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Purification, cDNA cloning and expression of a cadmium-inducible cysteine-rich metallothionein-like protein from the marine sponge *Suberites domuncula*

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ABSTRACT: During the last few years sponges have been proven to be suitable model organisms for biomonitoring programmes. Previously it was reported that the marine sponge *Suberites domuncula* reacts to cadmium, both under controlled laboratory conditions and in the field with an increased expression of the heat-shock protein 70 and with DNA damage. In this study it is shown that this sponge contains a cadmium-binding protein with a M_r of 24 000. Cloning studies detected a gene in *S. domuncula*, termed *SDMTL*, which encodes a putative polypeptide, MTL_SUBDO, that shares high sequence similarity with metallothioneins (MTs) from Metazoa. The open reading frame displays a polypeptide with a calculated size of M_r of 17 103. Phylogenetic analysis revealed that the sponge molecule, termed MT-like (MTL) protein, is related to the metazoan MTs. Under laboratory conditions, no expression of the *SDMTL* can be detected. After 1 d, incubation of the sponges with cadmium (200 µg l^{-1}) already revealed a dramatic upregulation of the MTL gene in *S. domuncula*. Subsequent studies in the field, the area around Rovinj (Croatia), demonstrated a correlation between the environmental load with cadmium (accumulation of this metal in the animals) and the expression of the gene encoding the 'detoxifying' cadmium-binding protein, MTL, in the test animals. These data suggest that the discovered MTL gene from *S. domuncula* is a sensitive indicator for environmental, bioavailable cadmium.

KEY WORDS: Cadmium · Sponges · Porifera · *Suberites domuncula* · Croatia · Metallothionein · Biomonitoring · Biomarker

INTRODUCTION

Sponges (phylum Porifera) as suspension feeders are characterized by an amazingly high water-filtration rate. Some marine species filter 24 000 l kg^{-1} body weight d^{-1} (Vogel 1977) or pump a volume of water equal to their own body volume every 5 s (Reiswig 1974). Hence they must be provided with a powerful protection system against adverse compounds which are taken up from the environment. Studies revealed that sponges can cope with organic pollutants and organismic attacking invaders by (1) cellular defense systems, e.g. heat-shock proteins (Koziol et al. 1997), targeting chaperones like the 14-3-3 protein(s) (Wiens et al. 1998), multidrug resistance (MDR) (reviewed in Kurelec 1997) or thioredoxin (Wiens et al. 1999) and by (2) organismic protection, including a well-developed immune system

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(Müller et al. 1999) and bioactive secondary metabolites (Sarma et al. 1993, Proksch 1994). Recently, we also identified the second MDR-system, related to the POH1 [*p*ad *o*ne *h*omologoue] mechanisms (Spataro et al. 1997), which is thought to 'eliminate' pollutants via an enzymatic route (Krasko et al. 2000).

Besides organic compounds, sponges are also exposed to inorganic compounds, e.g. cadmium, that are not only hazardous for the sponges in particular but also for the ecosystem dynamics and for human health in general (Rand 1995, Clark 1997). Even though sponges, here with reference to the marine sponge *Suberites domuncula*, react apoptotically to high concentrations of cadmium (Wagner et al. 1998), they are able to accumulate this metal in the body to high levels. One field study in the area around Rovinj (Croatia) revealed (Müller et al. 1998) that *S. domuncula* specimens, which are exposed to 0.4 µg cadmium l^{-1} in the surrounding sea water (Mikulic 1994), are able to accumulate this metal ~20 000-fold in their bodies; similarly high levels were determined for a second sponge species *Halichondria panicea* (Olesen & Weeks 1994, Hansen et al. 1995). In an earlier study we found that an increase of cadmium in *S. domuncula* causes a simultaneous decrease of zinc (Müller et al. 1998). Based on this finding we speculated that the differential capacity of the sponge to bind metals might depend on the presence of heavy-metal binding protein(s), e.g. metallothionein(s) (MT).

