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Salt tolerance characteristics of *rhizobium* species associated with *Vigna marina*

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ABSTRACT

A study was conducted during 2003–04 to examine the characteristics of *rhizobia* associated with *Vigna marina* (Burm.) Merr. (syn. *V. retusa*. Walp) a wild legume of Andaman and Nicobar islands, which was found growing naturally in the sandy seashore and was able to survive high saline environment with prolific root nodules. The *rhizobia* associated with the root nodule of *V. marina* was collected, characterized and studied for their growth and salt tolerance properties. The plant gave more root/shoot ratio (0.76) and had runner type roots with more than 50% viable nodules. Salinity however did not hinder the nodulation process. The *Rhizobium* was found to be tolerant to antibiotics, like erythromycin, chloromphenicol, streptomycin and ampicillin. High membrane protein (14.98 µg/ml) was observed in the cells of *Rhizobium* VM1 indicating lesser degradation of protein under saline environment. Lipopolysaccharide profile revealed that the bacterium was closely related to *Rhizobium* of cowpea (*Vigna unguiculata* (L.) Walp) and blackgram (*Vigna mungo* (L.) Hepper). The bacteria showed resistance up to 600 mM NaCl and tolerance up to 1000 mM of NaCl *in vitro*.

Key words: Andaman Islands, *Rhizobium*, Salt tolerance, *Vigna marina*

Biological nitrogen fixation is an efficient source of fixed nitrogen (200–300 kg N/ha/year), which plays an important role in nitrogen enrichment of the land. Some members of leguminoceae with its rhizobial association accounts 80% of the biological fixed nitrogen and contribute 25–30% of the worldwide protein intake. In addition to crop legumes, wild legumes possess potential for nitrogen fixation. A novel, suitable wild legumes-*Rhizobium* association was useful in improving the degraded lands. The majority of rhizobial strains from wild legumes were identified as members of the genera *Rhizobium* and *Sinorhizobium*, but a few strains were classified into the genera *Mesorhizobium* and *Bradyrhizobium* (Zahran 2001). The salinity response of legumes varies greatly and high soil salinity can limit productivity by adversely affecting the growth of the host plant and the symbiotic development of root nodule bacteria (Salehi *et al*. 2008). The knowledge about stress responses in the *rhizobia* is relatively poor despite it is long known that stresses (soil acidity and salinity) affect rhizobial persistence and nodulation efficiency. El-Hamdoui *et al*. (2003) reported that even a salt concentration of 75 m*M* NaCl reduced plant growth and inhibited nodulation and nitrogen fixation of 'Argona' pea (*Pisum sativum* L.) grown in symbiosis with *Rhizobium leguminosarum*. The formation of effective nodules under such stress depends upon the presence of the effective strains of rhizobia and leguminous plants, which posses the ability to produce early nodules.

Free-living *Rhizobium* varies in their response to salt stress. Growth of a number of rhizobia was inhibited by 100 mM NaCl, while others are tolerant to 300–700 mM NaCl (Mohammad *et al.* 1991). Rhizobia isolated from sand dune legumes (Arun and Sridhar 2005) and tree legume *Leucaena leucocephala* (Ali *et al.* 2009) showed that some isolates were tolerant upto 2.5–3.5% salinity. The rhizobia isolated from *A. nilotica* showed tolerance to NaCl up to 850 mM and the same rhizobia formed effective N-fixing nodules on *Acacia* trees grown with 150 mM NaCl (Lal and Khanna 1995).

Vigna marina (Burm.) Merr. is a wild legume distributed throughout the tropics. It possesses succulent stems and leaflets, broader and glabrous legumes oval and reddish-brown seeds (Sonnante *et al.* 1997). This wild crop is closely related to another wild legume *V. luteola*. Both *V. luteola* and *V. marina* showed a limited level of allozyme variation closely related to *V. unguiculata*. Since this plant is found growing profusely near the sea coast of South Andaman Islands, the present study was undertaken to examine the salt tolerance characteristics of rhizobia associated with *Vigna marina*.

