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Phylogenetic Evaluation of the Genera of *Oscillatoria* and *Lyngbya* spp using Amplified Ribosomal DNA Restriction Analysis (ARDRA) and Enterobacterial Repetitive Intergenic Consensus (ERIC)

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The electrophoretic patterns for 12 cyanobacterial strains belonging to two genera (8 *Oscillatoria* strains and 4 *Lyngbya* strains) were used for molecular analysis using methods ARDRA and ERIC PCR. The cyanobacterial strains were placed in different groups in each method. *Lyngbya lagerheimii* and *Lyngbya major* got grouped separately in all the dendrograms. However *Oscillatoria acuminata*, *Oscillatoria acutissima*, *Oscillatoria formosa* and *Oscillatoria earlei* resulted more or less in the same group in all the dendrograms. In ARDRA, the two genera formed separate clusters. In ERIC PCR, only *Lyngbya lagerheimii*, *Lyngbya major* and *Lyngbya martensiana* formed separate minor clusters. In all dendrograms one strain from each genus namely *Lyngbya spiralis* and *Oscillatoria obscura* out-grouped in ARDRA. However the strain, *Lyngbya spiralis* was grouped together with *Oscillatoria formosa* and *Oscillatoria boryana*. Unique bands more prominently by ARDRA then ERIC PCR. The strain *Oscillatoria obscura* produced maximum number of unique bands (seven bands) but strains *Oscillatoria acuminata*, *Oscillatoria foreaui* and *Oscillatoria earlei* did not produce any unique bands when compared with both methods. Similarly the strain, *Lyngbya martensiana* produced 8 unique bands among the strains of *Lyngbya* in both methods. The dendrogram analyses clearly indicate the relatedness and distinction of the strains, which are morphologically similar.

Key words: Cyanobacteria, ARDRA, ERIC PCR, Phylogenetic analysis.

Cyanobacteria (blue-green algae) are an ancient group of prokaryotic microorganisms exhibiting the general characteristics of gram-negative bacteria (Rasmussen and Svenning, 1998). Cyanobacteria are unique among the prokaryotes due to their capacity for oxygenic photosynthesis and ability to fix atmospheric nitrogen both under free-living and symbiotic conditions. The morphology of cyanobacteria in laboratory cultures are known to be modified and the variation between same species become reduced due to the controlled culture conditions (Dores and Parker, 1988). For example many species of the genera *Oscillatoria*, *Lyngbya*, *Phormidium*, *Schizothrix, Plectonema* were included in *Schizothrix*

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calcicola, which was originally classified on the basis of sheath characterization and the presence or absence of false branching (Drouet, 1968; Gomont, 1892). Rippka *et al*., (1979) classified strains within the Oscillatoriales under a new group designated as LPP-group B (*Lyngbya*, *Phormidium* and *Plectonema*). Hence there is an increased interest in applying molecular techniques to resolve many of the issues and problems in cyanobacterial taxonomy (Giovannoni *et al*., 1988; Wilmotte and Golubic, 1991).

Molecular biological methods like Amplified Ribosomal DNA Restriction Analysis (ARDRA) etc. are now recognized as standard protocol for identification, characterization and taxonomy of many bacteria and higher organisms (Giovannoni *et al*., 1988; Wilmotte and Golubic, 1991). Molecular taxonomy can play a major role in identifying closely related species or even new organisms, when identifying them by classical methods becomes difficult (Giovannoni *et al*., 1988; Wilmotte and Golubic, 1991).

The sequence analysis of the 16S rRNA gene has proven to be one of the reliable methods among the many used (Clarridge, 2004). Nelissen *et al*., (1996) developed cyanobacteria specific primers, which could amplify 16S rRNA of many cyanobacteria. ARDRA where the amplicon of 16S rDNA was digested with different restriction enzymes and analyzed for homology. Each enzyme produces a distinct banding pattern. It can be used to differentiate different strains and construct phylogenetic tree to understand the similarities among the strains (Weisburg *et al*., 1991).

