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Evaluation of the Antibacterial Activities of Actinomycetes Isolated from Bhitarkanika Mangrove Forest Soil against Human Pathogens

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Actinomycetes of marine environment are one of the most productive and novel micro organism having potential to produce a large number of substances of economic importance. The current research emphasizes on isolation, screening, physiological and biochemical characterization of actinomycetes form different areas of Bhitarkanika mangrove forest soil and study of their antagonistic effect against few human pathogens. Actinomycetes were isolated by serial dilution followed by plating on actinomycetes isolation agar and starch casein agar media. A total of thirty five (35) actinobacterial isolates were selected on the basis of colony characteristics (colour, morphology) on the above isolation medium and were primarily screened against 10 human pathogens ie., *E.coli* (ATCC 25922), *Pseudomonas aeruginosa* (MTCC 741 & ATCC 27583), *Klebsiella pneumoniae* (MTCC 109), *Staphylococcus aureus* (MTCC 902 & ATCC 25923) *Salmonella typhi* (MTCC 733), *Salmonella enterica* (ATCC 35640), *Shigella flexneri* (MTCC 1457) *and Micrococcus luteus* (MTCC 1538) by cross streak method. Nineteen isolates showed antagonistic activity against the test pathogens, whose cell free supernatant were again screened by agar well diffusion method. Eight (S1, S7, S8, S16, S21, S22, S24 and S25) isolates were found positive against most tested pathogens were further studied secondarily for their antibacterial activity by agar well diffusion method.

Key words: Mangrove, actinomycetes, antibacterial activity, starch casein agar, actinomycetes isolation agar.

Mangrove ecosystem acts as a bond between marine and terrestrial ecosystem norturing unique microbiata. Among the most productive ecosystems in the world, mangroves have ecological, economic and societal importance (Alongi, 2002). Bhitarkanika mangrove forest is an exuberant stimulating ecosystem with diverse group of microbes, performing complex interactions for nutrient recycling and balance of the ecosystem. Marine environments are largely untouched sources for the isolation of new potential microorganisms, ability to produce secondary metabolites (Baskaran *et al*., 2011). Among such microorganisms, actinomycetes are of special interest, since they are known to produce chemically diverse compounds with a wide range of biological activities (Bredholt *et al.*, 2008). The demand for new antibiotics continues to grow due to the rapid emergence of multiple antibiotic resistant pathogens causing life threatening infection. Traditionally, actinomycetes have been isolated from the terrestrial sources only and the first report of mycelium forming actinomycetes being recovered from marine sediments appeared several decades ago (Weyland, 1969). Recently, the marine derived actinomycetes are recognized as a source of novel antibiotic and anticancer agent with unusual structure and properties (Jensen *et al.*, 2005).

Almost 80% of world's antibiotic production, obtained mostly from the genera of *Streptomyces* and *Micromonospora* (Kumar *et al*., 2010) .Thus screening and isolation of promising strains of actinomycetes having potential of

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antibiotic production is a thrust area of search since many years (Hacene *et al.*, 2000; Rabah *et al.*, 2007). The present study emphasises on the isolation and load of actinobacteria from the Bhitarkanika mangrove forest soil having ability to exhibit antimicrobial activity

MATERIALS AND METHODS

Soil samples were collected in triplicates from different areas of Bhitarkanika mangrove forest. The media used in the study were procured from Hi-Media, Mumbai and prepared as per manufacture's instruction. All the experiments were carried out in triplicates.

Sample Collection

Bhitarkanika mangrove forest (20 º 4' to 20 º 8' North Latitude 86 º 45' to 87º 50' East Longitude) is situated along the east coast of Bay of Bengal in the Kendrapara district of Odisha, India. Soil samples were collected from the rhizospheric region of different areas of Bhitarkanika mangrove forest from a depth of 1.0 to 5.0 cm after removing the surface soil with a sterile spatula, mixed properly, kept in sterile polythene bags and transported to the laboratory, Department of Microbiology, O.U.A.T. for analysis.

The pH and salinity of the soil sample were studied. From the above collected soil sample 10.0 gm of soil was added to 20.0 ml of sterile distilled water, vortexed properly for pH and salinity measurement by pH meter and salinometer (Eutech instrument, Malaysia) respectively.

