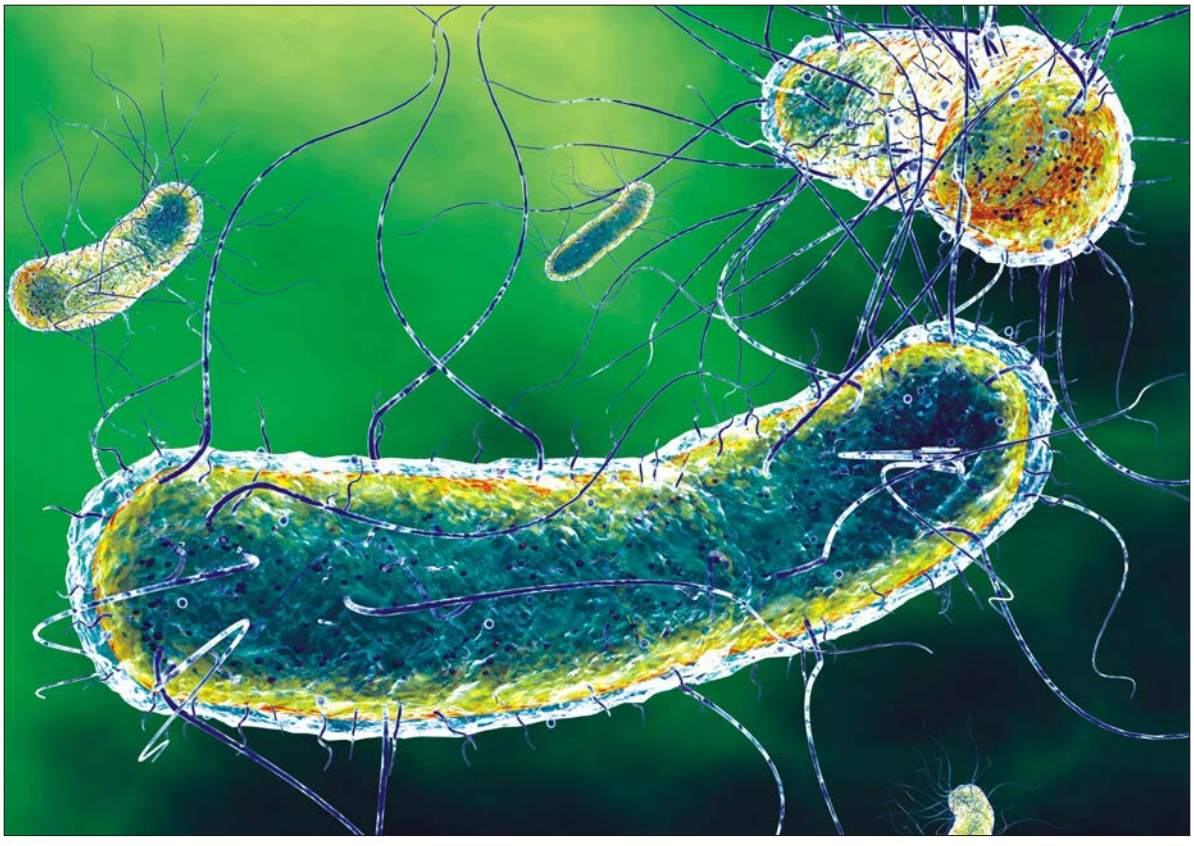

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Screening of Microbes for Their Metal, Antibiotic Resistance and Plant Growth Promoting Activity

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ABSTRACT

Heavy metal contamination has accelerated due to the rapid industrialization worldwide. Accumulation of metals in excess can modify the structure of essential protein or can replace an essential element. Aim of this study is to check the bacterial species for their tolerance towards multiple metals as well as antibiotics and further check whether these metal resistant microbes are producing any plant growth promoting substances. Bacterial strains were isolated from the soil sample collected from the industrial area of Lagos, were tested for their tolerance to both heavy metals and antibiotics by agar plate dilution method and by the disc diffusion method, respectively. Plant growth promoting activity was checked by the standard methods. All of the isolates showed tolerance to lead, zinc and copper. All the isolates showed maximum tolerance towards lead and zinc which was followed by copper. Bacterial species also showed tolerance towards antibiotics, 80c/o of the isolates were tolerant to Ampicillin, Cotrimozazole, Gentamycin, Colistin, Streptomycin and Tetracycline whereas 20% of the isolates were tolerant to Nalidic and Nitrofurantoin. Bacterial strains were also tested for their Plant Growth Promoting (PGP) substances, all the isolates were positive to ammonia, HCN and Phosphate solubilization.

