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Marine Pollution Bulletin 44 (2002) 726–732

MARINE POLLUTION BULLETIN

www.elsevier.com/locate/marpolbul

Reports

Mercury and lead tolerance in hypersaline sulfate-reducing bacteria

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Abstract

Sulfate-reducing bacteria (SRB) HSR1, HSR4, and HSR14 isolated from the salt pans of Goa grew best at 90–100‰ salinity on substrates like formate, acetate, lactate, butyrate, ethanol and benzoate. They were gram negative, non-sporulating, non-motile rods lacking in desulfoviridin and cytochromes. Examination of these isolates for heavy metal tolerance and response studies in terms of growth and sulfate-reducing activity (SRA) were carried out using $HgCl_2$ and $Pb(NO_3)_2$ at final concentration of 50, 100, and 200 and 100, 200 and 500 $\mu g\,ml^{-1}$ respectively. With Hg, HSR1 showed ≈80% of the control's growth at 100 and 200 $\mu g\,ml^{-1}$ but SRA reached only 60% of the control values at the end of 14 days. HSR14 could reach >100% of the control's growth at 200 $\mu g\,ml^{-1}$ but the SRA reached only up to 60% of the control without metal at 100 $\mu g\,ml^{-1}$. Though the concentration of Pb was double that of Hg, HSR4 could grow and respire better than the control, the growth being stimulated by 160% and respiration by 170% in the presence of 500 $\mu g\,ml^{-1}$ of $Pb(NO_3)_2$. It is probable that some hypersaline SRB are more tolerant to heavy metals than the mesohaline counterparts and could be more effectively used for precipitating these metals in bioremediatory measures. Further examination of their responses to varied concentration of metals under different salinities would indicate their range of applicability.

Keywords: Mercury; Lead; Toxicity; Sulfate-reducing bacteria; Hypersaline

1. Introduction

Microorganisms contribute to the biogeochemical transformations of metals and organic matter. The anaerobic sulfate-reducing bacteria (SRB) play a significant role in coastal ecosystems where more than 50% of biodegradation is through sulfate-reducing activity (SRA). Saltpans are sites where different ions, including metals, become concentrated and halophilic bacteria evolve, suppressing the less halophilic and halotolerant forms. As these systems also tend to concentrate the metals in seawater, it is pertinent to understand how bacteria interact with heavy metals. Saltpans along the coast of Goa draw seawater during the post monsoon from the adjacent coastal region during the high tide. The sea water is trapped in large enclosures made of low mud walls and allowed to crystallize and form salt by solar evaporation. The anaerobic forms in these systems could have a more important contribution than the aerobic forms not only because the solubility of oxygen is less but also because the water is generally stagnant. Though the general solubility becomes less with increased salinity, there is a tendency of metals to accumulate either in the soluble form until their salts reach a saturation point, or they get adsorbed/absorbed to particulate organic matter (POM) in saltpans. These metals also undergo other physical changes such as bacterial transformation in both aerobic and anaerobic layers of sediment and water. It is therefore possible that anaerobic processes can contribute to considerable flux of the elements in the environments. Most often bacteria transform the toxic metal to an innocuous state. Heavy metals such as mercury and lead have industrial uses and have been found to be widely distributed in the marine and estuarine environments (Sanzgiry et al., 1988; George, 1988).

Studies on metal microbe interactions are generally restricted to aerobic bacteria (Foster, 1983; Aiking et al., 1985), anaerobic consortia (White and Gadd, 1996) and sometimes to mesophilic SRB (Loka Bharathi et al., 1990). The present work has examined the interaction of a few hypersaline isolates of SRB with Hg and Pb at elevated concentrations of the metal salts, as saltpans

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PII: S0025-326X(02)00174-1

tend to concentrate metal. Hypersaline SRB have the ability to grow in high salt concentration of 80–250‰. Studies on metal tolerance of hypersaline SRB is as yet unexplored and the present work attempts to evaluate the metal tolerance limits of a few isolates of SRB from saltpans.