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MATERIALS AND METHODS

Vigna marina plants were collected from the seashores with its root and soil intact and transported to the glass house of Central Agricultural Research Institute, Port Blair, Andaman and Nicobar Islands. The matured seeds were collected and a part of the seeds were raised in the pots under glass house condition and partly stored in the laboratory for further studies. Seeds were surface sterilized with 90% ethanol, 10% Sodium Hypochlorite and 3% H_2O_2 and were soaked in sterile distilled water for 30 min. Seeds were inoculated with 2 ml of bacterium containing 10^6 cfu/ml, which was isolated as described later. Seeds were then germinated on wet filter paper by incubating at 30°C for 2–3 days until radicals were 2–3 cm long and root hairs appeared. Two seedlings were planted into cement pots containing autoclaved white sand. Plants were watered regularly with nutrient solution and sterile distilled water. Plants were harvested at the time of flowering (45 days after planting) with roots intact. Number of nodules, root and shoot length, fresh and dry weight of root, shoot and nodules and number of viable nodules were recorded.

The soil around the root zone of *V. marina* was collected, screened in 2 mm sieve and gravel content was discarded. The remainder was utilized for determination of *p*H, electrical conductivity (EC), cation exchange capacity (CEC) and exchangeable sodium percentage (ESP) following soil survey laboratory methods manual of USDA (USDA). The soil was found to be sandy with *p*H 8.1, EC 4.15 dSm, CEC 5.4 meq/100g and ESP 20%. This shows the saline nature of the habitat and the ability of the plant to grow under such adverse saline condition. The neutral *p*H and moderate EC of the soil under the present study may be due to predominance of sulphates in the soil and immediate percolation of the ingressed salt water owing to sandy texture of soil.

The *V. marina* plants from the native growth place were collected at various stages with the roots intact. Each plant was decapitated and the root and shoots were weighed. The roots were washed gently under tap water and blot dried. Nodules from each individual root were collected, counted and weighed. The nodules were cut open to find the viability and then air dried at room temperature until the weight became constant and weighed. The shoot and roots were dried in oven at 80°C and the dry weight was recorded.

Plants were uprooted with nodules intact, washed several times with sterile distilled water and placed in a sterile tube to avoid cross-contamination. The nodules were separated, surface sterilized with 95% (v/v) ethanol and 5% H_2O_2 for about 5 min. Nodules were thoroughly washed with six changes of sterile water, placed in drops of 0.85% (w/v) NaCl on Petri-dishes, crushed with a glass rod and the exudates streaked onto Yeast Mannitol Agar (YMA). Two weeks after incubation, representative bacterial colonies were selected on the basis of colony morphology and purified several times on same medium. Single colonies were stored on slants and checked by Gram staining. The isolates were used for inoculation of seeds. Reference strains of *Rhizobium* were collected from Agricultural College and Research Institute, Madurai, Tamil Nadu and used for the characterization involving lipopolysaccharide profile. The *Rhizobium* isolates used in the present study were VM1 (isolate of *Vigna marina*), CP2 (isolate of *Vigna unguiculata*), BG3 (isolate of *Vigna mungo*), GN4 (isolate of *Arachis hypogea*) and SS1 (isolate of *Sesbania sesban*). The former two isolates were collected from Port Blair and latter three were collected from Madurai.

The bacterial strain isolated was streaked onto YMA plates. Plates were incubated for 7–14 days at 28°C and examined during 3–5 days for the fast growing strains, during 5–7 days for slow growing strains, and again at 14 days for both types. The colony structure and slime production were recorded as growth parameter.

The antibiotic sensitivity assay was performed by measuring inhibition zone (Gunasekaran 1996). Fresh solutions of filter-sterile antibiotics were added to melted YMA medium to attain concentration (µg/ml) as follows: Chloramphenicol, 5 and 15; erythromycin, 10 and 20; penicillin, 0.1 and 0.3; streptomycin sulfate, 2.5 and 10; ampicillin, 0.1 and 0.3; and tetracycline 0.1 and 0.2. Bacterial culture was replicated twice for each antibiotic assay by dispensing 20 µl (10^{-5} dilution)/Petri-plate. Plates were incubated at 28°C and scored after 3 days. A swab of rhizobial strain was inoculated on surface of YMA and the surface was allowed to dry. The antibiotic disc was placed over the agar plate at least 15 mm from the edge of the plate and incubated for 24–48 hr. The zone of inhibition around the antibiotic discs was recorded for each antibiotic.