Key words: Heavy metal resistance, antibiotic resistance, bacterial species, plant growth promoting activity

INTRODUCTION

Contamination of the environment by heavy metals is the wide spread problem due to their use in industries and agricultural purposes (Fernandes and Henriques, 1991). It adversely affect about 12% of the world's agricultural land (Moffat, 1999). Heavy metal pollution has increased from the start of the industrial revolution. The primary source of this pollution includes the industrial operations such as, smelting, mining, metal forging, manufacturing of alkaline storage batteries and combustion of fossil fuel and sewage sludge of industrial/domestic origin (Ibekwe *et al*., 1995). The application of sewage sludge in agronomic practices is often the most economical means of disposal. It is beneficial because it increases the organic matter content and water holding capacity of soil (Pagliai *et al*., 1981) and also provides plant with sufficient nutrients. . However, sewage sludges from industrial sources, often contain variable amounts of potentially toxic heavy metals, such as, lead, nickel, copper and zinc (McGrath, 1987). When these sludges are repeatedly applied to agronomic lands, heavy metals accumulate and persist in the top cultivated layer (0-20 cm) (McGrath, 1987). The persistence of these metals in soil adversely affect the agro-ecosystem

(McIlveen and Negusanti, 1994; Broos *et al*., 2004, 2005). Despite the reports of availability of larger quantities of heavy metals in sewage water, it is widely applied in agronomic practices for irrigation purposes. Though, a large number of reports on the effects of sewage sludge having multiple metals on microbial communities (McGrath *et al*., 1988; Giller *et al*., 1998) and plants (Ibekwe *et al*., 1995) are available, yet there is discrepancy in the reported results (Ramirez *et al*., 2008). And hence, a firm conclusion on the toxicity of heavy metals on plants including legumes and their symbiotic partners or plant growth promoting rhizobacteria cannot be drawn. Moreover, the majority of the adverse effects observed in sludge treated soils are possibly due to the factors other than metals (e.g., contaminants, excess N supply) which increases the toxicity. The elevated concentration of such metals adversely affects the quantitative and qualitative composition of microbial communities in soil including those bacterial populations that aggressively colonize plant roots and termed as Plant Growth Promoting Rhizobacteria (PGPR) (Kloepper and Schroth, 1978), leading to an altered microbial equilibrium in rhizosphere (Gray and Smith, 2005). Also, the enhanced concentrations of metals affect growth, metabolisms and consequently the total biomass of naturally occurring beneficial microbes.

To survive under metal stress conditions, plant growth promoting bacteria have evolved several mechanisms to tolerate the uptake of heavy metal ions (Nies, 1999). These mechanisms include precipitation of metal as insoluble salts by chemical transformation, accumulation and sequestration of the metal ions inside the cell, biotransformation-transformation of toxic metal to less toxic forms (Thacker and Madamwar, 2005). Almost all known bacterial resistance mechanisms are encoded on plasmids and transposons (Silver and Walderhaug, 1992) and the bacteria acquire resistance to heavy metals either by the gene transfer or spontaneous mutation. Metal ions are known to cause oxidative stress, stress response genes are induced as metal ion concentrations increase from starvation to toxic level. It is studied that genes are expressed under specific metal stress (Singh *et al*., 2001). For example, in Gram-negative bacteria (e.g., *Ralstonia eutropha*), the czc system is responsible for the resistance to Cd, Zn and Co. The czc-genes encode for a cation-proton antiporter (CzcABC) which exports Cd, Zn and Co. Similarly in *Alcaligenes xylosoxidans*, ncc system was found which is resistant to Ni, Cd and Co. On the contrary, Cd-efflux ATPase was responsible for Cd resistance in Gram-positive bacteria (e.g., *Staphylococcus*, *Bacillus* or *Listeria*). Cu resistance systems observed in *Pseudomonas syringae* pv. tomato is cop whereas, in *Escherichia coli* it is pco which encode for different Cu-binding proteins which allow Cu to bind in the periplasm or in the outer membrane. In contrast, the pco system is an ion-dependent Cu antiporter (Kunito *et al*., 1997). Additionally, a few naturally occurring microorganisms with high metal resistance in plant rhizosphere significantly reduced metal toxicity (Kamaludeen and Ramasamy, 2008). Generally, plant growth promoting rhizobacteria release plant growth promoting substances (Sheng and Xia, 2006; Ahmad *et al*., 2008), vitamins, enzymes, siderophores and antibiotics (Noordman *et al*., 2006; Burd *et al*., 2000) which result in the overall improvement in the growth of the plants. They also promote the growth of plants by alleviating the stress induced by ethylene-mediated impact on plants by synthesizing 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Madhaiyan *et al*., 2007; Glick *et al*., 2007). The present study was therefore undertaken to check the resistance pattern of plant growth promoting rhizobacteria to heavy metals and antibiotics and their phytohormone production.

