RECORDS OF PHARMACEUTICAL AND BIOMEDICAL SCIENCES

Red Sea Sponges of the Genus *Hyrtios* as a Source of Symbiotic Fungi with Antimicrobial Activities

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Abstract

The diversity of the symbiotic fungi associated with two Red Sea sponges of the genus *Hyrtios* (*H. erecta* and *H. erectus*) was investigated. A total of 20 isolates were purified from these sponges. Using the morphological properties, 10 fungal strains were identified to the genus level from each sponge. Strains isolated from H. erecta were identified to the species level using the Internal Transcribed Spacer ITS-rDNA sequences. Most of the identified fungal strains belong to the genera *Penicillium* and *Aspergillus*. The antibacterial activities of organic extracts of these fungi were investigated against three pathogenic microbes including *E. coli, S. aureus and C. albicans.* All of the fungal extracts displayed moderate to high levels of antimicrobial activities against the three pathogens. Several fungal strains of the genera *Penicillium* and *Aspergillus* displayed strong antibacterial activities provide potential for future investigation of these strains to identify the bioactive leads in these fungal extracts.

1. Introduction

Marine microorganisms have become an important source of pharmacologically active metabolites. Published reviews show the importance of these organisms as potential sources of future pharmaceutical leads (Liberra and Lindequist, 1995; Fenical,1997; Pietra, 1997; Bernan et al., 1997; Verbist et al., 2000; Höller et al., 2000; Jensen et al., 2000; Jensen and Fenical, 2002). The fact that some marine microorganisms are easily cultured and that they had long been neglected by many marine natural product chemists has led to an increased research effort in this area during the last 10 years. Research is focusing mainly on marine bacteria, fungi and microalgae as reflected by the number of natural products described from each group of organisms. As interests have turned to marine microorganisms, the fungi have begun to be recognized as a likely source of potentially useful natural products.

Substrates used for the isolation of marine-derived fungi for chemical investigations are very diverse. Besides marine sponges, predominantly algae (Chen et al., 1996; Belofsky et al., 1998; Numata et al., 1993; Takahashi et al., 1994) crab shell (Sugano et al., 1991), fish (Shigemori et al., 1991), mangroves (Poch et al., 1991), sea hare (Numata et al., 1997), tunicate (Wang et al., 1997), and sediment samples (Onuki et al., 2004) were used as sources for marine fungi.

Bugni and Ireland reviewed marine fungi as a source of new compounds and compared the number of compounds obtained from each source (Bugni and Ireland, 2004). Fungi derived from sponges account for the highest number (33%) of compounds (Bugni and Ireland, 2004), and have the overall highest number of novel compounds. Algicolous fungi come

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in second place and account for 24% of the total number of metabolites, but represent a slightly higher percentage (27%) of new metabolites. Sponges are filter feeders and harbor large numbers of spores and/or fungal hyphae that stay dormant until appropriate nutrient conditions are encountered for growth. Fungi obtained from sponges, algae, or wood substrates account for the majority of chemistry described (70%) (Bugni and Ireland, 2004).

Among the classical substrates for the isolation of marine fungi are marine algae. While algae have long been known for the occurrence of specifically adapted marine fungi (Kohlmeyer and Kohlmeyer, 1979), sponges as a source of fungi only recently came into focus. This stands in contrast to symbiotic bacteria and cyanobacteria (Vacelet, 1975,; Bergquist and Sponges, 1978; Rai,1990) some of which seem to play an important role in the production of biologically active secondary metabolites found in the host animals (Elyakov et al.,1991; Unson et al.,1994). As sponges are filter-feeding organisms, spores and mycelium fragments of terrestrial fungi, washed into the sea, are likely to be present in these animals. The presence of these propagules in the sea water all over the world is well established, and they are known to be able to germinate and grow under laboratory conditions (Roth. et al., 1964; Miller and Whitney, 1981). Thus, isolates obtained from sponges may represent such terrestrial strains, which are also suggested by the taxonomy of the fungal strains in question, e.g. *Aspergillus niger* and *Trichoderma harzianum*.

