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Potential biological role of laccase from the sponge *Suberites domuncula* as an antibacterial defense component☆

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ABSTRACT

Background: Laccases are copper-containing enzymes that catalyze the oxidation of a wide variety of phenolic substrates.
Methods: We describe the first poriferan laccase from the marine demosponge *Suberites domuncula*.
Results: This enzyme comprises three characteristic multicopper oxidase homologous domains. Immunohistological studies revealed that the highest expression of the laccase is in the surface zone of the animals. The expression level of the laccase gene is strongly upregulated after exposure of the animals to the bacterial endotoxin lipopolysaccharide. To allow the binding of the recombinant enzyme to ferromagnetic nanoparticles, a recombinant laccase was prepared which contained in addition to the His-tag, a Glu-tag at the *N*-terminus of the enzyme. The recombinant laccase was enzymatically active. The apparent Michaelis constant of the enzyme is 114 μM, using syringaldazine as substrate. Exposure of *E. coli* to the nanoparticles, coated with Glu-tagged laccase, and to the mediator 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) in the presence of lignin, as the oxidizable substrate, resulted in an almost complete inhibition of colony formation. Quantitative studies of the effect of the laccase-coated iron oxide nanoparticles were performed using *E. coli* grown in suspension in reaction tubes within a magnetic nanoparticle separator.
Conclusions: This newly designed magnetic nanoparticle separator allowed a removal of the nanoparticles after terminating the reaction. Using this system, a strong dose-dependent inhibition of the growth of *E. coli* by the laccase iron oxide nanoparticles was determined.
General significance: From our data we conclude that the sponge laccase is involved in the anti-bacterial defense of the sponge organism.

1. Introduction

Sponges [phylum: Porifera] are the first animal taxa which branched off from the common metazoan ancestor about 800 MYA [1]. As active filter feeders which are devoid of any blood vessels, they rely on an efficient aqueous canal system through which they suck in organic particles and microorganisms that serve as nutrients. The water filtration system of sponges is amazingly efficient; the water pumping rates range from 0.002 to 0.84 $cm^3\ s^{-1}$ per cm^3 of sponge tissue [2]. During the passage of the environmental water which contains high amounts of bacteria ($>10^6\ mL^{-1}$ [3]) and viruses (likewise $\approx 10 \cdot 10^6\ mL^{-1}$ [4]), the sponges eliminate all particulate or cellular particles.

In order to control the adverse influences of microorganisms sponges have developed an efficient immune system which is able to discriminate between self–self and self–non-self. Allograft and xenograft studies revealed that these basal animals can distinguish between different species, e.g. between *Microciona prolifera* and *Haliclona occulata* [5] and between individuals, e.g. shown for *Callyspongia diffusa* [6–8]. The immune system of the Porifera and Cnidaria is restricted to innate defense mechanisms. These systems rely on receptors and their downstream molecules [9]. It was Metchnikoff [10] who succeeded first in demonstrating that sponges eliminate bacteria by phagocytosis through their macrophage-related archeocytes. The molecular biological basis for the sponge-microorganism defense systems has been worked out by using the demosponges *Suberites domuncula* and *Geodia cydonium* as model organisms (reviewed in: [1]). *S. domuncula* has been shown to recognize Gram-positive bacteria through binding to the

☆ Laccase cDNA: The cDNA of encoding the *Suberites domuncula* laccase (termed *SDLACC-1*) is deposited under the accession number LM994828 at EMBL/GenBank.

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bacterial proteoglycan surface [11]; the sponge cells respond with an increased synthesis of lysozyme and with an enhanced endocytotic activity. Similarly efficient is the repertoire of sponges that recognize and eliminate Gram-negative bacteria. Our group demonstrated that in *S. domuncula*, the mitogen-activated protein kinase pathway is activated after binding of lipopolysaccharide (LPS) to cells [12,13]; the p38 kinase and the c-Jun *N*-terminal kinase are rapidly phosphorylated. Furthermore, *S. domuncula* responds to lipopolysaccharide [LPS] with an up-regulation of the defense molecule tachylectin, a D-GlcNAc-binding lectin [14]. It had been proven that sponges can recognize and react to the interaction with fungi via a cell surface receptor that recognizes (1 → 3)-β-D-glucans [15]; in turn, a signal transduction pathway is activated that causes an increased expression of genes encoding a fibrinogen-like protein and an epidermal growth factor. Finally, like in vertebrates [16], also Toll-like receptor(s) [TLRs] are involved in sponges to detect and eliminate microbes [17]. Finally, after activation of TLRs by LPS, the mitogen-activated protein kinase and the NF-κB pathways are activated [18], and interferon production increases. In *S. domuncula*, three end point molecules involved in these processes have been identified: the mitogen-activated protein kinase [19], NF-κB, and the (2–5)A polymerase [20]. Like in vertebrates the LPS–TLR interaction involves the pattern recognition receptor which interacts with MyD88 [21].

Since the discovery of the first clinically relevant secondary metabolite isolated from the sponge *Cryptotethya crypta* (phylum Porifera) [22], 1-β-D-arabinofuranosylthymine [ara-T], a cornucopia of unique chemical compounds has been identified in sponges. They have been proven to act potently and specifically on target receptors/enzymes of attacking organisms [23]. In contrast to the secondary metabolites, the proteinaceous bioactive substances have been given less attention despite their presumed higher biological and biotechnological importance [24]. This changed gradually with the first cloning of such a bioactive polypeptide, the hemagglutinin from *G. cydonium* [25] and, more recently, the ASABF [*Ascaris suum* antibacterial factor]-type antimicrobial peptide from *S. domuncula* [26]. Since their genetic blueprints can be identified in a straightforward way, the proteinaceous compounds have the advantage over secondary metabolites in that they can be modified by molecular biological techniques [27].

In vertebrates, the production of free radicals, e.g. during the downstream reaction pathways of the (1 → 3)-β-D-glucan receptor, is an efficient system, especially in macrophages and during inflammation, to eliminate microorganisms [28]. This defense/response system via free radicals has not been studied thoroughly in sponges, in spite of the fact that free radical detoxification enzymes are abundant in these organisms and have been identified on the molecular level [29]. By screening the *S. domuncula* EST (expressed sequence tag) database (http://spongebase.genoserv.de/) it is striking that ESTs encoding the enzyme laccase are frequently found. This enzyme, together with its "mediator", catalyzes the oxidation of non-phenolic and phenolic substrates via free radical mechanisms [30]. The mediators are simultaneously substrates for the enzyme, like the compound to be oxidized [31]. This property that the laccase can act in different combinations with substrates and mediators makes this enzyme attractive for clean bioprocessing and remediation procedures [32]. At present, it is especially the lignolytic activity that is under intensive biotechnological attention, since lignin is nature's dominant aromatic polymer, found in large amounts in most terrestrial and marine plants and might represent a natural source for the generation of value-added products [33]. Besides its (potential) role in lignin degradation, this enzyme plays a crucial role in fungal developmental and morphogenetic processes, as well as in detoxification [34–36]. In the marine ecosystem, lignin is an important cause of eutrophication and ecotoxicity [37,38].

In the present study we show that the lowest metazoan taxon, the Porifera, owns the laccase which it uses to metabolize lignin, a process during which free radicals can be formed [30,39]. While this enzyme is apparently absent in mammals, several isoforms had been described from molluscs [40]. In turn, the sponges have the potential to utilize the laccase for the detoxification and elimination of lignin-derived products, but also very likely in combination with a mediator(s) as a system to kill bacteria.

2. Materials and methods

2.1. Sponge and exposure to LPS

Live specimens of *S. domuncula* (Porifera, Demospongiae, Hadromerida) were collected by SCUBA near Rovinj (Croatia) from depths between 20 and 35 m. After the transfer of the animals to Mainz (Germany) they were kept in aquaria (10^3 L) at 17 °C under continuous aeration for more than 6 months prior to use in the experiments. In the natural environment *S. domuncula* is abundantly found, in the region of Rovinj, at a depth of ~20 m in the disphotic zone.

