

Plasmid profiles of mercuric chloride tolerant rhizobia from horse gram (*Macrotyloma uniflorum*)

Author Details

Prabhavati Edulamudi (Corresponding author)	Department of Botany and Microbiology, Acharya Nagarjuna University, Guntur - 522 510, India e-mail: prabha_anumicro@rediffmail.com
Antony Johnson A. M.	Department of Virology, Sri Venkateswara University, Chittoor, Tirupati - 517 502, India
Venkata Ramana Sai Gopal Divi	Department of Virology, Sri Venkateswara University, Chittoor, Tirupati - 517 502, India
Veera Mallaiah Konada	Department of Botany and Microbiology, Acharya Nagarjuna University, Guntur - 522 510, India

Abstract

Thirty two rhizobia were isolated from the fresh healthy root nodules of horse gram. They were found to be highly salt tolerant. They were identified as rhizobia by cultural, biochemical and 16S rRNA sequence. The sequences of the four selected isolates were deposited in the NCBI GenBank. The obtained accession numbers were GQ483457, GQ483458, GQ483459 and GQ483460. All the rhizobia were able to grow at 10 ppm mercuric chloride concentration. Four isolates HGR-11, 16, 30 and 31 were used to study the effect of different concentrations of mercuric chloride on the growth of rhizobia. These isolates were able to grow at 30 ppm concentration also. In these isolates, HGR-11 and HGR-30 showed maximum growth at 20 ppm than at control. These isolates contained one mega plasmid (~ 22 kb) at 20 ppm mercuric chloride concentration.

Key words

Metal tolerance, Mercuric chloride, Rhizobia, Plasmids, *Macrotyloma uniflorum*

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Introduction

Heavy metal contamination in the environment has become a serious problem due to the increase in the addition of these metals to the environment. Some heavy metals (cadmium, mercury, lead etc.) have no biological role and are detrimental to the organisms even at very low concentration. However, at high levels both of the essential and non-essential metals become toxic to the organisms (Rathnayake *et al.*, 2009). These heavy metals influence the microbial population by affecting their growth, morphology, biochemical activities and ultimately resulting in decreased biomass and diversity (Roane and Pepper, 1999).

In this perspective many approaches have been used to assess the risk posed by the contaminating metals in soil and water bodies etc. (Rathnayake *et al.*, 2009). At present the tolerance of soil bacteria to heavy metals has been proposed as an indicator of the potential toxicity of heavy metals to other forms of biota (Hassen *et al.*, 1998). Therefore, there is a dramatic increase in the interest

on studying the interactions of heavy metals with microorganisms. The favoured approach now is selecting the organisms that can be used to develop tools to assess the metal levels in the environment (Rathnayake *et al.*, 2009).

Among the metals, mercury is known to be highly toxic to microorganisms. Gadd (1993) reported that mercury is not essential for biological functions and is a strong inhibitor of microbial metabolism even at low concentration. Metal resistant microorganisms may be useful as indicators of potential toxicity to other forms of life and are important in studies of mechanisms, determinants and genetic transfer of microbial metal resistance (Jayasekar *et al.*, 2008). Slow rates of metal accumulation over the years favoured adaptation of the rhizobia to the metal rather than elimination of metal sensitive organisms and the selection of a few pre-/ existing metal tolerant organisms. There have been a few studies (Rafia Azmat *et al.*, 2005; Delorme *et al.*, 2003; Scott *et al.*, 1991) on metal resistance in rhizobia.

The present work was taken up to study whether salt tolerant strains of horse gram rhizobia exhibit tolerance to mercuric chloride and analysis of plasmid content in the mercury tolerant strains.

Materials and Methods

Thirty two rhizobia were isolated (Vincent, 1970) from the fresh healthy root nodules of horse gram plants growing in different soil samples collected from various parts in Andhra Pradesh. They were designated as HGR-1 (Horse Gram Rhizobia) to HGR-32. They were identified as rhizobia by 16S rRNA sequence.

Rhizobia from horse gram were tested for their tolerance to metals on yeast extract mannitol agar (YEMA) medium supplemented with 10 ppm mercuric chloride. The plates were incubated at room temperature. Colony diameter was recorded for every 24 hr up to 96 hr. One ml of the inocula was added to 10 ml of yeast extract mannitol (YEM) broth containing different concentrations of mercuric chloride (10, 20 and 30 ppm). Bacterial growth was monitored after 72 hr by measuring optical density (OD) of the cultures at 610 nm using spectrophotometer (ELICO mini spec). Two replicates were maintained for each concentration. One set of the media without metal was also inoculated and kept as control.

