The Uncultured Gamma Proteobacterium And Culturable Associated-Bacteria From Tunicate Herdmania Momus

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The Uncultured Gamma Proteobacterium and Culturable Associated-Bacteria from Tunicate *Herdmania momus*

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Abstract. Tunicate associated bacteria have been widely known as secondary metabolite producers. Beside the culturable bacteria, little is known about uncultured tunicate associated bacteria. This research aims to know the uncultured bacteria from tunicate *Herdmania momus,* to screen for antimicrobial activity from culturable *Herdmania momus* associated bacteria, and to test the crude extract for antimicrobial activity. The tunicate was successfully identified as *Herdmania momus* with accession number LC456724. The isolation of culturable bacteria resulted in 14 strains. The result 3 of the showing antimicrobial activity against gram-negative (MDR *E. coli*) and gram-positive (MRSA, MSSA, MDR *B. cereus*) bacteria. The strongest activity was from the crude extract of strain TSC 20. The 16S rDNA identification revealed the strain has the closest similarity with *Bacillus subtilis,* and the accession number is LC455706. While from the DNA of *Herdmania momus* discovered one uncultured gamma proteobacterium DNA fragment.

INTRODUCTION

Tunicates (sea squirts, ascidian) are marine sessile protochordates which take up food by filter feeding. Therefore, tunicates harbour rich microbial communities inside their body. The latest review stated that around 21 genera of bacteria from 16 families (four phyla) are cultivable form tunicates, those are genus *Acinetobacter, Agrobacterium, Candidatud* Endoecteinascidia, *Candidatus* Endolissoclinum, *Endozoicomonas, Halomonas, Hasllibacter, Pseudovibrio, Ruegeria, Stappia, Pseudomonas, Vibrio, Bacillus, Paucisalibacillus, Paenibacillus, Staphylococcus, Exiguobacterium, Rubritalea, Labilibacter, Tenacibaculum* [1]. Another research reported some additional genera, i.e. *Pseudoalteromonas, Salinicola, Pantoea,* and *Virgibacillus* [2]. Besides bacteria, there are other microorganisms such as fungi which consist of 25 genera and actinobacteria for around 16 genera [1]. The number of microbes, including bacteria, fungi, and actinobacteria cultured from tunicates, was increasing [3]. That number will keep increasing due to the development of culture-independent methods [4].

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Many types of research have been reported microbial community living in association with tunicates, with several benefits to the organisms. The potential source of natural products seemed to represent tunicate-associated microorganisms [5]. About ~8% natural products that were initially thought to originate from tunicates are produced by ascidian-associated microorganisms [6]. One of them even being used commercially for sarcoma cancer treatment by EMEA, Yondelis (Trabectedin or ET-743) which originally isolated from the colonial tunicate, *Ecteinascidia turbinata* [7]. *But from the latest research revealed the real producer is the associated microbe Candidatus Endoecteinascidia frumentensis* [8]. This microbe is unculturable using standard laboratory techniques, that's why to test the hypothesis should be using a metagenomics approach using analysis of biosynthetic gene cluster to know the real producer [9].

Another compound is Didemnin B which initially isolated from tunicate *Trididemnum solidum,*[10]*, but later known that the real producer is culturable* bacterial strains *Tistrella mobilis* and *Tistrella bauzanensis* rather than the tunicates[11,12]. Compounds which have been isolated from tunicate-associated microorganisms have many biological activities such as antimicrobial activity [13-15]. One of an antimicrobial compound which produced by tunicate associated bacteria is indole-2,3-dione (isatin), produced by a gram negative bacterium *Pseudoalteromonas rubra* TKJD 22 in association with tunicate *Atriolum* sp. [2].

In this study reported the potential of the marine bacteria associated with tunicate for the production of natural products against human pathogenic Multidrug-resistant bacteria and the discovery of uncultured bacterium in tunicate *Herdmania momus.*

EXPERIMENTAL DETAILS

Specimen Collection

The tunicate specimen was collected from Nusa Laut Sea, Maluku, Indonesia on September 2018 at depth 12.6 m by scuba diving. The sampling coordinate was 3°38.7470'S and 128°48.7090E. The specimen was stored in sterile zip-lock plastic, which previously documented and labelled. The DNA extraction and isolation of associated culturable bacteria were conducted in Tropical Marine Biotechnology, Diponegoro University, Semarang.

