JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Nov. 2013. Vol. **7**(Spl. Edn.), p. 107-116

Gas Chromatography and Mass Spectrum Analysis of the Selective Diversity of Actinobacteria Isolated from Extreme Environments in Saudi Arabia

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(Received: 26 August 2013; accepted: 19 October 2013)

In this research fifty Actinobacteria isolates were obtained from soil samples collected from desert sites in the Kingdom of Saudi Arabia. The isolates were purified and exposed to a variety of biochemical and physiological tests to determine their tentative taxonomy. Color group was determined for all 50 strains and a total of 13 different groups were allocated as follows: 24% gray, 10% chalky white, 12% light gray, 12% dark gray, 10% light brown, 2% yellow, 2% whitish pink, 2% whitish yellow, 2% dark red, 2% pale gray, 6% pale yellow, 6% pale white and 8% grayish white. Bioactive compounds were extracted from each isolate using methanol as solvent; these were then tested against *Staphylococcus aureus* ATCC 13076, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 2592, *Pseudomonas aeruginosa* ATCC 27583, *Salmonella suis* ATCC 13076, *Shigella sonnei* ATCC 11060 and *Candida albicans* ATCC 10231. Whole extracts of the isolates which exhibited inhibitory activities against the seven microorganisms were chosen for gas chromatography–mass spectrometry (GC-MS) analysis. This showed that the predominant inhibitory compound in all extracts was 2, 3-butanediol, [R-(R*, R*)], while cyclobutanol and octadecanal were also present in most isolates. Based on the cultural, physiological and biochemical tests as well as scanning electron microscopy studies, all the selected isolates were members of the Genus *Streptomycetes*.

Keywords: Actinobacteria, *Streptomyces*, antimicrobial, pathogenic bacteria, Gas chromatography- mass spectrometry.

Bacteria are typically classified by the Gram stain in to two groups, i.e. Gram positive and Gram negative species. The Gram-positive bacteria include two major branches which are: the low G+C (Guanine + Cytosine) organisms that comprise genres such as *Bacillus*, *Clostridium*, *Staphylococcus* and *Streptococcus*. These organisms DNA typically has fewer guanine (G) and cytosine (C) DNA bases than adenine (A) and thymine (T) when compared to other bacteria; the DNA of high G+ C organisms in comparison is rich in guanine and cytosine about (57 – 75%)[1]. Members of the genus *Streptomyces* produce 75% of all known commercial and medical antibiotics[2]. Many are mycelial at some stage in their life cycle, but others are not; mycelial growth is however regarded as being typical of the phylum, Actinobacteria.

Actinobacteria which include the *Streptomyces* belong to the subdivision of Gram-positive filamentous bacteria. They are abundant in warm, dry soils[3], and also in freshwater[4,5],

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although they are often not native to this environment, but instead arrive in runoff from the land[6,7]. Actinobacteria produce two kinds of branching mycelium, i.e. a) so called "aerial mycelium" which grows above the growth medium, and b) substrate mycelium which grows beneath the medium; aerial mycelium is particularly important because it is generally the main source of spores.

Soil Actinobacteria produce a volatile compound called geosmin, chemically known as 1, 2, 7, 7-tetramethyl-2-norborneol, which is responsible for the earthy smell associated with the growth of these bacteria[8]. Geosmin is responsible for a significant number of taste and odor problems in drinking water supplies[9,10], which can be miss-attributed to the growth of Cyanobacteria[9,10].

The genus *Streptomyces* comprises a large group of microorganisms with some unique characteristics, notably their complex fungal like life cycle and earthy odor; they occur widely and show a higher diversity in color formation than most other bacteria. This genus is defined by both chemotaxonomic and phenotypic characters, notably the presence of LL-DAP, an isomer of diaminopimelic acid, which is present in the cell wall peptidoglycan; this feature and the characteristic substrate and aerial mycelium are diagnostic for *Streptomyces*.

MATERIAL AND METHODS

Soil sample collection

A total of 4 soil samples were obtained from the desert environment in the Kingdom of Saudi Arabia. The samples were obtained from a depth of 15 cm, after removing approximately 3 cm of the soil surface. The soil samples were placed in sterile polythene bags, closed tightly to avoid external contamination, labeled and transported to the laboratory. Here, they were they were dried at room temperature (25°C) for 1 week and then stored in sterile plastic bags until required.