Plasmid isolations and agarose gel electrophoresis were carried out according to the method described by Sambrook and Russell (2001). *Rhizobium* cultures were grown in 3 ml of YEM broth for 12 hr. After overnight incubation, the cells were pelleted by centrifugation at 10,000 rpm for 4 min at 4°C. To the pellet 300 µl of Milli Q water was added and centrifuged at 10,000 rpm for 4 min at 4°C. The supernatant was discarded and to this pellet 300 µl of P1 solution (25 mM Tris HCl, pH 8.0 and 10 mM EDTA, pH 8.0 containing 0.1 µg ml⁻¹ of RNase) was added and vortex the pellet. 300 µl of P2 solution (0.2 M NaOH and 1% SDS) was added and the tubes were inverted and incubated at room temperature for 5 min. After that 300 µl of P3 solution (1 M potassium acetate, pH 5.2) was added. The tubes were inverted and incubated for 5 min at -20°C. After centrifugation, supernatant was taken to this 600 µl of isopropanol was added and the tubes were inverted and centrifuged at 14,000 rpm for 30 min at room temperature. The obtained pellet was washed by adding 500 µl of 75% alcohol. The pellet was dissolved in 10 µl of distilled water / T₁₀E₁ (Tris-10 M and ethylene diamine trichloro acetic acid 1 M) buffer and stored at 4°C.

Agarose gel electrophoresis of the plasmid DNA was performed. The DNA bands were observed in gel documentation apparatus (Alpha Innotech, USA) and the gel picture was photographed.

Results and Discussion

The isolated bacteria were identified as rhizobia basing on their characterization tests. The cells were mostly pink in colour and appeared as rod shaped bacteria. The cell size of all the isolates varied from 2 to 2.3 µ. These rhizobia showed lot of variation in their cultural, biochemical and enzymatic activities. All the isolates

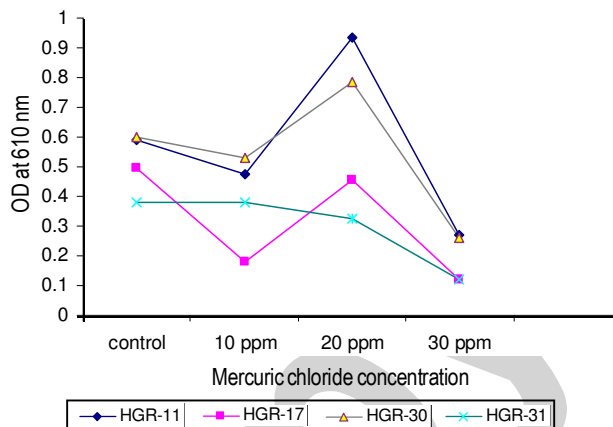


Fig. 1: Effect of mercuric chloride on the growth of four horse gram rhizobia

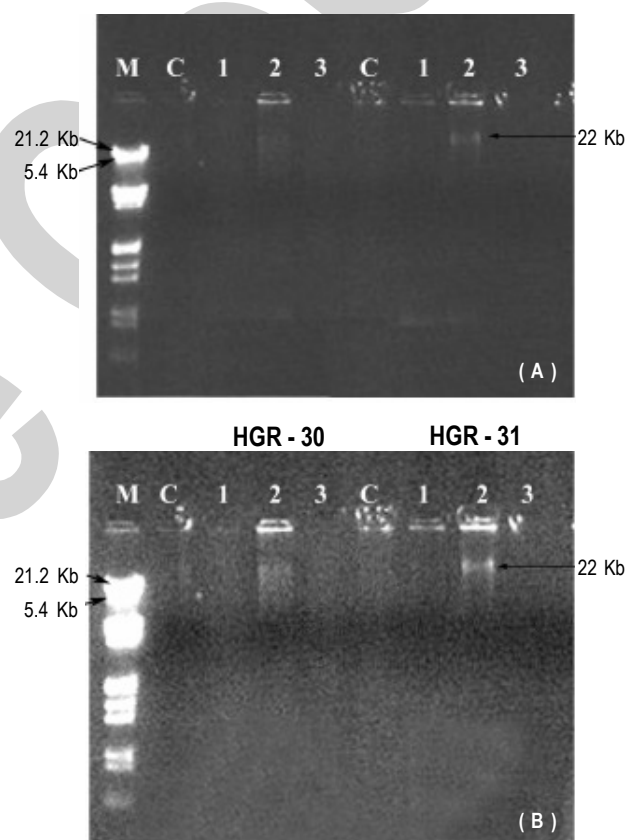


Fig. 2: Plasmid profiles of mercuric chloride tolerant Horse gram rhizobia (a) HGR-11, 16. (b) HGR-30 and 31, M- Marker Lane: Fermentas I EcoRI + HindIII double digest, (C) at control, 1 (10 ppm), 2 (20 ppm), 3 at (30 ppm)

