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Antifungal Potential of Actinomycetes Isolated from Region of Bhilai, Chhattisgarh, India

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Abstract:

Actinomycetes due to their unique repertoire of antimicrobial secondary metabolites can be a good source to control human pathogens. In our research plan, soil samples were collected from Bhilai, India. The strains were isolated using yeast extract-malt extract agar medium and identified based on their morphological, physiological, and biochemical characteristics. The antifungal activity of isolates was examined by the perpendicular streak plate as well as the agar well diffusion method. Out of 14 isolates, only 4 isolates (28.5%) showed antifungal activity. Further, one isolate showed the highest antifungal activity and was identified as *Streptomyces antibioticus* A8 based on PIB-Win software and the identification score was 0.99. Antifungal activity of new isolate *Streptomyces antibioticus* A8 against the *T. rubrum* MTCC 296, with a zone of inhibition 28±0.0 mm, whereas minimum activity was recorded against *A. niger* MTCC 872 with the zone of inhibition 9±0.0 mm. antifungal activity against *C.albicans* ATCC 10231, *C.albicans* ATCC 90028, *C. albicans* ATCC 24433, *C.albicans* MTCC 183, *C.tropicalis* MTCC 184, *A.alternata* MTCC 1779 was recorded as 20±0.0mm, 17±0.0 mm, 17±0.0 mm, 15±0.5 mm, 14±0.0 mm and 11±0.0 mm respectively by agar well diffusion method. Further, discovering new antimicrobial-producingmicrobes probably will be helpful for uncovering novel therapeutic agents against a broad range of pathogenic organisms.

Keywords: *Streptomyces sp*., Screening, Antifungal activity, Pathogens

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Introduction

The discovery of a novel antimicrobial agent to combat fatal infectious diseases brought on by microbes was one of the greatest accomplishments of the preceding seven decades [1]. The misuse or improper application of antifungal drugs has led to an increase in antifungal resistance that is occurring globally. One of the main factors contributing to morbidity and mortality in immunocompromised people is antifungal resistance. Despite more awareness and better treatment methods, the prevalence of antifungal medication resistance in clinical settings contributes to the rising death toll from mycoses. Due to this, it became necessary to screen fresh microorganisms from natural sources in order to create novel antibiotics with fresh biomolecules that are effective against a variety of diseases [2-3]. Actinomycetes are fungal-like gram-positive bacteria. These categories of microbes are proficient in producing extracellular enzymes, novel antibiotics, and other bioactive metabolites with variousantimicrobial activities[4]. Mycotic infections or mycoses are not only created economic burdens but also created psychological burdens during the course of inefficient treatment [5]. Mostly, antimycotic substances show signs of toxic effects in humans and animals during their mode of action. For instance, amphotericin B caused lipid peroxidation, and ketoconazole inhibits cytochrome P450 3A4 (CYP3A4) and P-glycoprotein (P-gp) [6-7]. Consequently, the pharmaceutical industries are looking for non-toxic, cost-effective, and broad-spectrum antimycotic compounds which can be used safely. A variety of antimycotic substances have been extracted from *Streptomyces* sp. for instance, allylamine from *Streptomyces* sp. KH-F12 is effective against multiple fungi [8]. Numerous studies have been conducted to look for novel microbial strains that could provide novel and diverse antimicrobial properties from previously undiscovered and various geographical places that may have evolved in a different manner from those that had previously been examined [9-10]. Keeping the above facts in mind current studyaims for the isolation of actinomycetes yielding novel bioactive antifungal metabolites from unique and unidentified habitats of Chhattisgarh[11]. Hence, the recent research was intended to discoveractinomycetes acquiring antifungal activities from the soil of different biological niches of Bhilai, India.

Materials and methods:

Collection of soil samples

Samples were collected from the soil of different biological niches of Bhilai of Chhattisgarh, India for the isolation of antifungal metabolite-producing *Streptomyces* sp. The rhizosphere of plants and agricultural soil was among these ecosystems. The samples were obtained from a depth of up to 20 cm after approximately 3.0 cm of dirt had been removed from the surface. These soil samples were obtained, packed tightly in sterile plastic bags, and stored at 4°C in the refrigerator for further testing.

pH determination of soil samples

Using an electronic pH meter, the pH of the collected soil samples was assessed in the slurry system. In order to make the slurry, 5.0 g of sieved, air-dried soil was suspended in 5 ml of

distilled water. Using pH 4.0 and 7.0 reference buffers, the pH meter was calibrated. After rapidly stirring the slurry for a short while and letting it stand for 30 minutes, the pH of the slurry was measured [12].