For the experiments the animals were separately kept in small 500 mL incubation beakers (under aeration) and exposed to lipopolysaccharide [LPS] from *E. coli* 055:B5 (#L2880 [41]; Sigma, Taufkirchen; Germany) at a concentration of 3 μg/ml. Then tissue samples were taken and processed for immunohistological analyses, as well as for the quantification of gene expression by reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR).

2.2. Laccase gene from S. domuncula

The complete laccase cDNA, termed *SDLACC-l*, was obtained by using expressed sequence tags [EST] from the EST database (http://spongebase.genoserv.de/) as the start. The sequence was completed by application of the 3′- and 5′-racing technique [12,15]. The total nucleotide sequence of 2612 nt, encoding the complete open reading frame [ORF], was obtained and spanned 2355 bp. The ORF $nt_{130–132}$ to $nt_{2482–2484[stop]}$ encodes a 785 aa-long polypeptide with a calculated size of 87,230 Da and a theoretical isoelectric point [pI] of 4.80. The cDNA/ORF was found to be complete, as proven by Northern blot analysis (2.8 kb; results not shown). The deduced sponge protein was termed LACC-l_SUBDO. The similarity/identity of the *S. domuncula* laccase to the corresponding sponge laccase-17-like sequence from *Amphimedon queenslandica* (accession number gi 340378577) is 42%/26%.

2.3. Sequence analysis and phylogenetic relationship of the sponge laccase

Homology searches were performed via the European Bioinformatics Institute, Hinxton, United Kingdom and the National Center for Biotechnology Information (NCBI), Bethesda, MD [42]. The sequence alignment was performed with ClustalW version 1.6 [43]. After aa sequence alignments, applying the Neighbor-Joining method to distance matrices that were calculated using the Dayhoff PAM matrix model [44,45], the phylogenetic tree was constructed. The degree of support for internal branches was assessed by bootstrapping [46]. Finally, the graphical output of the bootstrap figures was processed through the "Treeview" software [47] and GeneDoc [48]. Potential domains, subunits, patterns, and transmembrane regions were predicted searching the Pfam [49] or the SMART database [50].

2.4. Heterologous expression of sponge laccase with Glu-tag in E. coli

The Gateway System from Invitrogen (Karlsruhe; Germany) was used to facilitate subcloning of the laccase cDNA *SDLACC-l*. With respect to the cDNA, the intracellular part of the laccase encoding cDNA, without signal peptide was amplified by two step polymerase chain reaction [PCR]. The primers for the first amplification round were designed in Vector NTI program and forward primer contained the 8× Glu-tag encoding sequence. Forward primer (laccase_Glu_frw) for the Glu-tag encoded sequence 5′-GAAGAAGAGGAAGAGGAAGAAGAGTTTCAGCC GATTGTTGCAG-3′ (the 8× Glu-tag is underlined) and reverse primer

(laccase_Glu_rv) 5′-CTCTGCAGATGTTTCGTTCACCA-3′. The resulting PCR product was purified from the gel with "NucleoSpin Extract II kit" (Macherey-Nagel, Düren; Germany), according to the instructions of the company. The purified DNA was used as a template for the second PCR round, with primers carrying the *att*B recombination site. The forward primer: (*att*B1 SP/laccase Glu-tag) 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAAGAAGAGGAAGAGGAA-3′ (*att*B1 extension) was used. The sense primer for PCR amplification of the region (nt_1 to nt_{185}) of "laccase for expression 3 domains Glu-tag" for Gateway BP reaction and the reverse primer (*att*B2 ASP/laccase Glu-tag) 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTACTCTGCAGATGTTTCGTT-3′ (*att*B2 extension had been selected). The antisense primer for PCR amplification of the region (nt_1 to nt_{1815} 1–1815) of "laccase for expression 3 domains Glu-tag" for Gateway BP reaction was used. The *att*B PCR Product (1876 bp, including 61 bp of the *att*b recombination sites) was purified from the gel using the "NucleoSpin Extract II kit" (Macherey-Nagel), according to the manufacturer's instructions. The BP recombination reaction using the obtained *att*B PCR product and the empty *att*P pDONR 221 entry vector (Invitrogen) allowed the generation of *att*L, containing the entry clone *SDLACC-l* in pDONR 221. After transformation in TOP10 cells (Invitrogen) the colonies were analyzed by checking PCR with the forward vector specific primer [M13 forward sequencing primer (−20)] and the reverse gene specific primer (*att*B2 ASP/laccase Glu-tag). Positive clones were isolated for the subsequent LR recombination reaction using the *att*R containing pDEST 17 destination vector (Invitrogen); by that the expression clone laccase with the Glu-tag in pDEST 17 was constructed. After transformation in TOP10 cells the clones were analyzed by checking PCR with the forward vector specific primer T7 and the reverse gene specific primer (*att*B2 ASP/laccase Glu-tag), followed by sequencing. The positive expression clones were transformed into competent BL21 AI One Shot cells (Invitrogen), which were specially designed for the recombinant protein expression from any T7-based expression vector. The cells were incubated in LB-medium at 37 °C with vigorous shaking until the OD_{600} reached a value of 0.6–0.8. After adding the inductor L-arabinose at a final concentration of 0.2%, the cells were incubated overnight. The cell pellet was obtained by centrifugation and analyzed on 12% SDS-PAGE. The expected molecular weight of the recombinant laccase rec-LACC-l_SUBDO with Glu-Tag is 74.0 kDa.

The purification of the recombinant laccase, rec-LACC-l_SUBDO, was achieved by application of the PROFINIA purification system (Bio-Rad, Hercules, CA; USA) as described [51]. In brief, the clear lysate (30 ml) was purified in 1 ml Bio-Scale Mini Profinity IMAC Cartridges, using the following buffer steps: (*i*) 50 mM KH_2PO_4, pH 8.0; 5 mM imidazole, 300 mM KCl, (*ii*) 50 mM KH_2PO_4, pH 8.0; 10 mM imidazole, 300 mM KCl, and (*iii*) 50 mM KH_2PO_4, pH 8.0; 250 mM imidazole, 300 mM KCl.

2.5. Antibodies against the recombinant laccase

Polyclonal antibodies (PoAbs) were raised against the recombinant protein, rec-LACC-l_SUBDO, in female rabbits (New Zealand White) as described [52]; the PoAbs were termed PoAb-rec-LACC-l. In control experiments, 100 μL PoAb-rec-LACC-l were adsorbed to 20 μg of recombinant LACC-l_SUBDO (30 min; 4 °C) prior to use. Those adsorbed antibody samples did not give any signal in the immunohistological application.

2.6. Western blotting

The recombinant protein LACC-l_SUBDO (5 μg) was dissolved in loading buffer (Roti-Load; Roth, Karlsruhe; Germany), boiled for 5 min and then subjected to SDS/PAGE (12% polyacrylamide and 0.1% SDS) [53]. The gels were stained with Coomassie Brilliant Blue. In parallel, the proteins in the sample were transferred to poly(vinylidene difluoride) membranes (Millipore-Roth) and incubated with PoAb-rec-LACC-l (1:1000 dilution); the immune complexes were visualized by incubation with anti-rabbit IgG (alkaline phosphatase conjugated), followed by staining with 4-chloro-1-naphthol. Multi-Tag-Markers (Roche, Mannheim; Germany) were used as size markers.

