order to optimize the structure. The resulting model was checked for major deviations from the template and for unrealistic conformations using WHATCHECK (http://www.sander.embl-heidelberg.de/whatcheck).

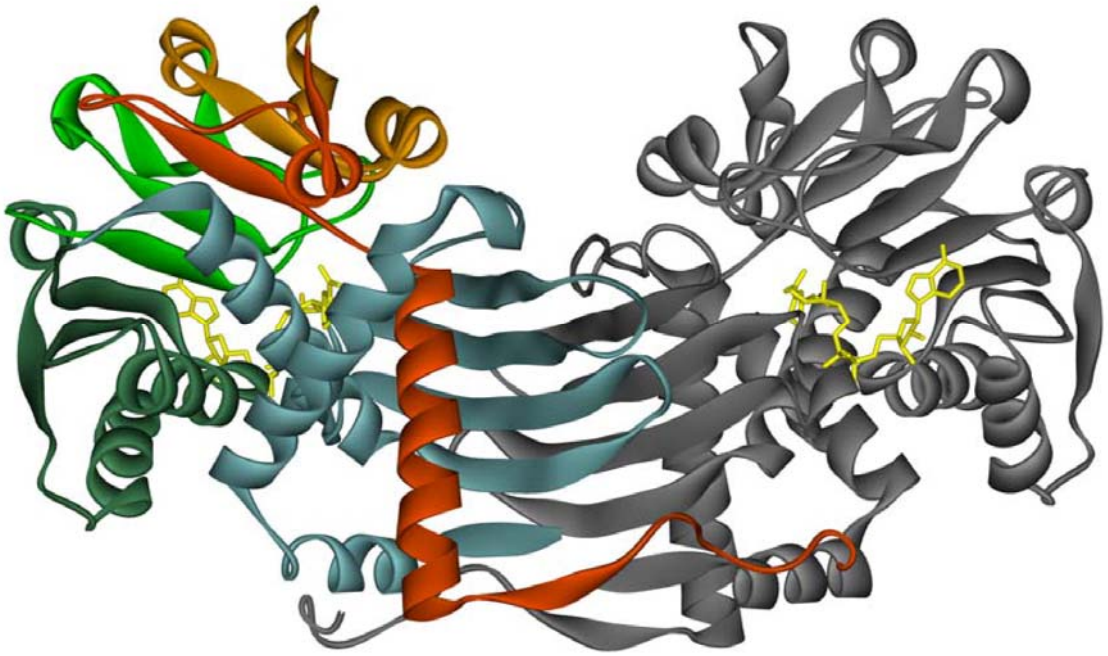


Fig.29. *Ribbon diagram of S. domuncula strombine dehydrogenase dimer with the bound NAD(H) represented as sticks model (yellow). One subunit (on the left) of this homodimer is colored from blue (at the N terminus) through green, brown and red to indicate sequence order of the structural elements. The figure was prepared using the UCSF Chimera package (Pettersen et al. 2004).*

Only lysine (Lys) residue at the position 252 showed slightly unrealistic conformation due to the fact it is located in the variable C-terminal part of the protein. Clashes between side chains and/or the c-alpha backbone were not found. Interestingly, the model has more amino acids in most favored confirmations than the template which may be an artifact of the protein crystallization process. Ramachandran plots and statistics for template and model are given in Fig.30. In order to validate the correct model fold a superimposition of side chains in a four angstrom distance around the bounded NAD ligand was done. The maximum deviations between related side chains in the template and model were determined at ~1.7 angstrom. This close mapping of binding site relevant amino acids showed clearly the correct fold of the model (Fig.31).

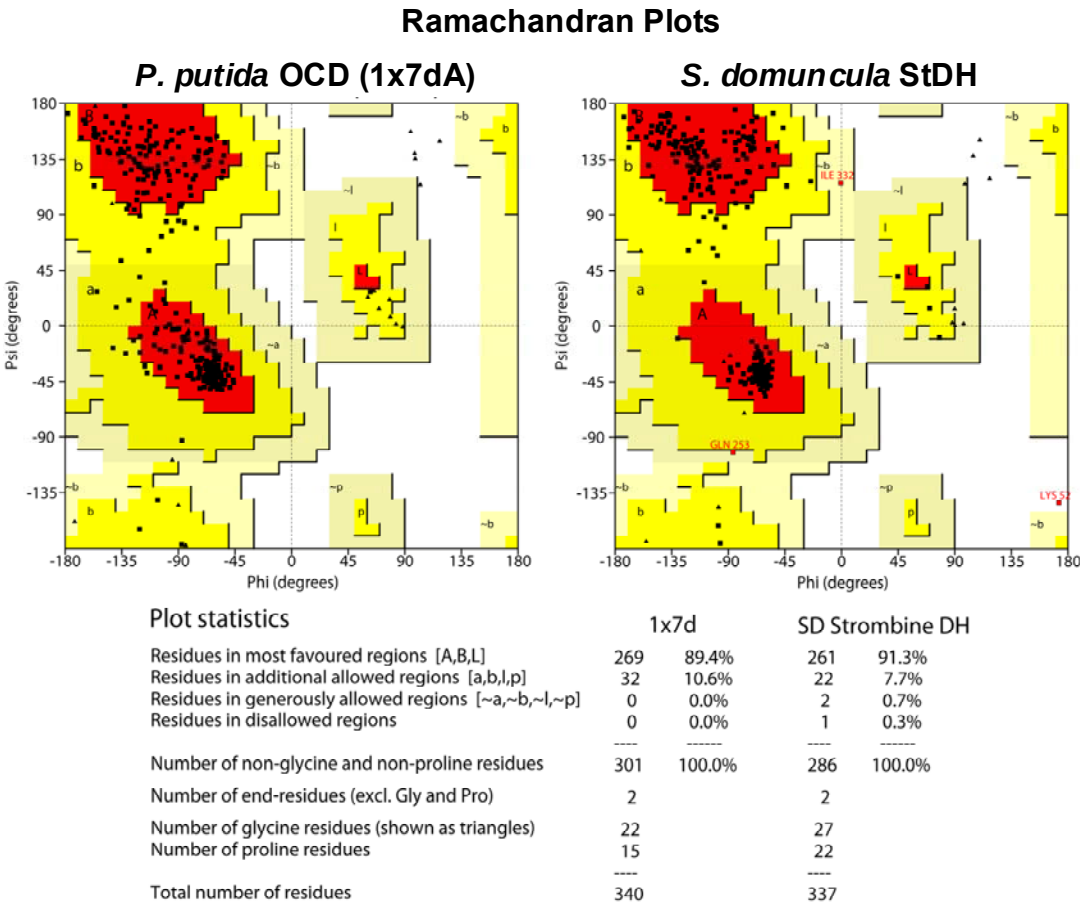


Fig.30. *Ramachandran plot of P. putida ornithine cyclodeaminase structure (PDB ID: 1x7d) and S.domucula strombine dehydrogenase model. Lysin 252 in the model is located in the variable C-terminal tail.*

