

Plasmid profile and curing analysis of *Pseudomonas aeruginosa* as metal resistant

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ABSTRACT: The isolate *Pseudomonas aeruginosa* exhibited resistance to heavy metals such as cadmium, chromium, nickel and lead. Plasmid DNA was isolated from *P. aeruginosa* and designated as pBC15. The size of the plasmid DNA was approximately 23 kb. *Escherichia coli* DH5 α was transformed with plasmid pBC15 subseqired resistance to nickel and ampicillin. The same size of the plasmid was isolated from *E. coli* transformant and separated on 0.7 % agarose gel electrophoresis. The restriction analysis of pBC15 showed that the plasmid DNA has single site for *Bam* HI and *Eco* RI and three sites for *Xho* I which were compared with 1 Kb DNA and λ *Hind* III digest molecular markers. Therefore, the size of the plasmid DNA of pBC15 was confirmed to be 23 kb. Curing was carried out by ethidium bromide, acridine orange, novobiocin, sodium dodecyl sulphate and elevated temperature (40 °C). Transformation and curing results suggest that nickel and ampicillin resistance gene was conferred by plasmid DNA. Cadmium resistant gene was present on chromosomal DNA along with the gene for chromium resistance. Lead resistance gene was shown to be present on the chromosomal DNA rather than the plasmid DNA as the cured and uncured cultures remained similar in lead resistance. Therefore, the ability of *P. aeruginosa* resistant to nickel and ampicillin is plasmid mediated and transferable to other strains whereas cadmium, chromium and lead could be chromosomal encoded. The heavy metal and antibiotic resistances of *P. aeruginosa* can be used to exploit for clean up industrial wastewater and bioremediation of heavy metal contaminated soil.

Keywords: Antibiotics, *E. coli*, heavy metals, wastewater, bioremediation

INTRODUCTION

Environmental pollution with toxic heavy metals is spreading throughout the world along with industrial progress. The important toxic metals cadmium, nickel and lead find its way to the water bodies through wastewater (Ajmal *et al.*, 1998). Hazardous characteristics of the pollutants cause renal dysfunction, bone degeneration, liver, lungs and blood damage (Ebdon *et al.*, 2001). Cadmium is the most dangerous metal ion for human health due to its hazardous characteristics and non-biodegradability. It has been reported that cadmium damages the cells by a broad spectrum of effects on cell metabolism. It is known to bind with essential respiratory enzymes (Nies, 2003), cause oxidative stress (Banjerdki *et al.*, 2005) and inhibits DNA repair (Jin *et al.*, 2003). Nickel is the most abundant heavy metal contaminants of the environment due to its release during mining and smelting practices (Prasad and Strazalka, 2000). Nickel is required as an essential co-factor in several bacterial

enzymes, which carry out metabolic functions (Mulrooney and Hausinger, 2003), but it disrupts processes when it is present in excess (Babich and Stotzky, 1983). Lead is a hazardous waste and is highly toxic to humans, plants and animals (Low *et al.*, 2000). In humans chronic lead exposure produces neurotoxicity, anaemia and kidney damage and acute lead toxicity can be fatal (Rensing *et al.*, 1998). Similarly chromium (VI) is the toxic form of chromium released during many industrial processes, including electroplating, leather tanning and pigment manufacture (Prasenjit and Sumathi, 2005). Chromium (VI) is extremely toxic, mutagenic (Cheng and Dixon, 1998), carcinogenic (Shumilla *et al.*, 1999) and teratogenic (Asmatullah *et al.*, 1998) which effects on biological systems. The role of microorganisms in bioremediation is important because of their ability to degrade hazardous compounds into harmless ones. *Pseudomonas* species are found ubiquitously in nature and abundantly in water, soil and plants. Many species of *Pseudomonas* are metabolically versatile and a large

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number of organic compounds can be used as unique carbon and energy sources (Romling, 1994). This versatility allows them to be present in many environments as natural autochthonous microflora with a high potential for bioremediation of pollutants (Rossello-Mera, 1994). The ability of a genetic marker for transferring from one bacterium to another through conjugation or transformation provides a good presumptive evidence for the involvement of plasmid, particularly if the frequency of transfer is high. Moreover, loss of certain genetic markers as a result of treatment of bacterial cell to plasmid curing agents also suggests for the plasmidial nature of the marker (Mesas *et al.*, 2004). Some heavy metal resistance determinants move from plasmid to chromosome (or in the reverse direction). This makes plasmid encoding heavy metal resistance an important aspect of environmental research. The plasmid can be the source of resistance genes for cloning purpose which have potential use in biotechnology such as the manufacture of biosensors and bioremediation processes (Collard *et al.*, 1994). An attempt was made to identify plasmid or chromosome mediated determinants of *P. aeruginosa* to confer resistance to heavy metals and antibiotics.

The isolate *P. aeruginosa* employed in the present study was isolated from oil mill industry waste water in Virudhunagar District, South India. This research was carried out from September 2006 to November 2007.