Isolation of Actinobacteria from soil

The serial dilution technique[11] was used to isolate Actinobacteria from the soil samples. Air dried soil (1g) was suspended in a tube containing 10 ml of sterile distilled water, each tube was then caped and stirred for 1minute using a vortex mixer (VTX-3000L). The soil suspension (1ml) was then serially diluted in sterile distilled water (9ml) to provide dilutions of 10^{-1}, 10^{-2} and 10^{-3}.

Tap water agar (TWA) media[12] was prepared [containing 22g agar (WINLAB, UK) and 1L tap water]. Aliquots (0.1ml) of each of the three final dilutions were transferred to the isolation medium in petri dishes and spread over the surface using a sterile glass spreader; no antibacterial or antifungal agents were added to the medium. The plates were then incubated at 30°C for 2 weeks.

Colony enumeration

After incubation, the numbers of colonies from each sample were determined using the colony count technique[13]; the resultant colony count is shown in (Table 1**).**

Color group determination

The isolates were purified on yeast starch agar (YSA) and then cultured on oatmeal agar (OA) media for color group determination[14]. Oatmeal agar contained [30g oatmeal (Quaker), 15g agar (WINLAB, UK) and 1L distilled water]. All the plates were incubated at 30°C for 1 week. After the incubation period, colony colors of the isolates were visually determined using an ISCC-NBS color chart[15].

Cultivation of Actinobacteria for secondary metabolites production

Each Actinobacteria isolate was inoculated into a flask (250 ml) containing 100 ml of starch glucose yeast (SGY) broth[16,17]; the broth had the following composition (per liter): 10g starch soluble (Avonchem, UK), 10g glucose (Fluka, UK), 10g glycerol (Winlab, UK), 2.5g corn flower (Riyadh food), 5g peptone (Biochemical, UK), 2g yeast extract (Winlab, UK) , 3g $CaCo_3$ (Winlab, UK) and 1L distilled water.

Secondary metabolites extraction

Secondary metabolites were recovered from the broth by solvent extraction method[18,19]. Methanol (Panreac, E.U) was added (1:1 v/v) to the medium containing the growing isolate. The flasks were then returned to the shaker for 3 extra days which after the contents were filtered to separate the mycelium from liquid. The filtrate was next transferred to a hot air oven in order to evaporate the methanol to dryness. Two drops of distilled water was the added to the residue and the resultant crude extract was tested for antimicrobial activity.

Antimicrobial activity test

The test microorganisms used were: Gram-positive bacteria: *Staphylococcus aureus* ATCC 13076, *Bacillus subtilis* ATCC 6633, *Shigella sonnei* ATCC 11060, Gram-negative bacteria: *Escherichia coli* ATCC 2592, *Pseudomonas aeruginosa* ATCC 27583, *Streptococcus suis* ATCC 13076 and one fungus**:** *Candida albicans* ATCC 10231.

The well diffusion method[20] was used to study the antimicrobial activity. Each test organism was inoculated onto the surface of Mueller-Hinton agar medium (MHA). Wells were then made in the medium and filled (0.5 ml) with the extract. The plates were then incubated at 37°C for 24 h, after which time the size of any resultant inhibition zones were measured.