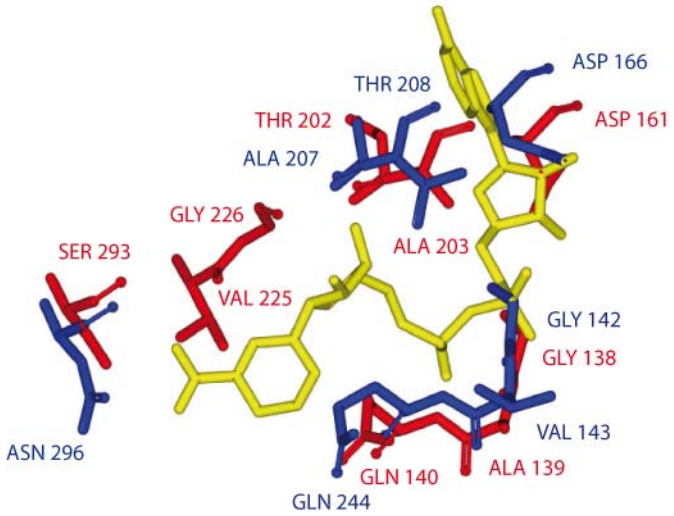


Fig.31. *Superimposition of side chains in a four angstrom distance to the bounded NAD ligand in P. putida ornithine cyclodeaminase (red - template) and S. domuncula strombine dehydrogenase (blue – model).*

Analysis showed that StDH from *S. domuncula* comprises a dimer of identical subunits. The refined model contains residues 1-335 of each chain. Each subunit folds into two functional different domains, the smaller dimerization/catalytic domain 1 (residues 1-128 and 299-337) and larger NAD-binding domain 2 (residues 129-298). Both termini belong to domain 1, where they are in close proximity. Domain 2 contains a classical dinucleotide-binding folding unit with the NAD-associated P-loop sequence motif GxGxxG/A present as residues 140-145. The putative active site determined on the mutual influence/position of NAD-binding site is in domain 1 and comprises Lys46, Asn57, Lys73, Arg116, Thr117 and Asp303. With the exception of Asn 57 these sites are well conserved among OCD/mu-crystallin family.

6 DISCUSSION

It is well accepted today that newly discovered opine production pathways, catalysed by opine dehydrogenases (OpDHs), in marine invertebrates are analogues to the classical anaerobic glycolytic pathway (lactate production pathway), which is predominant in vertebrates (Sato et al. 1993; Livingstone 1991). In this study identification, analyses and characterization of OpDH cDNA/gene in the *S. domuncula*, more specifically strombine dehydrogenase (StDH), on genomic and transcriptional level is described. So far described OpDHs from metazoans have several properties in common: octopine/nopaline dehydrogenase domain (Endo et al. 2007), similar lengths ranging from 396 to 409 amino acid residues and molecular weight of around 40 kDa, monomeric structure, identical reaction mechanisms (Gäde and Griesheber 1986; Ulrich 1994) and similar enzymatic properties. Regardless of their different substrate specificity all OpDHs from marine invertebrates were believed to be homologues (Gäde 1988; Kanno et al. 1999). Despite the distribution of OpDHs in a wide range of marine invertebrate phyla (Livingstone 1983; Livingstone et al. 1990; Sato et al. 1993) available sequence data is limited mostly on OpDHs from mollusks. Recent studies on the demospongiae *Halichondria japonica* (Kanno et al. 2005) and this study on demospongiae *Suberites domuncula* are implying that sponge OpDHs are unique enzymes that differ in several aspects from other OpDHs. Sponge OpDHs showed no significant amino acid sequence similarity to the seven so far described OpDHs from the metazoans (principally mollusks and the annelid). Previously described metazoan OpDHs share a protein sequence identity among each other more then 40%. The amino acids sequences of octopine dehydrogenases (OcDHs) from phylogenetically close species, such as within the three cephalopods species and also between the two scallops (both belonging to the phylum Mollusca), have over 90% amino acid identity (Tab.3). Even though, sponge OpDHs share 86% similarity (68% identity) among each other, comparison with other available sequence data from marine invertebrates revealed similarity of less then 28% (identity $\leq$ 10%) on the amino acid level as pointed out in Tab.3.

	S. domuncula StDH	*H. japonica* TaDH	*H. discus hannai* TaDH	*A. tricolor* TaDH	*L. vulgaris* OcDH	*L. opalescens* OcDH	*M. yessoensis* OcDH	*P. maximus* OcDH	*S. officinalis* OcDH
S. domuncula StDH		68%	10%	9%	8%	8%	9%	9%	8%
H. japonica TaDH	86%		9%	8%	8%	8%	9%	9%	9%
H. discus hannai TaDH	25%	23%		44%	51%	52%	48%	48%	50%
A. tricolor TaDH	24%	28%	64%		41%	41%	45%	45%	39%
L. vulgaris OcDH	24%	22%	68%	61%		93%	46%	46%	89%
L. opalescens OcDH	25%	23%	69%	61%	96%		46%	46%	89%
M. yessoensis OcDH	25%	24%	65%	65%	64%	64%		95%	47%
P. maximus OcDH	25%	24%	66%	65%	65%	65%	97%		47%
S. officinalis OcDH	24%	23%	68%	61%	97%	96%	65%	65%	

	S. domuncula StDH	*A. tumefaciens* OCD	*P. putida* OCD	*A. fulgidus* AlaDH	*R. meliloti* OCD	*R. solanacearum* OCD	*H. sapiens* THBP
S. domuncula StDH		18%	19%	28%	33%	30%	22%
A. tumefaciens OCD	37%		45%	23%	16%	18%	18%
P. putida OCD	41%	58%		23%	17%	19%	18%
A. fulgidus AlaDH	48%	39%	44%		24%	25%	26%
R. meliloti OCD	53%	31%	34%	45%		23%	18%
R. solanacearum OCD	49%	34%	36%	47%	42%		18%
H. sapiens THBP	38%	31%	35%	42%	34%	39%	