Lipopolysaccharide profile of rhizobia was analyzed following de Maggd *et al.* (1988). Bacterial cells were harvested by centrifugation at 5 000g for 10 min. at 4°C, washed once in PBS (10 mM sodium dihydrogen phosphate-hydrogen phosphate and 0.9% NaCl, *p*H 7.4) and suspended in 5 ml of 50 mM Tris hydrochloride (*p*H 8.5). All subsequent procedures were carried out at 0 to 4°C. The cells were disrupted by sonication in three to five bursts of 30 sec. each with 15 sec. intervals by using a sonifier with water-jacket cooling. The remaining undisrupted cells and large fragments were removed by centrifugation at 900 g for 20 min. After the addition of 0.2 mg of lysozyme/ml of supernatant and incubation of 30 min. at room temperature, 2 M KCl was added to a final concentration of 0.2 M and membranes were pelleted by centrifugation for 60 min. at 12 000 g. The membranes pellet was suspended in a small volume of 2 mM Tris hydrochloride (*p*H 7.8). Samples of cell envelopes were heated for 10 min at 95°C, cooled to 60°C, incubated for 60 min. at 60°C with 0.2 mg of Proteinase K/ml, and diluted 15-fold with sample buffer without ß–mercapto ethanol. Before electrophoresis protein content of the membrane was determined by following Bradford method. The samples

equivalent to 5µg protein were electrophoresed in Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE) following Laemmli, 1970. The stacking gel and resolving gel contained 4% and 12% acrylamide, respectively. Electrophoresis was carried out at a constant current of 30 V till the dye reaches the resolving gel and thereafter it was carried out at constant current of 80 V till the dye reaches the end. The gel temperature was maintained at 4°C to avoid overheating. After electrophoresis, the gel was removed and visualized by silver staining. The gel was rinsed twice with distilled H_2O (d H_2O) to remove electrophoresis buffer and fixed in 10% ethanol by gentle shaking for 10 min. on a rocking platform. Ethanol was removed by suction and 0.7% nitric acid was added just enough to cover the gel. The gel was shaken gently on a rocking platform for 6 min. Nitric acid was removed with suction and rinsed the gel with two changes of dH_2O. Sufficient volume of 0.2% silver nitrate was added to cover the gel and shaken for 30 min. The gel was rinsed with dH_2O thrice. To 100 ml of developer, 125 µl of formaldehyde was added and poured on gel and shaken in a tray in dark. When the solution turned yellow and on appearance of dark precipitates the developer/formaldehyde was replaced with a fresh 100 ml of the same solution. The gel was continuously shaken and the appearance of bands and background was monitored. The developer was removed and 250 ml of 3% acetic acid was added to the gel and rocked for 5 min. The solution was then removed and gel was stored for 2 min in a fresh 10% ethanol. The gel was photographed in a gel documentation system (Vilber lourmat, France, Bio 1D ++ version 99.04). Dendrogram was constructed by UPGMA method (Nei and Li 1979) using NTSYSPc (2.02) software.

The YMA medium was prepared by adding varying concentrations of NaCl (50, 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 mM). The medium containing each concentration of salt was poured into the sterile Petri-plates and allowed to solidify. The fresh culture of *Rhizobium* isolate was streaked on to the solidified medium and incubated at room temperature. The number of colonies produced in each salt concentration was recorded at 24, 48 and 72 hr after inoculation. The YMA without salt acted as control. Another set of experiment was done by pour plate technique. At mid-exponential growth phase, 0.5 ml of broth culture was transferred to sterile Petri-plate and then poured with YMA medium with various concentrations of NaCl as mentioned above. The number of colonies produced in each salt concentration was also recoded.