Pretreatment of soil

Soil samples collected from different areas were pretreated with the following physical and chemical methods to eliminate very common microbes [13].

Physical treatment

To separate spores from vegetative cells, test tubes containing dilutions of soil samples in the range of 10^{-2} to 10^{-6} were heated in a water bath (SONAR, INDIA) at 45°C for 16 hours.

Chemical treatment

In order to prevent the growth of fungi, cycloheximide and nystatin were added to the isolated growing medium at a concentration of 50 µg/ml each.

Isolation and Maintenance of Isolates

In order to prepare 10-fold serial dilutions aseptically in the test tube (10^{-1} to 10^{-7}), one gram of dried composite soil sample was dissolved in 9 ml of 0.85% normal saline solution. In triplicate, aliquots of 100 µl from each sample were transferred and evenly disseminated on the surface of Yeast Extract Malt Extract Glucose Agar (ISP-2) in an aseptic manner. After appropriate incubation, the number of actinomycetes colonies was observed on plates. Pure isolate colonies with distinctive morphologies and patterns of growth were selected, transferred to the appropriate agar slants, and then cultured once more for 1-2 weeks at 28 °C to promote healthy growth before being stored at 4 °C. Composition of yeast extract malt extract glucose agar (pH-7.2): Glucose, 4 g; yeast extract, 4 g; malt extract, 10 g; $CaCO_3$, 2 g; distilled water, 1000ml, 20g agar added for agar medium.

Test organisms used for antifungal activity

Fungaltest organisms such as *C.albicans* ATCC10231, *C.albicans* ATCC 90028, *C.albicans* ATCC 24433, *C.albicans* MTCC 183, *C.tropicalis* MTCC 184, *A.niger* MTCC 872, *A.alternata*, MTCC 1779, *P.citrinum* MTCC 1751, *T.rubrum* MTCC 296, C.*terreus* MTCC 1716, *R.oryzae* MTCC 2162 were obtained from the Microbial Type Culture Collection & Gene Bank (MTCC), IMTECH, Chandigarhto assess the antifungal activity of isolates.

Inoculum preparation and standardization

To test antifungal activity, inoculums preparation standardized by CLSI guidelines for testing yeast and filamentous fungi was followed and prepared accordingly. For spores forming fungi, an inoculum of spores was adjusted spectrophotometrically 0.4-0.5 x 10^4cfu/ml whereas for yeast inocula was adjusted to 1-5x 10^6cfu/ml [14].

Primary screening

Actinomycetes were isolated and identified from soil samples, and then their antifungal activity was tested against fungal test pathogens. Antifungal activity was assessed on Potato Dextrose Agar (PDA) media against fungal test organisms using the perpendicular streak method. Each isolate was streaked onto a plate, placed in the middle of the plate, and then cultured for 5–7 days at 37°C. Then, parallel to the actinomycete isolates, fresh sub-cultured test organisms were streaked [15]. Then, fungal inhibition was observed by incubating all of the plates for roughly 48–72 hours at 28 °C. After incubation, a millimeter (mm) measurement of the zone of inhibition (ZoI) was made.

Secondary screening:

Based on the zone of inhibition after initial screening, isolates with the highest antimicrobial activity were chosen for liquid-state fermentation and extraction, and the fermented broth was then assessed using the agar well diffusion method [16]. Actinomycete isolates that displayed potential features were cultured in yeast extract, malt extract, and glucose broth in a 500 ml flask at 37°C for 5-7 days while being shaken at a speed of 100 rpm. After incubation, the surface of potato dextrose agar was swabbed with a sterilized cotton swab to access the antifungal activity, and 6 mm diameter wells were cut using a sterile cork borer on the same potato dextrose agar plate. Each well-received 100ul of fermented broth was then filled and refrigerated for 30 minutes to allow the metabolite to diffuse. The plates were then kept at 28 °C for 48–72 hours. The zone of inhibition (ZoI) was measured following incubation to express the antifungal activity.