Tab.3: *Amino acid sequence homology of S. domuncula StDH with OpDHs from marine invertebrates and with the OCD/mu-crystallin protein family. Homology is displayed in percentages: blue letters indicate identity and red letters indicate similarity.*

TaDH from *H. japonica* and StDH from *S. domuncula* comprise within their ORFs the characteristic single ornithine cyclodeaminase/mu-crystallin family domain and differ by this from the dominant domain (octopine/nopaline dehydrogenase) found in other marine invertebrate species (Endo et al. 2007). FASTA and BLASTp searches with the SUBDO_StDH amino acid sequence revealed similarity to proteins of the ornithine cyclodeaminase (OCD)/mu-crystallin family. This family includes OCDs, lysine cyclodeaminase from *Streptomyces hygroscopicus,* AlaDH from *A. fulgidus*, mammalian mu-crystallins, NADP-regulated thyroid hormone-binding protein (THBP) in human (Vié et al. 1997) and many putative OCDs. OCD is an unusual enzyme that converts L-ornithine directly into L-proline and releases ammonia. It has limited distribution among

the specialized-niche eubacteria and archaea (Soto et al. 1994). OCD is one of the enzymes that participate in the octopine or nopaline catabolism pathway in Agrobacteria that infect plants by tumor inducing plasmid-mediated gene transfer (Sans et al. 1987). In other bacteria, OCD is thought to be one of enzymes involved in the arginase pathway, a pathway of arginine catabolism (Cunin et al. 1986).

In addition, individual pair-wise comparisons of SUBDO_StDH revealed highest sequence similarity with OCD of the: *Rhizobium meliloti* (53%)*, Ralstonia solanacearum* (49%) and *Agrobacterium tumefaciens* Ti-plasmid pTiC58 (37%). These plant pathogenic bacteria have genetic loci coding for opines located on Ti -plasmid. The role of *Agrobacterium tumefaciens* involving opines is well described by Schell et al. (1979). This natural occurring phenomenon represents a "genetic colonization" of the plant cell, by bacterial plasmid DNA, which is forced to produce and secrete, opines that bacteria can use as a carbon and/or nitrogen source. The Ti plasmids are examples of plasmids that, in essence, create new bacterial species when they are acquired, so drastic are the phenotypic variations they beget. Furthermore, SUBDO_StDH showed 38% similarity with human mu-crystallin (THBP). The function of the human THBP protein is still not clear. Mousses et al. (2002) have implicated this protein in prostate cancer and in deafness. High levels of expression were found in inner ear tissue and two specific mutations (one adding five extra residues at the C terminus, the other changing the C-terminal Lys314 to Thr) were found to be linked with non-syndromic deafness (Abe et al. 2003). Highly conserved regions within OCD/mu-crystallin protein family (including sponges) comprises the segments at positions 86-128, 191–207, 272–282 and 292-303, respectively, which may be involved in substrate binding. Classical NAD-associated P-loop sequence motif, known to be conserved among NAD(P)-dependent dehydrogenases (Rossmann et al. 1975; Wierenga et al. 1985), appears as G139-T-G141-V-Q-G144 in *S. domuncula* StDH. This glycine-rich sequence within the mu-crystallin family appears as GxGxQA/S, with the QA in most but not in all members and is located in the equivalent position as in sponge (Fig. 32).