2.7. Immunohistochemistry

Fresh sponge tissue from the Adriatic Sea was fixed in 2% paraformaldehyde as described [54]; the samples were embedded in Technovit 8100 (Heraeus Kulzer GmbH, Wehrheim; Germany), as published [7,55]. Sections of 6 μm thickness were prepared, blocked with 1% BSA [bovine serum albumin] in $NaCl/P_i$ and incubated with PoAb-rec-LACC-l overnight at 4 °C. The antibodies were used at a 1:750 dilution in 0.5% BSA in $NaCl/P_i$ buffer. Rhodamine-conjugated goat anti-rabbit immunoglobulin (Dako, Carpinteria, CA; USA) was used as a secondary antibody. The preimmune rabbit serum was used as a control and found not to react with any structures in the sections. Light microscopic studies (immunofluorescence analysis) were performed with an Olympus AHBT3 microscope equipped with an AH3-RFC reflected light fluorescence attachment using the excitation light wavelength 334/365 nm. In parallel, the nonstained sections were inspected directly using Nomarsky interference contrast optics or were reacted with DAPI [4′,6-diamidino-2-phenylindole dihydrochloride; #D9542, Sigma, Taufkirchen; Germany] as described [56].

2.8. Reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR)

The technique of RT-qPCR was applied to determine the expression levels of the following genes in *S. domuncula* tissue. Details were given recently [57].

The specimens were kept separately for up to 3 d in 500 mL incubation beakers. They remained either in the absence or presence of 3 μg/ml of LPS for up to 5 d. After termination of incubation tissue samples were taken and RNA was isolated and subjected to RT-qPCR reaction. Each reaction contained ~5 μg of total RNA, 0.5 mM dNTPs, 100 pmol of $oligo(dT)_{18}$ and 400 U reverse transcriptase [RT; Invitrogen, Darmstadt; Germany] in RT buffer. After incubation (42 °C for 1 h), the RT was inactivated (65 °C, 15 min). The following primer pairs have been used: for the sponge laccase (*SDLACC-l*) Fwd: 5′-CCATACTCTCTTCCTGATCCAG-3′ [nt_{880}–nt_{901}] and Rev: 5′-TGTATCTCCATCCTCCAAAATTC-3′ [nt_{984}–nt_{1006}; product length 127 bp] and as reference gene GAPDH [glyceraldehyde 3-phosphate dehydrogenase; GenBank accession number AM902265] Fwd: 5′-TCCAAACCAGCCAAGTACGATG-3′ [nt_{816}–nt_{837}] and Rev: 5′-AGTGAGTGTCTCCCCTGAAGTC-3′ [nt_{945}–nt_{924}; 130 bp]. By that cDNA was prepared and the RT-qPCR reactions were performed using an iCycler (Bio-Rad, Hercules, CA; USA) with the respective iCycler software. After determination of the C_t values the expression of the respective transcripts was calculated [58].

2.9. Laccase activity determination

The enzyme activity was determined as described [59–61]. The spectrophotometric procedure measures the oxidation of syringaldazine [#177539, Sigma] at 520 nm, as described [62]. The reaction mixture contained in a final volume of 1 mL·100 μl of protein sample (50 μg of purified enzyme) and 30 μM of syringaldazine, prepared from a 3 mM stock solution in methanol [63], in 100 mM Britton-Robinson buffer (pH 6.7). Enzyme activity was expressed as micromoles of substrate oxidized per milligram of protein per minute.

2.10. Preparation of ferromagnetic nanoparticles

The magnetic iron oxide oxide nanoparticles [Fe-nanoparticles] were prepared as described [64–66]. In brief, the iron oleate complex was synthesized from 2 mmol iron(III)acetyl acetonate [$Fe(acac)_3$] that was suspended in 20 mL of benzyl ether. After addition of 10 mmol 1,2-hexadecanediol, 6 mmol oleic acid and finally 6 mmol oleylamine

the reaction was started under argon atmosphere and heated to 250 °C under reflux for 30 min. The resulting dark-brown solution was cooled to room temperature; during this process the magnetite nanoparticles are produced; they have a size of ≈7 nm, are monodisperse, and can be conveniently attracted as well as translocated in the organic as well as the aqueous environment by using a circular magnet 18 × 10 mm.

2.11. Functionalization of ferromagnetic nanoparticles with laccase

The hydrophobic nanoparticles were processed to water-dispersible particles by replacing the oleic acid layer surrounding the particle surface [67]; the particles (500 mg) were suspended in hexane, containing 0.1% acetic acid, under rotation (72 h) to facilitate the ligand exchange reaction. After suspending the samples were briefly sonicated and the precipitate formed was collected. The nanoparticles were suspended with 5 μg mL^{-1} of Glu-tagged laccase, dissolved in 50 mM Tris/HCl buffer. After an incubation period of 24 h the Glu-tagged laccase iron oxide nanoparticles [laccase-Fe-nanoparticles] were recovered and purified using a magnet-based separator (MagnaRack CS15000; Invitrogen).

In order to demonstrate that the laccase-Fe-nanoparticles are indeed covered by the enzyme the sample was smeared onto a glass slide and reacted with polyclonal antibodies (1:1000 dilution), directed against laccase (PoAb-rec-LACC-l). After washing and blocking with goat serum (Invitrogen) the sample was incubated with the anti-laccase antibodies. Then the immunocomplexes could be visualized by reaction with fluorescently labeled (Cy3 [green]; Dianova, Hamburg; Germany) secondary antibodies (dilution 1:3000). Antibodies, adsorbed with recombinant laccase, did not show any reaction to the laccase-Fe-nanoparticles (not shown). The samples were inspected with an Olympus AHBT3 microscope/equipped with a light fluorescence attachment unit.

2.12. Transmission electron microscopy

Transmission electron microscopy (TEM) was performed as previously described [68], using a Philips EM-420:120-kV microscope equipped with a CCD camera.

2.13. Magnetic nanoparticle separator

In the home-made apparatus the Eppendorf reaction tube (volume 2 mL) was connected to a small docking platform. The cover of the tube was removed in order to allow the connection to a socket into which a stainless coil stirrer was fitted. The nanoparticles were put inside the reaction tube, containing the culture medium with the *E. coli* bacteria. The bacterial cell suspension was stirred via a motor and the connected stirrer. At the end of the incubation the nanoparticles were collected from the incubation assay in the reaction tube.

2.14. E. coli culture

E. coli (strain TOP10; Invitrogen) was cultivated in LB-Medium (Luria/Miller #X968.1; Roth, Karlsruhe; Germany). For starting the experiments, a cell density of 0.2 OD_{480} had been chosen. The bacteria were grown on 2 ml LB-Medium/LB-Agar in 35 mm Petri dishes (CELLSTAR; Greiner/Sigma). The growth medium was supplemented with 10 μg/ml of lignin (471003 Aldrich; low sulfonated; Mw ~ 10,000), containing phenolic and non-phenolic moieties [69]. The incubation assay remained either without any additional component, or had been supplemented with laccase-Fe-nanoparticles. Where indicated, the nanoparticle-supplemented culture agar had been dosed with 1 mM of the laccase mediator ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)]. Laccase-Fe-nanoparticles were added at concentrations between 3 and 100 μg/mL.

For quantitative assessment of the toxicity of laccase-Fe-nanoparticles on *E. coli*, in the presence or absence of ABTS, the turbidity of the culture broth at the end of incubation was determined. Here, the optical densities (OD) of the growing cultures were followed at 480 nm using a SmartSpec Plus spectrophotometer (Bio-Rad); the determinations have been performed against LB-medium. At this value the minimum of absorption for oxidized ABTS is recorded [70]. The experiments were started with a cell density of 0.2 OD_{480}; the microorganisms were allowed to grow in LB-Medium. Where indicated in the "Results" *E. coli* were incubated in medium supplemented with 10 μg/ml of lignin and – as indicated – in the absence or presence of 1 mM ABTS and or 10 μg/ml or 30 μg/ml of laccase-Fe-nanoparticles. After the indicated period of incubation the cultures were removed and the optical density was determined.

2.15. Further methods

The results were statistically evaluated using the paired Student's *t*-test [71]. For quantification of protein the Bradford method (Roti-Quant solution — Roth) was used [72].

