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Sponge-Associated Actinobacteria: Morphological Character and Antibacterial Activity against Pathogenic Bacteria

Aktinobakteri yang Berasosiasi dengan Spons: Karakter Morfologi dan Aktivitas Inhibisi terhadap Bakteri Patogen

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Sponge-associated actinobacteria may diverse and have potency to produce bioactive compounds. Diversity and antimicrobial activity of indigenous sponge-asccociated actinobacteria isolated from marine ecosystem in Indonesia have not much been explored. This work aimed to assess morphological and antibacterial activity of sponge-asccociated actinobacteria. The morphological characteristics were examined based on their color of aerial and substrate mycelia, and pigmentation, while antibacterial activities were assayed using antagonist technique. Selected actinobacterial isolate was identified using 16S rRNA gene. Various sponge-associated actinobacteria were successfully isolated from *Hyrtios* sp., *Callyspongia* sp., and *Neofibularia* sp. sponges. A total of 62 actinobacterial isolates were obtained, and each isolates showed variety of morphological characters, which could be seen in aerial mass color, substrat mass color, and pigmentation. Actinobacterial isolates were tested against human pathogenic bacteria, i.e. *Staphylococcus aureus* and Methicilline Resistant *S. aureus*, representing Gram-positive, and *Escherichia coli* EPEC K1-1 and *Shigella dysenteriae*, representing Gram-negative. Most of actinobacterial isolates had antimicrobial activities at least against one of pathogenic bacteria. High activity was shown by NOHa.2, isolated from *Neofibularia*, and HRHa.5 isolated from *Hyrtios*. The NOHa.2 showed the highest antimicrobial activity against *S. dysenteriae*, meanwhile, HRHa.5 showed antimicrobial activity against 3 of 4 tested bacterial pathogens. These data showed diversity of sponge-asccociated actinobacteria from marine ecosystem in Indonesia, and several of them have potency as source of antibacterial compounds.

Key words: *Actinobacteria, antibacterial activity, morphological colony, 16S rRNA gene, sponge*

INTRODUCTION

Actinobacteria is a group of filamentous, Gram-positive bacteria which are well known to produce secondary metabolites, that can function as antifungal, antibacterial, antitumor, anticancer, antiviral, herbicides, antidiabetic, anthelmintic as well as antiprotozoal (Zheng *et al.* 2000; Solanki *et al.* 2008; Basha *et al.* 2009; Sunaryanto *et al.* 2010). Actinobacteria can be found in both terrestrial and aquatic environments. Aquatic or marine actinobacteria have also known to produce secondary metabolites which can be developed as microbial bioactive compound producer for health purposes.

Sponges are an invertebrate belongs to phylum *Porifera*, which is an important component of benthic communities in aquatic environments. More than 6,000 species of sponges inhabit various ecosystems of sea water and fresh water, and found in tropics, subtropics and polar areas. These days, sponge has been the focus of much scientific study, for its associations with various microbes (Taylor *et al.* 2007). Sponge are also known as one of marine animals that have a great potential to generate new bioactive compounds with different efficacy. A total of 200 new bioactive compounds have been isolated from sponges (Abdelmohsen *et al.* 2010). More than 190 compounds with broad biological activities were isolated in *Haliclona*. These compounds include anti-fouling, antimicrobial, antifungal, antimalarial and cytotoxic activity (Kennedy *et al.* 2008).

Sponge is one of marine animals that can be associated with actinobacteria. More than 30 genus of sponges are known to associate with actinobacteria (Xi *et al.* 2012). Sponge-associated actinobacteria is suspected to be the dominant producer of bioactive compounds. Several genera of actinobacteria which known to associate with sponge were *Mycobacterium,*

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Micrococcus, Micromonospora, Microbacterium, Brevibacterium, Kocuria, Corynebacterium, Rhodococcus, Brachybacterium, Rubrobacter, Streptomyces, Dietzia, Salinispora, Actinokineospora, Gordonia, Arthrobacter, Nocardiopsis, Rothia (Abdelmohsen *et al.* 2010). Actinobacteria which inhibit aquatic environment allegedly produces different secondary metabolites compared with actinobacteria in terrestrial environment. A total of 4 new polyketide compounds i.e. Salinipirones A and B; and Pacificanones A and B were found in *Salinispora pacifica* (Oh *et al.* 2008).