Gas chromatography (GC) – mass spectroscopy (MS) analysis

Depending on the antibacterial activity only 20 isolate extracts were selected for gas chromatography-mass spectroscopy (GC-MS) analysis[21] in order to identify the active antimicrobial compound present. Analysis was conducted using the database of National institute Standard and Technology (NIST), with the spectrum of the unknown components being compared with the spectrum of known components stored in the NIST library. Before identifying the compounds in the extracts 0.1ml of each extract was mixed with 1ml of ethanol (HPLC grade) (WINLAB, UK) and filtered using a 0.22µ Millipore filter (Millex-HV) so as to obtain a crystal clear solution. The identification of the compounds was done by injecting 1µl of crystal clear sample into an RT x -5 columns (30 × 0.32 nm) of GC-MS model (Perkin Elmer, Clarus 500, USA) and helium (3 ml/min) was used as a carrier gas. The following temperature gradient program was used (75°C for 2 min followed by an increase from 75 to 175°C at a rate of 50°C per min and finally 7 min at 175°C). The m/z (mass - to - charge ratio) peaks representing mass to charge ratio characteristics of the antimicrobial fractions were compared with those in the mass spectrum library of the corresponding organic compounds[22]. The chemical components of the extracts were analyzed in the central laboratory of King Saud University, Riyadh, Saudi Arabia. Identification of the chemical constituents of extracts were performed using a Perkin Elmer (Clarus 500, USA) gas chromatograph coupled with (Clarus 500, USA) mass spectro-meter (MS). In addition, the molecular weights of all the identified substances were confirmed by GC/MS, using MeOH as CI ionizing.

Morphological characterization of the isolates using scanning electron microscopy

Bacterial cells were prepared for scanning electron microscopy (SEM) (JEOL, JSM, 3060) at King Saud University Central Laboratory, Saudi Arabia according to the initial fixation and dehydration steps was recommended[23]. The cells were fixed at 24°C overnight with 3% glutaraldehyde (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany), washed with distilled water 3 times each for 10 minutes , fixed again with 1% Osmium tetroxide overnight , washed once more with distilled water as before ,dehydrated with a serial ascending concentrations of ethanol as follows (30% for10 min, 50% for 10 min, 60% for 10 min, 70% for 10 min, 80% for 10 min, 90% for 10 min, 100% for 10 min, 100% again overnight); finally they were dried using a critical point dryer (HCP-2; Hitachi Co.). The dried cell samples were then gold coated using a gold sputter (JEOL, JFC 1600) and examined using a SEM (JEOL, JSM, 3060)[24].

Physiological characterization of the isolates

Growth at various temperatures (30-50) and ability to grow over a range of pH (4-10) and NaCl concentrations (7-10%) was determined[25,26,27] on YSA media.

Biochemical characterization of the isolates

The Actinobacteria isolates were characterized using seven different tests, namely: gelatin hydrolysis, urease production[28], catalase production[29], casein hydrolysis[30,31], citrate utilization, H_2S production and ability to utilize carbon sources such as glucose and lactose[25]. All isolate characteristics were determined following incubation for 7 days at 30°C on YSA medium. After incubation the growth, aerial mycelium color and diffusible pigmentation were also noted.

RESULTS

Actinobacteria isolation on water agar

As a result of isolation on TWA, 50 colored Actinobacteria isolates were obtained; 20 of those were chosen for further study.

Numbers of colonies isolated

Table 2 shows the CFUs per ml obtained from the second dilution of the environmental samples.

Color grouping

The twenty purified strains were cultured on OA media in order to determine the color groups of each strain; Fig. 1 shows the color groups of the selective strains under the study.

Antimicrobial activity of the isolate extracts

All Actinobacteria isolates were tested for their antimicrobial activity against *S. aureus* ATCC 13076, *B. subtilis* ATCC 6633, *S. sonnei* ATCC 11060, *E. coli* ATCC 2592, *P. aeruginosa* ATCC 27583, *S. suis* ATCC 13076 and *C. albicans* ATCC 10231. Only twenty of the isolates showed antimicrobial activity against at least one of the seven pathogens under the study; these strains were designated: M3, M7, M10, M11, M12, M13, M15, M16, M19, M20, M24, M25, M27, M28, M29, M33, M36, M38, M42 and M47. The antimicrobial activity of these strains is detailed in Table 3.

Scanning electron microscope studies

SEM analysis showed that all twenty isolates exhibited different morphologies. Arial mycelium was produced and was seen to be highly branched and coenocytes; spores appeared to be smooth, spiny or wart-like.