Characterization of Actinomycetes isolates

After preliminary studies, the isolate was identified by morphological, cultural, physiological, and biochemically [17]. Microscopic observation was done under 1000x using gram stain. Utilization of different carbon such as D-glucose, D-fructose, D-mannitol, D-sorbitol, D-lactose, D-maltose, meso-inositol, Dextran, Cellobiose and nitrogen sources such as L-arabinose, D-melibiose, L-arginine, L-asparagine, D-melezitose, DL-α-amino-n-butyric acid, L-serine, L-cysteine, L-histidine, L-rhamnose was also performed. Determination of cell wall amino acids, gelatin hydrolysis, urea hydrolysis, starch hydrolysis, hydrogen sulfide production test, indole test, motility test, citrate utilization test, methyl red test, Voges-Proskauer (VP) test, catalase test, oxidase test, nitrate reduction, lipid (Tween 20), pectin degradation, growth at 1% phenol, growth at 0.01 % sodium azide, egg yolk (lecithin), xanthine, triple sugar iron (TSI) agar test was performed. Additionally, varying pH levels, NaCl concentrations, and incubation temperatures were used to evaluate the growth features of isolates that produce antimicrobials. All the characteristics were entered on PIBW in (probabilistic identification of bacteria) software, a windows version of a DOS program is one of the excellent databases developed for the identification of microbes based on numerical taxonomy [18].

Results:

Isolation of Actinomycetes

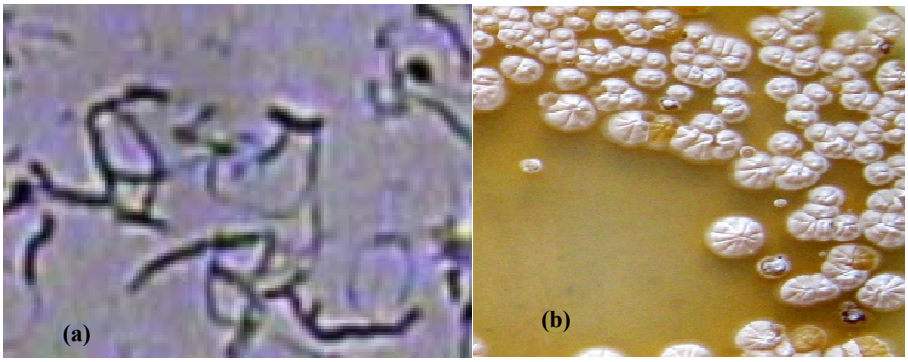
Overall, 14 isolates were isolated from soil of the Chhattisgarh region of Bhilai. The pH of the soil samples analyzed for the dilutions ranged from 6.9 to 7.5. Following 5-7 days of incubation at 10^{-2} to 10^{-6} dilutions on yeast extract malt extract glucose agar media, plates were evaluated for the growth of actinomycetes colonies based on their morphological characteristics. For further examination, colonies were purified, transferred to the appropriate agar slants, and kept refrigerated at 4℃.

Characterization of Selected Isolate

Morphological and Cultural Characterization

Isolates morphological characteristics were evaluated as per the standard procedures of the International *Streptomyces* Project (ISP) [19]. Isolate A8 showed the highest antifungal activity and was selected for further activity. Morphological characteristics were recorded for the isolate A8 on yeast extract malt extract glucose agar medium (ISP-2), isolate was gram-positive with the branches hyphae when examined under the microscope, aerobic, non-motile with aerial and substrate mycelium, spore with gray color, spore chains were rectiflexibles. Microscopic observation of the isolates A8 under 1000x confirmed branched hyphae mycelium-like structure (Fig 1a). Isolate exhibited higher growth on ISP-2 medium growth pattern was granular with the white surface and reverse side color was dark brown (Fig 1b).