Bacterial infectious diseases is one of the leading causes of death which are prevalent in tropical countries like Indonesia with a high number of patients. The emergence of this infectious disease allegedly caused by socio-economic factors, such as environment and ecological factors. Bacterial pathogens that cause human infections, for example are *Escherichia coli, Shigella dysenteriae, Staphylococcus aureus*, and Meticillint resistant *S. aureus*. In Indonesia, one of bacterial infectious diseases that often attack people is diarrhea. In 2014, more than 8 million cases of diarrhea were reported (Kementerian Kesehatan, 2014).

Indonesia is an archipelago country which is rich in diversity of marine biota, including variety of sponges. However, there are little information or reported data about sponge-associated actinobacteria and their potency. Based on these phenomenon, the research of potential sponge-associated actinobacteria in controlling human pathogenic bacteria is an important strategy.

MATERIALS AND METHODS

Materials. Materials used were sponge samples collected by SCUBA diving in shallow waters at a depth of 4 to 8 meters in Bira Island (5° 23' - 5° 40' S, 106° 25' 106° 37' E) which is an island in *Taman Nasional Kepulauan Seribu* (5° 24 '- 5° 45' S, 106° 25' - 106° 40' E).

Isolation and Characterization of Sponge-Associated Actinobacteria. Sponge samples were cut and crushed in a sterile mortal and weighed. A total of 1 g sample was put into 9 mL of sterile sea water and homogenized. Dilution is done up to 10-3 dilution. A total of 0.1 mL suspension was plated on a sterile growth medium and incubated for 2-8 weeks. The growth media used were Humic acid with Vitamin (HV), HV with sea water, and sea water agar (M5-S). Actinobacteria isolates which were grown on that media, were then purified on Yeast Starch Agar (YSA). The media was supplemented with antibiotics cicloheximide (100 mg/mL), nystatin (25 mg/mL), and nalidixic acid (25 mg/mL). Morphological characterization was conducted by observing aerial mass color, substrate mass color, elevation, and pigmentation on the media (Dharmaraj *et al.* 2010).

Antagonistic Test of Actinobacteria against Pathogenic Bacteria. Pathogenic bacteria used in this study were *Shygella dysenteriae*, *Escherichia coli* EPEC K1-1, *Staphylococcus aureus* and Meticilline Resistant *S. aureus* (MRSA). The MRSA and *S. dysenteria* were obtained from Faculty of Medical Microbiology, University of Indonesia. The MRSA have resistancy to methicillin antibiotic. *E. coli* EPEC K1-1 was obtained from Microbiology Laboratory of Bogor Agricultural University. *E. coli* EPEC K1-1 has a resistance to ampicillin antibiotics.

Pure actinobacterial isolates were obtained after around 7-14 days grown on YSA as the purification media. Actinobacterial isolates were taken with a cork borer and placed on media Nutrient Agar (NA) which had previously been inoculated with the testing bacteria (10^6cfu/mL). The plate was then incubated at 37ºC for 18-24 hours. Actinobacteria with the most potential was then used for further testing (Kitouni *et al.* 2005).

Identification of Actinobacteria based on 16S rRNA Gene. DNA was isolated using a DNA extraction kit (Geneaid). The DNA obtained was then amplified using PCR. Primers used were 20F (5' – GATTTTGATCCTGGCTCAG - 3') and 1500R (5' - GTTACCTTGTTACGACTT - 3') (Weisburg *et al.* 1991). PCR steps were pre denaturation for 5 min at 94°C, denaturation for 44 seconds at 94°C, annealing for 1 minute at 54°C, elongation for 90 seconds at 72°C, post-elongation for 7 minutes at 72°C and cooling for 15 minutes at 4°C. This process was performed with total of 30 cycles (Zhang *et al.* 2013).

Visualization of the 16S rRNA gene from PCR were seen in 1% agarose gel electrophoresis in TAE buffer (Tris Acetic EDTA) 1x. Gel electrophoresis then placed on a UV illuminator to see the DNA band (visualization). PCR products were sequenced using a sequencing services company. Analysis of the sequence data was performed by doing BLAST of nucleotide sequences analysis, based on the available data at the GenBank, NCBI http://www.ncbi.nlm-nih.gov/. Furthermore, the similarity relation could be seen by constructing phylogenetic trees based on Neighbor-Joining method, with a bootstrap value of 1000x.