MATERIALS AND METHODS

Collection of samples: Soil samples for the isolation of bacteria were collected from the soils of the the industrial area of Lagos State, Nigeria.

Microbial diversity in metal polluted soils: The rhizospheric soil samples collected from metal polluted soils of Lagos were used to determine total bacterial populations and fungal populations using standard microbiological methods (Holt *et al*., 1994). The diluted soil suspension was spread on nutrient agar (for total bacterial populations) and Martin's medium (for fungal populations). Each sample was replicated three times and incubated at 28±2°C for 24 h and three days for total bacterial and fungal population, respectively.

Evaluation of bacterial strains for metal tolerance: Bacterial strains were isolated from the soils of the industrial area of Lagos State, Nigeria. Bacterial strains were tested for their resistance to three metals like lead, zinc and copper by agar plate dilution method (Holt *et al*., 1994) using nutrient agar medium. The freshly prepared agar plates amended with increasing concentration of lead (0-1200 μg mL^{-1}), zinc (0-800 μg mL^{-1}) and copper (0-800 μg mL^{-1}) were spot inoculated (100 μL) with 10^8 cells mL^{-1}. Plates were incubated at 28±2° for 3-5 days. Lowest concentration of metals inhibiting bacterial growth on nutrient agar plate was defined as a minimum inhibitory concentration. Each experiment was replicated three times.

Determination of antibiotic resistance: To determine resistance to antibiotics, the plant growth promoting bacterial strains were tested for their sensitivity to eight antibiotics. The reactions to antibiotics were determined by the disc diffusion method (Bauer *et al*., 1966). Bacterial species were grown in nutrient broth respectively, at 28±2°C for 24 h. A 0.1 mL of the overnight grown culture was spread on the surface of nutrient agar. The antibiotic discs of known potency were then placed on the agar surface and the plates were incubated at 28±2°C for 24 h and the zones of inhibition around the antibiotic discs were measured to the following antibiotics: tetracycline (25 μg), streptomycin (25 μg), Colistin (25 μg), nitrofurantoin (200 μg), nalidixic acid (30 μg), gentamycin (10 μg), Cotrimoxazole (25 μg) and ampicillin (25 μg).

***In vitro* assay of HCN and ammonia:** Hydrogen cyanide production by bacterial isolates was detected by the method of Bakker and Schippers (1987). For HCN production, the bacterial strains were grown on an HCN induction medium (5 g tryptic soy broth, 0.88 g glycine,3 g agar) at 28±2°C for four days. For each bacterial isolate, 100 μL of 10^8 cells/1 mL was placed in the center of the petri plate. A disk of whatman filter paper No. 1 dipped in 0.5% of piciric acid and 2% Na_2Co_3 was placed at the lid of the petri plate. Plates were sealed with paraflim. After four days incubation at 28±2°C, an orange brown colour of the paper indicating HCN production. For the production of ammonia, the bacterial strains were grown in peptone water (g L^{-1}). Peptone 10 g, Nacl (5 g Ph7) and incubated at 30±2°C for four days. One milliliter of Nessler reagent was added to each tube and the development of yellow colour indicating ammonia production was recorded (Dye, 1962).