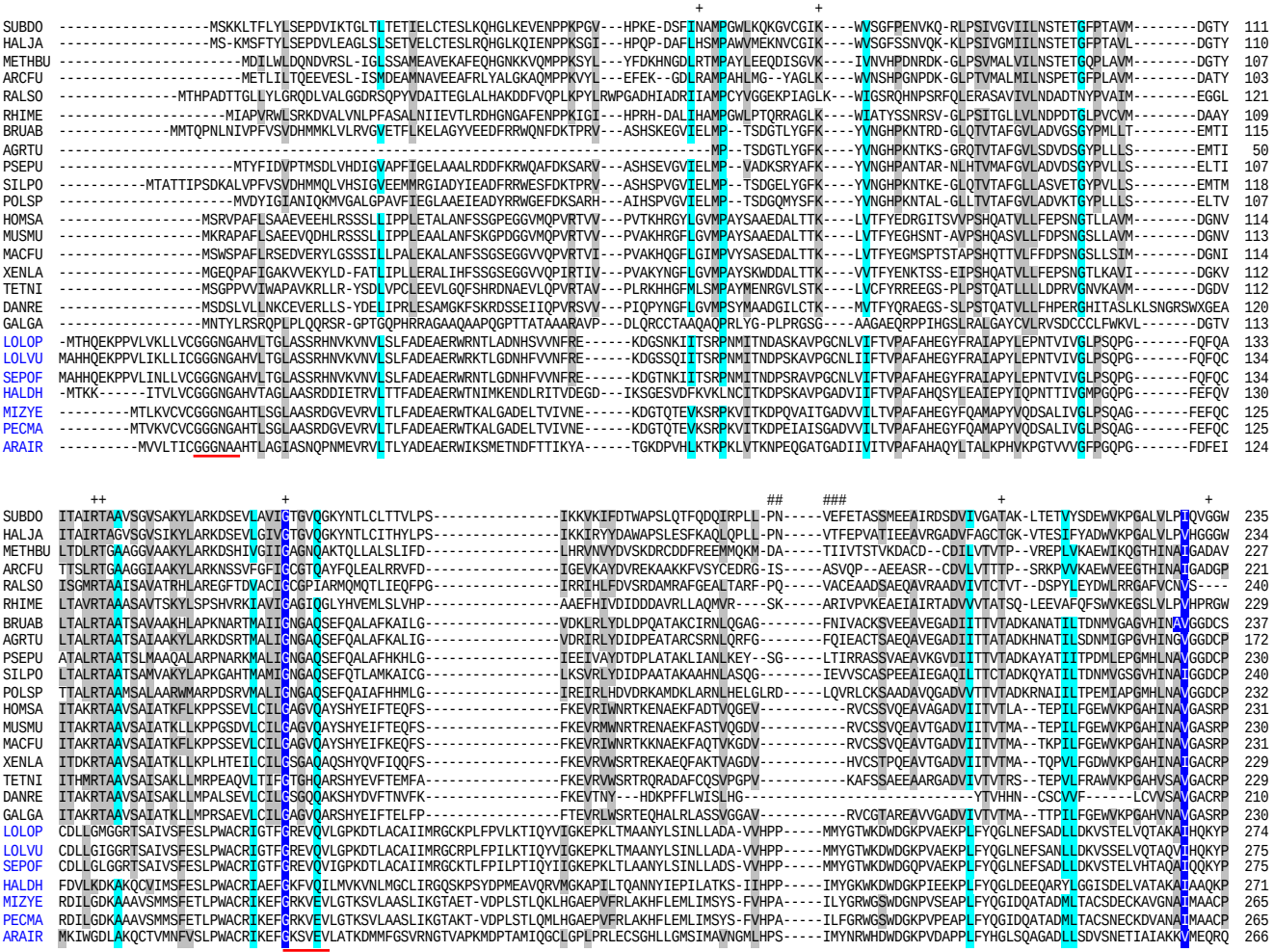


Fig.32. *Multiple sequence alignment of the mu-crystallin protein family with OpDHs included. First two sequences are poriferian and the last 7 sequences marked with blue letters are marine invertebrates OpDHs. GxGxxG/A motif is underlined in red below the sequence. Key residues involved in catalytic activity in A. fulgidus and P. putida are marked with plus (+) above the alignment. Insertion of 5 residues in S. domucula sequence is marked by #.*

In marine invertebrates NAD-binding site is located at the N-terminus whereas in the mu-crystallin protein family it is in the central part of the sequence. Alignment of the sponge and human sequences shows that there is only one large (more then three residues) insertion, of five residues at position 185 as pointed out in the Fig.32 (marked with #). Due to the strong sequence conservation across the mu-crystallin family the present study provides an approximate model for bacterial cyclodeaminase enzymes, mammalian lens proteins and human thyroxine-binding protein.

Recently, the structures of two enzymes of this protein family have been analyzed by X-ray crystallography: OCD from *Pseudomonas putida* (the GenBank Acc. No. NP745670; The Protein Data Bank Acc. codes, 1U7H and 1X7D; Goodman et al. 2004) and alanine dehydrogenase (AlaDH) from *Archaeoglobus fulgidus* (The Protein Data Bank Acc. code, 1omo; Gallagher et al. 2004). Although the overall amino acid homology between *P. putida* OCD and sponge OpDHs is low (similarity 41%) conservation of key residues involved in catalytic activity, similarity in biological function and the results of fold-recognition methods, suggests molecular relationships between the bacterial/archeal and sponge enzyme. *P. putida* OCD has a dimeric structure and belongs to the OCD/mu-crystallin family (Gallagher et al. 2004). Sequence alignments of SUBDO_StDH with PSEUP_OCD indicated that active site residues are highly conserved. Several residues which have been suggested as a probable candidate for the catalytic mechanisms in *P. putida* are highly conserved in sponge StDH. This set is made of Thr117 which binds the catalytic hydride acceptor atom, Gly140 which is part of the NAD-binding motif and Lys73 (active site: potential substrate ligand), Arg116 (active site: nicotinamide ligand and likely substrate ligand), Thr208 (adenine ligand) and Asp303 (bidentate coordination of active site). Asp228 in *P. putida* OCD (Asp219 in *A. fulgidus* AlaDH), which has been proposed to interact directly with the leaving of amino group, is replaced with Gly231 in Halj-TaDH or Gly233 in SUBDO_StDH. In addition, Arg52 in *A. fulgidus* AlaDH that has been presumed to interact with the amino group of L-alanine through a water molecule is replaced with His56 in Halj-TaDH and Asn58 in SUBDO_StDH. This replacement may be related to the difference in substrates (NH_3 vs. taurine vs. strombine). The presence of an extended C-terminus in bacterial OCDs (with notable exception of plant symbionts *R. solanacearum* and *R. meliloti*) was not observed among

OCD family members included in the alignment (neither protein orthologues in vertebrates nor the archeal) as shown in Fig.32. Although sponge OpDHs have a short extended C-terminus a basic residue equivalent to Lys331 in *P. putida* OCD is not found. These amino acid differences should prove to be useful in the differential classification of OCDs and OpDHs within the mu-crystallin family (Fig. 32; marked with +).

Comparission of OpDHs showed that the molecular mass of sponge OpDHs are smaller than those of the other known OpDHs (usually 39.5–45 kDa) from marine invertebrates (Gäde and Grieshaber 1986). This is significant because OCD and related proteins are about 330 amino acids long, whereas all of the known mollusk/annelid-type OpDHs are about 400 amino acids long. The most striking characteristic demonstrated in this study is that SUBDO_StDH has a dimeric structure. Application of native and seminative PAGE in combination with Western Blot analysis revealed a band at ~ 75 kDa indicating dimeric structure. These results were confirmed with tertiary and quaternary structure prediction using *P. putida* OCD as a template. Superimposition of side chains in a four angstrom distance around the bounded NAD ligand showed clearly the correct fold of the model. Looking at OpDHs, dimeric structure was observed only in *A. fulgidus* alanine dehydrogenase. This unusual OpDH, that catalyzes the reversible conversion of L-alanine to pyruvate and NH3 with NAD(+) as a co-substrate, is not homologous to known bacterial dehydrogenases and belongs to the OCD/mu-crystallin family (Gallagher et al. 2004). Knowing that all so far described Metazoan OpDHs are monomeric proteins and taken into account all the differences previously described, we can say that *S. domuncula* StDH has a novel structure. Sponge OpDHs may be specific enzymes having a limited distribution in sponges, which have no developed muscular tissues and do not seem to require such a large energy production that other invertebrates possessing developed muscular tissues do during muscular activities.

Having reached this step the question arises about the potential function of the StDH in sponges. The StDH enzyme uses glycine as substrate to produce strombine in a NAD(+)-dependent reaction (Gade and Grieshaber 1986). Glycine is a non-essential amino acid found in proteins of all life forms. This simple amino acid is of major importance in the synthesis of proteins, peptides, purines, adenosine triphosphate (ATP), nucleic acids, porphyrins, hemoglobin, glutathione, creatine, bile salts, one-carbon fragments, glucose,