Gas chromatography (GC)-mass spectroscopy (MS) analysis of the extract

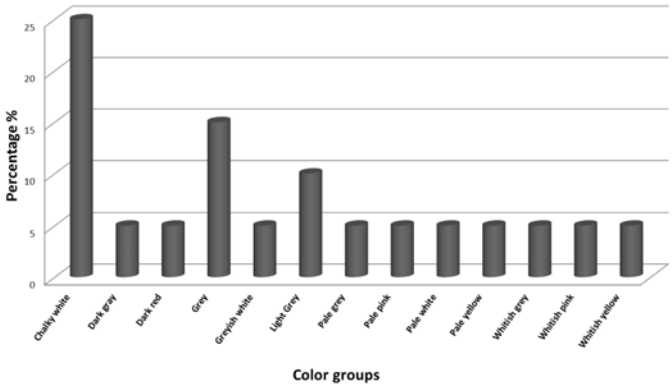
The crude extracts were analyzed using GC-MS technique. A large number of compounds were detected for each isolate some of which are known to possess antibacterial activity. Some of the compounds appeared in all extracts at a variety of concentrations while others were found in only some isolates. Details of the compounds identified

Table 2. The CFUs per ml for each soil sample collected from the extreme environment in the Kingdom of Saudi Arabia

Sample	Colonies	CFUs
Thumamah	150 colonies	1500×10^2 CFUs/ml
Kharj	187 colonies	1870×10^2 CFUs/ml
Madina	144 colonies	1440×10^2 CFUs/ml
Taif	125 colonies	1250×10^2 CFUs/ml

Table 1. The colony count obtained from the third dilution of all soil samples

Sample's origin	Dilutions		
	10^{-1}	10^{-2}	10^{-3}
		Colonies	
Thumamah	Many colonies to count (more than 300 colonies)	150 colonies	Less than 30 colonies
Kharj		187 colonies	
Madina		144 colonies	
Taif		125 colonies	



The color groups presented in the actinomycetes isolates and the percentage of each color

Fig. 1. The twenty strains grouped into specific color groups based on their mycelium color

Table 3. Antibacterial inhibition zones (mm) produced by the extracts

Extract Code	Pathogenic microorganism inhibited						
	Gram negative bacteria		Gram positive bacteria				Yeast
	(9) *S. suis* ATCC 13076	(10) *E. coli* ATCC 2592	(11) *S. sonnei* ATCC 11060	(12) *P.aeruginosa* ATCC 27583	(13) *B. subtilis* ATCC 6633	(14) *S. aureus* ATCC 13076	(15) *C. albicans* ATCC 10231
M3	-	-	-	8mm	-	8mm	10mm
M7	-	-	-	10mm	-	7mm	8mm
M10	12mm	-	17mm	8mm	15mm	16mm	8mm
M11	8mm	-	12mm	15mm	-	-	7mm
M12	8mm	11mm	-	-	13mm	-	-
M13	-	13mm	-	-	15mm	-	-
M15	10mm	13mm	8mm	12mm	15mm	-	-
M16	-	7mm	7mm	-	9mm	21mm	7mm
M19	-	-	10mm	9mm	6mm	7mm	10mm
M20	-	-	-	-	13mm	10mm	9mm
M24	-	-	-	-	10mm	6mm	-
M25	12mm	12mm	-	12mm	19mm	-	10mm
M27	10mm	-	-	-	15mm	-	-
M28	13mm	12mm	-	-	19mm	-	-
M29	-	10mm	-	9mm	29mm	-	-
M33	13mm	10mm	6mm	10mm	17mm	6mm	10mm
M36	8mm	10mm	9mm	10mm	18mm	-	8mm
M38	8mm	-	8mm	9mm	19mm	8mm	-
M42	-	9mm	-	-	19mm	-	-
M47	-	8mm	-	-	16mm	9mm	6mm

and their percentage appearance in all twenty isolates are shown in Table 4.

Physiological and biochemical characterization of the isolates

The isolates grew well at 30 and 45°C and 45, at pH 4, 7 and 10, and at NaCl concentrations up to 7%. Almost all of the strains hydrolyzed gelatin and coagulated milk a many showed the ability to bring milk peptonization, urea hydrolysis, and were able to utilize citrate utilization. Some of the strains were positive for glucose fermentation and others for lactose but only three produced H_2S (Table 5).