MTs represent a diverse family of metal-detoxifying proteins that are found in animals, plants, fungi, and prokaryotes (Hamer 1986). They are grouped according to their primary structure (Hamer 1986, Kagi & Schaffer 1988, Unger et al. 1991) into several classes. Class I MTs have the equine renal MT as a prototype (Kagi 1993). They include all vertebrate, some invertebrate (Lerch et al. 1982) and fungal MTs (Munger et al. 1987) and are characterized by the conservation of the following patterns: Cys-Xxx-Cys, Cys-Xxx-Xxx-Cys and Cys-Cys. Class II MTs have the sea urchin MT (Nemer et al. 1985) as an example, while Class III MTs comprise the non-translationally synthesized MTs which are found in plants and some fungi (reviewed in Unger et al. 1991). MTs in the strict sense have a molecular mass of 6000 to 7000 Da, are rich in the aa cysteine (23 to 33 mol%), have no aromatic aa or histidine, bind 4 to 12 atoms of metal mol^{-1}, and are heat stable (reviewed in Rand 1995).

Here we report on the first MT from a sponge, *Suberites domuncula*, which was at first enriched, then cloned, and finally used for expression studies *in vitro* and in the field. This sponge MT-like (MTL) polypeptide follows the characteristics known from other MTs, but differs in the sense that it is composed of a tandem repeat.

MATERIALS AND METHODS

Materials. Phenylmethylsulfonyl fluoride (PMSF) was obtained from Sigma (St. Louis, MO, USA), DEAE-cellulose (DE-52) from Whatman Chemical Separation Ltd. (Maidstone, UK) and Sephacryl S-100 from Pharmacia/LKB (Uppsala, Sweden). Restriction endonucleases and other enzymes for recombinant DNA techniques and vectors were obtained from Stratagene (La Jolla, CA, USA), QIAGEN (Hilden, Germany), Boehringer Mannheim (Mannheim, Germany), Gibco-BRL (Grand Island, NY, USA), Amersham (Amersham, UK), USB (Cleveland, OH, USA), DUPONT (Bad Homburg, Germany), Epicentre Technologies (Madison, WI, USA) and Promega (Madison, WI, USA). *Taq* DNA polymerase, DIG [digoxigenin] DNA labeling kit, DIG-11-dUTP, Fab fragment of anti-DIG alkaline phosphatase, and CDP [disodium 2-chloro-5-(4-methoxy-spiro{1,2-dioxetane-3,2'-(5'-chloro)-tricyclo[3.3.1.$1^{3,7}$] decan}-4-yl)phenyl phosphate] were from Boehringer Mannheim.

Sponges. Specimens of *Suberites domuncula* Olivi (Demospongiae: Tetractinomorpha: Hadromerida: Suberitidae: Suberites) were collected near Rovinj (Croatia; Mediterranean Sea: Adriatic Sea) and were either immediately frozen in liquid nitrogen until analysis or further exposed to cadmium.

Determination of cadmium concentration was performed as published earlier (Müller et al. 1998).

Exposure of *Suberites domuncula* to cadmium. Prior to the experiments the sponges remained under controlled conditions in the aquarium for 5 mo. Then, (1) in the studies for the isolation of the MTL protein from *Suberites domuncula*, the sponges were exposed to 100 µg l^{-1} of cadmium (corresponding to 163 µg l^{-1} of $CdCl_2$) for 5 d. Or, (2) for the studies to determine the response to cadmium on the level of gene expression, the sponges were exposed to 200 µg l^{-1} of cadmium in seawater for 0 to 6 d in 20 l aquaria at 17°C under continuous aeration as described before (Müller et al. 1998). After exposure, the sponges were immediately frozen in liquid nitrogen and stored at −80°C.

Enrichment of *Suberites domuncula* MTL. All procedures were performed at 4°C; a modification of the procedure described by Berger et al. (1995) was used. Sponge tissue (15 g) was homogenized with 30 ml of a 25 mM of Tris/HCl buffer (pH 7.5; 150 mM NaCl, 20 mM 2-mercaptoethanol, 50 µM PMSF) and centrifuged (90 min; 80 000 × *g*). The supernatant obtained was supplemented with 5 g of DEAE-cellulose and stirred at low speed for 30 min. The DEAE-cellulose was removed by centrifugation (10 min; 1200 × *g*); a 20 ml aliquot of the supernatant was applied onto a Sephacryl S-100 column (size 2 cm × 25 cm) and elution with was performed with the above-mentioned

Tris/HCl buffer at a flow rate of 5 ml min^{-1}. Fractions of 5 ml were collected and the absorbance measured at 280 nm to estimate the overall protein pattern.