It is hypothesized that hypersaline SRB can contribute to metal detoxification by biotransformation and that their contribution could be greater than that of the general mesophilic SRB. Earlier studies on the effect of Cd, Hg and Pb on mesophilic SRB have shown that the inhibitory concentrations of Hg and Pb were 100 and 200 $\mu g\,ml^{-1}$ of $HgCl_2$ and $Pb(NO_3)_2$, respectively (Loka Bharathi et al., 1990). The present study has shown that the hypersaline SRB have higher levels of tolerance and detoxification.

2. Materials and methods

2.1. Bacterial strains

HSR1, HSR4 and HSR14 were isolated from 2–5, 5–10, and 5–7 cm depths of sediment cores from the salt pans of Goa, purified and maintained on modified Hatchikian's medium prepared in sea water of 90–100‰ salinity (Hatchikian, 1972; Loka Bharathi and Chandramohan, 1985).

2.2. Inoculation and incubation

Sterile and complete media were distributed into screw-capped test tubes and filled almost to the brim. The test strains were grown to exponential phase, centrifuged and drained. The cell pellet was suspended in a small quantity of the spent medium. Cell suspensions of 500 μl volume were used as inocula. The tubes were incubated in triplicate sets in the dark at room temperature (28 ± 2 °C) for 10–12 days. Negative controls without inocula and positive controls without metal salts were always included.

2.3. Measurement of growth

Growth was measured by increases in OD at 480 nm using a Shimadzu 1601 spectrophotometer. Precipitation in the medium was corrected by the use of controls. These included un-inoculated media containing test concentration of metal salts. However, sometimes extracellular precipitation after incubation led to an over estimation of growth.

2.4. Estimation of sulfide

Sulfide was measured as outlined by Pachmayr and cited by Truper and Schlegel (1964).

2.5. Metals

$HgCl_2$ and $Pb(NO_3)_2$ were used from 3% and 5% stocks to give a final concentration of 50, 100, and 200 and 100, 200 and 500 $\mu g\,ml^{-1}$, respectively. The upper limits were set at saturation concentration of metal salts at 95‰ salinity for both the metal salts.

Inoculation was carried out in triplicate and controls for each metal salt and inoculum were maintained throughout.

3. Results

The isolates were Gram negative, non-motile, short rods varying in size from 0.56 to 0.84 μm in width and 1.21 to 1.96 μm in length and lacked desulfoviridin and cytochrome. They were able to grow on a range of substrates like formate, acetate, butyrate, lactate, benzoate and ethanol (Table 1). However, HSR14 could not use ethanol. The cultures best between 5% and 15% NaCl concentration and maximum SRA was observed between 3% and 15% NaCl concentration (Fig. 1a and b).

The values for the growth of cultures of HSR1, HSR4 and HSR14 without metal, at the end of 7 days were 3.91, 2.36 and 3.14 od_{480} units respectively. The corresponding 14 day values were 3.80, 2.35 and 3.82 respectively. The SRA for these cultures at the end of 7 days were 54.1, 139.8 and 240.3 $mg\,S^{2-}\,l^{-1}$ respectively. The corresponding 14 day values were 56, 151.8 and 355.2 $mg\,S^{2-}\,l^{-1}$ respectively.

3.1. Effect of Hg on growth and SRA

The effect was different on different strains. HSR1 showed over 80% of the control's growth at 100 and 200 $\mu g\,ml^{-1}$ at the of 7 days. However, activity reached only 60% of the control's value at the end of 14 days at 50 $\mu g\,ml^{-1}$. HSR14 could reach up to 114% of the control's growth at 200 $\mu g\,ml^{-1}$ by 7 days but the SRA reached

Table 1
Growth of hypersaline SRB on various substrates

Substrate	Isolates		
	HSR1	HSR4	HSR14
Formate	++[a]	++	+[b]
Acetate	+	+	+
Butyrate	+	+	+
Lactate	++	+	+
Ethanol	−[c]	+	+
Benzoate	ND[d]	+	++

[a] Good growth.
[b] Growth.
[c] No growth.
[d] Not done.