RESULT

Morphological Diversity of Sponge-Associated Actinobacteria. A total of 62 isolates of sponge-associated actinobacteria were successfully isolated. Amongst them, there were 36 isolates from *Hyrtios* sp. (Table 1), 13 isolates from *Callyspongia* sp. (Table 2), and 13 isolates from *Neofibularia* sp. (Table 3) which show various morphological characteristics based on aerial and substrate mycelia, pigmentation and colony elevation. The dominant color of aerial mycelium color was cream and some isolates looked gray. Meanwhile he dominant color of substrate mycelium was light brown. The actinobacterial isolates also generally did not produce pigmentation in medium.

Table 1. Morphological characteristics of Sponge-Associated Actinobacteria with *Hyrtios* sp.

Isolates	Aerial mycelium	Substrate mycelium	Pigmentation	Elevation
HRHa.1		Light brown	-	Raised
HRHa.2		Light brown	-	Raised
HRHa.3		Dark brown	-	Flat
HRHa.4		Brownish yellow	-	Flat
HRHa.5		Light brown	-	Flat
HRHa.6		Grey	-	Raised
HRHa.8		Light brown	-	Raised
HRHa.9		Light brown	-	Raised
HRHa.10		Light brown	-	Raised
HRHa.12		Light brown	-	Flat
HRHa.13		Light brown	-	Raised
HRHa.14		Light brown	-	Raised
HRHa.1\5		Light brown	-	Raised
HRHs.1		Light brown	-	Raised
HOHs.1		Grey	-	Raised
HOHs.2		Grey	-	Raised
HOHs.3		Greyish purple	-	Raised
HOHs.4		Dark brown	-	Raised
HOHs.5		Grey	-	Raised
HOHs.6		Grey	-	Raised
HOHs.7		Light brown	-	Raised
HOHs.8		Light brown	-	Raised
HOHs.9		Light brown	-	Raised
HOHa.1		Light brown	-	Raised
HOHa.2		Light brown	-	Raised
HOHa.3		Yellowish brown	-	Flat
HOHa.4		Yellowish brown	-	Flat
HOHa.5		Light brown	-	Raised
HOHa.6		Light brown	-	Raised
HOHa.7		Light brown	-	Raised
HOHa.8		Brwonish yellow	-	Flat
HOHa.9		Light brown	-	Raised
HOHa.10		Dark brown	-	Raised
HKHa.1		Pink keabu-abuan	-	Flat
HKHa.2		Black	-	Raised
HKHa.3		Grey	-	Raised

Table 2. Morphological characteristics of Sponge-Associated Actinobacteria with *Callyspongia* sp.

Isolates	Aerial mycelium	Substrate mycelium	Pigmentation	Elevation
CRHa.1	Cream	Light brown	-	Rised
CRHa.2	Cream	Light brown	-	Rised
CRHs.1	Cream	Light brown	-	Rised
CRHs.2	Cream	Dark brown	-	Flat
COHa.1	Grey	Grey	-	Rised
COHa.2	Cream	Light brown	-	Rised
COHa.3	Cream	Light brown	-	Flat
COHa.4	Cream	Light brown	-	Flat
COHa.5	Black	Black	-	Rised
COHa.6	Cream	Light brown	-	Rised
COS.1	Cream	Light brown	-	Rised
COS.2	Cream	Light brown	-	Rised
CKHs.1	Grey	Black	Dark brown	Rised

As can be seen in Figure 1, actinobacteria isolated from those three sponges, which were originated from Bira Island, have various morphological characters. They produced abundance of aerial mycelia.

Table 3. Morphological characteristics of Sponge-Associated Actinobacteria with *Neofibularia* sp.