Isolation of Actinobacteria from soil sample

For selective isolation of actinobacteria, the collected soil samples were dried at room temperature for a week. The dried samples were powdered with a mortar and pestle, passed through a 2.0mm sieve filter to remove gravel and debris. For selective isolation of the actinobacteria individually 10.0 gm of finely powered soil was transferred to sterile petriplates, kept at 55ºC for 10 minutes (Pisano *et al.*, 1986, Balagurunathan *et al.*, 2010). 1.0g of the dried soil sample was added to 50% sterile sea water (Kim *et al*., 1994) which acts as mother culture for the isolation of actinobacteria following the 10 fold serial dilution using 9.0 ml of sterile distilled water. From the above serially diluted sample upto 10^{-6}, about 100 µl from each dilution were spreaded uniformly with a sterile L-shaped glass rod over the surface of actinomycetes isolation agar(AIA) and starch casein agar (SCA)plates (Wellington and cross, 1983) supplemented with filter sterilized antibiotics i.e. 80 µ g/ml cycloheximide (Baskaran *et al.*, 2011) and 50 µ g/ml nystatin (Porter *et al*., 1960 ; Istianto *et al.*, 2012) to retard the growth of bacterial and fungal contaminants. The plates were incubated aerobically at 28 ±2ºC and observed intermittently during incubation. The actinobacterial load of different soil samples was determined (CFU/g) after 5 days of incubation. Pure cultures of the isolates were done on restreaking on the isolation medium following by streaking on slants.

Test human pathogens

Selective human pathogens used in the study were: *E.coli* (ATCC 25922), *Pseudomonas aeruginosa* (MTCC 741 & ATCC 27583) ,*Klebsiella pneumoniae* (MTCC 109),*Staphylococcus aureus* (MTCC 902 & ATCC 25923) *Salmonella typhi* (MTCC 733), *Salmonella enterica* (ATCC 35640),*Shigella flexneri* (MTCC 1457) *and Micrococcus luteus* (MTCC 1538)

***In vitro* primary screening of the actinobacterial isolates for antimicrobial activity**

Cross streak method (Balagurunathan *et al*., 2001, Ravikumar *et al*., 2010)

Actinobacterial isolates were streaked as parallel lines on modified nutrient agar plates and Muller Hinton agar plates followed by incubation at 28±2 ºC for 5-7 days. After observing a good actinobacterial growth, the bacterial pathogens were streaked at right angles to the original streak of actinomycetes and incubated at 28±2ºC for 24 to 48 hrs. The inhibition zones were measured. The isolates showing good zone of inhibition were considered further for screening using agar well diffusion method.

Agar well diffusion method (Pandey *et al.*, 2004)

Overnight nutrient broth cultures of the test pathogens were swabbed on Muller Hinton agar plates. Wells of 6.0 mm diameter were bored on the sterilized agar plates by using sterile cork borer followed by addition of 100 µl of 7 days old actinomycete culture of each isolate grown in tryptone soya broth (pH 7.2±0.2) to the well and incubation at 28±2°C for 24-48 hr. Then the inhibition zones (in mm) were measured against the pathogens.

Characterization of Actinomycetes

The antagonistic actinomycetes selected after secondary screening were characterized following morphological, physiological and biochemical test results.

Morphological characterization by macroscopic method

The colony characteristic of the above actinobacterial isolates were observed by spot inoculating on starch casein agar (SCA) plate. Morphological and cultural characteristic such as colony morphology, reverse side and diffusible pigmentation, pigment production (Pridham *et al.*, 1957), absence or presence of aerial and substrate mycelium etc. were observed (Shirling and Gottlieb, 1966).