Cadmium analysis. Cadmium-containing fractions were analyzed by atomic absorption spectrometry using a Perkin-Elmer 2380 machine (GF-AAS).

Field studies. For studies to determine the level of gene expression, *Suberites domuncula* specimens were taken from 5 different sites (Fig. 1A) which had been previously used in an earlier study (Müller et al. 1998). They are in the close vicinity of fixed sampling sites used in earlier environmental studies (Ugarkovic et al. 1990, Bihari & Batel 1994) characterized by a distinct gradient of pollution. Site S-1 is a small bay at Rovinj (influence of a fish cannery); Site S-2 is at the rim of the harbor area of Rovinj (direct influence of the urban runoff and a tobacco factory); Site S-3 is near the island of S. Giovanni in Pelago (uncontaminated); Site S-4 is the entrance to the Limski Kanal, placed close to the tourist camps Blesicka in the North and Valalta in the South (tourist waste) and Site S-5 is 2 km from the end of the Limski Kanal (uncontaminated and only occasionally under the influence of a fish farm).

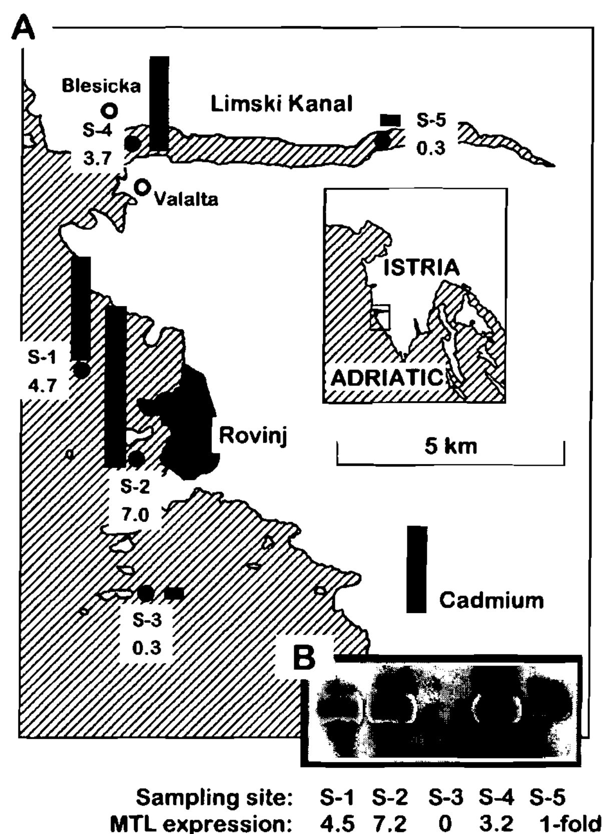

Fig. 1. Selected sites for the field study. (A) Sampling sites for *Suberites domuncula* in the Rovinj area (Northern Adriatic, Mediterranean Sea). The sites are termed Site S-1, Rovinj (fish cannery); Site S-2, Rovinj (harbor area); Site S-3, S. Giovanni in Pelago; Site S-4, entrance to the Limski Kanal; and Site S-5, end of the Limski Kanal. (B) Sponges were collected and RNA immediately extracted. Four µg of total RNA from sponges from each of the 5 indicated sites were analyzed. After RNA extraction, the samples were size separated and after blot transfer hybridized with the *SDMTL* probe. The Northern blot with the references to the sites indicated under (A) is shown in (B); further details are given in 'Materials and methods'. In (B) the relative level of expression of the gene for *SDMTL* is indicated. The bars [and the numbers below] in (A) mark the concentration of cadmium in the sponges (given in mg kg^{-1} wet weight)