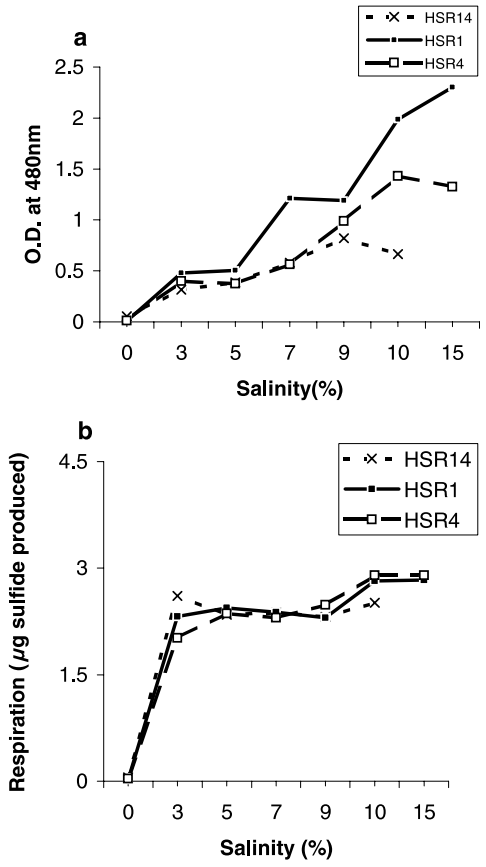


Fig. 1. Effect of sodium chloride on the growth and respiration of hypersaline SRB: HSR1, HSR4 and HSR14.

only up to 60% of the control's activity at 100 $\mu g\,ml^{-1}$. Growth of HSR4 reached 130% of the control's growth at 100 $\mu g\,ml^{-1}$ after 7 days, but SRA was 70% of the control value at 100 $\mu g\,ml^{-1}$ at the end of 14 days (Fig. 2a–f).

3.2. Effect of Pb on growth and SRA

Pb was less toxic than Hg for hypersaline SRB. HSR1 accounted for less than 20% of the respiration in the control but growth reached 80% after 7 days at 200 $\mu g\,ml^{-1}$ concentration. HSR14 showed very low growth and respiration as compared to control. HSR4 could grow and respire better than the control even after 14 days' incubation with 200 and 500 $g\,ml^{-1}$ concentrations of the metal (Fig. 3a–f). The stimulation of HSR4 for growth and respiration was 157% and 171% respectively.