Morphological characterization by cover slip culture method (Kawato and Sinobu, 1979)

The details of morphology were observed by Gram's staining (Gram, 1884) of the colonies on the coverslip. Culture was observed under microscope in order to recognize true branched substrate mycelium, aerial mycelium and sporulation using 100 X magnification. The colonies on slide were also stained with methylene blue or Giemsa's stain (Health Protection Agency, 2009) as some times a Gram-stained preparation may not be helpful in visualising actinobacteria because it is too dark and obscures crucial morphologic details. The vegetative cells and spores were Gram's stained and Giemsa's stained for studying the morphology and Gram's reaction under inverted microscope [400X]

Physiological and Biochemical characterization

Physiological and biochemical characteristics of the organisms were checked following the standard methods. (Shirling and Gottlieb, 1966; Collins and Lyne, 1970). Effect of pH (5.0-9.0), temperature (20-60 °C) and salinity was studied. The susceptibility of the isolates to different antibiotics was determined using different antibiotic disc (Hi Media). Purified isolates of

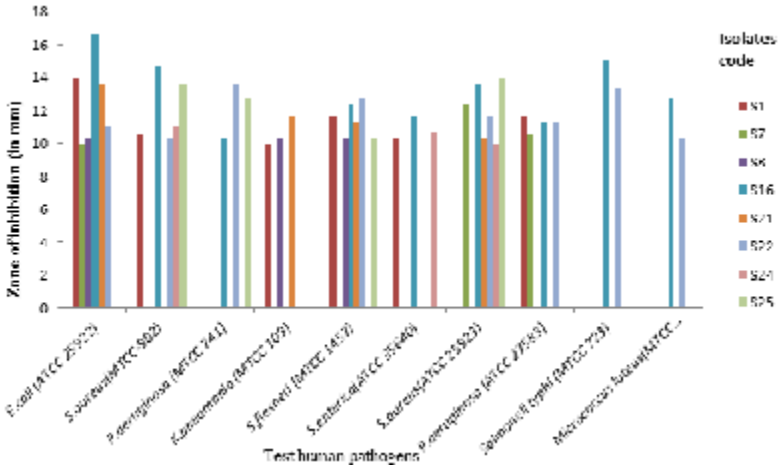

Fig.1. Zone of inhibition of the potent isolates

Table 1. Physicochemical parameters of the soil and actinobacterial load

Soil Sample	pH	Salinity(ppt)	Actinobacterial load in CFU/g	
			AIA	SCA
BDS1	7.18	0.50	1.8×10^4	1.3×10^4
BDS2	7.45	0.50	2.1×10^4	1.5×10^4
BDS3	7.16	0.60	1.9×10^4	1.1×10^4
BDS4	7.68	0.50	2.6×10^4	1.5×10^4
BDS5	7.34	0.50	2.9×10^4	1.7×10^4
BDS6	7.78	0.60	2.8×10^4	2.1×10^4

Table 2. Antimicrobial activity of isolates against human pathogens

Isolate code	Zone of inhibition (mm)									
	E.coli (ATCC 25922)	*S.aureus* (MTCC -902)	*P.aeruginosa* (MTCC 741)	*K.pneumoniae* (MTCC 109)	*S.flexneri* (MTCC 1457)	*S.enterica* (ATCC 35640)	*S.aureus* (ATCC 25923)	*P.aeruginosa* (ATCC 27583)	*S.typhi* (MTCC 723)	*M.luteus* (MTCC 1538)
S1	14.00	10.60	0	10.00	11.66	10.33	0	11.66	0	0
S7	10.00	0	0	0	0	0	12.33	10.60	0	0
S8	10.33	0	0	10.33	10.33	0	0	0	0	0
S16	16.66	14.66	10.33	0	12.33	11.66	13.66	11.33	15.00	12.66
S21	13.60	0	0	11.66	11.33	0	10.33	0	0	0
S22	11.00	10.33	13.66	0	12.66	0	11.66	11.33	13.33	10.33
S24	0	11.00	0	0	0	10.66	10.00	0	0	0
S25	0	13.60	12.66	0	10.33	0	14.00	0	0	0

0-No zone of inhibition was observed

Table 3. Profile of morphological and cultural characteristics of actinobacterial isolates at different incubation period