Polymerase chain reaction (PCR) cloning of *Suberites domuncula* MT cDNA *SDMTL*. The complete sponge cDNAs, encoding the MTL protein MTL_SUBDO, termed *SDMTL*, was isolated from the *Suberites domuncula* cDNA library (Kruse et al. 1997) by PCR (Ausubel et al. 1995). The degenerate forward primer, directed against the conserved aa segments found in the sequences from the MT of the crab *Carcinus maenas* (MT_CARMA, P55948 [Pedersen et al. 1994]; aa_{30} to aa_{41}) 5'-TTGT/CAAA/GTGT/CGGA/G/T/CGAA/GGAT/CTGT/CAA-3' was used in conjunction with the 3'-end vector-specific primer. The PCR reaction was carried out at an initial denaturation at 95°C for 3 min, then 30 amplification cycles at 95°C for 30 s, 52°C for 45 s, 74°C for 1.5 min, and a final extension step at 74°C for 10 min. The reaction mixture was as described earlier (Wiens et al. 1998). The longest fragment of ~500 bp was used and completed by primer walking as described by Ausubel et al. (1995). The clone obtained had a size of 713 nucleotides [nt] (excluding the poly(A) tail). The clones were sequenced using an automatic DNA sequenator (Li-Cor 4200).

Sequence comparisons. The sequences were analyzed using the computer programs BLAST (Pearson & Lipman 1988) and FASTA (Lipman & Pearson 1985). Multiple alignments were performed with CLUSTAL W Ver. 1.6 (Thompson et al. 1994). Phylogenetic trees were constructed on the basis of aa sequence alignments by neighbour-joining, as implemented in the 'Neighbor' program from the PHYLIP package (Felsenstein 1993). The distance matrices were calculated using the Dayhoff PAM matrix model as described by Dayhoff et al. (1978). The degree of support for internal branches was further assessed by bootstrapping (Felsenstein 1993). The graphic presentations were prepared with GeneDoc (Nicholas & Nicholas 1997).

Northern blotting. RNA was extracted from liquid-nitrogen pulverized sponge tissue with TRIzol Reagent (GibcoBRL) as recommended by the manufacturer. An amount of 4 µg of total RNA was electrophoresed through a formaldehyde/agarose gel and blotted onto a Hybond N^+ membrane following the manufacturer's instructions (Amersham). Hybridization experiments were performed with the following probes: the ~500 bp fragment of *SDMTL* from *Suberites domuncula* and

the *S. domuncula* β-tubulin (authors' unpubl. data) *SDBTUB* (~800 bp). Hybridization was performed with the antisense DIG-labeled probes at 42°C, following the instructions of the manufacturer (Boehringer) and as recently described by Krasko et al. (1999). After washing, DIG-labeled nucleic acid was detected with anti-DIG Fab fragments and visualized by the chemiluminescence technique using CDP-Star. The signals of the Northern blots were quantitated by the chemiluminescence procedure (Stanley & Kricka 1990). The screen was scanned with a GS-525 Molecular Imager (Bio-Rad). The relative values for the expressions of the *SDMTL* and *SDBTUB* genes in *S. domuncula* tissue were correlated with the intensities of the bands measured for the expression of the tubulin gene.

RESULTS

Procedure for the enrichment of MTL

Sponge extracts were prepared and adsorbed to DEAE-cellulose as described in 'Materials and methods'. Subsequently, the resulting supernantant was subjected to gel-permeation chromatography and the cadmium content in each fraction was analyzed by atomic absorption. The cadmium-binding fractions eluted from the Sephacryl column at a position equivalent to about 24 kDa (Fig. 2). The protein fraction with the highest cadmium content (fraction 29) consisted of 0.45 µg cadmium 8 mg^{-1} protein; determined according to Lowry et al. (1951). Taking into account the estimated M_r of 24 000 a total of about 1.4 g of atom cadmium is bound per mol of cadmium-binding protein in fraction 29. For the molluscan MTs a binding capacity of 7 g mol^{-1} was measured (Roesijadi et al. 1989), suggesting that the sponge preparation was—as expected—not yet pure. Cadmium could not be measured in any other fractions than those between 27 and 34.