The REP and ERIC sequences were originally described for the family *Enterobacteriaceae* but later found in several gram-negative bacteria and close relatives in the same phyla (De Bruijn, 1992). ERIC sequences, also described as intergenic repetitive units differ from most other bacterial repeats in being distributed across a wider range of species. ERIC sequences were first reported in *E. coli* and other members of Enterobacteriaceae as well as *Vibrio cholerae* (Sharples and Lloyd, 1990; Hulton *et al*., 1991; Lindsay Wilson; Paul Sharp, 2006). Lyra *et al*., (2001) reported the use of ERIC PCR for the genotyping of cyanobacterial strains. Widespread distribution of these repetitive DNA elements in the genomes of various microorganisms should enable rapid identification of bacterial species and strains. (James *et al*., 1991).

Among the cyanobacteria, the family Oscillatoriae consists of uniseriate and differentiated trichomes (with or without mucilage sheath). In these cultures there is always a difficulty in identification and it was considered to analyze the molecular features with available tools to understand their relatedness. In the present study, eight strains of non-heterocystous, filamentous *Oscillatoria* spp. and four strains of *Lyngbya* spp. were isolated from fresh water bodies in different places and maintained at Cyanobacterial Culture Collections of CAS in Botany, University of Madras were used. Taxonomic analysis based on ARDRA and ERIC PCR was carried out for the phylogenetic characterization of these strains.

MATERIAL AND METHODS

Cyanobacterial cultures and culture conditions

The cultures used in this study were obtained from Cyanobacterial Culture Collection of CAS in Botany, University of Madras. The cultures were axenized by Imipenem (Merck), a broad-spectrum β-lactam antibiotic (Ferris and Hirsch, 1991). All the isolates were maintained in BG 11 medium under a light intensity of 40µE m^{-2} s^{-1} and 25 ± 1ºC. The culture rack was fitted with Sangmo Weston S650 313F automatic model timer to provide alternative light and dark phases of 12 h each. Batch cultures of the isolates were maintained in BG 11 medium in 250 ml Erlenmeyer flasks. Sixteen days old cultures were used for the study.

Extraction of total genomic DNA from cyanobacterial strains

Total DNA was isolated using the protocol by Wu *et al*., (2000) with few modifications. 5ml of exponentially growing cyanobacterial cells were harvested by centrifugation, washed twice in wash buffer (Tris buffer (pH 8.0) – 100 mM, EDTA (pH 8.0) - 50 mM, NaCl, - 100 mM) and resuspended in 200 µl of Solution I (Tris buffer (pH 8.0) – 25 mM, Glucose-50 mM, EDTA (pH 8.0) - 10 mM) followed by the addition of 2 mg of lysozyme and incubated at 37° C for 1 hour. SDS was added to a final concentration of 2% and mixed vigorously and 100 µL of 5M NaCl was added and mixed,

left at -20°C for 10 min. The mixture was centrifuged at 12000 rpm for 5 min and to the supernatant, equal volume of phenol: chloroform (1:1) was added and the contents mixed well and centrifuged at 12000 rpm for 5 minutes followed by one chloroform wash and DNA was precipitated with 2.5 volumes of 95% ethanol. The pellet was dissolved in 25 µl of STE buffer, followed by RNase (5µg) treatment and then reprecipitated with 95% ethanol after phenol treatment. The DNA was resuspended in 0.1X TE and used for further analysis.

Polymerase Chain Reaction (PCR) Primers

All PCR reactions were carried out in 0.5 ml tubes in either a Perkin Elmer 9600 thermal cycler or in a MJ research PTC 100 cycler. The programs used for amplification are mentioned in detail later along with individual experiments. The List of primers used for the different PCR reaction and their sequences are given in Table 1. All primers were obtained from Sigma Genosys, Bangalore.

Table 1. Primers used for ARDRA and ERIC analysis of cyanobacterial strains

Name of the Primers	Sequence (5'-3')
16S F	AGAGTTTGATCCTGGCTCAG
16S R	GTACGGCTACCTTGTTACGAC
ERIC1	ATGTAAGCTCCTGGGGATTCAC
ERIC2	AAGTAAGTGACTGGGGTGAGCG