The Red Sea sponges of the genus *Hyrtios* have proven to be a rich source of biologically active secondary metabolites (Youssef et al.,2005; Youssef et al.,2002; Youssef, 2005; Sauleau et al.,2006; Youssef et al.,2004). Investigation of several *Hyrtios* species in our laboratory afforded different classes including alkaloids, sesterterpenes, sesquiterpenes and sterols (Youssef et al.,2005; Youssef et al.,2002; Youssef, 2005; Sauleau et al.,2006; Youssef et al.,2004). The compounds displayed a diverse array of bioactivities such as antimycobacterial, cytotoxicity, anti-PLA2, and antimicrobial activity (Youssef et al.,2005; Youssef et al.,2002; Youssef, 2005; Sauleau et al.,2006; Youssef et al.,2004). These results encouraged us to look at the symbiotic fungal biodiversity in the Red Sea sponges *Hyrtios erecta* (Youssef et al.,2005; Youssef et al.,2002) and *Hyrtios erectus* (Youssef, 2005; Sauleau et al.,2006) and evaluate the antimicrobial activities of these fungi as a potential and future source of drug leads. Herein we report the fungal isolation and identification as well as the antimicrobial activities of fungal extracts from this sponge. The diversity of symbiotic fungi was examined based on morphological observations and DNA analysis of the internal transcribed spacer (ITS) region of the 10 isolated fungal strains. Antimicrobial activities of the fungal extracts were investigated with three pathogenic microorganisms including Escherichia coli ATCC 25922, Staphylococcus aureus ATCC 6538 and *Candida albicans* ATCC 14053.

2. Materials and methods

2.1. Sponge Materials

Fungal host sponges, H. erecta and H. erectus were collected from the Red Sea, Hurghada, Egypt in 2007 at depths between 13 and 20 m by SCUBA. The samples were transferred directly to a sterile plastic bag without seawater. Latex gloves were worn during collection of the specimen. The samples were stored immediately at 4 °C on ice and brought to our local laboratory after 4 hours of collection where the purification process of the fungi began immediately. Specimens of the sponge material were kept in our Red Sea marine invertebrates collection at Suez Canal University under the registration number DY07-19 and DY07-20. The sponge specimens were kindly identified by Dr. Rob van Soest.

2.2. Fungi Isolation

In order to ensure fungal isolates to be endophytic when obtained, a surface sterilization of sponges were performed. The sponge samples were disinfected with 5% sodium hypochlorite, followed by 70% ethanol, (Li and Wang, 2009) to ensure that epiphytic fungi were destroyed by the washing while fungal symbionts (if any) were not affected. In all cases, approximately 2 cm3 of inner tissue of each sponge material was homogenized using a sterile mortar and pestle containing 10 mL of sterile artificial sea water under aseptic conditions.

The resulting homogenate was diluted with sterile seawater at three dilutions (1:10, 1:100, and 1:1000). For fungi cultivation, 100 µL of each dilution was plated in quadruplicate onto four plates of each of the following media (**Table 1**); Czapek-Dox Yeast Agar medium (NaNO3 3 g, KCl 0.5 g, K_2HPO_4 0.1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $FeSO_4$ 0.01 g, sucrose 30 g, agar 20 g, pH 6.7); Malt Agar medium (Malt extract 17 g, peptone 3 g, agar 20 g) and Sabouraud Dextrose Agar medium. All media were amended with 2% NaCl and 0.25% chloramphenicol as antibacterial agent to

Table 1. Antimicrobial Activity of the Extracts from fermentation broth and mycelia of the fungi derived from the Red Sea marine sponges *H. erecta* and *H. erectus*