MATERIALS AND METHODS

Pseudomonas aeruginosa strain BC15 isolated from oil mill industry wastewater sample was identified and characterized (Edward Raja *et al.*, 2006). Plasmid DNA from bacterial strains was isolated using alkaline lysis method (Sambrook *et al.*, 1989). *P. aeruginosa* plasmid DNA was extracted with STE solution. The size of the plasmid DNA was determined by 0.7 % agarose gel electrophoresis with λ *Hind* III digest used as marker. The plasmid DNA was used to transform *E. coli* DH5 α by transformation using calcium chloride. The transformation procedure was adopted as described by Sambrook *et al.* (1989). The transformants were selected on LB agar plates containing nickel (100 μ g/mL) or ampicillin (100 μ g/mL). Restriction analysis of BC15 plasmid DNA was digested with restriction enzymes such as *Bam* HI, *Eco* RI and *Xho* I. The digestion products were analyzed by electrophoresis in 1 % agarose gel and the size of the plasmid DNA fragment was determined with that of known molecular

size marker. The overnight culture of *P. aeruginosa* was inoculated in Luria-Bertani (LB) medium supplemented with a curing agents such as ethidium bromide 0.5 mM, followed by acridine orange 0.5 mM, novobiocin 100 μ M, 10 % sodium dodecyl sulphate (SDS) and elevated temperature at 40 °C were incubated for 48 h. The culture was serially diluted and plated on LB agar plates. The isolated colonies were tooth picked and transferred to Luria-Bertani (LB) agar plates with and without 100 μ g/mL nickel. The colonies which were unable to grow on the LB agar plates supplemented with nickel were counted and their numbers were compared with those present in the LB agar plates to verify plasmid curing. Antimicrobial susceptibility of wild type, cured, transformant and *E. coli* DH5 α was performed on Mueller-Hinton agar plates by disc diffusion method (Bauer *et al.*, 1966) and using antimicrobial discs. The zone of inhibition was observed after 48 h of incubation at 37 °C. The following antibiotics were used in the present study: Ampicillin (10 μ g), tetracycline (10 μ g), chloramphenicol (30 μ g), streptomycin (10 μ g), kanamycin (30 μ g) and erythromycin (50 μ g). Growth studies of wild type, cured, transformant and *E. coli* DH5 α was carried out in LB medium supplemented with varying concentration of heavy metals 2 mM cadmium, 0.75 mM chromium, 2.5 mM nickel and 1.75 mM lead. Cultures were grown separately in 25 mL LB medium in 100 mL conical flasks at 37 °C for 30 h. The growth of the bacteria was determined in terms of turbidity in the culture medium by measuring the absorbance at 600 nm spectrophotometrically. *P. aeruginosa* wild and cured type cultures were cultured in LB medium and incubated at 37 °C with agitation for 12 h. Cells were harvested by centrifugation at 1, 200 g for 15 min at 4 °C. The pellet was washed twice with sterile milliQ water. Samples for AFM analysis were mounted on cover glass and air-dried as described earlier (Bolshakova *et al.*, 2001) and mounted directly on the specimen metal disc using a double adhesive tape. Samples were scanned at different areas using AFM (Shimadzu SPM 9500-2J). For high resolution, contact mode micro cantilever was used for the analysis. Digital images were stored in the computer and processed.

RESULTS AND DISCUSSION

Contaminated environments like those in the vicinity of industries or industrial dump grounds accumulate a heavy load of toxic metal ions, organic ions, organic

wastes and antibiotics (Haq *et al.*, 1999). Microorganisms resistant to antibiotics and tolerant to metals appears to be the result of exposure to metal contaminated environment that cause co-incident selection for resistance factors for heavy metals and antibiotics (Ramteke, 1997). *Pseudomonas* species show that resistance to a variety of toxic substances, heavy metals and antibiotics, can use various compounds as carbon sources. Hence they have generated a high degree of interest in the area of environmental bioremediation (Bruins, 2003). A variety of plasmids have been isolated from *Pseudomonas* species such as R-factor plasmids, which inactivate certain antibiotics and S-factor plasmids capable of initiating chromosomal gene transfer (Chakrabarty, 1976). Plasmids in *Pseudomonas* species are also known to code for heavy metal resistance and degradation of organic compounds (Chakrabarty, 1976). In the present study, the plasmid DNA was isolated from *P. aeruginosa* BC15 which revealed the presence of single plasmid and was designated as pBC15. The size of this plasmid was approximately 23 kb and *E. coli* DH5 α was transformed with plasmid pBC15.