Fig No. 1(a) Microscopic observation under light microscope 1000x(b) Growth of isolating *Streptomyces antibioticus* A8 on yeast extract malt extract glucose agar (ISP-2) medium



Physiological and Biochemical Characteristics

Physiological and biochemical characteristics of isolate A8, exhibitedstrong antifungal activity, is given in (**Table 1).** Isolate A8, was able to produce gelatinase, starch hydrolysis, urease, catalase, oxidase, lipase (Tween 20), and pectinase enzymes and was able to degrade esculin, adenine, guanine, and xanthine. Diaminopimelic acid was present in the cell wall. The isolate was able to

utilize D-glucose, D-fructose, D-mannitol, D-sorbitol, D-lactose, D-maltose, cellobiose, *meso*-inositol, L-arabinose, D-melibiose, L-arginine, L-asparagine, D-melezitose, DL-α-amino-n-butyric acid, L-serine, L-cysteine, dextran, L-rhamnose, and L-histidine. The growth characteristics of the selected antimicrobial-producing isolate were examined at different pH levels, incubation temperatures, and salt concentrations. The isolate was able to grow between temperatures 15-45°C, between pH 4-10, and able to grow with 1-7% NaCl and 0.001% potassium tellurite. No growth was observed with 0.01% sodium azide and 0.1% phenol. The isolate showed a positive reaction for nitrate reduction, methyl red test, citrate utilization, Voges-Proskauer test, and triple sugar iron (TSI) whereas negative reaction for motility and in dole test. A8 was identified as *Streptomyces antibioticus* with a PIB-Win identification score of 0.99.

Table 1: Physiological and Biochemical Features ofisolate A8

Isolate A8		
Physiological and Biochemical Features		
Utilization of 'C' Sources	D-glucose	3+
	D-fructose	2+
	D-mannitol	2+
	D-sorbitol	1+
	D-lactose	3+
	D-maltose	3+
	meso-inositol	2+
	Dextran	1+
	Cellobiose	+
Utilization of 'N' Sources	L-arabinose	2+
	D-melibiose	1+
	L-arginine	2+
	L-asparagine	2+
	D-melezitose	1+
	DL-α-amino-n-butyric acid	1+
	L-serine	1+
	L-cysteine	2+
	L-histidine	+
	L-rhamnose	1+
Enzyme production	Gelatinase	+
	Starch hydrolysis	+
	Urease	+
	Catalase	3+
	Oxidase	2+

	Lipase (Tween 20)	1+
	Pectinase	1+
	Lecithinase (egg yolk)	-
Degradation of	Esculin	2+
	Adenine	2+
	Guanine	3+
	Xanthine	1+
Growth at temperature	15°C	+
	28°C	3+
	35°C	2+
	45°C	+
Growth at pH	4	+
	6	1+
	7	3+
	9	1+
	10	-
Growth at	1% phenol	+
	0.01 % sodium azide	-
	0.001% potassium tellurite	+
Growth at NaCl	1%	3+
	2%	3+
	3%	3+
	4%	2+
	5%	2+
	6%	1+
	7%	+
Others	Hydrogen sulfide production test	+
	Diaminopimelic acid (DPA) in cell wall	+
	Indole test	-
	Motility	-
	Nitrate reduction	+
	Methyl red test	+
	Citrate utilization	+
	Voges-proskauer test	+
	Triple sugar iron (TSI)	+

(−), No growth/ Negative; (+), Positive; (+1), Poor growth; (+2), Moderate growth; (+3), Heavy growth.

Antifungal activity study of the isolates

Primary screening:

The perpendicular streak method was employed to screen all 14 typical actinomycete isolates isolated from soil in Bhilai, India, against all of the test fungi utilized in the study to determine their antagonistic activity. Of the total isolates, 4 (28.5%) had antifungal activity whereas 8 (57.1%) isolates did not show any activity against any test organisms. Plates were observed for antagonistic activity after 48-72 h of incubation and the zone of inhibition was recorded in millimeters (mm) shown in **(Table 2)**. In primary screening only three isolate namely A2, A7 and A8 showed antifungal activity. Isolate A8 showed the highest activity and was selected for further secondary screening.