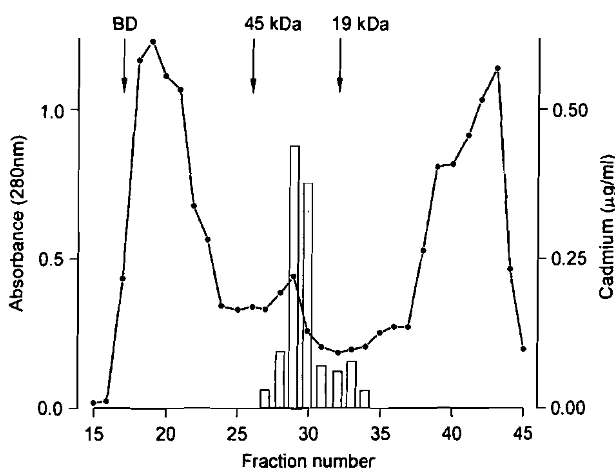

Fig. 2. Enrichment of MTL protein in *Suberites domuncula* tissue. Sephacryl S-100 column chromatography of *S. domuncula* extract, which had been pretreated by DEAE-cellulose adsorption. An aliquot was applied to the gel-permeation chromatography column as described in 'Materials and methods'. The fractions were analyzed at an absorbance at 280 nm (•——•) and for cadmium concentration (open bars). Calibration of the column was performed with Blue Dextran (M_r 2000 kDa [V_o]; BD), chicken egg albumin (45 kDa) and myoglobin (18.5 kDa) in parallel runs

Cloning of the MTL cDNA SDMTL from *Suberites domuncula*

The cDNA encoding the MTL from *Suberites domuncula* was isolated and characterized. The 713 bp long sequence has an open reading frame of 516 nt. Northern blot analysis revealed a single band of 0.9 kb, indicating that the clone is of full length (Fig. 3).

The predicted translation product of 172 aa, named MTL_SUBDO (Fig. 4), has a calculated size of M_r 17 103 and a pI of 7.69 (PC/GENE Data Banks 1995). The instability index is 44.26, suggesting that the sponge polypeptide is unstable (PC/GENE Data Banks 1995). Thirty Cys-rich patterns, characteristic for MTs of Class I are present in the sponge sequence (Fig. 4).

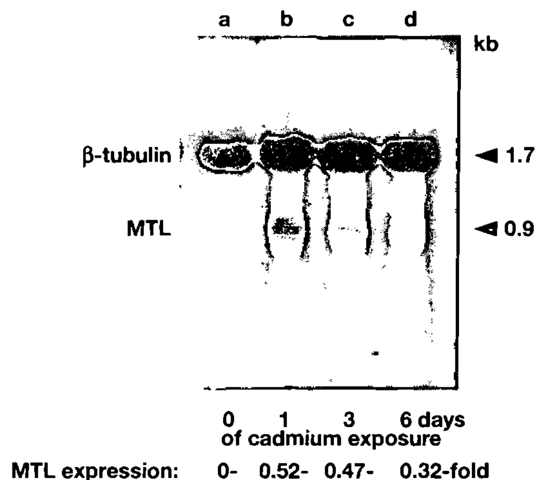

Fig. 3. Effect of cadmium on the expression of the sponge *SDMTL*. Tissue samples were incubated for 0 d (lane a), 1 d (lane b), 3 d (lane c) or 6 d (lane d) in the presence of 200 µg ml^{-1} of cadmium. Northern blot analyses to estimate the level of expression of the genes were then performed using the probes for the MTL protein, *SDMTL*. RNA was extracted and 4 µg of total RNA was size separated; after blot transfer hybridization was performed with the *SDMTL* (signal at 0.9 kb) and the *SDBTUB* probe (1.7 kb). The intensities of the transcripts for *SDMTL* are correlated with the expression of β-tubulin (probe added together with *SDMTL* during the hybridization procedure)