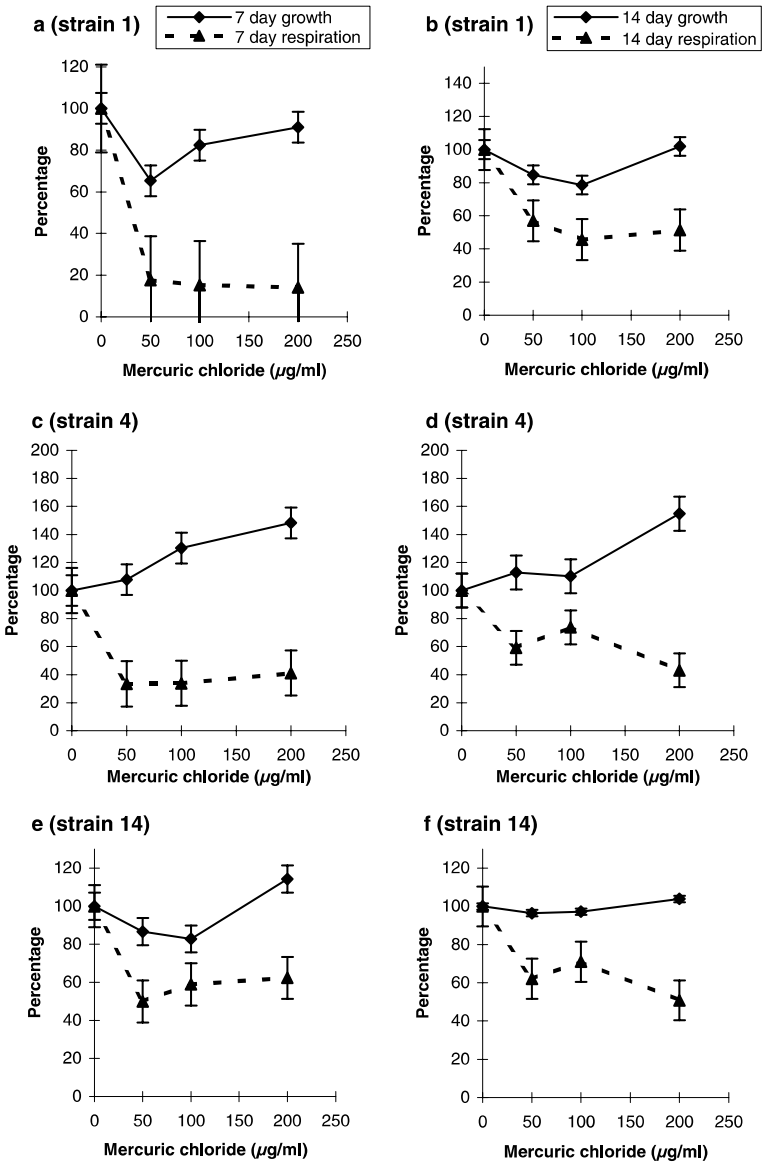
4. Discussion

Use of non-biodegradable materials including heavy metals in industry is increasing, as is the possibility of hazardous exposure. Though this aspect was realized as early as 1975 (Beliles, 1975), the hazards have been increasing in the current years. Heavy metals influence even microorganisms by affecting their growth, morphology or biochemical activities in concentrations as low as 5–10 ppm. They tend to accumulate in sediments by complex formations (Mooney et al., 1978; Appanna and St Pierre, 1997) and chemical combination with organic and inorganic matter. Microbes have different defense strategies including complexing, extra-cellular precipitation, impermeability, or reduced transport of metals across cell membrane. Metal binding proteins metallothioneins are also synthesized (Atlas and Bartha, 1997). The capacity of bacteria for biomethylation, volatilization, biopolymerisation, bioprecipitation, biosorption and intracellular traps are responsible for developments of resistance towards the toxic heavy metals. Hg-resistant *Bacillus* strains detoxify Hg to volatile elemental Hg via mercuric reductase. The detoxification is generally plasmid mediated (Komura et al., 1970).

Robinson and Tuovinen (1984) have discussed at length the anthropogenic sources of mercury and mechanisms of microbial resistance and detoxification. Hughes and Poole (1991) state that in the study of biological effects it is imperative that full account be taken of the speciation or chemical form of the metal ion in the organism's environment, the bioavailability and hence reactivity. Thus though lead exists both in organic and inorganic forms (Hoffman et al., 1995), Organo-lead compounds are generally more toxic to marine organisms than inorganic forms (Kennish, 1998). Both chemical and physical forms of lead influence its distribution and behavior in environment. Likewise, mercury is mostly present as organomercury. Inorganic forms of mercury are generally less toxic than organic forms, however within inorganic forms the toxicity increases with solubility in water (Hoffman et al., 1995). The concentration of heavy metals in the seawater off West Coast of India is quite low. Mercury levels ranged from 0.05 to 1.32 $\mu g\,l^{-1}$ (Sanzgiry et al., 1988) and Pb from 1.03 to 1.44 $\mu g\,l^{-1}$ (George, 1988). Previous metal analyses of the saltpan water and salt using ICP-AES and AAS respectively showed the lead concentration to be 20 and 23 ppm respectively (unpublished results) The concentration of mercury was found to be negligible.

Solubility checks of metal salts in hypersaline sea water showed that while 200 $\mu g\,ml^{-1}$ was the saturation limit for Hg, it was 500 $\mu g\,ml^{-1}$ for Pb. Thus metal concentrations tested included highest concentration that was soluble in the test medium. Growth and respiration of SRB at these concentrations or below showed that the hypersaline SRB could express different responses.

Though the isolates HSR1, 4 and 14 were from the same saltpan they originated from different depths. While 1 and 14 were from shallower depths originally



	Growth Units of optical density	Respiration mg $S^{2-}.l^{-1}$
HSR1 7th day	3.91	054.1
HSR1 14th day	3.80	056.0
HSR4 7th day	2.36	139.8
HSR4 14thday	2.35	151.8
HSR 14 7th day	3.14	240.3
HSR 14 14th day	3.82	355.2

Fig. 2. Effect of $HgCl_2$ on the growth and respiration of HSR1, HSR4 and HSR14 (a–f). Bars represent ± standard error of the mean ($n = 3$) 100% growth and 100% respiration in controls of HSR1, HSR4 and HSR14 on 7 and 14th day are shown.

isolated on formate, 4 was from 8 to 10 cm depth, initially isolated on benzoate. Pore waters from deeper layers tend to be relatively more reduced and more saline than from the surficial layers. It is perhaps for these reasons HSR4 has a competitive edge over the other two isolates.