Table No.2 Screening of actinomycete isolates for their antifungal activity using the perpendicular streak method

Isolates	*C.albicans* ATCC10231	*C.albicans* ATCC90028	*C. albicans* ATCC24433	*C. tropicalis* MTCC184	*C.albicans*MTCC183	*A.niger*MTCC872	*A. alternate*MTCC 1779	*P. citrinum* MTCC 1751	*T. rubrum* MTCC296	C. *terreus* MTCC 1716	*R.oryzae*MTCC2162
	Zone of Inhibition (mm)										
A1	-	-	-	-	-	-	-	-	-	-	-
A2	11	-	-	-	-	-	-	-	-	-	-
A3	-	-	-	-	-	-	-	-	-	-	-
A4	-	-	-	-	-	-	-	-	-	-	-
A5	-	-	-	-	-	-	-	-	-	-	-
A6	-	-	-	-	-	-	-	-	-	-	-
A7	-	9	11	-	-	-	-	-	-	-	-
A8	16	14	10	11	14	12	8	-	18	-	-
A9	-	-	-	-	-	-	-	-	-	-	-
A10	-	-	-	-	-	-	-	-	-	-	-
A11	-	-	-	-	-	-	-	-	-	-	-
A12	-	-	-	-	-	-	-	-	-	-	-
A13	-	-	-	-	-	-	-	-	-	-	-
A14	-	-	-	-	-	-	-	-	-	-	-

Secondary screening

Inoculated *Streptomyces antibioticus* A8 grows on yeast extract malt extract glucose broth in a 500 ml flask at 28 °C for 5-7 days while being agitated at a speed of 100 rpm. After fermentation, the broth was centrifuged at 8000 rpm for approximately 15 minutes, and the supernatant obtained was utilized to access extracellular antifungal activity. *Streptomyces antibioticus* A8 exhibited highest antifungal activity against the *T. rubrum* MTCC 296, with the zone of inhibition 28±0.0 mm, whereas minimum activity was recorded against *A. niger* MTCC 872 with the zone of inhibition 9±0.0 mm, antifungal activity against *C.albicans* ATCC 10231, *C.albicans* ATCC 90028, *C. albicans* ATCC 24433, *C.albicans* MTCC183, *C.tropicalis* MTCC 184, *A.alternata* MTCC 1779 were recorded as 20mm, 17mm, 17±0.0mm, 15±0.5 mm, 14±0.0mm and 11±0.0mm respectively whereas no activity was recorded against *P.citrinum* MTCC 1751, *C.terreus* MTCC 1716, *R. oryzae* MTCC 2162 **(Graph 1 & Figure 2).**

Graph 1 Antifungal activity of *Streptomyces antibioticus* A8

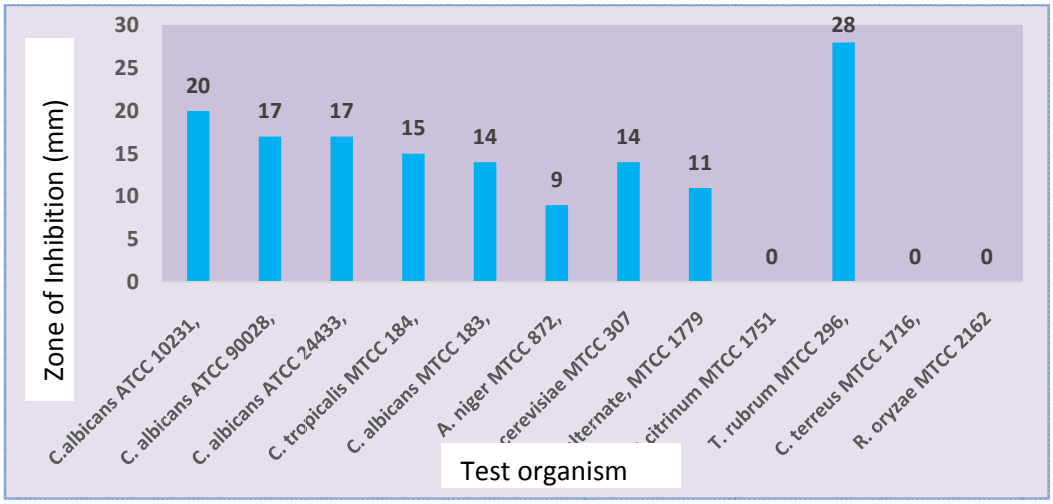
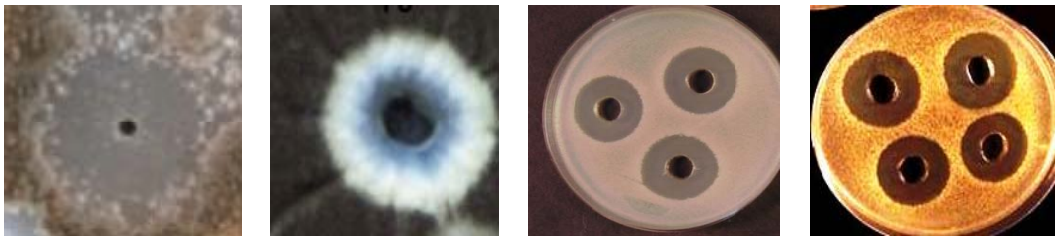