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Full-length 16S rRNA gene was obtained by PCR amplification with the conserved eubacterial primer16S F and 16S R as described by Weisburg *et al*., (1991). The amplification was carried out with 50 µL reaction mixture containing, 16S F (10X) (2 µM/µL) - 1.5 µL, 16S R (10X) (2 µM/µL) - 1.5 µL, 10X Buffer - 5.0µL, 2 mM dNTP Mix-5.0 µL, *Taq* DNA polymerase (3U/µL) - 1.0µL, Template DNA (50ng) - 2.0 µL, Sterile distilled water - 34.0 µL. PCR was carried out on PTC-100 Thermal cycler (MJ Research Inc.) with following conditions initial denaturation at 95°C for 5 min followed by 30 cycles of 94°C for 60sec, 56°C for 45sec and 70°C for 60sec and a final extension at 70°C for 10 min. The amplified products were separated on 1% agarose gel, visualized and documented using a Vilber Lourmat gel documentation system with Bioimage software.

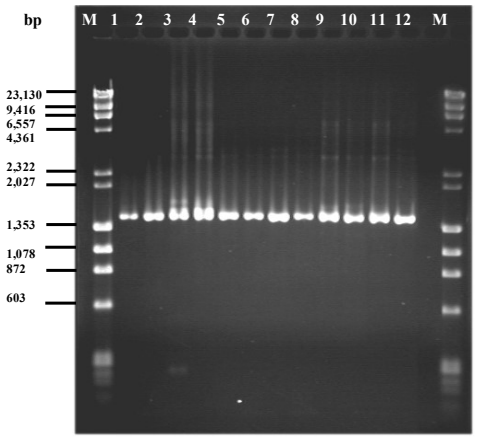
The restriction digestions were carried out with 7 µL of the PCR reaction mixture and 10U of the *Hae* III and *Msp* I (at 37°C in a total volume of 20 µL) incubated for overnight. The digested samples were resolved on 2% agarose gel electrophoresis. Banding patterns were visualized and documented using a Vilber Lourmat gel documentation system.

Enterobacterial Repetitive Intergenic Consensus (ERIC)

ERIC PCR was carried out using ERIC 1 and ERIC 2 primers designed based on Rasmussen and Svenning (1998). The amplification was carried out in a 20 µL reaction mixture containing ERIC 1 (2 µM/µL) - 2.0 µL, ERIC 2 (2 µM/µL) - 2.0 µL, 10X Buffer -2.0 µL, 2 mM dNTP Mix - 3.0 µL, *Taq* DNA polymerase (3U/µL) - 0.6 µL, Template DNA (50ng) - 2.0 µL, Sterile distilled water-8.4 µL. Amplification was carried out with PTC-100 Thermal cycler (MJ Research Inc.) with following cycling conditions 7 min initial denaturation at 95°C followed by 30 cycles of 94°C for 1min, 52°C for 1min, 65°C for 8 min and a final extension at 65°C for 16 min (De Bruijn, 1992). The amplified products were separated on a 2.0% agarose gel. The gel was stained with ethidium bromide and the amplified products were visualized and documented using a Vilber Lourmat gel documentation system.

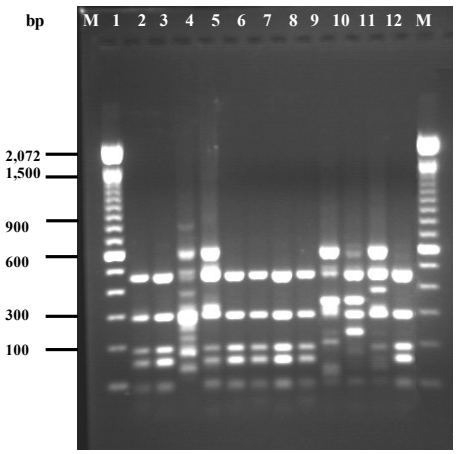
Dendrogram analysis

The data obtained from ARDRA and ERIC was used separately to construct dendrogram for each. The presence or absence of a band in each gel was used to construct a binary matrix for each of the method to generate a dendrogram. The dendrogram was obtained using NTSYS software and UPGMA (Unweighted Pair–Group Method using Arithmetic Averages) programme.