glycogen, L-serine and other amino acids. Glycine is also a neurotransmitter in the central nervous system (CNS). Glycine can be synthesized via two main pathways, both of which occur in mitochondria. The main pathway is formation of glycine from CO_2, NH_4^+, and N_5N_{10}-methylene tetrahydrofolate (THF) in a reaction catalysed by glycine synthase or from serine, by way of serine hydroxymethyl transferase. As it is pointed out with enzymatic assays obtained in this study, *S. domuncula* StDH has strict substrate specificity for glycine and pyruvate and no detectable activity with alanine, arginine, taurine neither with ornithine. This high affinity for pyruvate and glycine or taurine in case of *H. japonica* (Kanno et al. 2005) is one of the sponge OpDHs characteristics which distinguish them from OpDHs from other animals. In addition, sponge OpDHs are subjected to potent substrate inhibition by pyruvate. Comparable low Km values and the potent substrate inhibition have never been recorded for OpDHs from other animals. *S. domuncula* StDH showed a specific requirement for NAD(H), which is common to all OpDHs characterized so far (Gade and Grieshaber 1986). Natural respiration rates and oxygen saturation of suspension feeders are still poorly known. Sponges have complex water circulating system connecting inhalant and exhalant canals (Möhn 1984). This aquiferous system is required for supply of nutrients (Simpson 1984) and of oxygen (Gatti et al. 2002). Oxygen saturation in the sponge tissue is generally affected by flow regime, culturing conditions and pumping activity of sponges (Gatti et al. 2002). Oxygen supply is crucial for sponge metabolism in general and for morphogenetic events (Perović et al. 2003) or the support of bacterial growth (Müller et al. 2004) in particular. Concentrations of oxygen in adult sponges are strongly dependent on the surrounding flow regime, on sponge pumping activity as well as on culturing conditions. Oxygen concentrations in the tissue of adult sponges are usually between 50 – 60% of the surrounding water (Gatti et al. 2002). In order to cover minimal energy requirements marine suspension feeders are required to filter more than 10 l of water per ml of oxygen consumed (Jörgensen 1975). Exposure to reduced oxygen tension at the bottom of muddy waters forces sponge *S. domuncula* to survive hypoxic or even anoxic conditions in the environment. Reduced pumping activity can lead as well to anoxia in parts of the tissue and even in the canal- and exhalant water. It is reported from many sponges in situ to reduce or even stop pumping for several hours at irregular intervals (Reiswig 1971;

Vogel 1977; Gerodette and Flechsig 1979; Pile et al. 1997). The main roles for the oxygen sensing and signal transduction system appear to include coordinate down-regulation of energy demand and energy supply pathways in metabolism. By this means, hypoxia tolerant cells stay in energy balance as they down regulate to extremely low levels of ATP turnover. The main ATP demand pathways in normoxia (protein synthesis, protein degradation, glucose synthesis, urea synthesis, and maintenance of electrochemical gradients) are all depressed to variable degree during anoxia or extreme hypoxia. Duration and completeness of recovery from anaerobic metabolism is of important functional consequence in invertebrates. Two basic processes occur during recovery: (1) recharging of the phosphagen and ATP pools and (2) disposal of end products by excretion, oxidation or conversion back to anaerobic substrates. Anaerobic metabolism is involved not only in situations when oxygen uptake is blocked (natural anaerobiosis), but also when the energy demand can not be met by the respiratory capacity alone. Sponge StDH has a similar role like the lactate dehydrogenase (Grieshaber et al. 1994; Kanno et al. 2005). The major pathway in both, marine and terrestrial animals, for the regeneration of NAD(+) from NADH under anaerobic conditions is the conversion of pyruvate to lactate under consumption of NADH, through the lactate dehydrogenase. The formed NAD(+) is essential for the maintenance of the glycolytic pathway on the level of glyceraldehyde 3-phosphate. However, no transcript could be identified in *S. domuncula* cDNA/EST (expressed sequence tag) library, comprising 30000 ESTs, that encodes lactate dehydrogenase. The closest related enzyme, which could be identified in this database, was malate dehydrogenase which converts oxaloacetate to malate under consumption of NADH. Since the latter enzyme occurs, like all enzymes from the glycolytic pathway, in the cytosol it is most likely that both enzymes, StDH and malate dehydrogenase, are the key enzymes for the regeneration of NAD(+) under anaerobic conditions; see scheme in Fig. 33.