Qualitative and quantitative assay of phosphate: The bacterial strains were inoculated into Pikovskaya medium and incubated at 28±2°C for seven days and observed for halo formation. The colony forming a clear halo around the bacterial growth was considered phosphate solubilizers. The colony forming clear halo around bacterial growth indicating P solubilization as counted and further used to determine the relative P solubilizing efficiency (RPSE) in liquid Pikovskaya medium. For the quantitative measurement of P, 100 mL of Pikovskaya broth containing tricalcium phosphate (TCP) was inoculated with one mL of 108 cells mL^{-1} of each culture. The flasks were incubated for seven days with shaking at 120 rpm at 28±2°C. A 20 mL culture broth from each

flask was removed and centrifuged (15000 rpm) for 30 min and the amount of water soluble P released into the supernatant was estimated by the chlorostannous-reduced molybdophosphoric acid blue method (King, 1932; Jackson, 1967). To 10 mL of supernatant, 10 mL chloromolybdic acid and 5 drops of chlorostannous acid was added and volume was adjusted to 50 mL with distilled water. The blue colour developed was read at 600 nm. Amount of phosphate solubilized was calculated using the calibration curve of KH_2PO_4. Each independent experiment was repeated twice after several subcultures.

Statistical analysis: Data of three replicates were subjected to statistical analysis using pair samples T test with significance level of $p<0.05$. The values indicate the mean±S.D of three replicates.

RESULTS

Microbial diversity of metal polluted soils of lagos: The metal polluted soils of Lagos were subjected to microbial analysis. Generally, the bacterial population (800×10^4 CFU g^{-1} of soil) was more than the fungal population (05×10^4 CFU g^{-1} of soil).

Tolerance of plant growth promoting rhizobacteria to metals: The selected plant growth promoting rhizobacterial strains were tested for their ability to tolerate various concentrations of heavy metals like copper, zinc and lead using agar plate dilution method. Generally, the Plant Growth Promoting Rhizobacterial (PGPR) strains showed a varied level of tolerance to heavy metals (Fig. 1). Among the bacterial strains, strain PA6 showed highest tolerance to most of the metals. Strain PA6 tolerated a concentration of 800, 800 and 1200 μg mL^{-1} of copper, zinc and lead, respectively, amended in agar plates whereas strain PA3 showed a tolerance level of 800, 800 and 1000 μg mL^{-1} to copper, zinc and lead, respectively, added to solid plates.

Antibiotic resistance of plant growth promoting rhizobacteria: Resistance to antibiotics among metal tolerant rhizobacterial strains differed considerably (Table 1). Among bacterial spp, 100% of strains were resistant to tetracycline, colistin, nitrofurantoin, gentamycin and cotrimoxazole, 83.33% were resistant to ampicillin whereas 66.66% were resistant to each streptomycin and nalidixic acid.

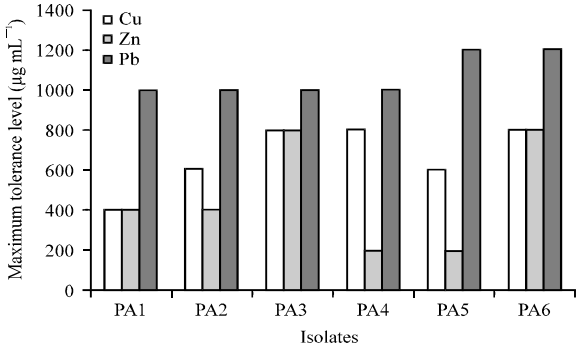

Fig. 1: Maximum tolerance level shown by different bacterial isolates

Table 1: Resistant pattern of bacterial species to various antibiotics

Antibiotics used	Concentrations (μg mL^{-1})	No. of resistant isolates (%)
Tetracycline	25	6 (100)
Streptomycin	25	4 (66.66)
Colistin	25	6 (100)
Nitrofurantoin	200	6 (100)
Nalidixic acid	30	4 (66.66)
Gentamicin	10	6 (100)
Cotrimoxazole	25	6 (100)
Ampicillin	25	5 (83.33)