The *Rhizobium* grown on slants was transferred to Yeast Mannitol (YM) broth and cultured flasks were incubated on rotary shaker at 150 rpm. At mid exponential growth phase, 0.5 ml of broth culture was transferred in triplicate to 250 ml flasks of YM (50 ml) broth with varying concentrations of NaCl. The inoculated flasks were held in a rotary shaker at 150 rpm. Optical density was measured using a spectrophotometer at 660 nm after 1, 3, 5 and 7 days after inoculation (DAI). The YM broth without salt was used as control. Cell dry weights were determined following Herbert *et al*. (1971). Two 10 ml aliquots of cell suspension were centrifuged at 1500g for 15 min; each pellet was washed once with 10 ml of sterile 150 mM NaCl, re-suspended in 1 ml of 150 mM NaCl, and combined in a tared 2 ml microcentrifuge tube. The combined pellets were centrifuged at 11 000g for 5 min., the supernatant was discarded, the pellet dried at 100°C, cooled to room temperature and then weighed.

RESULTS AND DISCUSSION

Growth and nodulation pattern in V. marina

Growth and nodulation patterns were observed in young seedling and matured plants growing naturally on the seashores. Total number of nodules, viable nodules, and nodule fresh and dry weight were found to be higher in matured plants than younger seedlings (Table 1). In mature plants 50% nodules were viable and salt did not hinder the nodulation process. Generally, salinity reduces the total number of nodules/plant (Salehi *et al.* 2008). This is mainly due to the adverse effects of salinity on the process of nodule initiation, an event in *Rhizobium*-legume symbiosis, which is highly vulnerable to osmotic stress (Thrall *et al*. 2008). However, several species of *Prosopis* were found to be salt tolerant forming effective symbiosis with rhizobial strains at salinity equivalent to seawater (Velarde *et al*. 2003). This is in conformity with our findings. El-Hamdoui *et al*. (2003) reported that salt concentration of 75 m*M* NaCl reduced plant growth and inhibited nodulation and nitrogen fixation of 'Argona' pea grown in symbiosis with *Rhizobium leguminosarum.* This, however, is in contrast with the present findings, where the *Rhizobium* VM1 tolerated even the salinity of seawater, which gets inundated during high tide regime.

In mature plants of *V. marina*, the root length was recorded

Table 1 Nodulation pattern and growth characters in *Vigna marina*

Character	Plant growth stage*	
	Young seedling	Mature plant
Number of nodules	30±2	32±2
Number of viable nodules	3±0.2	18±2
Fresh weight of nodules (g)	0.09±0.01	0.37±0.02
Dry weight of nodules (g)	0.02±0.005	0.11±0.006
Root length (cm)	28.8±1.2	130.3±6.6
Root fresh weight (g)	1.15±0.06	21.25±0.8
Root dry weight (g)	0.25±0.02	3.91±0.08
Shoot length (cm)	64.0±3.6	107.4±8.2
Shoot fresh weight (g)	2.58±0.5	32.3±2.6
Shoot dry weight (g)	0.54±0.02	5.12±0.4
Root/shoot based on dry weight	0.46±0.02	0.76±0.03

up to 130.3 cm and shoot length was up to 107.4 cm. The root/shoot ratio was 0.76, indicating greater shoot biomass under saline environment. Salinity normally reduces plant weight in several legumes (Salehi *et al*. 2008). However, in the present study the plant was found to produce greater biomass even in the high saline environment. The salt effect on shoot dry weight was more pronounced than on root dry weight thereby increasing the root/shoot ratio. In plants grown with NaCl, shortening of the internodes was observed in addition to the smaller leaves (Corodovilla *et al*. 1999) leading to more root/shoot ratio. This was in conformity in *V. marina* also due to the production of runner type of roots with indeterminate nodules. Indeterminate nodules were produced by some legumes, which are more salt tolerant than legumes producing determinate nodules. It has been implicated that former type of nodules has more potential to regeneration affected by stress environments.

Characteristics of rhizobia isolated from V. marina

The rhizobia isolated were fast growing, white pigmented, mucilaginous and Gram-negative. The bacterial colony was circular, convex and semi-translucent on YMA. The rhizobia isolated showed properties corresponding to the general character of genus *Rhizobium* (Rana and Krishnan 1995, Holt 1984). Among the eight antibiotics tested by pour plate (Table 2), isolate VM1 was found to be tolerant to erythromycin (20 µg/ml), chloromphenicol (15 µg/ml) and ampicillin (0.3 µg/ml). This test showed that it is different from *Rhizobium* sp. SIN1 which was sensitive to most of the antibiotics tested (Rana and Krishnan 1995) including chloromphenicol and erythromycin.