Host Sponge	Fungal Strain Genus	Strain	Culture Media	*E. coli* Broth	*E. coli* Mycelia	*S. aureus* Broth	*S. aureus* Mycelia	*C. albicans* Broth	*C. albicans* Mycelia
Hyrtios erecta	*Penicillium*	S001	CZYB	−	−	−	−	++	++
	Penicillium	S002	CZYB	−	−	−	−	++	++
	Penicillium	S003	CZYB	−	−	−	−	++	++
	Aspergillus	S004	CZYB	−	−	−	−	+	+
	Fusarium	S005	CZYB	−	−	++	++	++++	++++
	Penicillium	S006	SDB	+++	+++	+++	+++	++++	++++
	Aspergillus	S007	SDB	−	−	−	−	++	++
	Choiromyces	S008	SDB	−	−	−	−	++	++
	Penicillium	S009	MEB	++	++	−	−	++	++
	Aspergillus	S010	MEB	++	++	++	++	++	++
Hyrtios erectus	*Penicillium*	S011	SDB	−	−	+++	+++	−	−
	Penicillium	S012	CZYB	−	−	+++	+++	++++	++++
	Fusarium	S013	CZYB	++	++	++	++	++++	++++
	Penicillium	S014	CZYB	++	++	++	++	++++	++++
	Aspergillus	S015	SDB	++	++	+++	+++	++++	++++
	Penicillium	S016	MEB	++	++	−	−	++	++
	Penicillium	S017	SDB	+++	+++	+++	+++	++++	++++
	Aspergillus	S018	SDB	−	−	++	++	++++	++++
	Aspergillus	S019	MEB	++	++	−	−	++	++
	Aspergillus	S020	SDB	++	++	+++	+++	++++	++++

Note: CZYB: Czapek-Dox Yeast Broth; MEB: Malt Extract Broth; SDB: Sabouraud Dextrose Broth.

Extracts tested 200 µg/6 mm disc; Inhibition zone in mm including disc; Growth inhibition diameters were used to define the categories of bacterial growth inhibition: −: no inhibition was detected; +: growth inhibition diameter less than 7 mm; ++: between 7 and 10 mm; +++: between 10 and 15 mm; ++++: more than 15 mm.

prevent bacterial growth and to enrich fungi growth. Plates were wrapped in parafilm, incubated at 28 ºC for 1–3 weeks until the morphology of fungi could be distinguished and checked after fungi growth. Many purification steps were done until pure fungal isolates were obtained.

3.3. Extraction of Genome DNA from Cultured Fungal Isolates S001–S010

The distinct fungi isolates (S001–S010) from the sponge H. erecta described in the above paragraph were cultured in corresponding broth at 28 ºC for 2–5 days. The mycelia were harvested separately by using vacuum filtration and dried with two layers of paper towel. The resulting mycelial mat was ground into powder with liquid nitrogen. The fungal DNA was extracted using QIAamp DNA Mini Kit (Qiagen) according to manufacturer's instructions.

2.4. Amplification of Fungal ITS-rDNA Fragments of Isolates S001–S010

The genomic DNA of the strains S001–S010 were used as the template to amplify fungal ITS-rDNA fragments using the primers ITS1 (5′-TCCGTAGGTGAACCTGCG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al, 1990) (which were synthesized by the University of Utah DNA/peptide synthesis core facility. The reaction mixture for PCR amplification contained 5 µL of 10 × reaction buffer with 15 mM MgCl2 (Invitrogen), 2 µL of 2.5 mM dNTPs, 0.5 µL of 10 µM each primer, 4 µL of fungal DNA, 0.3 µL of Taq DNA polymerase (5 U·µL−1, Invitrogen), and 39.7 µL of H_2O. PCR conditions included an initial denaturation at 94 ºC for 4 min followed by 30 cycles of denaturation at 94 ºC for 50 s, annealing at 51 ºC for 50 s, and elongation at 68 °C for 1 min, with a final elongation at 68 ºC for 10 min. PCR products were purified using the Agarose Gel DNA Purification Kit (Qiagen) and sequenced in at the University of Utah DNA sequencing facility.