were positive for citrase, nitrate reductase, tryptophanase, asparaginase, catalase and the production of ammonia, indole acetic acid, siderophores and bacteriocin. The rhizobia associated with horse gram were found to be highly salt tolerant (Prabhavati and Mallaiah, 2007). In these thirty two isolates, four representative isolates (HGR-4, 6, 13 and 25) having the most representative characters from the previous characterization tests (cultural, biochemical, enzymatic activities and molecular characterization) were selected for 16S rRNA sequence. The sequences were

submitted to the NCBI GenBank. The obtained accession numbers are GQ483457, GQ483458, GQ483459 and GQ483460.

Tolerance to mercuric chloride differed each of the *Rhizobium* isolate involved. All of the rhizobia showed growth on YEMA plates containing 10 ppm mercuric chloride concentration. The diameter of the colonies ranged from 3.0 mm (HGR-22) to 10.0 mm (HGR-1, 11 and 17). Almost all the isolates (except four) tolerated 10 ppm concentration of mercuric chloride in YEM broth. Four isolates (HGR-11, 16, 30 and 31) were able to grow even at 30 ppm concentration also. Each experiment was repeated twice and found to produce similar results. These strains showed resistance against mercuric chloride from 10 to 30 ppm. The isolates HGR-11 and HGR-30 showed maximum growth at 20 ppm mercuric chloride concentration than at control (Fig. 1). At 30 ppm concentration, the growth of these two isolates rapidly declined but still they were able to grow. The growth of the isolate HGR-31 slowly decreased with increase in mercuric chloride concentration whereas the isolate HGR-17 showed maximum growth at 20 ppm concentration but it was low when compared to the control. The bacteria growing at 10 ppm mercury concentration was considered as mercury resistant bacteria (Gadd, 1993). Generally, rhizobia are highly sensitive to mercuric chloride but these rhizobia (HGR-11, 16, 30 and 31) showed tolerance to mercuric chloride even at 30 ppm. At 40 ppm concentration the growth of these rhizobia rapidly declined.

Some of the heavy metal resistant bacteria are shown to possess specific plasmids (Nies, 1992; Osborn *et al.*, 1997). The isolates HGR-11, 16, 30 and 31 contained one mega plasmid (~22 kb) at 20 ppm mercuric chloride concentration (Fig. 2). Rasmussen and Sorensen (2001) noticed high levels of self transmissible mercury resistance plasmids in bacterial communities from a mercury contaminated site.

There are some reports that rhizobia are highly sensitive to some of the metals like cadmium and copper. Silver and Phung (1996) reported that copper and cadmium are highly toxic to rhizobia. The growth of *Rhizobium leguminosarum* and *Agrobacterium tumefaciens* was affected by copper treatment (Alexandre *et al.*, 1999).

In the present study, horse gram rhizobia showed tolerance to mercuric chloride up to 30 ppm concentration and it is high for the survival in metal polluted soils. Ravel *et al.* (1998 and 2000) reported that Gram-negative isolates are more resistant to mercury than Gram-positive bacteria. Heavy metal resistant microorganisms do not arise by chance and that there must be selectional factors like environmental contamination by heavy metals (Ramaiah and Jayasankar, 2003). Organisms able to grow under extreme environments offer good potential as indicators of pollution and as biosorbents (Sarma, 2001) and in bioremedial measures.

Even though *Rhizobiaceae* are generally very sensitive to metals, some isolates may show resistance. But it is an exceptional character. Rhizobia showing such exceptional characters are

considered as very distinct even taxonomically, for example a rhizobial isolate which can reduce selenium salt was described as a separate species to the genus in two legumes *Rhizobium viz. Rhizobium selenitereducens* (Hunter *et al.*, 2008).

These extremely halotolerant *Rhizobium* sp. also exhibit tolerance to mercuric chloride and hence could be used as agents for abatement of metal pollution in reclamation of saline as well as non-saline soils. Analysis of plasmid content of these mercury tolerant strains will be helpful for plasmid transfer studies.

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