Isolates code	Colony Morphology	Arial mycelium	Substrate mycelium	Reverse side pigmentation	Soluble pigments	Spore chain morphology	Incubation period(hrs.)
S1	Concentric rings ,powdery, non-sticky, dry, ash white colour	Grey-white	Brown	Brown	-	Flexibilis	72
S7	Pale yellow, flat sticky, uneven colony	Creamy	Yellow	Yellow	-	Fragmented	72
S8	Concentric rings , leathery, non-sticky, dry ash, white colour, small colony	Grey-white	Brown	Brown	-	Flexibilis	72-96
S16	Dry, creamy- white, bulging out, pin point colonies.	Creamy white	Light yellow	-	-	Fragmented	72
S21	Light pink, leathery, small, bulging out colony	Light pink	Dark brown	Dark brown	-	Rectus or straight	72
S22	Ash grey, large, uneven colonies	Grayish	Pale yellow	Pale yellow	-	Spore in chain attached to hyphae	72
S24	Leathery, sticky white colour colonies becomes black gradually	White	Light brown	Light brown	-	Spiral	72-96
S25	Leathery ,ash white small colonies	Grayish	Yellow	Yellow	Yellow	Spiral	72

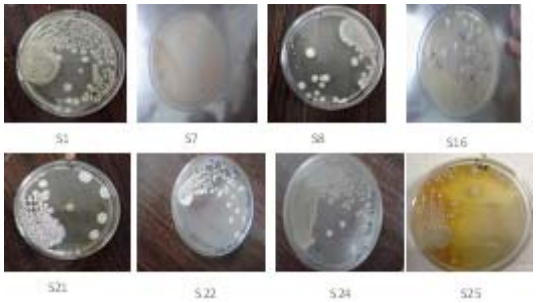

Fig.1. Colony morphology of isolates on starch casein agar

actinomycetes were identified using Bergey's Manual of Determinative Bacteriology (2000, Actinomycetales 9th edition).

RESULTS AND DISCUSSION

The soil (Bhitarkanika mangrove forest) was analysed for pH & salinity and presented in Table-1. Isolation of actinomycetes has always been a matter of concern due to their long incubation period. Colonization of actinomycetes has been inhabited by bacterial and fungal contaminants (Williams & Cross 1971); so

Table 4. Biochemical profile of the actinobacterial isolates

	Different biochemical Tests	S1	S7	S8	S16	S21	S22	S24	S25
	Indole	-	-	-	-	-	-	-	-
	MR	-	-	-	-	-	-	-	-
	VP	-	-	-	-	-	-	-	-
	Citrate utilization	-	+	-	-	-	+	-	+
	Urease	-	-	-	-	-	-	-	-
	Motility	-	-	-	-	-	-	-	-
	Mannitol utilization	-	-	-	-	-	-	-	-
	Melanin production	-	-	+	-	+	-	-	-
	onPeptone yeast extract iron agar								
	Nitrate reduction	+	+	-	+	-	+	+	-
	Catalase	+	+	+	+	+	+	-	-
	Oxidase	+	+	+	+	+	+	+	-
	ONPG	-	+	+	+	+	-	-	-
	H2S production	+	+	+	+	+	+	-	-
	Casein Hydrolysis	-	-	-	+	-	-	-	-
	Amylase	+	+	+	+	+	+	-	+
	Protease	+	-	+	+	+	-	-	-
	DNAse	-	-	-	-	-	-	-	-
	Chitinase	-	-	-	+	-	-	-	-
	Pectinase	-	-	-	+	-	-	-	-
	Gelatin liquification	+	+	+	+	+	-	-	-
Amino acid utilization	Esculin	+	+	+	+	+	-	-	-
	L-histidine monohydrochloride	-	-	+	-	-	-	-	-
	L-lysine monohydrochloride	+	-	-	-	+	-	-	+
	Ornithin	-	-	-	-	-	-	-	-
Carbon source utilization test	Fructose	-	-	-	-	-	-	-	-
	Sorbitol	-	-	-	-	-	-	-	-
	Inositol	-	-	-	+	-	-	-	-
	D-Glucose	-	-	-	+	+	-	-	-
	Salicin	-	-	-	-	+	-	-	-
	Mannose	-	-	-	-	+	-	-	-
	Galactose	-	-	-	-	+	-	-	+
	Raffinose	-	-	-	-	-	-	-	-
	Arabinose	-	-	-	-	-	-	-	-

pretreatment of the soil was employed for the selective isolation of the actinomycetes whereas the isolation without pretreatment of the soil resulted in the dominance of other contaminants as explained earlier (Hayakawa & Nonomura, 1987; Hayakawa *et al.*, 1991; Jensen *et al*., 1991; Kim *et al*., 1994 ; Seong *et al*., 2001 ; Bhaskaran, 2011).The present study revealed that the actinobacterial ecology of the sampling sites of the Bhitarkanika is influenced by the chemical nature of the soil. A very typical pattern of soil characteristics of almost all the 6 samples from different locations were observed (Table 1). There was no marked variation in the pH and salinity. The soil pH was ranging