Isolates	Aerial mycelium	Substrate mycelium	Pigmentation	Elevation
NOHa.1	Grey	Grey	-	Raised
NOHa.2	Cream	Dark brown	-	Flat
NOHa.3	Cream	Dark brown	-	Raised
NOHa.4	Cream	Dark brown	-	Raised
NOHa.5	Cream	Light brown	-	Flat
NOHa.6	Cream	Light brown	-	Flat
NOHs.1	Cream	Dark brown	-	Raised
NOHs.2	Cream	Light brown	-	Flat
NRHa.1	Cream	Light brown	-	Raised
NRHa.2	Cream	Dark brown	Brown	Raised
NRHs.1	Cream	Light brown	-	Flat
NRHs.2	Cream	Light brown	-	Raised
NRHs.3	Cream	Light brown	-	Raised

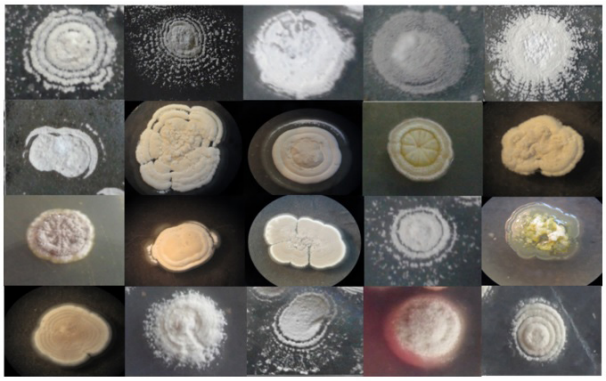

Figure 1. Sponge-associated Actinobacteria colonies grown in YSA medium for 10-14 days

The Inhibition Activity of Sponge-Associated Actinobacteria against Pathogenic Bacteria. Sponge-associated actinobacteria were generally able to inhibit the growth of *S. dysenteriae*, some isolates were able to inhibit the growth of *E. coli* and *S. aureus,* but no isolates were able to inhibit the growth of MRSA (Table 4, 5 and 6). Two isolates that had the highest activity were HRHa.5 from *Hyrtios* sp.which capable of inhibiting the growth of *E. coli* (5 mm), *S. dysenteriae* (5 mm), and *S. aureus* (1 mm) (Table 4), and NOHa.2 from Neofibularia sp. which had the highest inhibitory activity (14 mm) against *S. dysenteriae* (Table 6). The antibacterial activity of selected actinobacterial isolates were found to be varied.

Identification of Sponge-Associated Actinobacteria Based on 16S rRNA Gene. Molecular work in identifying the HRHa.5, showed that the PCR product containing 16S rRNA gene can be amplified and visualized on an agarose gel (Figure 2). Comparation of the sequence of 16S rRNA gene of HRHa.5 with the available reference strains in Genbank showed similarity to *Streptomyces sampsonii* strain NRRL B12325 (94%), *S. champavati* B-5682 strains (94%), and *S. albidoflavus* DSM 40455 strains (93%).

The phylogenetic tree analysis showed that HRHa.5 had closed relationship wth *Streptomyces* spp. genus (Figure 3). In this phylogenetic tree

Table 4. The inhibition potency of Actinobacteria associated with *Hyrtios* sp.

Isolate	Diameter of clear zone (mm)			
	S. dysenteriae	*E. coli*	*S. aureus*	*MRSA*
HRHa.2	4	-	-	-
HRHa.3	4	-	-	-
HRHa.5	5	5	1	-
HRHa.9	7	3	1	-
HRHa.10	7	-	-	-
HRHa.14	6	-	-	-
HRHa.15	4	-	-	-
HOHa.1	4	-	-	-
HOHa.2	4	4	1	-
HOHa.3	9	-	1	-
HOHa.5	3	-	-	-
HOHa.6	4	-	-	-
HOHa.7	5	-	1	-
HOHa.10	5	-	-	-
HOHs.2	-	3	-	-
HOHs.4	4	-	1	-
HOHs.7	6	3	1	-
HOHs.8	4	-	1	-
HOHs.9	5	4	1	-

Table 5. The Inhibition Potency of Actinobacteria Associated with *Callyspongia* sp.

Isolate	Diameter of clear zone (mm)			
	S. dysenteriae	*E. coli*	*S. aureus*	*MRSA*
CRHa.1	5	-	-	-
COHa.2	6	-	-	-
COHa.3	4	-	-	-
COHa.4	5	-	-	-
COHa.9	9	-	1	-
CRHs.1	8	-	-	-
CRHs.2	12	-	-	-

Table 6. The inhibition potency of actinobacteria associated with *Neofibularia* sp.