Specimen Identification

Extraction of DNA was conducted using Zymo Research Kit following the protocol from the supplier. PCR reaction conducted in 25 µl total reaction volume, consist of specimen DNA 1 µl, forward and reverse primers each 2.5 µl, mix PCR 12.5 µl (Lucigen master mix), and ddH_2O 6.5 µl. Primers used were according to Stefaniak et al. [16] Tun_forward 5' TCG ACT AAT CAT AAA GAT ATT AG 3' and Tun_reverse 5' AAC TTG TAT TTA AAT TAC GAT C 3', with program as follows; initial denaturation at 94 °C for 1 min, followed by 60 cycles of denaturation at 94 °C for 10 sec, annealing at 50 °C for 30 sec, elongation at 72 °C for 50 sec and final elongation at 72 °C for 10 min. PCR products were examined on a 1% agarose gel and the result was visualized using UVIDoc HD5 (UVITEC Cambridge). The good PCR products were sent to 1st base for further sequencing.

PCR Reaction for Uncultured Gamma Proteobacterium

The whole DNA extract was amplified for COI gene. PCR was performed in 25 µL volume consist of 12.5 µL Lucigen master mix, forward primer 2.5 µL, reverse primer 2.5 µL, DDH_2O 6.5 µL, and DNA template 1 µL. Primers used in this research were universal COI primer LCO1490 5' GGT CAA CAA ATC ATA AAG ATA TTG G 3'and HCO2198 5' TAA ACT TCA GGG TGA CCA AAA AAT CA 3' [17]. They have found very few overlaps with the eukaryotic DNA barcode area. This study corroborates that the *COI* gene can be a DNA barcode marker for bacteria [18].

Isolation of Culturable Associated Bacteria

The specimen was cleaned and cut as many as 5 g to be ground, then homogenized in 5 ml sterilized seawater. The result was serially diluted until reach 10^{-3}, 10^{-4} and 10^{-5}, then were spread on to desired media Zobell 2216

Himedia and modified ISP2. All isolation plates were incubated at room temperature 29±2°C for 3 – 5 days depending on the growth condition of each isolate. The isolated were purified according to the morphological appearance, i.e. shape, colour, elevation and size.

Screening for Antimicrobial Activity

The pure isolates, which were obtained from the isolation process, were grown for 3 days for production of antimicrobial compounds in respective media (Zobell 2216 Himedia and modified ISP2). The human pathogenic MDR bacteria (MRSA, MSSA, MDR *E. coli* and MDR *B. cereus*) were grown a day before the test to reach 10^8 CFU (0.5 Mc Farland). The respective test bacteria were swapped on to soft strength *Nutrient Agar* (NA) medium. Agar blocks from the grown isolated were cut and placed on to it. Cultures were kept in 4°C for an hour before incubation in 37 °C for overnight. The isolates were considered as antimicrobial producers if the zone of inhibition more than 11 mm[19]. The isolates were classified as low, intermediate and strong antimicrobial producers as follows; low antimicrobial producer if zone of inhibition (ZOI) 7 – 11 mm, intermediate antimicrobial producer if ZOI 12 – 16 mm, strong antimicrobial producer if ZOI 17 – 21 mm and very strong antimicrobial producer if ZOI ≥21 mm [2].

16s rDNA Identification and Gram Staining

The isolate with the strongest antimicrobial producer was grown in the plate for overnight and prepared for DNA extraction. The colonies were picked into microtube-contained saponin. The whole step was following Chelex DNA extraction method with some modification [20]. PCR reaction was performed in 25 µl volume, consist of 12.5 µl Lucigen Master Mix, forward and reverse primer each 1 µl, ddH$_2$O 9.5 µl and DNA template 1 µl. The universal primer for 16S rDNA identification was PA 5'-AGA GTT TGA TCC TGG CTC AG-3' and PH 5'-AAG GAG GTG 146 ATC CAG CCG CA-3'. PCR Reaction was conducted in a MJ Mini Personal Thermal Cycler (BIO-RAD) using optimation consist of initial denaturation at 95 °C for 3 min, then followed by 34 cycles of denaturation at 95°C for 45 s each cycle, annealing at 50 °C for 1 min, extension at 72°C for 90 s, and followed by final extension at 72°C for 5 min [2]. PCR products were examined using agarose 1 % gel electrophoresis, and the result was visualized by using UVIDoc HD5 (UVITEC Cambridge).