Table 5. Physiological characteristics of the actinobacterial isolates

Isolates code		S1	S7	S8	S16	S21	S22	S24	S25
pH	5.0	+	-	-	-	+	+	+	+
	7 .0	+	+	+	+	+	+	+	+
	9 .0	+	+	+	+	+	+	+	+
	11.0	-	-	-	-	-	-	-	-
Temp.	4°c	-	-	-	-	-	-	-	-
	30°C	+	+	+	+	+	+	+	+
	37°C	+	+	+	-	+	+	+	+
	45°C	-	-	-	-	-	-	-	-
NaCl %	2	+	+	+	+	+	+	+	+
	4	+	+	+	+	+	+	+	+
	6	+	+	+	+	+	+	+	+
	8	+	+	+	+	+	+	+	-
	10	+	+	+	+	+	+	+	-
	12	-	+	-	-	+	-	+	-
	14	-	+	-	-	+	-	+	-
	16	-	-	-	-	+	-	-	-
	18	-	-	-	-	+	-	-	-
	20	-	-	-	-	+	-	-	-
Lysozyme	0.001%	+	+	+	+	+	+	+	+
concentration(w/v)	0.005%	+	-	+	+	+	+	-	+
	0.01%	-	-	-	-	-	-	-	-
sodium azide	0.001%	+	-	+	+	+	+	+	+
concentration(w/v)	0.005%	-	-	-	-	-	-	-	+
	0.01%	-	-	-	-	-	-	-	-

- No growth ,+ growth

Table 6. Antibiotic sensitivity test of the potent isolate

	Isolates code							
Antibiotics used(µg/disc)	S1	S7	S8	S16	S21	S22	S24	S25
Ampicillin(A)10	R	R	R	R	R	R	R	R
Ampicillin(A)25	R	R	R	R	R	R	S(24)	S(20)
Chloramphenicol(C)50	S(20)	S(24)	S (18)	R	R	S(15)	R	R
Ciprofloxacin(CIP)30	R	R	R	R	R	R	R	R
Erythromycin(E)15	R	S (22)	R	R	R	S(17)	R	R
Methicillin(Met)10	R	R	R	R	R	S(20)	S(18)	R
PenicillinG(P)10	R	R	R	R	R	R	R	R
Rifampicin(R)5	S (19)	S (17)	S(16)	S (16)	R	S(15)	R	R
Streptomycin(S)10	S(15)	S(24)	S(20)	R	R	R	R	S (17)
Vancomycin(Va)30	S(22)	S (19)	S(22)	R	R	S(25)	R	S(22)

R – resistant;S- sensitive

from neutral to slightly alkaline (pH range 7.16 – 7.78) having moderate salinity (0.50-0.60ppt).

In the present study, about 35 morphologically different (sticky, powdery, and leathery) actinobacterial colonies were isolated from different regions of Dangamal of Bhitarkanika mangrove soil. As compared to SCA, AIA was promising for the maximum isolation of actinomycetes ($28x10^{-4}$) than the former ($21x10^{-4}$).