2.5. Sequence Fungal ITS-rDNA Regions of Isolates S001–S010

For preliminary identification, sequences of fungal ITS-rDNA regions obtained from the marine sponges *Hyrtios erecta* were compared with related sequences in NCBI (National Center for Biotechnology Information). Fungal ITS-rDNA sequences acquired in this study were edited and aligned with the best n-BLAST hits from GenBank in the Clustal X (version 1.83) program (Thompson et al, 1997), and further manually adjusted using BioEdit software (Hall, 1999). The program MEGA 5 (Tamura et al, 2011) was applied to calculate the base composition of the fungal sequences. The identification of the fungal strains S001–S010 was shown in **Table 2**.

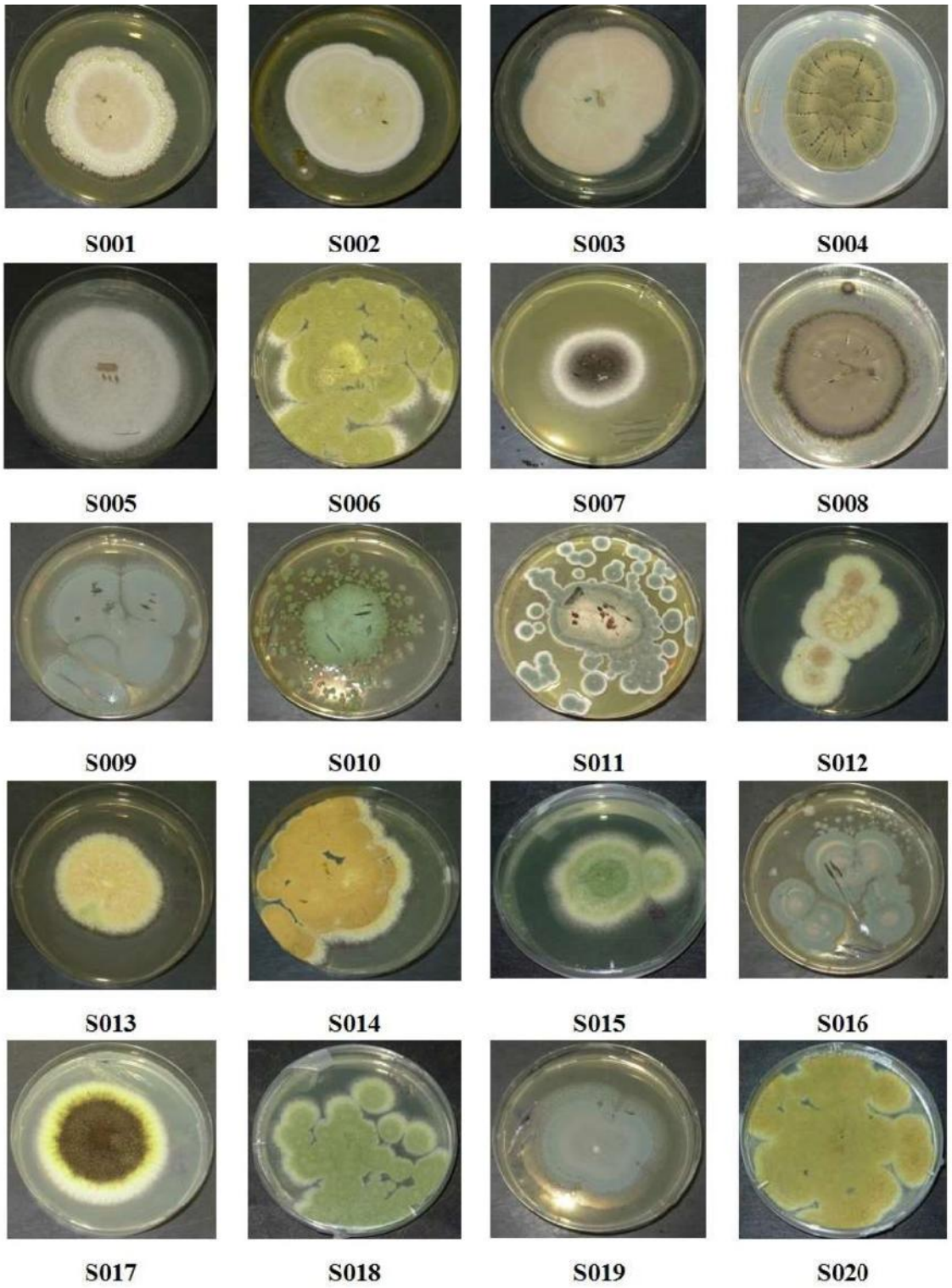

Figure 1. Morphological photos of the fungal isolates (S001 – S020) derived from the Red Sea sponges *Hyrtios erecta* and *Hyrtios erectus*

2.6. Bioassays

2.6.1. Preparation of Fungal Extracts

The fungal strains S001–S020 were inoculated into 250 mL Erlenmeyer flasks containing 50 mL of corresponding liquid media (**Table 1**) followed by shaking incubation at 28 ºC with 150 (rpm) in an orbital shaker continuously for 14 days. After the incubation, 50 mL of EtOAC were added to each flask and left overnight to stop cell growth. The fermented whole broth was filtered through cheese cloth to separate the supernatant and the mycelia. The supernatant layer was extracted 3 times (3 x 50 mL) with EtOAc. The organic portion (combined extracts) was evaporated under vacuum and the residues obtained were washed with water and then taken to dryness to obtain colored