Hg and Pb inhibited both HSR1 and 14 and Hg was more toxic than Pb. Levels of inhibition reduced with time with 14 days showing less inhibition than 7 days. HSR1 is more inhibited than HSR14 after 2 weeks' incubation reaching only 58, and 70% of the control value, respectively at 50 $\mu g\,ml^{-1}$. HSR4 is quite different in that, growth was stimulated at the end of 7 and 14 days. With Pb both growth and respiration were stimulated after 14 days. Stimulation was better with Pb even at saturation concentration of 500 $\mu g\,ml^{-1}$.

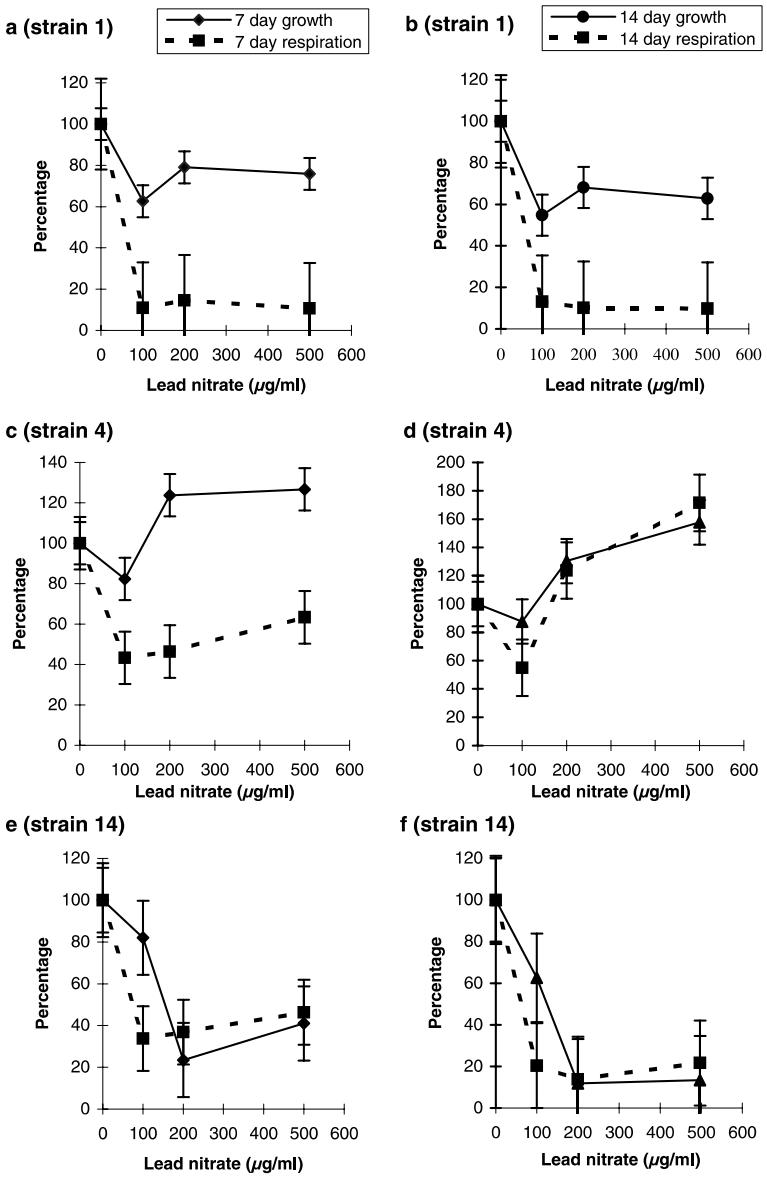


Fig. 3. Effect of $Pb(NO_3)_2$ on the growth and respiration of HSR1, HSR4 and HSR14 (a–f). Bars represent ± standard error of the mean ($n = 3$). 100% growth and 100% respiration in controls of HSR1, HSR4 and HSR14 on 7th and 14th day are the same as in the legend for Fig. 2.