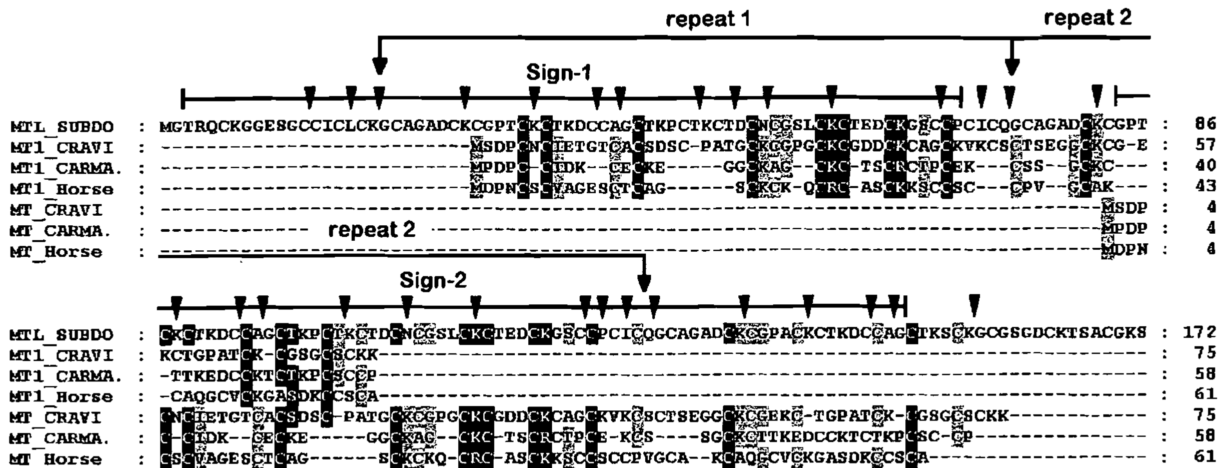

Fig. 4. Deduced MTL protein from *Suberites domuncula*. Alignment of the *S. domuncula* MTL protein (MTL_SUBDO) with the corresponding MT sequences from the oyster *Crassostrea virginica* (MT_CRAVI, accession number P23038 [Unger et al. 1991]), the crab *Carcinus maenas* (MT_CARMA, P55948 [Pedersen et al. 1994]) and the horse MT 1B (MT_HORSE, SMHOB [Kojima et al. 1976]). These 3 sequences, from the oyster, the crab and the horse, were aligned both with the NH_2-terminal as well as the COOH-terminal portion of the sponge MTL. Similar (with respect to physico-chemical properties) amino acids in all sequences are given in white on black and those in at least 3 sequences in black on gray. The characteristic Cys patterns: Cys-Xxx-Cys, Cys-Xxx-Xxx-Cys and Cys-Cys are indicated (arrow head). The location of the 2 vertebrate MT signatures (Sign-1 and Sign-2 [⊢—⊣]) and the 2 repeats (bordered by arrows) are delimited

Likewise characteristic for MTs is the aa compositon of the sponge polypeptide; 32 mol% of Cys and the absence of aromatic aa and of His (PC/GENE Data Banks 1995; Prosite). Two vertebrate MT signatures are present (aa_2 to aa_{71} and aa_{84} to aa_{151}). The fact that the sponge MT polypeptide comprises 2 identical repeats, ranging from aa_{20} to aa_{74} and from aa_{74} to aa_{129} (Fig. 4), is interesting. Since such an arrangement has not been described in other MTs, we termed the sponge sequence, a MTL protein; Fig. 4.

Phylogenetic position of *Suberites domuncula* MTL

The sponge MTL polypeptide is composed of 2 identical repeats. To demonstrate this fact the 3 sequences used for the alignment with the sponge MTL, the MT from the oyster *Crassostrea virginica* (Unger et al. 1991), the crab *Carcinus maenas* (Pedersen et al. 1994) and the horse (Kojima et al. 1976) were aligned both with the NH_2-terminal as well as the COOH-terminal portion of the sponge MTL (Fig. 4).