Membrane protein content of *Rhizobium* VM1 was significantly higher (14.98 µg/ml of culture) than other rhizobial isolates tested (Table 3). The electrophoretic mobility of Lipopolysaccharide on PAGE showed two distinguished patterns (Fig 1). The slow migrating LPS1 was predominant in all the tested rhizobia. The fast migrating LPS2 was observed in GN4 and SS1. Fifty per cent similarity was also observed with *Rhizobium* associated with groundnut and sesbania (Fig 2). Comparison of outer membrane protein and LPS profiles are used generally for strain identification in bacteria. It was reported that the level of total protein

Table 2 Antibiotic sensitivity of *Rhizobium* sp. VM1-Pour plate method

Antibiotic	Concentration (µg/ml)	No. of colonies
Erythromycin	10	Merged
	20	Merged
Chloromphenicol	5	Merged
	15	Merged
Streptomycin	2.5	20
	10	18
Tetracyclin	0.1	0
	0.2	0
Ampicillin	0.1	Merged
	0.3	Merged

Merged denote maximum growth in plate

Table 3 Membrane Protein content in *Rhizobium* sp. isolated from various legumes

Rhizobium isolate	Membrane protein content (µg/ml of culture)
VM1	14.98
CP2	6.52
BG3	5.70
GN4	2.18
SS1	2.69
CD (*P*=0.05)	0.15

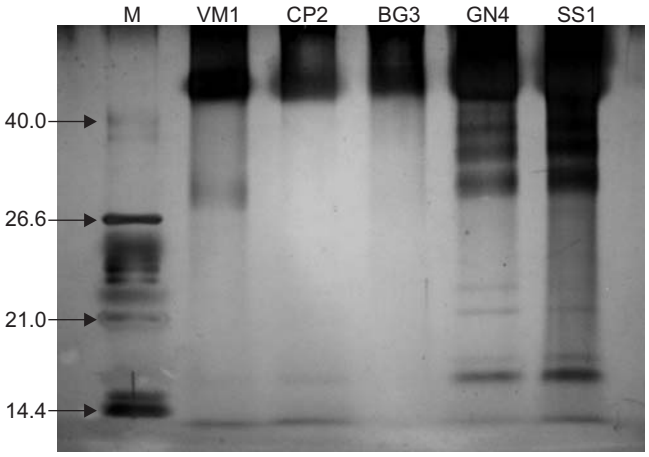

Fig 1 Lipopolysaccharide profile of *Rhizobium* isolates (M, Protein Marker in kDa; VM1, isolate from *V. marina*; CP2, isolate from cowpea; BG3, isolate from blackgram; GN4, isolate from groundnut; SS1, isolate from Sesbania)

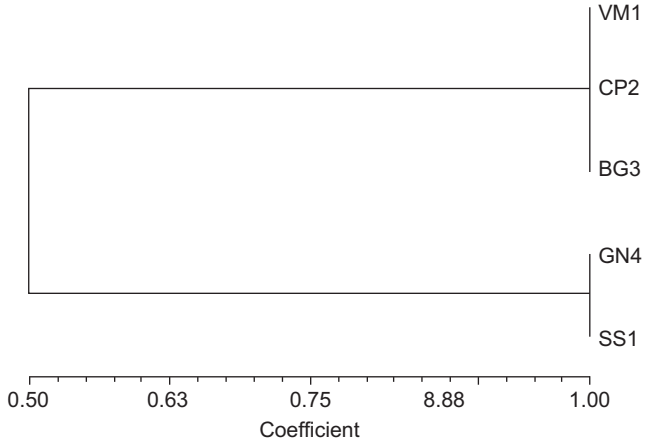

Fig 2 Dendrogram based on Lipopolysaccharide profile of *Rhizobium* isolates (VM1, isolate from *V. marina*; CP2, isolate from cowpea; BG3, isolate from Black gram; GN4, isolate from Groundnut; SS1, isolate from Sesbania)