3. Results

3.1. Sponge (S. domuncula) laccase

Several EST tags have been identified in the *S. domuncula* database; based on them the corresponding cDNA was worked out. The resulting cDNA (*SDLACC-l*) was found to encode a deduced polypeptide that shares highest sequence relationship with the *A. queenslandica* (GenBank accession number XP_003387804.1) laccase 17-like polypeptide [*E* value [73] of $1e^{-64}$], the postulated laccase from *Branchiostoma floridae* (GenBank accession number XP_002604762.1; $E = 1e^{-58}$) as well as the laccase-2 from *Crassostrea gigas* (GenBank accession number EKC25244.1; $9e^{-49}$). Therefore, we termed the deduced *S. domuncula* polypeptide laccase-like protein (LACC-l_SUBDO). This 785 aa-long polypeptide with a calculated size of 87.2 kDa and a predicted pI of 4.80 has a computed half-life of 30 h with an instability index of 41.4, indicative of an unstable protein.

The *S. domuncula* laccase was aligned with these three laccase/laccase-like polypeptides (Fig. 1). The comparison shows that the *S. domuncula* laccase, LACC-l_SUBDO, comprises the three characteristic multicopper oxidase homologous domains [74], starting with the Cu-oxidase_3 domain (aa_{102} to aa_{217}; $E = 4.8e^{-36}$), followed by the general Cu-oxidase domain (aa_{230} to aa_{419}; $E = 1.5e^{-15}$), and finally the Cu-oxidase_2 domain (aa_{552} to aa_{698}; $E = 1.4e^{-21}$). One transmembrane segment has been predicted within the aa_{751} to aa_{773} region and one signal peptide exists at aa_1 to aa_{24}. Like the laccase (EC 1.10.3.2; urishiol oxidase) from fungi and plants the sponge protein is a 3-domain enzyme, oxidizing different phenols and diamines [75].

The phylogenetic tree was constructed to disclose the position of the sponge laccase in the Metazoan kingdom. As expected, the sequence from the demosponge *A. queenslandica* shows the closest relationship, followed by the cnidarian *Hydra vulgaris* sequence. In addition, the *S. domuncula* laccase shares high sequence similarity with the insect enzymes, e.g. from *Bombus impatiens*, *Apis mellifera*, or *Acromyrmex echinatior*, and the nematodan *Caenorhabditis remanei*, *Ancylostoma ceylanicum* and *Haemonchus contortus* laccase-related sequences. Similarly high is the relationship to the molluscan enzyme, from *Lottia gigantean*, and the postulated laccase from the cephalochordate *B. floridae* (Fig. 2). Surprising is the fact that the laccase does not exist in vertebrates, but is present in yeasts, e.g. *Saccharomyces cerevisiae* with a high similarity ($E = 6e^{-26}$).

3.2. S. domuncula recombinant laccase and antibodies

The *SDLACC-l* sequence, lacking the expressed signal peptide, was expressed in *E. coli*. A construct was chosen which contained in addition to the His-tag (6x His-tag residues at the *N*-terminus) also 8 Glu-tag residues. The recombinant protein was expressed in pDEST 17 vector and *E. coli* strain BL21AI induced by L-arabinose (Fig. 3A). After affinity

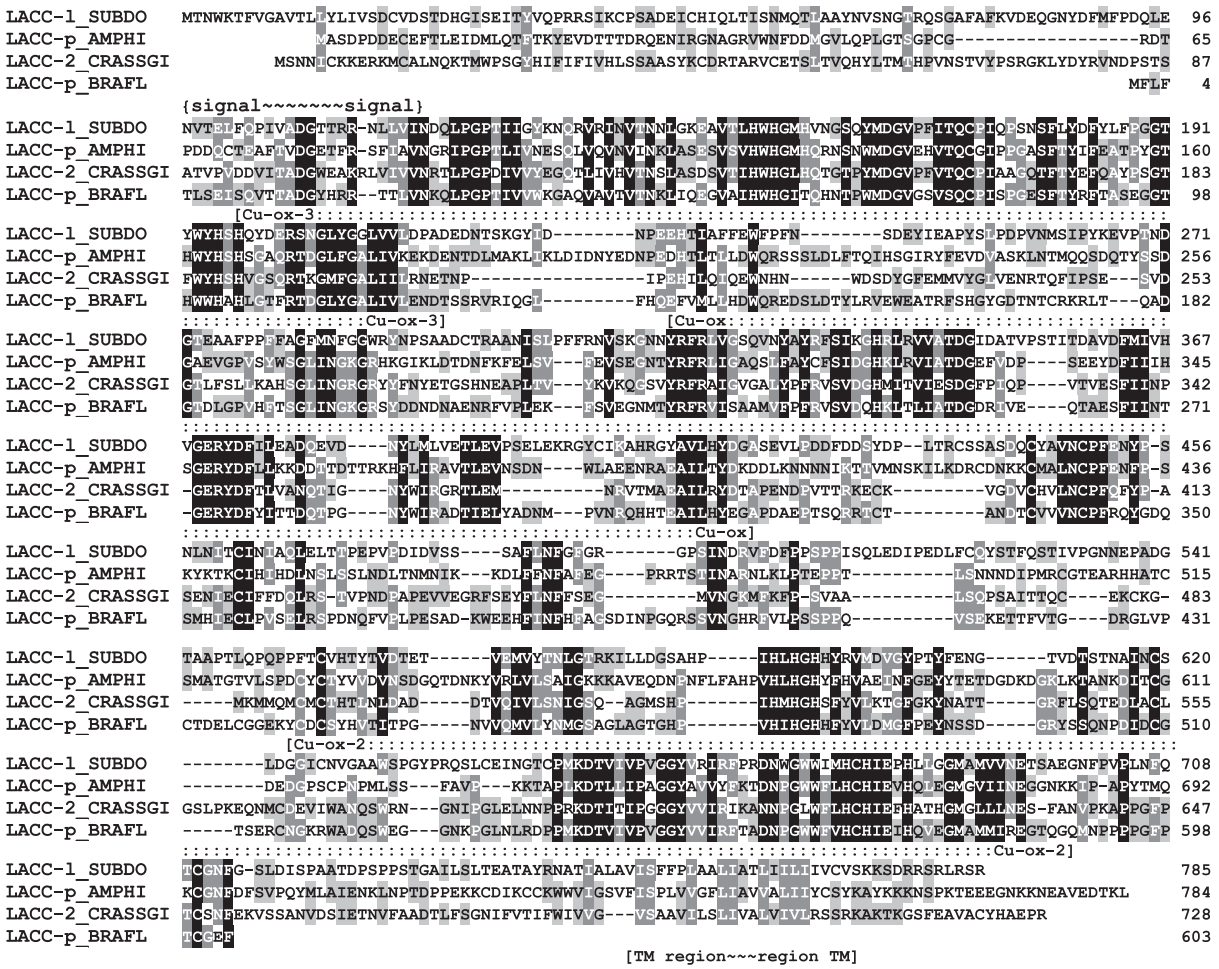

Fig. 1. The *S. domuncula* laccase; alignment. The similarities of the *S. domuncula* deduced laccase (LACC-l_SUBDO) with the next related sequences from *A. queenslandica* (LACC-p_AMPHI; GenBank accession no. XP_003387804.1), *B. floridae* (LACC-2_CRASSGI; GenBank accession no. XP_002604762.1) as well as *C. gigas* (LACC-p_BRAFL; GenBank accession no. EKC25244.1). These sequences were aligned. Residues conserved (identical or similar with respect to their physico-chemical properties) in all sequences are shown in white on black. Those sequences which share similarity to at least three residues are in white on gray and at least to two sequences in black on gray. The borders {signal} of the signal peptide, the transmembrane region (TM region) as well as the three characteristic domains, the Cu-oxidase_3 domain [Cu-ox-3], the general Cu-oxidase domain [Cu-ox], and the Cu-oxidase_2 domain [Cu-ox-2] are highlighted.

purification the 658 aa long laccase, LACC-l_SUBDO, comprising the C-terminal Glu-tag [1.05 kDa], as well as the His-tag [0.84 kDa], a total size of 74.08 kDa for the protein has been calculated and also experimentally found.