A databank search with the *Suberites domuncula* polypeptide MTL_SUBDO revealed similarity to metazoan MTs. The phylogenetic tree, constructed after alignment of the 4 sequences denoted in Fig. 4 together with the protostomian sequence from *Caenorhabditis elegans*, the mussels *Mytilus edulis* and *Dreissena polymorpha*, and the oyster *Crassostrea virginica*, as well as the deuterostomian MTs (from sea urchin *Sterechinus neumayeri*, carp *Cyprinus carpio*, frog *Xenopus laevis*, pigeon, human, mouse and bovine), revealed that the sponge MTL polypeptide forms the basis of all metazoan MTs (Fig. 5). It is notable that the sea urchin sequence clusters together with the protostomian sequences and not with the deuterostomian MTs. Evolutionarily more distantly related is the fungal MT (related) from *Candida glabrata*. The described cysteine-rich protein from the earthworm *Enchytraeus buchholzi* forms a separate branch and groups with the chosen outgroup, the plant MTL sequence from *Arabidopsis thaliana*.

Expression of *SDMTL* in *Suberites domuncula* tissue after cadmium exposure

Sponges used for these studies were kept in an aquarium for 5 mo in order to eliminate potential uncontrolled effects of heavy metal. As a concentration for the cadmium exposure studies 200 µg l^{-1} was selected. In a previous study this concentration was found to be suitable for the detection of a response of the sponge *Halichondria panicea* in *in vitro* experiments (Hansen et al. 1995). Tissue samples from *Suberites domuncula* were taken after 0 to 6 d; then the level of expression of *SDMTL* was determined by Northern Blot experiments. The results revealed that in the absence of cadmium, no expression of the gene encoding the MTL can be measured (Fig. 3). However, already after an incubation of 1 d in the presence of cadmium the expression of *SDMTL* increased strongly