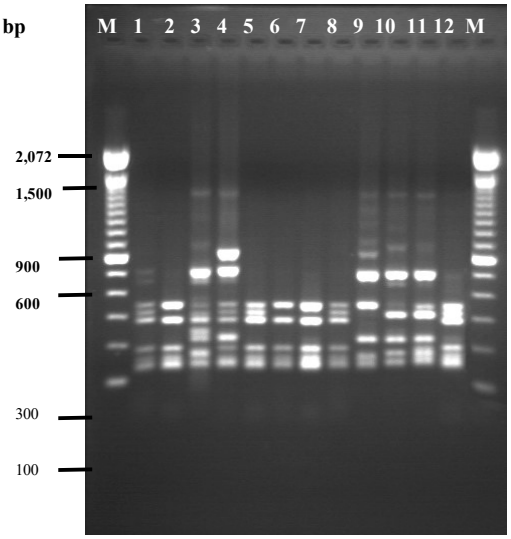
Lane M. λ DNA –*Hind* III digest and øX174 DNA *Hae* III digest Mix (Finnzymes), Lane 1. *Oscillatoria acuminata* Gomont, Lane 2. *Oscillatoria foreaui* Fremy , Lane 3. *Oscillatoria animalis* Ag, Lane 4. *Oscillatoria obscura* Bruhl et Biswas, Lane 5. *Oscillatoria acutissima* Kuff, Lane 6. *Oscillatoria formosa* Bory, Lane 7. *Oscillatoria boryana* Bory ex Gomont, Lane 8. *Oscillatoria earlei* Gardner, Lane 9. *Lyngbya lagerheimii* (Möbius) Gomont, Lane 10. *Lyngbya major* Meneghini ex Gomont, Lane 11. *Lyngbya martensiana* Meneghini, Lane 12. *Lyngbya spiralis* Geitler.

Fig. 1. PCR fingerprint amplification patterns of 16s rRNA gene



Lane M. Trackit TM 100 bp DNA Ladder (Invitrogen), Lane 1. *Oscillatoria acuminata* Gomont., Lane 2. *Oscillatoria foreaui* Fremy, Lane 3. *Oscillatoria animalis* Ag, Lane 4. *Oscillatoria obscura* Bruhl et Biswas, Lane 5. *Oscillatoria acutissima* Kuff, Lane 6. *Oscillatoria formosa* Bory, Lane 7. *Oscillatoria boryana* Bory ex Gomont, Lane 8. *Oscillatoria earlei* Gardner, Lane 9. *Lyngbya lagerheimii* (Möbius) Gomont, Lane 10. *Lyngbya major* Meneghini ex Gomont, Lane 11. *Lyngbya martensiana* Meneghini, Lane 12. *Lyngbya spiralis* Geitler.

Fig. 2. ARDRA (16S rRNA gene) digested with *Hae* III.



Lane M. Trackit TM 100 bp DNA Ladder (Invitrogen), Lane 1. *Oscillatoria acuminata* Gomont, Lane 2. *Oscillatoria foreaui* Fremy, Lane 3. *Oscillatoria animalis* Ag, Lane 4. *Oscillatoria obscura* Bruhl et Biswas, Lane 5. *Oscillatoria acutissima* Kuff, Lane 6. *Oscillatoria formosa* Bory, Lane 7. *Oscillatoria boryana* Bory ex Gomont, Lane 8. *Oscillatoria earlei* Gardner, Lane 9. *Lyngbya lagerheimii* (Möbius) Gomont, Lane 10. *Lyngbya major* Meneghini ex Gomont, Lane 11. *Lyngbya martensiana* Meneghini, Lane 12. *Lyngbya spiralis* Geitler.

Fig. 3. ARDRA (16S rRNA gene) digested with MspI

RESULTS

Molecular taxonomic analysis of 12 cyanobacterial strains belonging to the genera *Oscillatoria* and *Lyngbya* were carried out, using number of techniques like ARDRA and ERIC PCR. All the analyses were done with total DNA (Wu *et al*., 2000).