In order to localize the laccase within the sponge tissue, antibodies were prepared in rabbits. Western blotting revealed that only the antibodies from the challenged animals reacted with the recombinant LACC-l_SUBDO (Fig. 3B; lane b), while the preimmune serum did not (Fig. 3B; lane a). By that the specific 74 kDa protein was visualized.

3.3. Upregulation of laccase gene in sponge tissue after exposure to LPS

3.3.1. Immunohistology

Tissue slices were prepared from an animal, kept for 5 d with 3 μg/mL of LPS or, in the control, in the absence of the endotoxin. Then the slices were reacted with anti-laccase IgG (PoAb-rec-LACC-l). The analyses show that in tissue from the LPS-treated animal the brightest reactions (highest level of laccase) are in the surface zone; only a lower quantity of immunocomplexes is visualized in the central part of the animal (Fig. 4C). In parallel the histology is represented by Nomarsky interference contrast optics (Fig. 4A) or by DAPI staining (Fig. 4B). The control animal, not exposed to LPS, shows a significant lower expression of laccase (Fig. 4F); again the histology is visualized by interference contrast optics (Fig. 4D) and by staining with DAPI (Fig. 4E). In controls it was established that the pre-immune serum did not react with the structures within the slices (not shown).

3.3.2. RT-qPCR analysis

The *laccase* gene expression depends on the exposure to the endotoxin LPS. Specimens were kept separately for up 5 d in beakers in the absence or presence of LPS. Tissue samples were removed after 0 to 5 d, followed by RNA extraction. Then the steady-state expression level of *laccase* was determined by RT-qPCR and the values were normalized to *GAPDH* expression (Fig. 5). The data show that in tissue from a non-treated animal the level for *laccase* only slightly increased from 0.06 ± 0.01 (day 0) to 0.14 ± 0.02 (day 5). In contrast, in LPS-exposed specimens the increase of *laccase* is significantly higher, also if compared to the controls. At day 0 the expression level is 0.08 ± 0.02, while at day 5 the steady-state expression value is 0.26 ± 0.04.

3.4. Laccase activity of the recombinant enzyme

The enzymatic activity of the recombinant laccase (LACC-l_SUBDO) was determined in the spectrophotometric assay, as described under "Material and methods", by using syringaldazine as a substrate. In the presence of syringaldazine the sponge laccase has a V_{max} of 65 μM/min and an apparent Michaelis constant (K_m) of 114 μM. These values are somehow lower than those described for the bacterial laccase [60] but still close to the laccase from mushrooms, determined earlier [76,77].

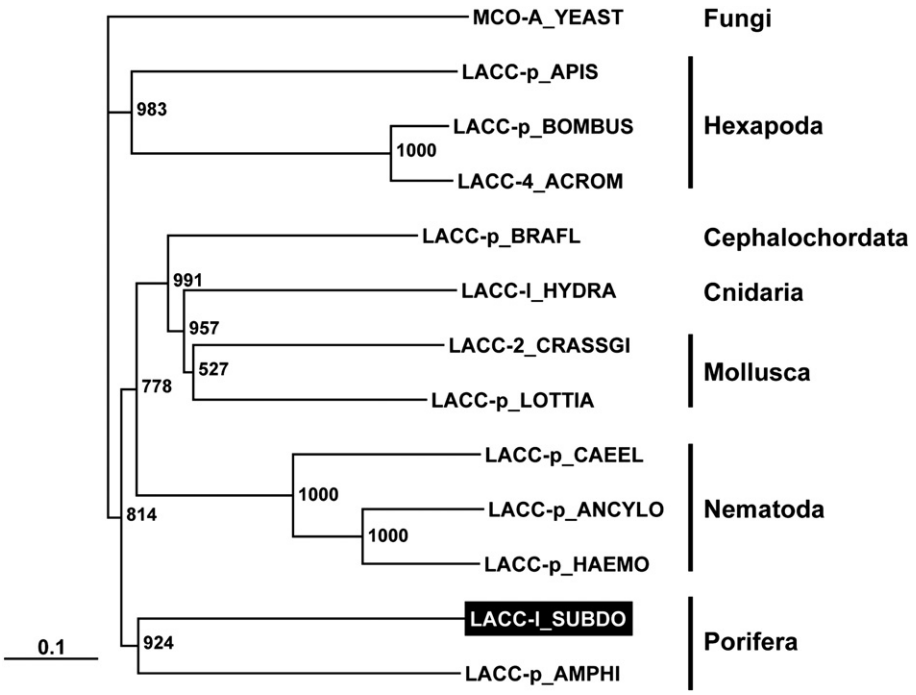

Fig. 2. The *S. domuncula* laccase; phylogenetic tree. A tree was constructed using, in addition to those sequences in Fig. 1, the sequences from the laccase-like protein from *H. vulgaris* (LACC-l_HYDRA; GenBank accession no. XP_002159531.2) and the laccase-postulated protein from *L. gigantean* (LACC-p_LOTTIA; GenBank accession no. ESO89911.1), *A. ceylanicum* (LACC-p_ANCYLO; GenBank accession no. EYB82076.1), *A. mellifera* (LACC-p_APIS; GenBank accession no. XP_001120790.2), *B. impatiens* (LACC-p_BOMBUS; GenBank accession no. XP_003490974.1) and *C. remanei* (LACC-p_CAEEL; GenBank accession no. XP_003092481.1). In addition, the multicopper oxidase domain containing protein from *H. contortus* (LACC-p_HAEMO; GenBank accession no. CDJ83113.1) and laccase-4 from *A. echinatior* (LACC-4_ACROM; GenBank accession no. EGI60467.1) were included. The multicopper oxidase from *S. cerevisiae* (MCO-A_YEAST; GenBank accession no. P38993.2) was included and used as outgroup to root the tree. The scale bar indicates an evolutionary distance of 0.1 aa substitutions per position in the sequence.

3.5. Preparation of nanoparticles, coated with recombinant laccase

The ferromagnetic nanoparticles were covered with Glu-tagged laccase. In the first step the iron oxide nanoparticles were prepared as iron oleate complex. The iron oxide nanoparticles formed have an average size of ≈ 7 nm (Fig. 6). They display strong magnetic properties which can be utilized to collect them back from the reaction mixture after incubating them with bacteria (see below). The hydrophobic surface residues were transferred to hydrophilic residues by acid treatment, allowing the coating of the particles with laccase. The particles are stained in darkish brown (not shown here). After transfer of the oleate ferromagnetic particles to acetic acid the particles were covered by Glu-tagged laccase. During this functionalization with laccase the particles increased in size to 14–18 nm (not shown here).

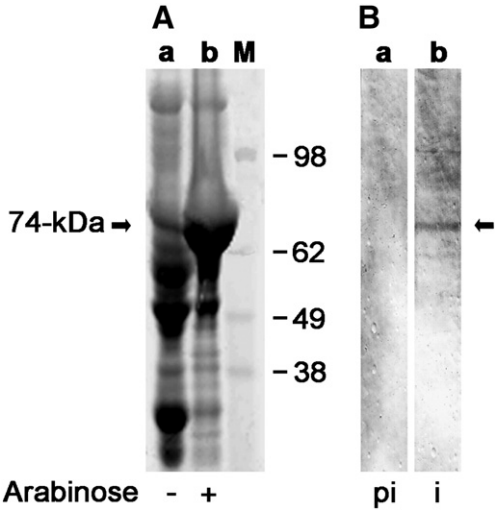

Fig. 3. The recombinant sponge laccase. The *S. domuncula SDLACC-I* was expressed in *E. coli*. (A) The bacterial protein extract, obtained from non-induced (lane a) or from arabinose-induced cultures (lane b) was size-separated by SDS/PAGE analysis (12% gels). The 74 kDa fusion protein is marked. (B) The purified fusion protein rec-LACC-l_SUBDO was used to raise antibodies. The resulting PoAb-rec-LACC-l protein (immune serum [i]) was found to react with a 74-kDa protein in the Western blot assay with the recombinant protein (lane b), while the pre-immune serum (pi) did not (lane a). The size markers (M) are given in (A).