The gram staining test was performed to identify the isolates belong to gram-positive or gram-negative bacteria. The method used was, according to Benson [21], prepared four solution Gram A (crystal violet), gram B (Iodine), gram C (decolourizer) and gram D (safranin). All solutions were provided from Gram Stain-Kit Himedia®.

Crude Extract Generation

The secondary metabolite extraction was started by preparation of preculture, from the plate to 150 ml Erlenmeyer flask containing 50ml of the respective medium. The precultures were incubated at room temperature 29±2 °C with 110 rpm for 24 h. Then, 1% (v/v) from pre-culture was transferred to 500 mL Erlenmeyer flask containing 200 mL of the same medium. The culture was fermented for four days with 110 rpm in the shaker. To generate extract, the same volume (1:1) of solvent Ethyl Acetate was poured on to the culture at harvest time, and the mixture was shaken thoroughly for short maceration. The organic phase was separated from the water phase using a separatory funnel, then evaporated using rotary evaporator until completely dried.

Antimicrobial Activity of Extract

Disk Diffusion assay: Mueller Hinton Agar (Oxoid) was prepared and the respective pathogen (MDR *E. coli,* MDR *Bacillus cereus,* MSSA and MRSA) were grown overnight. After that, as many as 0.5 Mc Farland (Himedia) of the pathogens were swapped on to MHA medium. 15 µL of an ethyl acetate extract (150 µg/disk) were added to a paper disk, then positioned on an agar plate. The plates were incubated in 37 °C for overnight. The resulted antimicrobial activity was measured using Vernier calliper, than being recorded. The isolates which have an inhibition zone greater than 11 mm were considered as having antimicrobial activity [19].

RESULTS

Specimen Identification

The specimen was collected from Nusa Laut Sea, Maluku, Indonesia, as documented in figure 1. According to COI gene identification, the solitary ascidian 18-SM-N-24 was closely related to *Herdmania momus* (table 1). But the ident value was only 82%, it might be that the specimen was in the same genus but different species. *H. momus* is one of the most commonly encountered species of tunicates. This solitary ascidian is sometimes referred to as the *red-throated ascidian* [22], as its two siphons are a vivid red due to the presence of tunichromes in the blood cells. Similar to all tunicates, *Herdmania momus* is a sessile filter feeder. It is commonly found attached to rocks from depths of 3–50 meters (10–160 ft). In this collection, *Herdmania momus* was found in depth of 12.6 m.

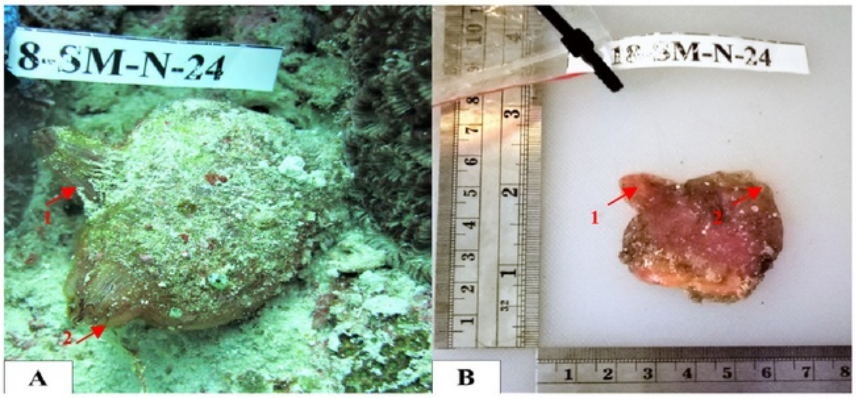

FIGURE 1. Underwater documentation (A) Outside water documentation (B). Whereas 1 (atrial siphon) and 2 (oral or branchial siphon).