Microorganisms adopt different modes of detoxification. Mercury can be sequestered as methyl mercury under fresh water conditions when sulfate can be limiting (Compeau and Bartha, 1985). Bacterial methylation of mercury can be stimulated under acidic or low alkalinity conditions or by increased availability of organic substrate (Hoffman et al., 1995). Under excess sulfate, mercury inhibits at 1000 ppm in sediment both during short and long term experiments, although the microbial biomass remained uninfluenced (Capone et al., 1983). Hg^{2+} ions can be reduced to less toxic Hg^0 enzymatically. In the present study Hg inhibits respiration more than growth. There was a certain amount of adaptation in growth with HSR1. With prolonged incubation, the isolate overcomes the inhibitory effect to an extent. This is evident from the reduced difference with control. With HSR14 inhibition was total. Earlier studies with mesohaline SRB showed stimulation in growth at 50 $\mu g\,ml^{-1}$ and inhibition at 100 and 200 $g\,ml^{-1}$ (Loka Bharathi et al., 1990). In the present study there was increasing adaptation in the growth of HSR4, the adaptation was fast and growth and respiration was better than control. A general mechanism of heavy metal toxicity is dependent on pH and organic matter concentration of the environment. Low pH mobilizes heavy metals and high pH precipitates them, thus reducing the toxicity. The detoxfication is most probably due to extra-cellular precipitation of HgS. As the pH of the medium was close to 8 and screw capped test tubes were used, loss of Hg as vapor (Aiking et al., 1985) was minimized. Webb

et al., 1998, have demonstrated such removal of metals by SRB, by production of sulfide. However, they noted that the rate of metal removal did not correlate with S^{2-} generation in all cultures.

Like Hg, Pb is also formed as metal sulfide. *Klebsiella* sp. also resort to the formation of PbS for detoxification (Aiking et al., 1985). Lead is more soluble and perhaps less toxic for the same concentration. Therefore the order of toxicity was Hg > Pb. *Escherichia coli* is also more tolerant to Pb than Hg (Ramteke, 1997). Pado et al. (1994) also showed SRB to be more tolerant to Pb than Hg. The low toxicity of Pb is probably due to the big ion radius of Pb and its considerable polarizability (Pado et al., 1994). As Capone et al. (1983) indicate, there is an initial inhibition followed by a period of stimulation in the case of HSR4. The stimulatory effect on growth could indicate Arndt Schulz effect rather than a requirement for the metal.

Thus the SRB are either inhibited or stimulated depending upon the strain, concentration of the metal and other environmental parameters. Under the conditions tested, it was seen that HSR4 was best adapted of the three isolates as it was able to detoxify up to 500 $\mu g\,ml^{-1}$ Pb. The ability to detoxify would perhaps have evolved with longer periods of incubation. Thus with Pb, the rate is faster even with higher concentration (500 $\mu g\,ml^{-1}$) than with Hg with lower concentration(100 $\mu g\,ml^{-1}$). As with other microbes, It could take longer for hypersaline SRB to detoxify as the metal Hg is more toxic than Pb.

5. Summary and conclusion

The study shows that HSR1 and 14 were tolerant of both the test metals up to a certain concentration. At the end of 14 days, growth was on par with the control in the presence of Hg. Although the concentration of Pb was double that of Hg, HSR4 could grow and respire better than control, the growth being stimulated by 160% and respiration by 170% in the presence of 500 $\mu g\,ml^{-1}$ of $Pb(NO_3)_2$. The hypersaline SRB have adapted to grow under extreme environmental conditions, although their habitat for isolation may not necessarily reflect their ability to tolerate and proliferate under adverse conditions (Lowe et al., 1993). However, in this study HSR4 was more adapted than the other two. Some of these forms could prove useful to mitigate high levels of toxic metals. Some highly toxic forms of elements and compounds can be biotransformed to innocuous forms through reductive metabolism by anaerobes. Inorganic sulfate can be reduced to H_2S by SRB which can result in the detoxification of metal ions or valence state alteration in toxic or soluble metals thus rendering them immobile and biologically unfavorable. Further examination of their responses to varied concentration of metal concentration under different salinities would indicate their range of applicability.

Acknowledgements

The authors are thankful to the Head, Marine Science and Marine Biotechnology Department, Goa University and Director NIO for all the facilities extended during the work. This is NIO contribution 3754.

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