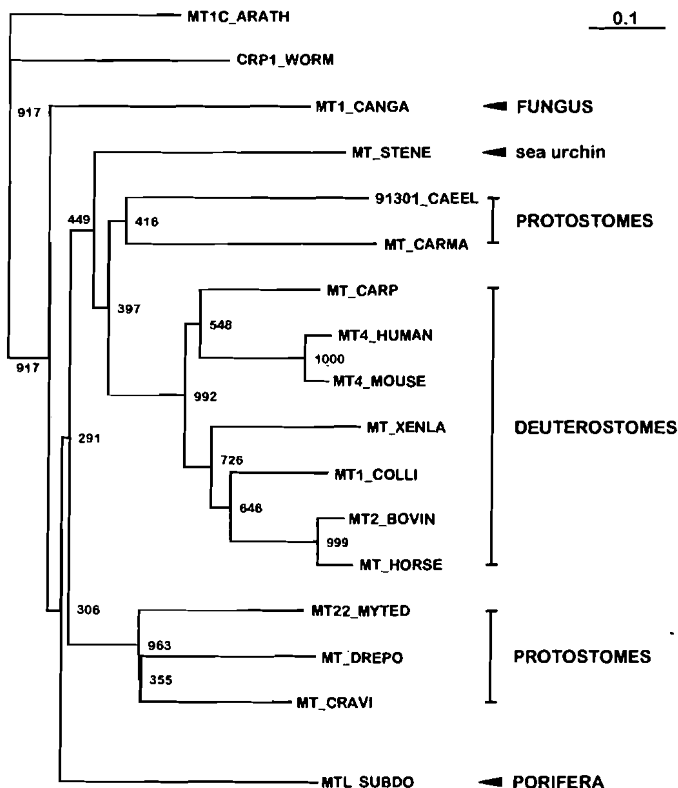

Fig. 5. Phylogenetic analysis of the sponge MTL protein. Rooted phylogenetic tree, constructed after alignment of the 4 sequences denoted under Fig. 4 together with the protostomian sequence from *Caenorhabditis elegans* (91301_CAEEL, CAA91301 [Wilson et al. 1994]), crab, the MT 20-II from the mussels *Mytilus edulis* (MT22_MYTED, P80252 [Mackay et al. 1993]), and *Dreissena polymorpha* (MT_DREPO, Q94550, [Scekan et al. 1996]) and the oyster, the deuterostomian MTs from the carp *Cyprinus carpio* (MT_CARP, AAB61577 [Chan & Chan 1997]), the frog *Xenopus laevis* (MT_XENLA, Q05890 [Saint-Jacques & Seguin 1993]), the pigeon type-I (MT1_COLLI, P15786 [Lin et al. 1990]), human type-IV (MT4_HUMAN, P47944 [Quaife et al. 1994]), mouse type-IV (MT4_MOUSE, P47945 [Quaife et al. 1994]), bovine type-II (MT2_BOVIN, P09579 [Munger et al. 1985]) and from horse as well as from the sea urchin *Sterechinus neumayeri* (MT_STENE, P55953 [Scudiero et al. 1997]). In addition, the MT (related) sequences from the fungus *Candida glabrata* (MT1_CANGA, P15113 [Mehra et al. 1989]) and the cysteine-rich protein CRP1 from the earthworm *Enchytraeus buchholzi* (CRP1_WORM, A55035 [Willuhn et al. 1994]) have been included. The plant MTL sequence from *Arabidopsis thaliana* (MT1C_ARATH, Q38804 [Zhou & Goldsbrough 1995]) was used as outgroup. The analysis was performed by neighbour-joining as described in 'Materials and methods'. The numbers at the nodes are an indication of the level of confidence—given in percentage—for the branches as determined by bootstrap analysis (1000 bootstrap replicates). The scale bar indicates an evolutionary distance of 0.1 aa substitutions per position in the sequence

and thereafter reached the maximum; at Days 3 and 6 the expression slowly reduced. On the same blot the β-tubulin probe was added; no significant differences were seen on comparing the different RNA samples, confirming that the same amount of RNA was applied (Fig. 3).

Correlation of *SDMTL* expression with the cadmium content in the sea

After having established that the expression of the *SDMTL* gene depends on the presence of cadmium under controlled laboratory conditions it was tempting to elucidate if the level of expression varies in sponges taken from defined sites from the sea as well. Those sites that had also recently been selected for the determination of the cadmium concentration in the same sponge species (Müller et al. 1998) were used. The analytical data by Müller et al. (1998) revealed that (S. Giovanni in Pelago) the level of cadmium in the sponges is lowest at Site S-3, with 0.29 mg kg^{-1}. Increasing levels of cadmium have been determined: Site S-5, end of the Limski Kanal (0.32 mg kg^{-1}) <Site S-4, entrance to the Limski Kanal (3.7 mg kg^{-1}) <Site S-1, Rovinj close to the fish cannery (4.7 mg kg^{-1}) <Site S-2, Rovinj at the harbor area (7.0 mg kg^{-1}); Fig. 1A.

Parallel with the determination of cadmium concentrations in the sponges collected from the selected sites, the level of *SDMTL* expression was determined. The experiments revealed that at Site S-3 no transcripts from the *SDMTL* have been detected. In areas of higher cadmium load transcripts of *SDMTL* are detected; the intensity of the band, which reflects the steady-state level of the *SDMTL*, increases from Site S-5 (1-fold expression [arbitrary unit]) in the following order: <Site S-4 (3.2-fold) <Site S-1 (4.5-fold) and <Site S-2 (7.2-fold); Fig. 1B. These results demonstrate a strong correlation between the environmental load with cadmium (accumulation of this metal in the sponges) and the expression of the gene encoding the 'detoxifying' cadmium-binding protein, MTL, in the test sponge.

DISCUSSION

The concentration of cadmium in marine sponges collected from the natural environment varies between 0.1 to 10 µg g^{-1} body weight (Olesen & Weeks 1994, Hansen et al. 1995, Müller et al. 1998); these are levels which are also found in other organisms from the marine fauna (Kennish 1994). If sponges, e.g. the species *Halichondripa panicea* or *Suberites domuncula*, are exposed to high levels of cadmium (1 to 5 µg ml^{-1}) (Hansen et al. 1995, Müller et al. 1998) they accumulate this metal by a factor of up to 20 000-fold. Since the rate and extent of cadmium accumulation in sponges is