Tunicates found in Nusa Laut Sea Maluku were different from what was found in Karimunjawa National Park, Jepara. Based on previous research conducted by Ayuningrum [2], none of the genus *Herdmania* was found in Karimunjawa but we found the other genera *Ascidia, Atriolum, Clavelina, Didemnum,* and *Lissoclinum.* Geographical distribution and water quality might affect the kind of species living in that place. The tunicates found in Nusa Laut specifically could not be found in Karimunjawa i.e. *Herdmania momus* and *Polycarpa aurata* [23].

The molecular identification of specimen 18-SM-N-24 supports the morphological characteristic which revealed the closest relativity of specimen 18-SM-N-24 as many as 82 % with *Herdmanis momus.* Because of the ident value of less than 90%, it might change the name of species in the future. Therefore, further research needed to follow up the species.

TABLE 1. BLAST homology result

Specimen	Closest relativity	Ident	Accession Number
18-SM-N-24	*Herdmania momus*	82%	LC455706

Herdmania momus (Savigny, 1816) belongs to the genus *Herdmania,* family *Pyuridae,* Order *Stolidobranchia* and Class *Ascidiacea.* The class *Ascidiacea* includes sedentary tunicates, which are simple/solitary or Compound/colonial in nature. Both siphons (branchial and atrial) are pointed upward in direction. *H. momus* is a solitary ascidian, with the tunic is tough and leathery, provides a habitat for several epibionts. The acicular spicules of the body wall concentrate mainly between the siphons and in the ventral region. The species are pink or light red in living condition [22]. The phylogenetic tree of specimen 18-SM-N-24 was shown in figure 2. Based on the phylogenetic tree, we can see that 18-SM-N-24 lied in the same clade with *H. momus.*

Phylogenetic tree

Species of *H. momus* known as antimicrobial producers. Mary et al [24] reported that crude extract of *H. momus* showed antimicrobial activity against human and shrimp pathogenic bacteria. Another research reported the antimicrobial activity from *H. momus* crude extract against human pathogen *Staphylococcus aureus, Escherichia coli* and *Candida albicans* [25]. Beside antimicrobial activity, some new methylsulfinyladenosine derivatives compounds having activity against a series of human pathogenic viruses [26].

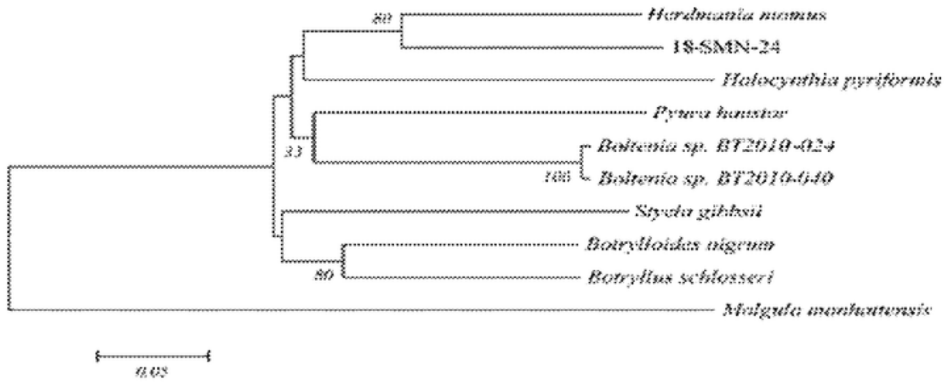

FIGURE 2. Phylogenetic tree of specimen 18-SM-N-24

Uncultured associated bacteria

The specimen also contained an uncultured *gamma proteobacterium* inside its body. BLAST homology revealed the identity value of the bacterium (table 1).

TABLE 2. BLAST homology result

Specimen	Closest relativity	Ident
18-SM-N-24	*Uncultured gamma proteobacterium*	86%

The discovery of uncultured *gamma-proteobacterium* detected by using COI gene primers indicate that in the *H. momus* lived an associated with bacteria which have the ability to do assimilation (autotrophic bacterium) [27]. Until now, most of drug-producing associated microbes still uncharacterized, because of> 99% of prokaryotic species currently no able of being cultured in the laboratory using standard protocol [9].

Cultivated bacteria and Screening for antimicrobial activity

The total of 14 bacterial strains was successfully cultivated from a tunicate *H. momus.* All strains were tested for antimicrobial activity against multi-drug resistant bacteria. A total of 3 strains (21%) were active against gram-positive bacteria (table 3).