Table 4 Growth of *Rhizobium* sp. VM1 on salt-amended medium

NaCl (mM)	Type of growth			No. of colonies		
	Time after inoculation (hr)			Time after inoculation (hr)		
	24	48	72	24	48	72
50	Poor	Heavy	Heavy	Merged	Merged	Merged
100	Poor	Heavy	Heavy	Merged	Merged	Merged
200	Poor	Heavy	Heavy	Merged	Merged	Merged
300	Poor	Heavy	Heavy	258	Merged	Merged
400	Poor	Heavy	Heavy	215	Merged	Merged
500	Poor	Heavy	Heavy	165	Merged	Merged
600	Poor	Moderate	Heavy	135	Merged	Merged
700	Poor	Poor	Moderate	117	334	Merged
800	No growth	Poor	Poor	53	162	389
900	No growth	Poor	Poor	41	84	273
1 000	No growth	No growth	Poor	12	45	120
Control	Poor	Heavy	Heavy	Merged	Merged	Merged

Merged denote maximum growth in plate

content decreased significantly with the increment of salt (Wahab *et al*. 2002), which is a typical response to salt stress and observed in many legumes (Cordovilla *et al*. 1999). This kind of response may be due to the presence of active proteases leading to protein digestion and formation of amino acids (Wahab *et al*. 2002). In the present case the membrane protein content of VM1 was very high indicating lesser degradation of protein owing to the inherent nature of salt tolerance. The Lipopolysaccharide profile study revealed that VM1 was closely related to *Rhizobium* sp. associated with cowpea and blackgram. It is well known that Lipopolysaccharide are important for the development of N-fixation and helping bacteria to overcome adverse environmental conditions. Lipopolysaccharides were also found to be protecting the rhizobia from salinity or other desiccation stresses (Vriezen *et al*. 2007).

Salt tolerance properties of Rhizobium VM1

Growth of rhizobial cells were found to be normal up to salt concentration of 600 mM NaCl in the media under streak plate and pour plate method (Table 4). The number of colonies, however, decreased in the media above 600 mm NaCl (2.9% NaCl). However, the colony growth was observed even up to 1 000 mM NaCl, albeit at a lower rate after 24 hr of inoculation and the colony growth was full after 72hr of inoculation. In broth culture the absorbance of bacterial suspension decreased in general with increase in concentration of NaCl at 1, 3, 5 & 7 days after inoculation (Fig 3). Increased absorbances were observed across all the salt treatments after inoculation, indicating multiplication of bacteria though at a slower pace.

Various workers reported low to massive growth of rhizobia on agar plates with increased salt concentration. Halotolerant *Rhizobium* isolated from sand dune legumes

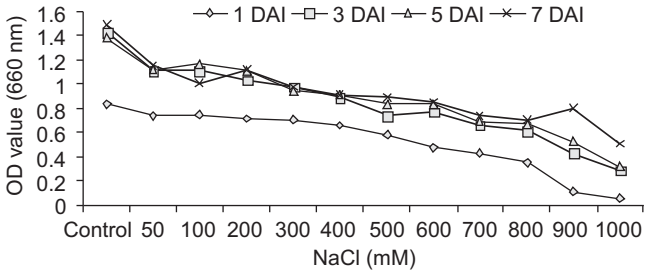

Fig 3 Growth of *Rhizobium* sp VM1 isolate in salt amended Yeast Mannitol Agar broth at 1, 3, 5 and 7 days after inoculation (DAI)

(Arun and Sridhar 2005) and tree legume *Leucaena leucocephala* (Lam.) de Wit (Ali *et al*. 2009) were able to grow in NaCl concentration of 2.5–3%. However, Ali *et al*. (2009) reported that salt tolerance of herb legumes (*Tephrosia purpurea* (L.) Pers and *Crotalaria medicaginea* (Wight and Arn.) Baker were reduced with salinity; whereas in the present study *V. marina* produced indeterminate nodules with 50% viability even at 4.5 dS/m. This highlights the salt tolerance ability of *Rhizobium* isolate VM1. The results of the present study showed immense ability of the rhizobia isolated from *V. marina* to grow at unusually high salt concentration. The ability of such salt tolerance strains of rhizobia to form effective N-fixing symbiosis in saline soil will improve soil fertility under such adverse condition. We do envisage that inoculation of this *Rhizobium* sp. into crop legumes of agricultural importance will be useful in improving the fertility status of degraded lands affected by salinity. To achieve the same a detailed study on cross inoculation of the strain is essential.

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