In order to demonstrate that the nanoparticles are indeed coated with laccase the particles were incubated with antibodies, directed against the laccase (PoAb-rec-LACC-l). The samples were smeared onto a glass slide (Fig. 7A); after applying the antibodies and incubating the antigen/antibody complexes with a labeled secondary antibody, the complexes strongly lighted up in green (Fig. 7B). In the controls, incubation with the preimmune serum, no signals were seen (not shown).

3.6. Antimicrobial effect of laccase

The effect of laccase that had been coated around iron oxide nanoparticles was directly checked under agar-growth conditions. An oxidizable substrate 10 μg/ml of lignin was added. A suspension of *E. coli* was plated onto the agar; where indicated the agar was supplemented with the laccase mediator ABTS and/or the laccase-Fe-nanoparticles. The results show that after an incubation period of 4 h the number of colonies that were formed in the control cultures, containing neither ABTS nor laccase, is relatively dense (Fig. 8). If ABTS and, especially, the laccase-Fe-nanoparticles are added separately to the bacteria a reduction of the number of bacterial colonies is observed. Co-addition of the mediator (ABTS) to the laccase-Fe-nanoparticles results in a dose-dependent strong inhibition of the *E. coli* growth. In controls it was established that lignin does not affect the growth of the bacteria (not shown).

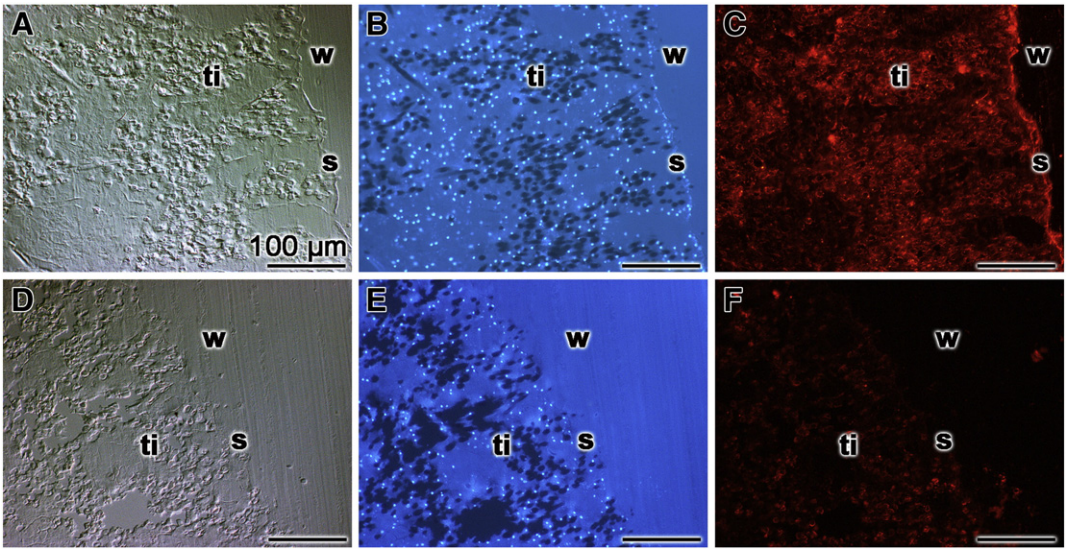

Fig. 4. Immunohistological detection of laccase in *S. domuncula* tissue. The specimen was kept for 5 d in the presence of 3 µg/mL of LPS (A, B, C) or in the absence of the endotoxin (D, E, F). Then slices through the tissue (ti) were prepared and inspected by Nomarsky interference contrast optics (A, D), or after DAPI staining (B and E) to highlight the cell nuclei, or after reaction with the laccase antibodies (PoAb-rec-LACC-l). In addition, the cell surface (s) and the surrounding water (w) environment are marked. Size bars are given.

3.7. Effect of laccase iron oxide nanoparticles in a magnetic nanoparticle separator

To analyze the effect of the laccase-Fe-nanoparticles on the growth of bacteria in suspension, a magnetic nanoparticle separator has been used. This device allows the incubation of the bacteria in a reaction tube under controlled stirring (Figs. 9A to C). A coiled stirrer that has been inserted into the reaction tube is connected with a motor, allowing a tuned mixing of the cultures. In the incubation chamber, used here, a hole has been inserted which allows the insertion of an ultraviolet lamp. The coiled stirrer allows the collection of the nanoparticles by electromagnetic forces (Figs. 9D and E). In control experiments it had been ascertained that the bacteria did not attach to the nanoparticles, surrounded by the laccase.

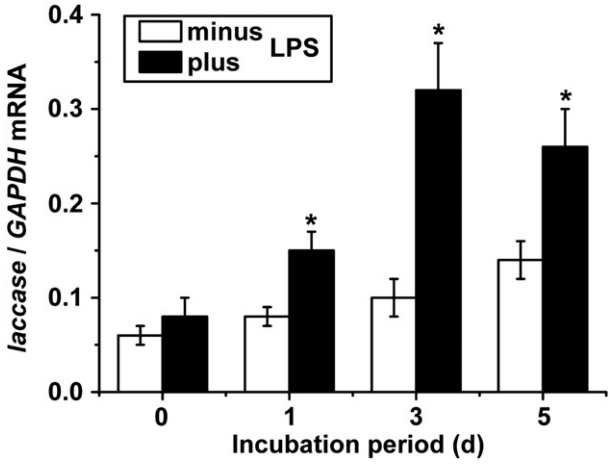

Fig. 5. Influence of LPS on the expression of the laccase gene in sponge tissue. In parallel experiments the sponge specimens were incubated in the absence (open bars) or presence of 3 µg/mL of LPS (closed bars) for 0 (start of the experiment), 1 d, 3 d or 5 d. Subsequently, RNA was extracted and subjected to RT-qPCR analysis for quantification of the steady-state-expression level of both *laccase* (*SDLACC-l*) and *GAPDH*. The expression level of *laccase* was normalized to the expression of *GAPDH*. Data are expressed as mean values ± SD for five independent experiments. Differences between two groups were evaluated using an unpaired *t*-test, $^{*}P < 0.05$.

A quantitative assessment of the effect of different concentrations of laccase-Fe-nanoparticles was performed in the magnetic nanoparticle separator, in which the bacteria were growing in the reaction tube in suspension. The medium was supplemented with the laccase substrate 10 µg/ml of lignin. As a measure for bacterial growth the OD_{480} was determined (Fig. 10). In the absence of either ABTS or laccase-Fe-nanoparticles the bacteria grew from ≈ 0.42 ± 0.5 to 1.73 ± 0.19 after 100 min incubation and to 2.14 ± 0.19 after 200 min. If the mediator ABTS is added a non-significant reduction is measured with 1.47 ± 0.17 (100 min) and 1.93 ± 0.21 (200 min), respectively. However, after exposure of the bacteria in the absence of ABTS to 10 µg/ml laccase-Fe-nanoparticles a significant reduction was measured with 1.12 ± 0.16 (100 min) and 1.47 ± 0.16 (200 min). Co-addition of ABTS and laccase-Fe-nanoparticles strongly increased the inhibitory activity of the enzyme; after 200 min the density of the bacteria measures only 0.62 ± 0.08 (ABTS plus 10 µg/ml of laccase-Fe-nanoparticles) and 0.28 ± 0.04 (ABTS plus 30 µg/ml of laccase-Fe-nanoparticles), respectively.