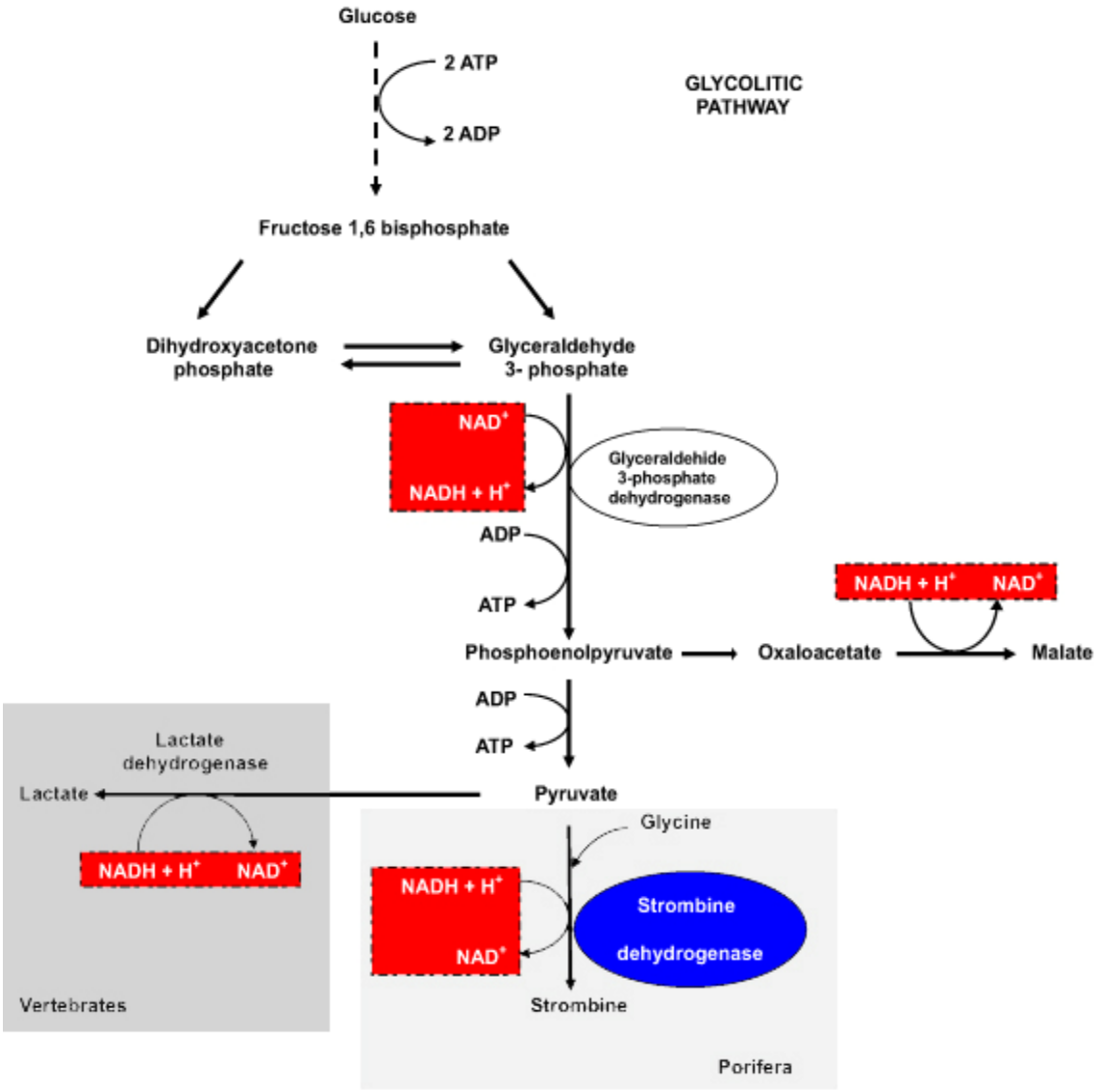


Fig.33. *Proposed function of StDH in sponge tissue under anaerobic conditions. During glycolytic pathway, glucose is catabolically converted to pyruvate under the consumption of NAD(+) [oxidation of glyceraldehyde 3-phosphate to phosphoenolpyruvate] and generation of ATP. In vertebrates, NAD(+) is regenerated during the lactate dehydrogenase reaction. This enzyme is absent in S. domuncula. As an alternative dehydrogenase, the enzyme StDH reacts with glycine and pyruvate to strombine under generation of NAD(+).*

In order to elucidate the proposition that under anaerobic conditions NAD(+) is regenerated by the StDH we asked in this study if this enzyme would be down-regulated on the transcriptional level in animals that were kept at more intense aeration (oxygen partial pressure). After exposure of *S. domuncula* to oxygen, expression of StDH gene becomes totally blocked as demonstrated by semiquantitative RT-PCR analysis. These finding indicated that in sponge *S. domuncula* the end product of the glycolysis pathway is not lactate but strombine. The production of opines in vivo is presumably restricted by the concentration of free amino acids in their tissues (Kreutzer et al. 1989) and glycine is often dominant amino acid in the amino acid pools of sponges (Bergquist 1978). The biological meaning of strombine formation is not fully understood. In invertebrates such as *Mytilus edulis* (Zachariassen et al. 1991) accumulation of strombine has been identified as a metabolic stress indicator. The data suggested that high concentrations of strombine contribute to the regulation of tissue osmolarity (Simpson et al. 1959). De Zwaan et al. (1983) have suggested that the main functional role of strombine accumulation is to meet the elevated energy demands during post anaerobic recovery. Apart from this it might be proposed that strombine is either excreted from marine animals or metabolized by bacteria. Opines are selective nutrients catabolized by a restricted group of bacteria (Dairi and Asano 1995; Beaulieu et al. 1983; Bouzar et al. 1987; Rossignol and Dion 1985; Tremblay et al. 1987; Moore at al. 1997; Paracer and Ahmadjian 2000) and most belong to genera recognized as root colonizers (Beauchamp et al. 1990). Opine-utilizing bacteria (*Agrobacterium, Rhizobacterium, Arthrobacter, Pseudomonas* and *Coryneform* bacteria) are carrying opine metabolism genes and can use opines as nitrogen and carbon source. The utilization of opine by Rhizobacteria could select for efficient catabolism of carbohydrate and amino acids, which could correlate with the ability to catabolize many organic compounds found in the rhizosphere. Although up to date there is no evidence for presence of these bacteria in sponge *S. domuncula*, homology on the amino acid level, could implicate mutual influences like potential horizontal gene transfer (HGT) or benefit from produced compounds. Nevertheless, further studies are necessary in order to investigate this relationship.

In view of these findings, (i) of high level of StDH at ambient oxygen pressure and low transcript levels during constant aeration (oxygen surplus) and (ii) of the potential of

some bacteria to metabolize glycine/strombine, *in situ* localization of the protein was performed. *S. domuncula* StDH polypeptide was prepared in a recombinant way in *E. coli* and used to raise specific antibodies which were applied for immunohistology analyses. These studies showed that a high expression level of the protein is seen in all cells of the *S. domuncula* tissue, which were maintained under ambient oxygen pressure, especially in the choanocyte chambers. However, if tissue from animals exposed to additional aeration was analyzed almost no signals were seen. These results were supported by the semiquantitative RT-PCR analyses implying down-regulation of StDH during constant aeration. In addition, under high expression levels of StDH an accumulation of the enzyme can be seen around bacteria. After maintenance of sponges for several weeks under controlled conditions bacteria are compartmented/encapsulated in special cells, the bacteriocytes (Bohm et al. 2001). Interestingly, the bacteria in those cells (Thakur et al. 2005) are presumably commensals, since they are not attacked by the innate immune response of the host; they can be easily distinguished from the extracellular bacteria by their dense accumulation in the tissue. The load with extracellular bacteria is reduced by maintaining the sponges in the aquarium for at least two-weeks (LePennec et al. 2003). Results from immunohistology analyses showed that around the bacteriocytes strong and specific signals can be seen when applying specific antibodies. Future studies must show if the commensalic/symbiotic bacteria, which exist in *S. domuncula* encapsulated in the bacteriocytes and which are surrounded by StDH, have an aerobic or an anaerobic metabolism and are able to convert in vitro strombine.

Taking into account all the specific characteristics that *S. domuncula* StDH showed the question arose is StDH really a sponge protein. Therefore, StDH gene was analyzed. Obtained results showed that StDH gene comprises two introns, the first one spans 1229 nts ($nt_{270-1498}$) and the second one 390 ($nt_{1617-2006}$) nts. Southern Blot analysis showed that sponge StDH is a single copy gene. Further polymorphism analysis, on the intron2, revealed two allelic variations represented as two size-classes, distinguished by 16 single nucleotide transitions or deletions resulting in alleles of 468-nts and 453-nts. This is the first report, on the gene level, about intraspecies divergences within OpDHs. In turn, it became possible to determine by PCR if the allelic frequency of this gene follows the Mendelian law. Analysis of the specimens revealed that the two alleles occur either in a

homozygotic (same size classes) or in a heterozygotic genotype (different size classes). These findings implicated that if StDH was acquired by HGT from bacteria this event may be ancient and the exogenous gene may have diverged quickly to fit genomic constraints or adaptive requirements of the recipient species.