Screening of actinobacterial isolates against human pathogens

Marine actinomycetes are regarded as potent producers of secondary metabolites but research is going on in this field in search of new findings. The incedence of multiple resistance pathogenic organisms to the clinically used drugs necessitates the discovery of new classes of antibiotics (Burgess *et al.*, 1999). All the actinobacterial isolates were screened for their antimicrobial activity against some human pathogens by cross streak and agar well diffusion methods. Out of 35 isolated strains, 19 (54.29%) isolates showed antimicrobial activity against atleast one of the test human pathogens by cross streak method on both modified nutrient agar and Muller Hinton agar and were again subjected to another screening test by agar well diffusion method. During the screening by agar well diffusion method 8 isolates showed broad spectrum antibacterial activity against most of the selected pathogens. The diameter of the inhibition zones were measured (Table 2). From the present study, it was observed that 75% of the isolates showed activity against *Shigella flexneri* (MTCC 1457), *E.coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923), 62.5 % showed activity against *Staphylococcus aureus* (MTCC 902), 50% were effective against *Pseudomonas aeruginosa* (ATCC 27583), 37.5% were active against *Pseudomonas aeruginosa* (MTCC 741), *K.pneumoniae* (MTCC 109) and *Salmonella enterica* (ATCC 35640), an activity of 25% was seen against *Salmonella typhi* (MTCC 733) and *Micrococcus luteus* (MTCC 1538). The isolate S16 showed activity against all test pathogens except *K.pneumoniae* (MTCC 109) and highest zone of inhibition was observed against *E.coli* (ATCC 25922) ie., 16.66mm. S22 also showed inhibitory activity against maximum pathogens used followed by S1, S21, S25, S24, S7 & S8 and others. Similar type of work has been carried out by Ellaiah *et al.*, 1987; Remya and Vijaykumar, 2008; Sathiyaseelan and Stella, 2011. They had observed the antimicrobial activity of actinobacteria isolated from marine sediments of Vishakhapatnam and west coast of India. The present study also correlates with study conducted in Turkey where actinomycetes isolated from 11 soil samples collected from terrestrial environment exhibited antibacterial activity against 7 indicator bacterial strains including multi drugs resistant bacteria. Five isolated *Streptomyces* had potent activities against methicillin resistant *Staphylococcus aureus.* (Livermore, 1989). However, actinobacteria from marine sediments were well acknowledged for their antimicrobial activity (Goodfellow and Williams, 1983).

Morphological physiological and biochemical characterization

The morphology of the actinomycetes plays a vital role in their identification. All 19 isolates showing antimicrobial activity were characterized based on their distinct colony morphology (sticky, powdery, and leathery) and Gram's reaction. Eight potent isolates having broad spectrum antimicrobial activity were characterized based on their morphological, physiological and biochemical characters, different colour of aerial, substrate mycelium and soluble pigments. Isolates S1 & S8 showed chalky white powdery coloured colony, S22 and S25 showed dry, leathery ash white or grey aerial mycelium. This type of colony characteristics, typical of actinomycetes has already been reported (Alimuddin *et al.*, 2011). Aerial mycelium with coiled spiral spores were distinctive to majority of marine *Streptomycete* (Augustine *et al.*, 2013). S16 was a dry cream coloured colony and S21 was light pink leathery colony. Two isolates S1 &S21 showed brown reverse side pigments; S22 and S25 showed yellow reverse side pigments and S16 were having yellowish brown reverse pigmentation. Basing upon this they were grouped into *streptomyces* (abundant aerial mycelium with powdery spores) (Table 3 & Fig. 2)

Physiological & biochemical characteristics of the isolates

Physiological and biochemical tests are necessary tools for classification and identification of the actinomycetes. Biochemical test of the

isolates (Table 4) revealed that all organisms except S24 & S25 produced catalase, one of the important characteristics of actinobacteria (Waksman, 1959). This finding was also supported and confirmed by the previous reports of Remya and Vijaykumar (2008). ONPG is a distinguishing factor between member of actinobacterias for identification (Flores *et al.,* 1990). All isolates except S1, S22, S24 and S25 were positive for O-nitrophenyl-β-D-galactopyranoside (ONPG) test. The physiological parameter study revealed that the isolates grow within a pH range of 7.5-8.0. All of them exhibited salt tolerance upto 10% but isolate S7 and S24 were tolerant upto 14%, S21 upto 20% salt concentration. S25 showed lowest tolerance i.e 6%. All the isolates were resistant to lysozyme at concentration of 0.001% except S7 and S24. All the isolates were active at 0.005% of lysozyme concentration but none remain active at 0.01%. Most isolates except S7 were active at 0.001% sodium azide whereas S25 showed activity at 0.005% of sodium azide concentration. (Table 5)

From the antibiotic sensitivity test (Table 6), it was observed that 2 isolates were sensitive to ampicillin(25), erythromycin and methicillin; 4 isolates were sensitive to chloramphenicol (50) and streptomycin(10) and 5 isolates were sensitive to rifampicin (5) and vancomycin(30). S21 was resistant to all antibiotics tested followed by S16(sensitive only to rifampicin(5).

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