Table 2: Plant growth promoting activities of metal resistant bacterial isolates

	Plant growth promoting activity			
			Phosphate solubilization	
Isolate No.	Ammonia	HCN	Zone on solid media (mm)	In liquid medium (μg mL^{-1})
PA1	+	+	5±0.4	180±16.4
PA2	+	-	4±0.3	160±14.7
PA3	+	+	5±0.5	193±18.2
PA4	+	+	3±0.4	177±16.9
PA5	+	+	2±0.3	171±13.3
PA6	+	-	6±0.5	221±18.2

Bioassay of plant growth promoting activities: The Plant Growth Promoting (PGP) substances like hydrogen cyanide and ammonia synthesized by the metal tolerant PGPR strains were assayed under *in vitro* experiments and are explained as follows.

***In vitro* assay of ammonia and HCN:** The metal tolerant plant growth promoting rhizobacterial strains were tested further for the synthesis of ammonia and hydrogen cyanide using peptone water and HCN induction medium, respectively. Generally, all PGPR strains were found positive for ammonia whereas PA1, PA3, PA4 and PA5 where positive for HCN (Table 2).

Phosphate solubilization on solid and liquid medium: The plant growth promoting rhizobacteria were further evaluated for their phosphate solubilizing potential, both on solid and liquid Pikovskaya medium. In the present study, all the PGPR strains showed the phosphate solubilizing activity, as detected by the formation of clear halo around their growth. Among the phosphate solubilizing PGPR strains, strain PA1, PA3 and PA6 produced the largest zone of P solubilization on solid Pikovskaya medium (Table 2). Further, a considerable amount of tri-calcium phosphate (TCP) was solubilized in liquid broth by PA1 (180 μg mL^{-1}), PA3 (193 μg mL^{-1}) and PA6 10 (221 μg mL^{-1}), respectively (Table 2).

DISCUSSION

Deposition of metal into soil over a long period of time results in high concentration of metal in the soil which adversely affects the microflora of the soil (Matsuda *et al*., 2002). Heavy metals in general show adverse effect on the soil microbial flora by blocking their functional groups or these metals modify the biological molecules in particular their active sites. But these metals when

present in low concentrations are important for the microbes as they supply the microorganisms with the essential co-factors for metallo proteins and enzymes (Nies, 1999). The metal-microbe interaction in natural environment is complex and is influenced by pH or organic matter content (Saeki *et al*., 2002). The ability to grow at concentration of metals is however, found in many plant growths promoting rhizobacteria including symbiotic nitrogen fixing bacteria (Lakzian *et al*., 2002) and may be the result of intrinsic or induced mechanism (Giller *et al*., 1998). There are reports that have shown a high level tolerance to heavy metals by rhizobia (Wani and Khan, 2010). Conflicting reports are, however, available in the literature on the tolerance level of rhizobia which could possibly be due to the variation in the tolerance level of rhizobia and growth conditions employed (Rajkumar *et al*., 2005). For instance, *Rhizobium leguminosarum* isolated from metal contaminated soil tolerated 92.9 μM of zinc (Delorme *et al*., 2003) while *Rhizobium* species isolated from nodules of *Trifolium repense* tolerated 300 mg kg^{-1} nickel and showed an effective symbiosis with its legume host, when grown in nickel amended soils (Smith and Giller, 1992). Similarly Luo *et al*. (2011), isolated *Serratia* sp. LRE07 from cadmium hyperaccumulator *Solanum nigrum* L. was resistant to to the toxic effects of heavy metals. In the present study, bacteria PA6 displayed a high resistance towards Lead (1200 μg mL^{-1}), zinc (800 μg mL^{-1}) and copper (800 μg mL^{-1}) whereas strain PA3 showed a tolerance level of 800, 800 and 1000 μg mL^{-1} to copper, zinc and lead, respectively. Bacterial strains showed a high tolerance to lead and zinc which was followed for copper, whereas, lead was found to be more toxic than the other heavy metals. The metal tolerant strains were characterized by physiological and biochemical methods. The strain PA1, PA3 and PA6 was characterized as *Pseudomonas* species while as PA2, PA4 and PA5 as *Bacillus* species. In other studies, bacteria have also shown resistance to cadmium and zinc salts. For instance, zinc tolerance by *Protobacteria*, *Actinobacteria* and *Bacteroidetes* were resistant to Zn (Kuffner *et al*., 2010). Nickel and zinc tolerance by *Rhizobium leguminosarum* biovar *trifolii* isolated from sewage sludge treated soil was also reported by Purchase and Miles (2001), who observed a metal tolerance of 0.24-0.26 mM Ni^{2+} and 6.0-8.0 mM Zn^{2+}. Similarly, metal tolerance by *Rhizobium*, *Bradyrhizobium* and *Azotobacter* (Pajuelo *et al*., 2008) and varying level of resistance among other PGPR (*Bacillus* and *Pseudomonas*) have also been reported (Yilmaz, 2003; Thacker *et al*., 2007; Wasi *et al*., 2008).