Isolate	Diameter of clear zone (mm)			
	S. dysenteriae	*E. coli*	*S. aureus*	*MRSA*
NRHa.1	6	-	-	-
NOHa.2	14	-	-	-
NOHa.3	11	-	-	-
NOHa.4	8	-	-	-
NOHa.5	5	-	-	-
NOHa.6	3	-	-	-
NRHs.1	10	-	-	-
NRHs.2	9	-	-	-
NRHs.3	12	-	-	-
NOHs.1	12	-	-	-
NOHs.2	9	-	-	-

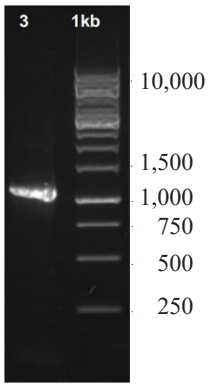

Figure 2. The electropherogram of amplified PCR product containing of 16S rRNA Gene.

construction, *Micromonospora* sp. has diferent group position, and the outgroup used was *Bacillus subtilis* strain KCTC 3135, a non actinobacteria.

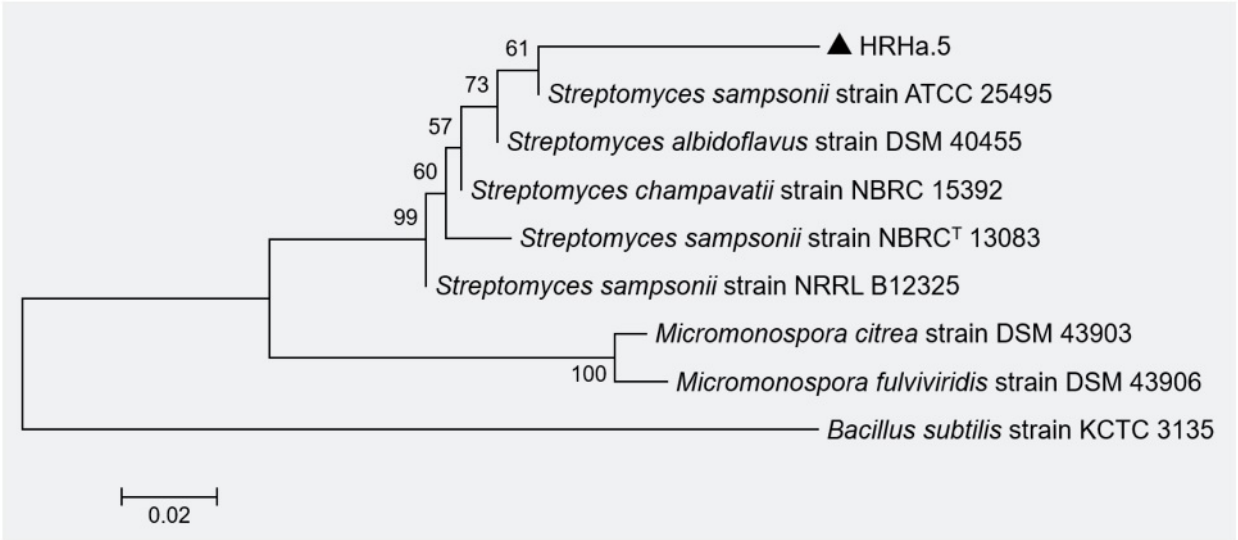

Figure 3. Phylogenetic tree of HRHa.5 based on 16S rRNA gene showing its position amongst others

DISCUSSION

Actinobacteria which were isolated from three sponges from Bira Island showed to have various morphological characteristics, based on their aerial and substrate mycelia, pigmentation and colony elevation. Based on these morphological characteristics, sponge-associated bacteria from Bira Island were diversed. It has been reported that difference color of mycelia produced by actinobacteria can be used to indicate their diversity. It has been reported that different species may produce different physiological characters (Remnya and Vijaykumar 2008; Dharmaraj *et al.* 2010) which can partly be represented by various color of mycelia, pigmentation and spore chain morphology. Most of the sponge-associated actinobacteria described here are able to produce abundance of aerial mycelia, which may relate to the *Streptomyces* spp. characters (Shirling and Gotlieb 1966). From three sponges used as actinobacterial host, the number of isolated actinobacteria were found higher, i.e. 36 isolates from *Hyrtios* sp., while each of *Calyspongia* and *Neofibularia* sp. yielded 13 actinobacterial isolates, respectively. The number and diversity of sponge ascociated actinobacterial community may be influenced by kind of sponge as host that provide micro-environment suitable for for the associative actinobacteria.