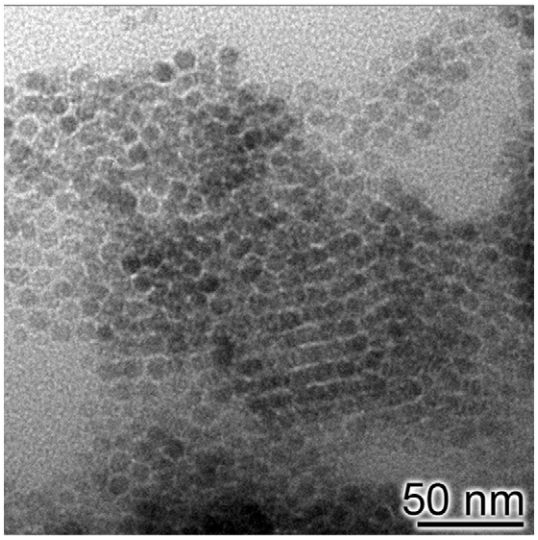

Fig. 6. Iron oxide nanoparticles were prepared from iron(III)acetyl acetonate and oleylamine.

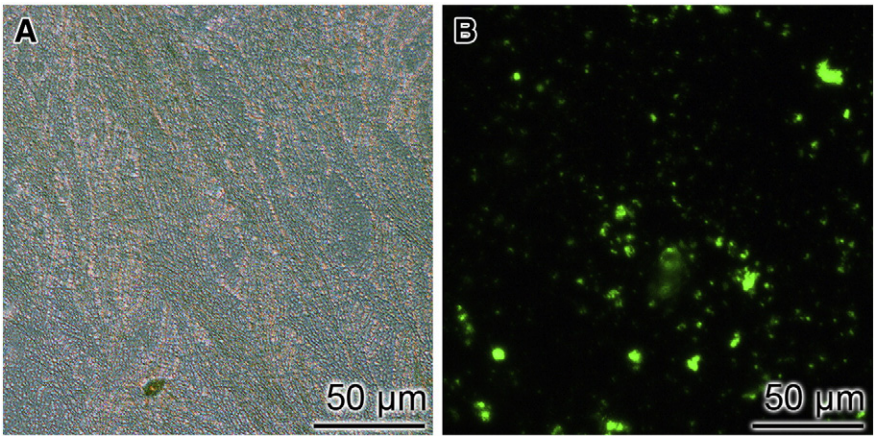

Fig. 7. Identification of laccase on a smear of laccase-Fe-nanoparticles. (A) An aliquot of laccase-Fe-nanoparticles was applied on a glass slide and (B) incubated with antibodies against sponge laccase. The immunocomplexes were visualized with a labeled secondary antibody.

4. Discussion

It could be indicative that the *S. domuncula* laccase with its domain structure and phylogenetic relationship comprises a high sequence similarity besides to other poriferan enzymes also to cnidarian laccases. While a significant, even though more distant relationship also exists to insect and molluscan laccase, no homologous or similar enzyme has been described for the evolved vertebrates. In sponges the laccase is apparently frequently present; in the demosponge species *A. queenslandica* several isoforms have been cloned (see e.g. GenBank accession number XP_003387804.1). This fact could imply that the laccase gene has been lost during vertebrate evolution. Such a gain and loss of a gene has been described to have happened for the strombine dehydrogenase, likewise in *S. domuncula* [78]. However, the existence of laccase in sponges and cnidarians could also signify that this enzyme plays an important role in the physiology of these basal metazoans. The laccase is also present in yeast [79,80]. Common to all of these laccases is the presence of three characteristic multicopper oxidase homologous domains that are crucial for the one-electron oxidation of a broad range of compounds, including substituted phenols, arylamines and aromatic thiols during which the corresponding radicals are formed [81]. It is very obvious that in sponges and cnidarians, laccase has a functional role in detoxification and elimination of lignin, coming from the plants that surround sponges, e.g. *S. domuncula*. In a future study we will determine why

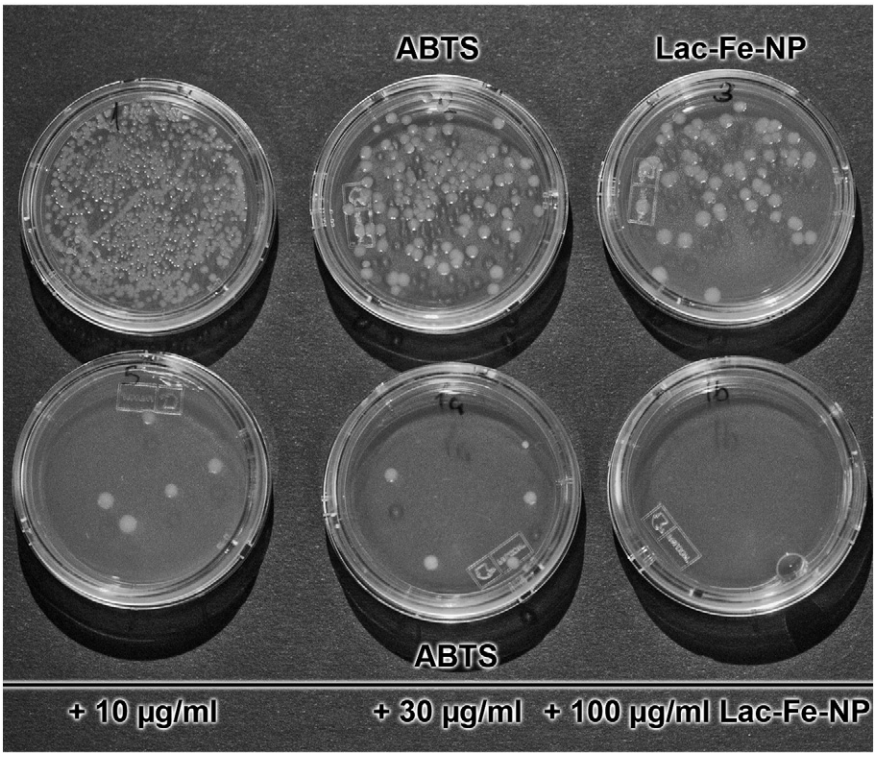

Fig. 8. Effect of laccase (added as laccase-Fe-nanoparticles [10 μg/ml]) and of the mediator ABTS [1 mM], in separate, on the colony formation of *E. coli* (upper row); the upper left assay contained neither laccase-Fe-nanoparticles nor ABTS. As oxidizable substrate 10 μg/ml of lignin had been added to all assays. It is apparent that if the medium is mixed with the mediator ABTS together with 10 μg/ml, 30 μg/ml or 100 μg/ml of laccase-Fe-nanoparticles [Lac-Fe-NP] an almost complete inhibition of bacterial growth is measured (lower row).

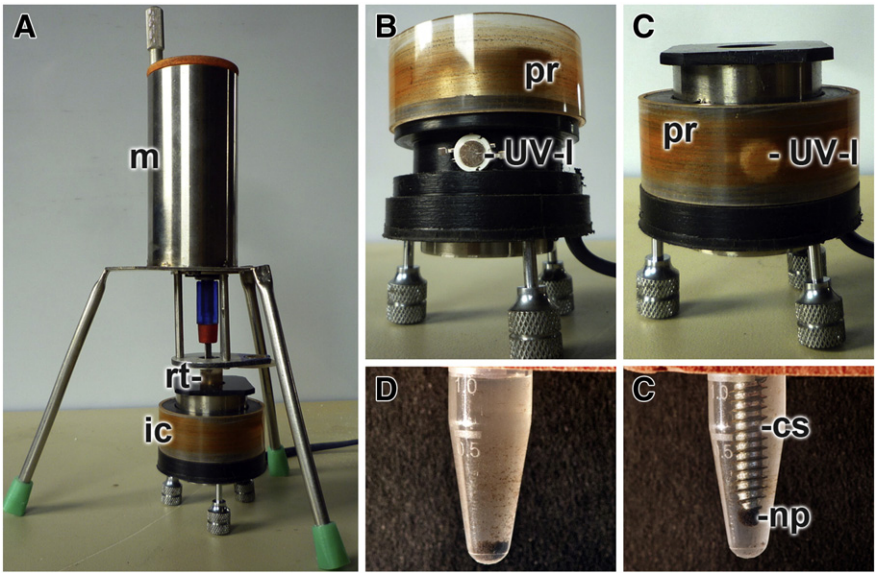

Fig. 9. Magnetic nanoparticle separator. (A) Separator which features in the functional center of the device a reaction tube (rt) into which the bacteria grow in suspension. The reaction tube is flush-mounted in an incubation chamber (ic). The stirring of the bacterial suspension is carried out by a coil stirrer that is linked to a motor (m). (B and C) Incubation chamber, at higher magnification, into which the reaction tube can be steeped in. A drilled hole allows the introducing of an ultraviolet lamp (UV-l). This lamp can be protected by a protection ring (pr) that can be put over the feeding hole. (D and E) Reaction tube harboring the bacterial cultures with the laccase-Fe-nanoparticles; a coil stirrer (cs) is shown in E. The nanoparticles (np) in (E) have been attracted by applying electromagnetic forces.