Bacterial resistance to antibiotics is an emerging problem these days. Resistance to antibiotics is acquired by a change in the genetic makeup of microbes which can occur by either a genetic mutation or by transfer of antibiotic resistant genes between organisms in the environment (Spain and Alm, 2003). Furthermore, the increased use of antibiotics in health care as well as in agriculture, is in turn contributing to the growing problems of antibiotic resistant bacteria. Products such as heavy metals used in industry along with antibiotics create a selective pressure in the environment that consequently leads to the mutation in organism that will allow them better to survive and multiply. Clustering of genes on a plasmid, are beneficial to the survival of that organism and its species because those genes are more likely to be transferred together in the event of conjugation. Thus, in an environment with multiple stresses, for example antibiotics and heavy metals, it would be more ecologically favourable in terms of survival for a bacterium to acquire resistance to both stresses. If the resistance is plasmid mediated, bacteria harbouring clustered genes are more likely to pass on those genes to other neighbouring bacteria which would then have a better chance of survival. With these considerations, the antibiotic resistance among PGPR was studied which differed from antibiotic to antibiotic for all the PGPR strains. Multiple antibiotic resistances shown by PGPR strains (e.g., Bacteria PA3, PA4) might be associated with a high

degree of tolerance to metals. In many studies, metal tolerance and antibiotic resistance have been reported (Wani *et al.*, 2009; Yilmaz, 2003; Verma *et al.*, 2001). It has been suggested that under environmental conditions of metal stress, metal and antibiotic resistant microorganisms will adapt faster by the spread of R-factors than by mutation and natural selection (Silver and Misra, 1988). Similar observations on antibiotics resistance by PGPR strains have been reported (Thacker *et al.*, 2007). The variation in the resistance to many tested antibacterial drugs (antibiotics) may possibly be due to the differences in growth conditions and exposure of PGPR to stress conditions or toxic substance as well as presence or absence of resistance mechanisms that could be encoded either by chromosome and/or R-plasmid (Spain and Alm, 2003).

In the present study bacterial strains were positive for plant growth promoting activities and produced substantial amount of HCN, ammonia and solubilised considerable amount of phosphate. Bacterial strains produce ammonia; this ammonia plays a signalling role when the plant growth promoting bacteria and plants interact with each other (Becker *et al.*, 2002). Moreover, the ammonia released by the bacterial strains are known to increase the glutamine synthetase activity (Sood *et al.*, 2002). In addition, ammonium transporters found in several plant growths promoting rhizobacteria are thought to be involved in the reabsorption of NH_4^+ released as a consequence of NH_3 diffusion through the bacterial membrane (Van Dommelen *et al.*, 1997). Similarly phytohormone production (Wani and Khan, 2010; Rajkumar *et al.*, 2006; Ahmad *et al.*, 2008). In other study, the heavy metal resistant *Bacillus* species are also known to produce considerable amount of plant growth promoting substances (Wani *et al.*, 2007).

CONCLUSION

This study concluded that the bacterial strains not only tolerated heavy metals but also antibiotics. Bacterial strains also produced substantial amount of plant growth promoting substances. Due to multifarious properties expressed by the bacterial strains, these strains could therefore, be used as bioinoculant to increase the performance of crops in soils contaminated with heavy metals.

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