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

16S rDNA gene was amplified using specific primers (16S F and 16S R), separated on 1% agarose and a band of size 1.5kb was obtained (Fig.1). These amplified products were digested individually with restriction endonucleases *Hae*III and *Msp*I and analyzed on agarose gel. About 26 different bands, ranging from 1.2 kb to 100 bp, were obtained for the cyanobacterial strains. Four monomorphic bands, 350 bp and 150 bp in case of *Msp*I digestion (Fig.2) and 450 bp and 300 bp in case of *Hae*III digestion (Fig.3) were observed. A unique fragment of molecular weight 275 bp was observed in *Oscillatoria animalis* with *Msp*I digestion. Similarly one unique fragment of 275

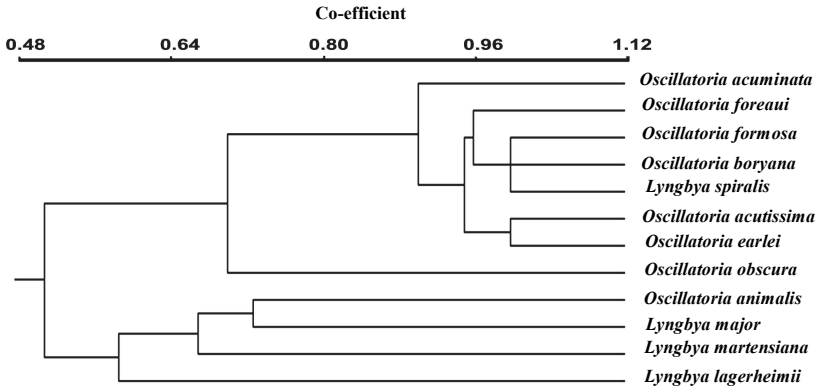
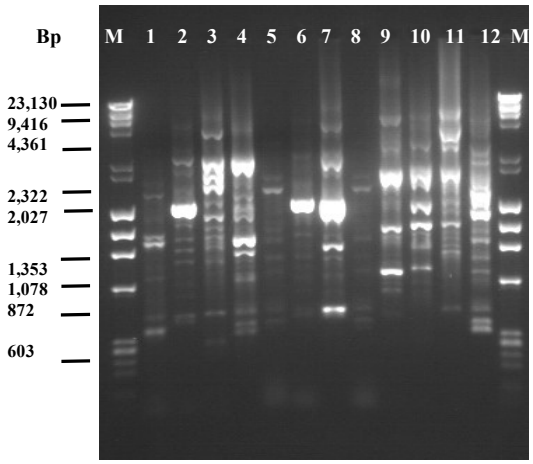


Fig. 4. Phylogenetic analysis of ARDRA.



Lane M. λ DNA–*Hind* III digest and øX174 DNA *Hae* III digest Mix (Finnzymes), Lane 1. *Oscillatoria acuminata* Gomont, Lane 2.*Oscillatoria foreaui* Fremy, Lane 3. *Oscillatoria animalis* Ag, Lane 4. *Oscillatoria obscura* Bruhl et Biswas, Lane 5. *Oscillatoria acutissima* Kuff, Lane 6. *Oscillatoria formosa* Bory, Lane 7. *Oscillatoria boryana* Bory ex Gomont, Lane 8. *Oscillatoria earlei* Gardner, Lane 9. *Lyngbya lagerheimii* (Möbius) Gomont, Lane 10. *Lyngbya major* Meneghini ex Gomont, Lane 11. *Lyngbya martensiana* Meneghini, Lane 12. *Lyngbya spiralis* Geitler.

Fig. 5. ERIC profile analysis of PCR amplification with primers ERIC 1 and ERIC 2

bp for *Lyngbya lagerheimii* and another of 325 bp for *Oscillatoria obscura* was observed in *Hae*III digestion. Phylogenetic tree constructed using UPGMA (NTSYS software) revealed (Fig.4) the strains belonging to the genus *Lyngbya* and *Oscillatoria* formed separate clusters. *Oscillatoria animalis* was grouped with *Lyngbya* cluster whereas *Lyngbya spiralis* was grouped with *Oscillatoria* cluster. *Oscillatoria obscura* and *Lyngbya lagerheimii* formed separate out-groups.