Table 4. Compounds found from the analysis of the Actinomycete extracts using GC-MS

No	Compound name	M.W	Formula	Similarity percentage
1	2,3-Butanediol, [R-(R*,R*)]-	90	$C_4H_{10}O_2$	100
2	Octadecanal	268	$C_{18}H_{36}O$	70
3	Cyclobutanol	72	C_4H_8O	65
4	Oleic acid	282	$C_{18}H_{34}O_2$	40
5	Oleyl alcohol	268	$C_{18}H_{36}O$	25
6	Eicosanoic acid	312	$C_{20}H_{40}O_2$	25
7	Erucic acid	338	$C_{22}H_{42}O_2$	25
8	Glycerin	92	$C_3H_8O_3$	15
9	Heptanal	114	$C_7H_{14}O$	10
10	Acetic acid	60	$C_2H_4O_2$	5

DISCUSSION

Actinoinobacteria, especially members of the genus of *Streptomyces*, are common and widely distributed in soils[32]. Their ability to produce a wide range of secondary metabolites and extracellular enzymes is of use in pharmacy, agriculture and industrial development. Actinobacteria can directly or indirectly affect the microorganism population in the soil because they produce many antimicrobial compounds, pathogenic fungal cell wall degrading enzymes, toxic hydrogen cyanide (HCN) and a range of siderophores[3,33,34]. In this study, 50 Actinobacteria isolates were obtained from desert soils of the Kingdom of Saudi Arabia using TWA medium. A wide range of media have been used to isolate members of this genus of bacteria, including most notably: starch casein agar[20,35] and starch-nitrate agar[36,37] however, also used TWA which resulted in the isolation of high numbers of Actinobacteria, a fact which led to this medium being selected for use here.

A large number of studies on the antimicrobial activity of Actinobacteria have been reported [38,39]. In the present study the fifty Actinobacteria isolates were tested for their ability to produce antimicrobial metabolites, but only twenty were chosen for further study. The twenty isolates chosen all showed antimicrobial activity against at least one of seven human test pathogens chosen, namely: *Staphylococcus aureus* ATCC 13076, *Bacillus subtilis* ATCC 6633, *Shigella sonnei* ATCC 11060, *Escherichia coli* ATCC 2592, *Pseudomonas aeruginosa* ATCC 27583, *Salmonella suis* ATCC 13076 and *Candida albicans* ATCC 10231.

Forty five per cent of the selected isolates showed antimicrobial activity against *Salmonella suis* ATCC 13076, while 55% of the strains exhibited marked activity against *Pseudomonas aeruginosa* ATCC 27583, with 35% of the strains showing reasonable activity against *Escherichia coli* ATCC 2592, Some 55% of the isolates inhibited the pathogen, *Shigella sonnei* ATCC 11060,while 80% of all strains were very effective against *Bacillus subtilis* ATCC 6633 and 50% inhibited *Staphylococcus aureus* ATCC 13076. Finally 55% percent of the isolates inhibited the growth of *Candida albicans* ATCC 10231. The most active of these isolates were then chosen for further study.

Table 5. Physiological and biochemical characterization tests results for the twenty Actinobacteria isolates

Strain	Temp.°C			pH			NaCl Conc.			Gelatin	Catalase	Casein hydrolysis		Urea	Citrate	Sugar fermentation		H_2S
No	30	45	50	4	7	10	1	7	10	hydrolysis		Coagulation of milk	Milk papton ization	hydrolysis	Utiliz-ation	Glucose	Lactose	produ-ction
M3	+	-	-	+	+	+	+	-	-	+	-	+	+	+	+	-	-	-
M7	+	+	-	-	+	+	+	+	-	+	+	+	+	-	-	+	+	-
M10	+	+	-	+	+	+	+	+	-	+	+	+	+	-	-	-	-	-
M11	+	+	-	+	+	+	+	-	-	+	+	+	+	+	+	+	+	-
M12	+	-	-	+	+	+	+	-	-	+	+	+	+	+	+	-	-	-
M13	+	-	-	+	+	+	+	+	-	+	-	+	+	+	-	-	-	-
M15	+	+	-	+	+	+	+	+	-	+	-	+	+	+	-	-	-	-
M16	+	+	-	+	+	+	+	+	-	+	-	+	-	-	-	+	+	+
M19	+	+	-	-	+	+	+	+	-	-	-	+	-	-	-	+	+	+
M20	+	+	-	+	+	+	+	+	-	+	-	+	+	-	-	-	-	-
M24	+	+	-	+	+	+	+	+	-	+	+	+	+	-	+	-	-	-
M25	+	+	-	+	+	+	+	+	-	+	+	+	+	+	-	-	-	-
M27	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	-	-	+
M28	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	-	-	-
M29	+	+	-	+	+	+	+	+	-	+	+	+	+	-	-	+	+	-
M33	+	+	-	+	+	+	+	+	-	+	+	+	-	+	+	+	-	-
M36	+	+	-	+	+	+	+	+	-	+	+	+	-	-	+	-	-	-
M38	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	-	-	-
M42	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-
M47	+	+	-	+	+	+	+	+	-	+	+	+	+	-	-	-	-	-