HGT has the potential to play an important role in the exploitation of new niches, particularly when recipient's organism is naive for functions associated with the newly acquired gene(s). HGT has been inferred in many biological processes including the emergence and spread of virulence-factors, resistance to antibiotics and the long-term maintenance of organelles (Doolittle 1998). The role of HGT in speciation, adaptation and evolution of life on earth has been studied intensively (Brown 2003) and there has been a growing body of evidence of gene transfers among species (Nelson et al. 1999; Koonin 2001; Gogarten et al. 2002) and transfers from organelles to nuclei (Martin et al. 1998; Huang et al. 2003; Bergthorsson et al. 2003). For genes to be horizontally transferred and successfully integrated into the recipient genome, the transferred genes would need to provide a selective advantage either to the recipient (sponge) or to donor (bacteria). Considering the HGT theory Monneuse and Rouze (1987) proposed two hypotheses for OpDHs origin: either they arose from the HGT of an already differentiated OpDH or they are the result of adaptive evolution of a gene coding for a different function (or, independently of two related genes). The two evolutionary mechanisms are not mutually exclusive, and could even have operated sequentially. One year letter Hochachka proposed that marine invertebrate OpDHs were acquired from *Agrobacterium* via horizontal gene transfer. This could be so taking into account that several marine *Agrobacterium* species have been described from the sea bottom sediments (Rüger et al. 1992) and capability of interkingdom genetic transfer of these bacteria to higher plants. Recent studies (Bulgakov et al. 2006) showed that *Agrobacterium* can transfer genetic information to animal such as sea urchin. The fact that extrachromosomal DNA is usually able to rearrange and to evolve much more rapidly than its bacterial chromosomal counterpart or the plant host nuclear genome could go into favor of HGT theory via plasimd. Given the phylogenetic distribution of the pathways in widely divergent living systems such as marine invertebrates and bacteria, HGT must have been a general phenomenon and occurred early in the evolution of the

Animalia (Livingstone 1991). Hopefully, results obtained in this study were a small contribution to the HGT theory. Moreover, bacterial OCD genes in total DNA extracted from the sponge were identified. By application of primers, designed against bacterial OCDs eight different sequences were obtained which could be attributed to *S. domuncula*-associated bacteria. Surely this result does not give an answer if these bacteria are symbiotic/commensalic microorganisms or only accidentally present in the sponge prior to DNA extraction. Nevertheless, the data demonstrate that *S. domuncula* harbors a series of bacteria, like previously published for *H. panicea* (Webster et al. 2001, Hentschel et al. 2002), thus providing the basis for a potential HGT. It should be stressed that the bacterial sequences code for OCDs, and not for OpDHs like in sponges, indicating that after the hypothetical HGT a change of the substrate specificity of the OpDH from a NAD(+)-dependent L-ornithine/L-proline cyclodeaminase (in bacteria) to a NAD(+)-dependent strombine dehydrogenase (sponges) must have occurred.

Taking into account that opine production pathway is the original ancestral pathway of the anaerobic glycolysis as suggested by Livingstone (1991), studies on sponges are interesting and necessary in order to understand better the evolutionary processes of anaerobic glycolisis. Unfortunately, research for example, on sponges was done only on *Halichondria japonica* and *Halichondria panacea* (Barrett and Butterworth 1981; Kanno et al. 2005) with sequence data provided only for the former. An understanding of the molecular relationship between marine invertebrate OpDHs will be possible when more sequence data will be available. Futures studies should address the question if the sponge StDH gene was acquired by HGT or was a result of a parallel evolutionary line emerging from bacteria OCDs.

7 SUMMARY

The study presented here encompasses identification, analysis and characterization of the strombine dehydrogenase (StDH) from the sponge *S. domuncula,* on the gene and protein level. StDH is an opine dehydrogenase which is involved in opine production pathways found mainly in marine invertebrates. These anaerobic pathways are regarded as analogues to the classical anaerobic glycolytic pathway (lactate production pathway), which is predominant in vertebrates. The StDH was previously annotated as a tauropine dehydrogenase (TaDH) on the basis of its 68% identity with the TaDH protein from *Halichondria japonica*. Subsequent enzymatic assays showed that *S. domuncula* opine dehydrogenase is in fact strombine dehydrogenase which possesses specific characteristics not found in other proteins of the same family. It is described here for the first time the StDH gene in Eukaryotes. Two allelic variants have been identified which are present in the different specimens either as a homozygotic or a heterozygotic. Phylogenetic analyses supported with enzymatic assays indicate that *S. domuncula* StDH is only distantly related to the opine dehydrogenases from marine invertebrates. StDH showed that the protein is highly specific to glycine and inhibited by the substrate pyruvate. Furthermore, *S. domunucla* StDH has a dimeric structure (~75 kDa) which is not observed in so far described OpDHs that are monomeric proteins. This enzyme showed similarities to the OCD/mu-cristallyin protein family. Results showed that a sponge StDH is unusual enzyme that belongs to the independent enzyme class. In addition, expression studies revealed that the StDH is down-regulated with aeration. Immunohistology analyses showed high expression of the protein in almost all sponge cells. A strong accumulation of the enzyme was seen around the bacteria indicating that under aerobic conditions the bacteria might metabolize strombine (end product of the reaction). In conclusion, the data documented here shed new light on the anaerobic pathways in marine invertebrates. Potential mutual influences between bacteria and sponge are discussed as well. Hopefully, these results could have a small but important contribution to the better understanding of the evolution in the animal kingdom.

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8 APPENDIX

Sequences used for construction of the phylogenetic tree, in Fig.10. The full name and accession numbers in the EMBL/GenBank, are annotated.