the phenolic avarol, a secondary metabolite from sponges, comprises antimicrobial activity but is not toxic to the host [82]. It might be anticipated that it is the laccase that "detoxifies avarol" in the sponge.

The broad substrate specificity of laccases is unusual for an enzyme [81] and suggested to us that the laccase in *S. domuncula* might have the function to kill bacteria, or control bacterial growth. In order to test this hypothesis the *S. domuncula* laccase was heterologously expressed in *E. coli*. In order to allow subsequent binding of the enzyme to iron oxide nanoparticles a Glu-tag was genetically engineered to the *N*-terminus of the enzyme [83]. The recombinant laccase obtained displayed enzymatic function, if the mediator syringaldazine was added to the enzyme reaction. The activity of the sponge recombinant laccase is in the range of activities measured for other recombinant enzymes [60] and natural enzymes [76,77,84]. The redox potential among the laccases vary drastically; there are both enzymes with a high redox potential, e.g. the mushroom *Trametes versicolor* laccase, and enzymes with a low redox potential, like the plant *Rhus vernicifera* enzyme [85]. Adapted to this inherent property the mediator has to be selected [81]. The recombinant sponge enzyme is significantly more active if the mediators ABTS and syringaldazine are added; both compounds are universal mediators for laccases [86]. The Michaelis constant (K_m) of the sponge enzyme is 114 μM, somewhat higher than the one found for bacterial laccase [60], but close to the one from the mushroom *Clitocybe maxima* [87].

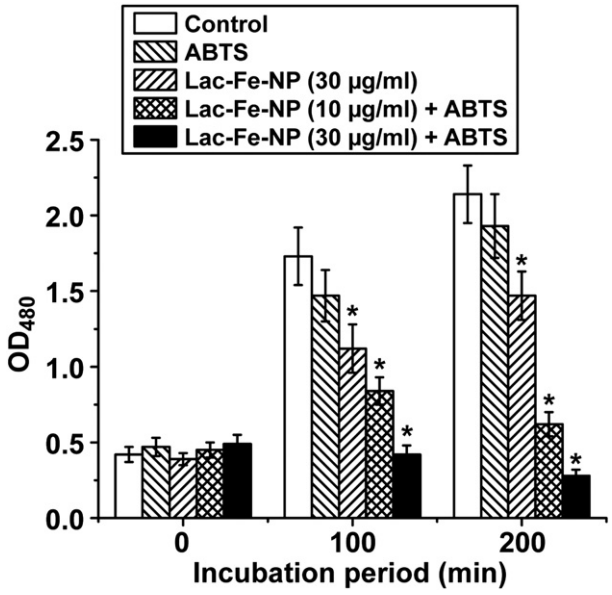

Fig. 10. Growth of *E. coli* cells, grown in 10 μg/ml of lignin, in the absence of ABTS and laccase-Fe-nanoparticles (control; open bars), or – in separate – either 1 mM ABTS (ABTS; shaded to the left) or 30 μg/ml laccase-Fe-nanoparticles (Lac-Fe-NP; shaded to the right). In two additional series ABTS and the nanoparticles, either 10 μg/ml of laccase-Fe-nanoparticles [Lac-Fe-NP (10 μg/ml + ABTS); cross hatched bars] or 30 μg/ml laccase-Fe-nanoparticles [Lac-Fe-NP (30 μg/ml + ABTS); filled bars]. After an incubation period of 0 min (start of the experiment), 100 min or 200 min the samples were taken and the optical density (OD_{480}) was determined. Values represent the means (±SD) from 10 separate experiments each (*$P < 0.01$).

Having obtained the recombinant sponge laccase the elucidation of the distribution of the enzyme within the sponge became possible. In continuation, immunohistological studies have been performed revealing that the enzyme is highly expressed at the rim of the animals. Interestingly enough, the level of tissue expression of the laccase is strongly upregulated if the animals are exposed to bacterial LPS. This finding underscores our hypothesis that the sponge laccase has a role in host-defense against microorganisms. Such a role has been proposed for the insect laccase [88]. A quantitative approach to assess the steady-state level of the sponge laccase by RT-qPCR revealed that the enzyme is highly expressed in response to the LPS load in the aqueous environment. Until now, this is the first report that in metazoans the *laccase* gene expression can be upregulated by LPS. From the functionally related enzyme, the phenoloxidase from sea cucumber *Apostichopus japonicas*, an increased expression has been measured after challenging the animals with LPS [89], also implying that this oxidoreductase is involved in defense against infection from bacteria or fungi.

Prior to a subsequent application of the sponge recombinant laccase in a biological assay the Glu-tagged laccase had been linked to iron oxide nanoparticles. The rational behind is the fact that the application of ferromagnetic nanoparticles is very efficient with respect to a cost-effective testing. The selection of these nanoparticles allows a future sustainable

application by the magnetic removal and recovery of the particles after terminating the enzyme reaction.

In order to further substantiate the view that the sponge laccase is involved in bacterial defense the enzyme reaction was directly coupled with the bacteria. An oxidizable substrate 10 μg/ml of lignin, as a source for free radicals during laccase reaction, was added to the broth in all assays. Lignin is non-toxic for *E. coli* [90]. Exposure of *E. coli* to the iron oxide nanoparticles coated with enzymatically active Glu-tagged laccase revealed that the bacterial growth is slightly inhibited by the nanoparticles and only a little by the mediator ABTS. However, if both components are added to the agar onto which the bacteria are growing an almost complete inhibition of growth is seen. A quantitative assessment of the effect of the laccase has been achieved by adding the laccase-Fe-nanoparticles to *E. coli*, growing in suspension in reaction tubes, hooked to a magnetic nanoparticle separator. In this system the bacterial growth, mediated by laccase in the presence of the mediator ABTS, is completely inhibited. This finding can be taken as direct proof that the sponge laccase displays anti-bacterial activity if the two components enzyme and mediator are brought into close contact with the microorganisms.

In conclusion, the data presented show that the *S. domuncula* laccase is most likely involved in the antibacterial defense of the sponge organism. The laccase had been immobilized to iron oxide nanoparticles to allow us to repeatedly and sustainably use the enzyme and to apply the laccase-Fe-nanoparticles together with a second set of nanoparticles that are surrounded by titania. Those titania-iron oxide nanoparticles allow the photocatalytic killing of the bacteria, if the cultures are exposed to UV-light (in progress). Again, the data on the *S. domuncula* laccase support the view that sponges have optimized their biosynthetic/biodegradation capabilities to the highest efficiency and diversity, especially if the stress-response system of the animals is challenged. The aquatic environment where sponges live is characterized by a much higher density of organismic populations in comparison to organisms living in the air, due to the higher density of the surrounding medium. This implies that these animals must have developed an efficient repertoire of defense strategies to cope with these adverse environmental conditions.

Acknowledgments

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