TABLE 3. Screening result of culturable *Herdmania momus* associated bacteria

No.	Strain	Activity against			
		MRSA	**MSSA**	**MDR *B.Cereus***	**MDR *E. coli***
1.	TSA 33	-	-	-	-
2.	TSA 34	-	-	-	-
3.	TSC 19	-	++	-	-
4.	TSC 20	++	++	++	-
5.	TSC 21	-	-	-	-
6.	TSC 22	+	+	+	-
7.	TSC 23	-	-	-	-
8.	TSC 24	-	-	-	-
9.	TSC 25	-	-	-	-
10.	TSC 26	-	-	-	-
11.	TSC 27	-	-	-	-
12.	TSC 28	-	-	-	-
13.	TSC 29	-	-	-	-
14.	TSC 30	-	-	-	-

The Test microorganisms: MRSA (Methylcillin Resistant *Staphylococcus aureus*), MSSA (Methylcillin Sensitive *Staphylococcus aureus*), MDR (Multi-drug resistant). Sign: *Disk diffusion, - = no Zone of Inhibition (ZOI), + = ZOI 7-11 mm, ++ = ZOI 12 – 16 mm, +++ = ZOI 17 – 21 mm, and ++++ = ZOI ≥ 21mm.

Molecular 16 S rDNA Identification, Phylogenetic Tree and Gram Staining

The most active strain, TSC 20, was closely related to *Bacillus subtilis* (table 4) as compared to the data of 16S rDNA sequencing analysis, which was deposited in NCBI.

TABLE 4. BLAST homology result

Specimen	Closest relativity	Ident	Accession Number
TSC 20	*Bacillus subtilis*	99%	LC456724

Bacillus subtilis TSC 20 is in the clade with *B. methylotropicus,* yet in the different clade with *B. velezensis, B. amyloliquifaciens* and *B. vallimortis* (figure 3). Genus *Bacillus* is wel known for its ability produce secondary metabolite functions as antimicrobial. *Bacilli* produce a wide range of natural products, including lipopeptides, polypeptides, macrolactams, fatty acids, polyketides, and isocoumarins, with a series of bioactivities kJ9 [28-29].

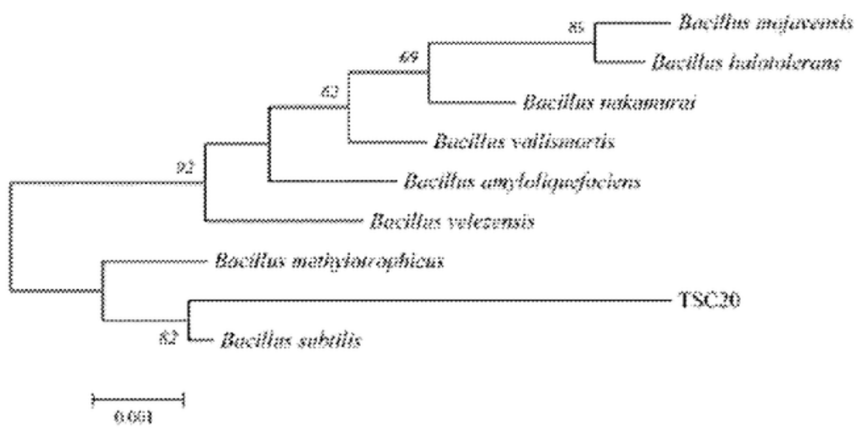

FIGURE 3. Phylogenetic tree of strain TSC 20

The gram staining result for *B. cubtilis* TSC 20 was shown in figure 4, which the cells stained purple (gram-positive bacterium).