Scanning electron microscope studies showed that all isolates exhibited aerial mycelium which was both coenocytic and significantly branched. Spore shapes differed between smooth, spiny and wart-like in chains; the isolates differed from one another and appeared all to be species of *Streptomyces*.

Many different color groups were seen among the isolates which could be divided, based on this criterion, into: white, gray, brown, yellow, creamy, pink and red groupings. This diversity colony coloration shows that extreme desert environment of Saudi Arabia produces a wide range of color groupings amongst the Actinobacteria.

Taxonomically, the strains were classified using phenotypic and chemotypic determinants. Good growth rates were seen on both YSA and OA medium. The varieties of colors present amongst isolates included chalky white (25% of isolates), light gray (10%), gray (15%), whitish pink, whitish yellow, pale white, dark gray, pale grey, greyish white, whitish grey, pale yellow, dark red and pale pink (5% of isolates) which made a total of 13 color groups in the twenty isolates studied; the most common colors displayed were however, chalky white and the a range of grays. Pigmentation was produced by only 15% of strains. The two strains M28 and M47 produced brown pigmentation while the strain M29 has shown a Maroon color of pigmentation in the media. The work suggests that most soil Actinobacteria is gray, white or brown with the highest percentage being white and gray[1,40].

All of the isolates studied here could grow in the presence of up to 7%, but not 10% of NaCl, making them slightly halotolerant[41,42]. However, three isolates failed to grow on this concentration and are thereby not considered halotolerent Actinobacteria. Actinobacteria are normally regarded as being thermotolerant and can grow at temperatures up to 50°C[43]. The results presented here disagree with this assumption since most of the isolates grew at 45°C (there were 3 exceptions), but none grew at 50°C.

Most soil Actinobacteria can liquefy gelatin[38], a fact supported by the findings reported here showing that only one isolate was incapable of gelatin solubilisation. All isolates were also able to produce catalase, thereby confirming the findings that this is a common characteristic of these bacteria[29]. Similarly, 95% of all isolates showed the ability to peptonize skimmed milk broth, while only 5% of these coagulated milk in milk agar plates. However, 70% of the isolates hydrolyzed casein, a common ability amongst *Streptomyces*. The results obtained from this study show that 55% of the isolates hydrolyzed urea, a finding which agrees with the work[44].Ten of the isolates also utilized citrate; similarly found that many Actinobacteria isolated from Saudi Arabian soil are capable of using citrate as a carbon source in the absence of sugar. Some 30% of all the twenty strains had also fermented glucose and 5% could ferment both glucose and lactose which was indicated by the yellow tube's bottom and red slant, the other 65% could not ferment either glucose or lactose but instead used amino acids as nutrient source in the media. Finally, only 20% of isolated were shown to produce H_2S as indicated by the formation of a black color in the media.

CONCLUSIONS

The extreme desert environment of the Kingdom of Saudi Arabia has a rich Actinobacteria population which shows diverse color groups and a wide variety of physiological and biochemical properties. Many of the isolates of these organisms studied here produce secondary metabolites which inhibit other microorganisms. It is hoped that further studies of the Actinobacteria isolated from extreme environments represented by Saudi deserts will lead to the discovery of new organisms capable of producing important new biotechnological products and novel lifesaving antibiotics.

ACKNOWLEDGMENTS

Authors extend their appreciation to the Deanship of Scientific Research at King Saud University for funding the work through the research group project No. RGP-VPP-332.

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