Table 2. Identification of fungal strains isolated from the Red Sea marine sponge *H. erecta* based on morphological characteristics as well as DNA analysis of the internal transcribed spacer (ITS) region. Closest relatives to fungal strains according to BLAST search are presented

Strain	Morphological Identification	Sequence Length	Related Strain (BLAST)	Similarity (%)
S001	*Penicillium* sp.	614	*Penicillium vinaceum*	99 %
S002	*Penicillium* sp.	613	*Penicillium granulatum*	98 %
S003	*Penicillium* sp.	527	*Penicillium chrysogenum*	98 %
S004	*Aspergillus* sp.	564	*Aspergillus oryzae*	95 %
S005	*Fusarium* sp.	1059	*Fusarium proliferatum*	96 %
S006	*Penicillium* sp.	611	*Penicillium citrinum*	99 %
S007	*Aspergillus* sp.	594	*Aspergillus flavus*	100 %
S008	*Choiromyces* sp	552	*Choiromyces aboriginum*	100 %
S009	*Penicillium* sp.	551	*Penicillium crustosum*	98 %
S010	*Aspergillus* sp.	532	*Aspergillus uniguis*	83 %

crude extracts (EtOAc Extract). The mycelia were extracted with MeOH three times, and the solvent was evaporated in vacuo to obtain colored crude extracts (MeOH Extract). The resulted EtOAc and MeOH extracts were lyophilized and stored for biological screening.

2.6.2. Antimicrobial Activity of Fungal Extracts of Isolates S001–S020

Three pathogenic microorganisms were used for the antimicrobial assay: *E. coli* ATCC 25922, *S. aureus* ATCC 25923 and *C. albicans* ATCC 14053. A standard paper disk/agar diffusion assay was used (Mitscher et al, 1972). Chloramphenicol, and erythromycin were used as positive controls for antibacterial tests and nystatin for antifungal tests. After overnight culture, each tested microbe was adjusted to 2 × 108 –5 × 108 colony forming units per mL, and 0.1 mL of each culture was spread on medium of Petri dishes (Φ 9 cm). *C. albicans* was grown on SDA while nutrient agar medium was used for E. coli and S. aureus.