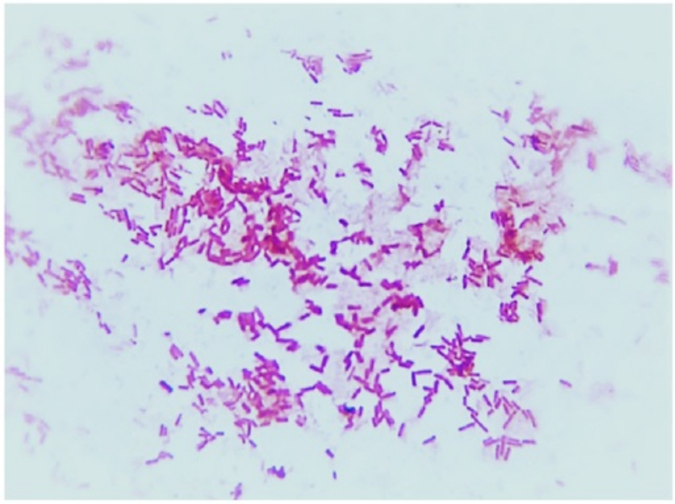

FIGURE 4. Gram staining result of TSC 20

Antimicrobial Activity from Extract

The crude extract from the active strains was tested again and resulted only *B. subtilis* TSC 20 retained the antimicrobial activity (figure 5). The concentration of crude extract was 150 μg/disk. Strain *B. subtilis* TSC 20 was able to inhibit MDR *B. cereus* with a zone of inhibition (ZOI) 13.00 mm, MRSA with ZOI 13.64 mm and MSSA with ZOI 13.24 mm. The diameter of the paper disk was 6 mm.

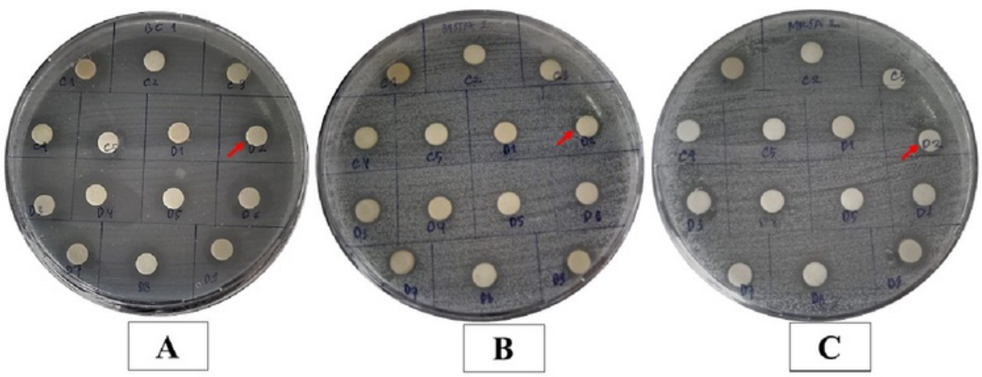

FIGURE 5. The antimicrobial activity result against (A) MDR *B. cereus,* (B) MSSA, and (C) MRSA.

In the present investigation, the metabolites produced by the marine bacteria associated with the tunicate *H. momus* was evaluated for anti-multidrug resistant activity. This research pointed out the potential of metabolites produced in combating the MDR *E. coli,* MDR *B. cereus, MRSA* and MSSA. Separation of individual compounds is underway to find out the exact compound responsible for its potential anti-multidrug resistant activity. As most of the tunicate-associated microorganisms derived metabolites are still not utilized at a great level for the development of drugs as well as for the infection caused by a bacterial pathogen. The present report on the bioprospecting of the bacteria associated with the tunicate *H. momus* will lead to the discovery of a single bioactive compound.

SUMMARY

The tunicate 18-SM-N-24 was collected from Nusa Laut Sea, Maluku which based on morphological and molecular identification using COI gene amplification showing 82% ident to Herdmania momus. A total of 14 bacterial strain were successfully cultivated on agar media Zobel 2216 and ISP2, which then prospected for antimicrobial activity test. The test resulted in three bacterial (TSC 19, TSC 20 and TSC 21) strains showing antimicrobial activity. From those three strain were the cultivated in liquid medium respectively to be extracted using ethyl acetate. The antimicrobial test resulted in one most outstanding antimicrobial activity from bacterial strain TSC 20. Based on morphological identification, it belonged to gram-positive bacterium. The 16S rDNA identification and BLAST analysis showed TSC 20 has 99% similarity with Bacillus subtilis. Another surprising result from this research is the discovery of DNA gene fragment of the uncultured gamma *proteobacterium* form this specimen. It is indicated that inside tunicate there has a lot of microbial symbionts whether it is culturable or not. Bioprospecting them for lead compounds discovery is an interesting future research topic, whether through direct isolation or dependent culture of independent culture using a metagenomics approach.

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