Actinobacteria were mostly obtained from HV medium compared with the other two media used in this study. The HV medium is a selective media for actinobacteria, supplemented with antimicrobial substances like nystatin and nalidixic acid, which can function to controll the growth of non actinobacteria. Moreover, humic acid in the medium can be utilized as source of nutrition (organic matters) for the growth of actinobacteria. The HV medium is one of the most commonly used media in isolating actinobacteria (Zhang 2011; Jiang *et al.* 2013). Only a total of 2 actinobacterial isolates were obtained from M5-S medium, which might be caused by the low nutrition present in the M5-S medium.

Based on the antinobacterial data obtained, a total of 37 isolates (59.67%) were able to inhibit the growth of at least one of tested pathogenic bacteria. This data suporting the research results reported by Kennedy *et al.* 2008, and Zheng *et al.* 2000 where 50% and 43.6% respectively; the actinobacterial isolates obtained showed antimicrobial activity. Moreover, the results of Xi *et al.* (2012) also showed that 27.5% of the isolates obtained showed antimicrobial inhibitory activity. The ability to inhibit the growth of pathogenic bacteria were different in each isolates. It might be occured due to the difference of metabolite compounds produced by the isolates.

Antimicrobial inhibitory activity can be broad-spectrum, narrow-spectrum, or representing activity against specific pathogens (Dharmaraj *et al.* 2009). The microbial activity produced by HRHa.5 may have a broad activity spectrum because it could inhibit both Gram positive and Gram negative. On the other hand, NOHa.2 showed to have a narrower spectrum or had activity against only for a particular pathogen. Antimicrobial compounds inhibit the groath of bacterial target can be based on various mechanisms, e.g. inhibiting synthesis of cell wall, membrane cell, DNA, or protein.

Hyrtios sp. has been reported to produce antimicrobial compounds and anticancer (Yousseff *et al.* 2013). Meanwhile, bioactive compounds produced by *Callyspongia* sp. may function as antimicrobes, antiprotozoa and antifouling (Dobretsov *et al.* 2004). For the bioprospecting of sponge-associated microbes, the sponges used were from the genus *Xestospongia, Halichondria, Haliclona*, and *Mycale* (Indraningrat *et al.* 2016). There was no or little data regarding the presence of sponge-associated actinobacteria from *Hyrtios, Callyspongia,* and *Neofibularia*. Therefore, these described data on morhological character which indicate diversity of sponge-associated actinobacteria from those three sponges, and the antibacterial activity against pathogenic bacteria can be considered as new information. The output of this study indicate diversity and antibacterial potency of sponge-associated actinobacteria from tropical marine environment.

Sequence identification of the 16S rRNA gene indicated that HRHa.5 has closed similarity to *Streptomyces sampsonii* strain NRRL B12325 with identities 1043/1115 (94%) and gaps 45-1115, *S. champavati* strain B-5682 with identities 1043/1115 (94%) and gaps 45-1115, and also with *S. albidoflavus* strain DSM 40455 with identities 1043/1116 (93%) and gaps 46-1116. The NOHa.2 had been previously identified by Simamora *et al.* (2016) showing that NOHa.2 had closed similarities with *S. sampsonii* strain NRRL B12325 (97%), *S. albus* strain J1074 (97%), and *S. resistomycificus* strain ISP 5133 (97%). The degree of similarity less than 97.5% based on 16S rRNA gene may have novel potency as a new species (Stackebrandt and Goebel 1994). Based on the morphology and 16S rRNA gene analysis, sponge-associated actinobacteria isolated from *Hyrtios* sp. and *Neofibularia* sp. showed to have relationship with Streptomyces spp. Moreover, they can be further examined for their novelty, which based on polyphasic study, e.g. morphology, cell wall composition, physiological characters and molecular identity. The output of this work showed that marine biota like sponges from tropical marine environment associated with various kind of sponge-associated actinobacteria.

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