Figure No.2Antifungal activity of *Streptomyces antibioticus* A8 against (a) *T. rubrum* MTCC296; (b) *A. niger* MTCC 872; (c) *C.albicans* ATCC90028; (d) *C. albicans* ATCC24433



(a)*T. rubrum* **MTCC296** **(b)** *A. niger***MTCC 872** **(c)** *C. albicans* **ATCC90028;** **(d)** *C. albicans* **ATCC 24433**

Discussion

At present, the threat posed by drug-resistant fungi to currently available antibiotics has become a problem around the globe, mostly in underdeveloped nations. Therefore, the hunt for a novel, powerful, and broad-spectrum antifungal medicines are urgently needed to combat infectious diseases derived from various fungal pathogens [20]. Actinomycetes in particular *Streptomyces* are the furthermost abundant sources of secondary metabolites, including new antibiotics, and have the potential to synthesize bioactive chemicals [21]. Therefore, the investigation of actinomycete bioactive metabolite chemicals isolated from uncharted areas has been essential for potential use in biotechnological, agricultural, and therapeutic applications [22-23]. Secondary metabolites obtained from *Streptomyces* sp.exhibited antifungal activity by various modes of action. Abdel Kader and Muharram 2017 have described the synthesis of the antifungal drug terbinafine which was promoted under the name "Lamisil" by *Streptomyces* sp.KH F12 [24]. According to Karthik et al. (2015), *Streptomyces sp*. chitinase exhibits antifungal action [24]. Polyolmacrolactones from *Streptomyces hygroscopicus* that are produced from naphthoquinone have been shown to have antifungal action reported by Alferova *et al.* (2018) [25]. The current study focused on isolating bioactive actinomycetes from the soil of Bhilai, India, and screening them for potent antifungal activity in light of this reason. Soil samples collected from various rhizosphere and agriculture soil of Bhilai showed a total number of 14 actinomycetes, out of them 4 were able to produce antifungal agents against pathogenic fungal strains *C.albicans* ATCC 10231, *C.albicans* ATCC 90028, *C. albicans* ATCC 24433, *C.albicans* MTCC183, *C. tropicalis* MTCC 184, *A.niger* MTCC 872, *S.cerevisiae* MTCC 307, *A.alternata* MTCC 1779, *P. citrinum* MTCC 1751, *T.rubrum* MTCC 296, C.*terreus* MTCC 1716, *R.oryzae* MTCC 2162 which indicated that rhizosphere soils had the respectable number of actinomycetes. Rhizosphere soils were useful for locating effective actinomycetes that contained bioactive antifungal agents [26]. Isolate characterized as *Streptomyces antibioticus* A8 was found to produce broad spectrum antifungal compound which needs further purification and characterization of the bioactive compound for details information and to check their toxicity as well.

Conclusion

In conclusion, the current research investigation proves that actinomycetes that have the capacity to produce bioactive compounds are present in soil samples taken from various rhizosphere regions of Bhilai, India. Our piece of research has proven that a soil-isolated strain of *Streptomyces* sp. is bioactive against a wide range of fungi that cause disease in both humans and animals. The bioactive substances produced from the potent isolate A8 need to be further identified and purified, according to our findings. Additionally, studies should concentrate on isolating actinomycetes that produce antimicrobials from various uncharted regions of Chhattisgarh.

Author contributions

Neha Singh designed the experimental scheme and performed the research and analysis. Manuscriptdesign, writing, and correction were done by Nikita Sherwani, Neha Singh, and Khushboo Bhange, Vibhuti Rai provided the facility to carry out research activities.

Data Availability

On request, data pertinent to the current investigation can be made available.

Conflicts of Interest

Author(s) do not have any conflicts of interest.

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