The ethyl acetate extracts of the culture broth as well as the methanol extracts of the mycelial biomass were tested against the three microbes. The lyophilized EtOAc and MeOH extract described in the above paragraph was dissolved in DMSO with a final concentration of 1 mg/mL. Assays were performed by placing 200 µg of the test extract onto a filter paper disk (Φ 6 mm). After drying, the disks were placed on the surface of the solidified agar layer of an assay plate. The petri dishes were placed into an incubator at an appropriate temperature and allowed to stand overnight. Activity is indicated by the presence of a clear zone of growth inhibition about the disk. Control disks were treated with solvent alone. Inhibition zones were measured in mm and the results are represented in **Table 2**.

3. Results and Discusion

3.1. Diversity of Culturable Fungi Derived from Red Sea Sponges H. erecta and H. erectus

Cultivation of fungi from the tissue of the Red Sea marine sponges H. erecta and H. erectus yielded a total of 20 isolates. Redundant isolates were excluded under the guidance of observation on morphological characteristics and 20 distinct isolates, 10 from each sponge, were identified (S001–S020; **Figure 1**, **Table 2**). There was no dominant morphotypes covering most of the strains, but strains from *Penicillium* sp. and *Aspergillus* sp. account for a large proportion of the total isolates. Twenty isolates were identified to the genus level based on morphological traits and from these only 10 isolates were cultured for genomic DNA extraction and sequencing analysis to identify the species.

3.2. Antimicrobial Activity of the Extracts from Fungal Broth and Mycelia

Antimicrobial activities of the extracts from the fermentation supernatant and mycelia of 20 isolated fungi were evaluated against a Gram-negative bacterium (*E. coli* ATCC 25922), a Gram-positive bacterium (*S. aureus* ATCC 25923) and a yeast (*C. albicans* ATCC 14053). Fungal extracts showed different levels of antimicrobial activities to at least one pathogen with their fermentation broth and/or mycelia (**Table 1**). It is worth pointing out that the

extracts of most strains displayed exceptionally high antifungal activities to *C. albicans* (growth inhibition diameters: larger than 15 mm). In addition, the extract of the strains (S006, S010, S013-S015, S017 and S020) exhibited high activities to all the pathogens (**Table 1**).

Microbes can secrete a wide variety of metabolites including intracellular and extracellular products (Konings et al, 1992). From the data in the present study, we found that some broth and/or mycelia extracts of fungi have the antimicrobial activities. This suggested that different fungi could produce intracellular bioactive metabolites or secrete extracellular bioactive compounds.

In addition, the results indicated that the fungal extracts exhibited higher inhibition activities to S. aureus than to E. coli, which might be a result of their different cell wall compositions. Many diseases are caused by Gram-positive bacteria, such as the infection of respiratory tract and skin (Kumar et al, 2007; Stulberg et al, 2002). Because of the efficacy of these extracts against S. aureus, these extracts might have the potential for drug discovery to treat these widespread diseases.

Our results of the antimicrobial assay revealed that fungi derived from the marine sponge *Hyrtios erecta* isolated in this study exhibited potential for the isolation of antibacterial and antifungal natural products.

4. Conclusions

From the Red Sea sponges *Hyrtios erecta* and *Hyrtios erectus* a total of 20 symbiotic fungi were isolated. The fungal strains were identified by morphological traits and ITS-rDNA sequences. Most of the isolated strains belong to the genera *Penicillium* and *Aspergillus*. These fungi displayed various levels of antibacterial activities to the pathogens *E. coli, S. aureus* and *C. albicans*, and some strains of the genera *Penicillium* and *Aspergillus* showed strong growth inhibition to these pathogens. The results of this study contribute to understanding microbial diversity in the sponges of the genus *Hyrtios*. Currently, there is great demand for the development of new drugs and dug leads to combat the emergence of drug resistance to the traditional antibiotics. The isolation of symbiotic fungi from the marine invertebrates and the biological evaluation of these fungi for different bioactivities will play a significant role in future research towards discovery of new drug leads.

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6. Conflict of interest

The authors report no declaration of conflict of interest.

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