Appendix 1: *The vertebrate OCD/thyroid-hormone binding proteins (THBP); the THBP from Homo sapiens (HOMSA; U85772), Macropus fuliginosus (MACFU; Q28488, kangaroo), Gallus gallus (GALGA; XP_424610, chicken), Danio rerio (DANRE; XP_693188.1, fish), Xenopus laevis (XENLA; AAH78518.1, frog) and the hypothetical OCD-related protein from Tetraodon nigroviridis (TETNI; CAG04436.1, pufferfish). The* bacterial OCDs from Brucella abortus (BRUAB; Q59175, α-proteobacteria), Polaromonas sp. (POLSP; *YP_551383 β-pr*oteobacteria), Pseudomonas putida (PSEPU; Q88H32, γ-proteobacteria), Rhizobium meliloti plasmi*d (RHIME; X64613.1, α-proteobacteria), Ralstonia solanacearum (RALSO; Q8XSP9, β-proteobacteria), Silicibacter pomeroyi (SILPO; Q5LRK6, α-proteobacteria), Agrobacterium tumefaciens (AGRTU; CAB44645.1, α-proteobacteria). The TaDH sequences from marine invertebrates Arabella iricolor (ARAIR; AB081841.1, segmented worm), Haliotis discus hannai (HALDH; AB085184.1, mollusc) as well as from the sponge Halichondria japonica (HALJA; BAD52445.1) are also included. The octopine dehydrogenase (OcDH) sequences from marine invertebrates Loligo opalescens (LOLOP; AJ278691.1, mollusc), Loligo vulgaris (LOLVU; AJ250884.1, mollusc), Mizuhopecten yessoensis (MIZYE; AB085183.1, mollusc), Pecten maximus (PECMA; AJ237916.1, mollusc), Sepia officinalis (SEPOF; AJ250885.1, mollusc); the alanopine dehydrogenase (AlDH) from Methanococcoides burtonii (METHBU; YP_565945, Archaea) and OCD from Methanosaeta thermophila (METTH; YP_843143, Archaea) fall in a separate branch. In addition, eight bacterial sequences, obtained from S. domuncula using degenerative primers, named SUBDO_BAC have been included: SUBDO_BAC1 (AM712890), SUBDO_BAC2 (AM712891), SUBDO_BAC3 (AM712892), SUBDO_BAC5 (AM712894), SUBDO_BAC6 (AM712895), SUBDO_BAC7 (AM712896), SUBDO_BAC8 (AM712897) and SUBDO_BAC9 (AM712898). Entamoeba histolytica OCD (ENTHI; XP_651745, Entamoeba) was used as outgroup.*

9 LIST OF ABBREVIATIONS

µl	Microliter
µg	Microgram
AA/BA	Acrylamid/Bisacrylamid
Acc. No.	Genbank *accession number*
Amp	Ampicillin
AP	Alkaline Phosphatase
APS	Ammonium persulfate
Aqua bidest.	(Aqua bidestillata)
Aqua dest.	Distillated water (Aqua destillata)
BCIP	5-Bromo-4-chloro-3-indolylphosphat
BSA	Bovine Serum Albumin
bp	*base pair(s)*
cDNA	*complementary DNA*
CDP-Star	*Disodium 2-chloro-5-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)-tricyclo[3.3.-1.13,7] decan}-4-yl)-1-phenyl phosphate*
C-terminal	carboxyterminal
Cy3	Indocarbocyanin 3
CMFSW	Calcium Magnesium free sea water
CMFSW+E	Calcium Magnesium free sea water plus EDTA
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
DNA	Desoxyribonucleinacid
ddNTP	Dideoxynucleotide Triphosphate
dNTP	Deoxyribonucleotide triphosphate
dTTP	desoxytyrosintriphosphate
DIG	Digoxigenin
DIG-UTP	digoxigenin-labeled uridine triphosphate
DMPC	Dimethylpyrocarbonat
DMSO	Dimethylsulfoxid
dsDNA	double-stranded DNA
E. coli	*Escherichia coli*
EDTA	Ethylendiamin-N,N,N´,N´-tetraacetic acid
ELISA	*Enzyme Linked Immunosorbent Assay*
EST	*expressed sequence tag*
g	gram
x g	times gravity (units of gravity)
GDP	Guanosindiphosphat
GTP	Guanosintriphosphat
HCl	hydrochloric acid
His	Histidin
dH_2O	Destiled water
h	Hour

IgG	Immunglobulin G
IPTG	Isopropyl-1-thio-β-D-galactopyranosid
kb	Kilobasis
kD	kilo-Dalton
L	Liter
LB	Luria-Bertani medium
M	molar (mol/l)
mA	Milli-Ampere
MgCl2	Magnesiumchlorid
MgSO4	Magnesiumsulfat
min	Minute
ml	Milliliter
mm	Millimeter
mM	millimolar
mRNA	Messenger ribonucleic acid
NaCl	Sodiumchlorid
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide hydrogen
NADPH	nicotinamide adenine dinucleotide phosphate hydrogen
NaOH	Sodiumhydroxid
NBT	4-Nitroblautetrazoliumchlorid
Nt, nt	Nucleotide
NTA	*Nitrilotriaceticacid*
ODx	Optical density
PAGE	Poly-Acrylamid-Gelelectrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	*Plaque forming units*
pH	*potentia hydrogenii*
PVDF	Polyvinylidendifluorid
rpm	rounds per minute
RNA	Ribonucleic acid
RNAse	Ribonuclease
rpm	rounds per minute
RT	Room temperature
RT	Reverse Transkription
SDS	Sodium Dodecyl Sulfate
sec	Second(s)
SM	suspension medium
SSC	*Saline-Sodium Citrate*
Tab.	Table
TBE	Tris-Borat-EDTA-Puffer
TEMED	N,N,N´,N´-Tetramethylendiamin
Tm	Melting temperature
Tris	Tris-(hydroxmethyl)-aminomethan
Tween 20	Poly(oxyethylen)20-sorbitan-monolaurat

U	Unit
UV	Ultraviolett
v/v	volume/volume (Vol.%)
via	by way of
vs	*versus* (against)
X-Gal	5-Bromo-4-Chloro-3-Indolyl-ß-D-Galactopyranosid

Abbreviation-code of amino acids and bases

Amino acids	Abbreviation-code
Alanine	Ala (A)
Cysteine	Cys (C)
Aspartic acid	Asp (D)
Glutamic acid	Glu (E)
Phenylalanine	Phe (F)
Glycine	Gly (G)
Histidine	His (H)
Isoleucine	Ile (I)
Lysine	Lys (K)
Leucine	Leu (L)

Amino acids	Abbreviation-code
Methionine	Met (M)
Asparagine	Asn (N)
Proline	Pro (P)
Glutamine	Gln (Q)
Arginine	Arg (R)
Serine	Ser (S)
Threonine	Thr (T)
Valine	Val (V)
Tryptophane	Trp (W)
Tyrosine	Tyr (Y)

Base	Abbreviation-code
Adenin	A
Cytosin	C
Guanin	G
Thymin	T
Uracil	U