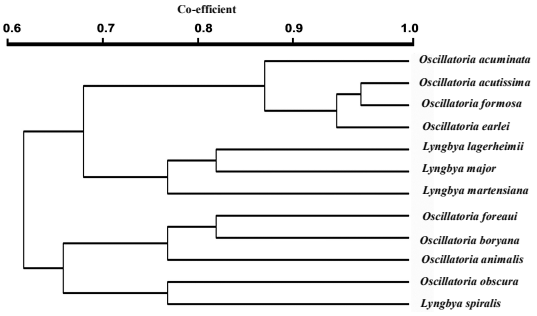


Fig. 6. Phylogenetic analysis of ERIC

Enterobacterial Repetitive Intergenic Consensus (ERIC)

ERIC PCR gave multiple bands of sizes ranging from 9.4 kb to 350 bp for the cyanobacterial strains analyzed (Fig.5). All the bands obtained were polymorphic. Two unique bands (1 kb and 700 bp) were observed in strains of *Lyngbya* whereas no such bands were found in strains of *Oscillatoria* (Fig 5). An amplified fragment of molecular size 9.54 kb was found to be unique for *Lyngbya martensiana*. Similarly unique band of 3.5 kb and 500 bp was observed in case of *Lyngbya spiralis* and *Lyngbya lagerheimii* respectively. The phylogenetic tree showed no clear demarcation between the two analyzed genera (Fig.6). The clusters had strains belonging to both genera except one minor cluster where *Lyngbya lagerheimii*, *Lyngbya major* and *Lyngbya martensiana* were grouped together.

DISCUSSION

Most of the strains got grouped according to their genus with exception of *Oscillatoria animalis* and *Lyngbya spiralis* grouping with *Lyngbya* and *Oscillatoria* respectively. There are instances where two closely related genera have been shown to group together (Giovannoni *et al*., 1988; Lyra *et al*., 1997; 2001; Palinska *et al*., 1996). Other examples include grouping of *Oscillatoria* with *Microcoleus* (Wilmotte *et al*., 1992), *Nostoc*152 with *Nodularia* PCC73104/1 (Lyra *et al*.,1997), *Nodularia* PCC73104 with *Anabaena cylindra* PCC 7122 (Wilmotte *et al*.,1992), *Anabaena* with *Nostoc* and *Oscillatoria* and *Planktothrix* strains belonging to family Oscillatoriaceae at 16S rRNA gene RFLP (Restriction Fragment Length Polymorphism) has been reported by Lyra *et al*.,(1997; 2001). Even different genera belonging to different families have been reported to form clusters. Lyra *et al*., (1997; 2001) has reported grouping of *Anabaena* strain and *Aphanizomenon* strain and *Oscillatoria acuminata* PCC6304 grouped with strains belonging to *Microcystis*, at 16S rRNA gene level. In the present study, the 16S rDNA that has been amplified was not sequenced but directly used for restriction analysis. Sequencing of the whole 16S rRNA gene may give better results as it has been shown that partial sequence and the complete sequence of the 16S rRNA gene classified R*hizobium galegae* into different groups (Willems and Collins, 1993; Nour *et al*., 1994). More number of restriction enzymes may have resulted in better polymorhic bands as only two enzymes were used in the present study.

ERIC has been shown to be very useful to differentiate two organisms that are closely related (Versalovic *et al*., 1991). Strains belonging to *Nostoc* and *Nodularia* were clearly resolved with REP and/or ERIC fingerprinting (Rasmussen and Svenning, 1998; Lehtimaki *et al*., 2000). Lyra *et al*., (2001) has reported that ERIC and REP fingerprinting can differentiate closely related strains like neurotoxic *Anabaena* and non-toxic *Aphanizomenon*, *Microcystis* strains and *Planktothrix* strains to a high degree. This was the reason for employing ERIC fingerprinting for differentiating strains in this study. As observed in ARDRA analysis, the major cluster in the ERIC phylogenetic tree had strains belonging to both genera. The reason could be the close relatedness of both genera, which belong to same family *Oscillatoriaceae*. The strains belonging to *Oscillatoria* and *Lyngbya* are morphologically differentiated only by the presence of the sheath around the trichomes in the later.

In conclusion, this paper shows preliminary attempt for classification of cyanobacteria using ARDRA and ERIC techniques. Overall, the strains got grouped more or less in accordance with genus in ARDRA, but in ERIC, only three strains of *Lyngbya* showed separate grouping. The results indicate that some strains of *Oscillatoria* and *Lyngbya* are genetically closer among all the tested strains. It is evident from the present study that ARDRA and ERIC PCR fingerprinting can be used successfully to differentiate closely related cyanobacterial strains.

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