Transformant harboured a plasmid of the same size as that in BC15 which is shown in Fig. 1. The similar size of plasmid DNA was identified from titanium ion resistant *P. aeruginosa* ATCC 10145 (Park *et al.*, 2006) and cadmium, lead resistance *Pseudomonas* species S8A isolated from mine tailing contaminated soil (Kassab and Roane, 2006). The restriction analysis profile showed that the plasmid DNA has one site for *Bam* HI, *Eco* RI and three sites for *Xho* I exhibited by three fragments corresponds to 11.5 kb, 7.5 kb and 5 kb positions of the 1 kb DNA marker. The aggregate of the fragments in *Bam* HI, *Eco* RI and *Xho* I was approximately 23 Kb and 23.5 kb, respectively. Therefore, the plasmid DNA of *P. aeruginosa* BC15 was approximately 23 kb (Fig. 2). Curing of plasmid was carried out with ethidium bromide, acridine orange, novobiocin, SDS and temperature at 40 °C (Fig. 3). Plasmid curing was achieved only by growing the strain treatment with SDS while other curing agents and temperature at 40 °C were not effective. A plasmid containing *E. coli* strain was treated with 10 % SDS leads to loss of a plasmid. The plasmid elimination was accompanied by drastic changes in antibiotic resistance

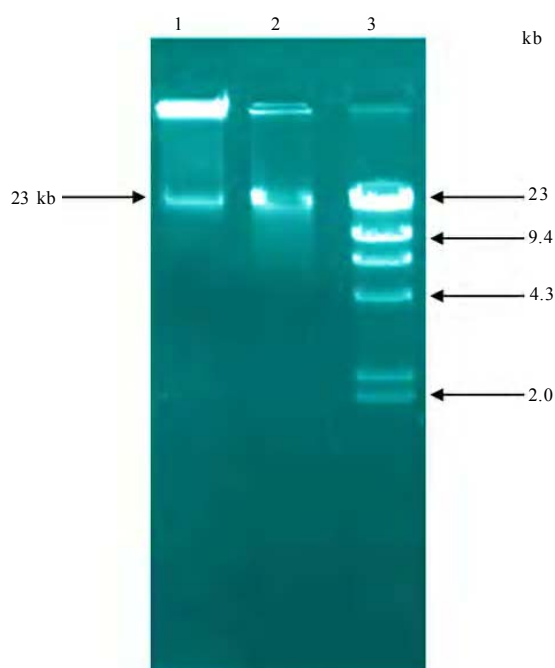


Fig. 1: Plasmid DNA profile of *Pseudomonas aeruginosa* BC15. Lane 1 = *P. aeruginosa* BC15 (pBc15); Lane 2 = Transformed *E. coli* DH5 α pBC15 and Lane 3 = λ *Hind* III digest molecular weight marker

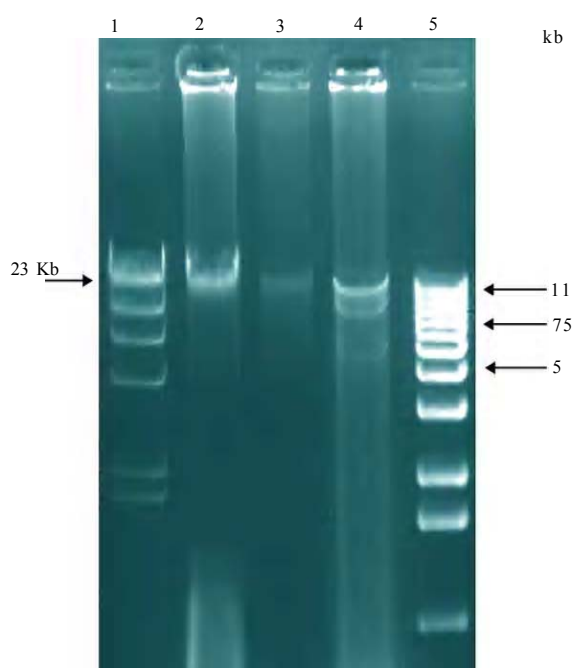


Fig. 2: Restriction analysis of *P. aeruginosa* BC15 plasmid DNA. Lane 1 = λ *Hind* III digest; Lane 2 = *Bam* HI digest; Lane 3 = *Eco* RI digest; Lane 4 = *Xho* I digest and Lane 5 = 1kb DNA ladder

Metal resistant *Pseudomonas*

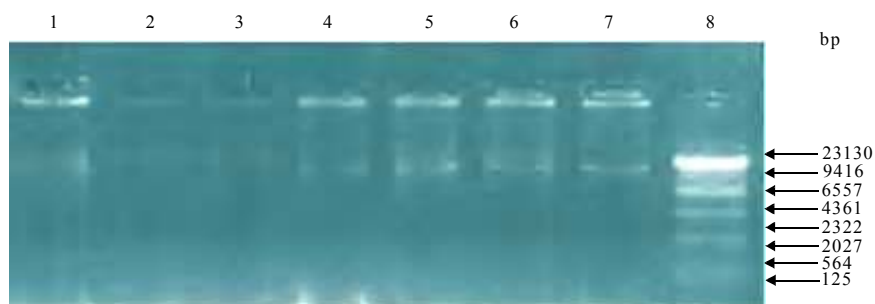


Fig. 3: Effect of curing agents on BC15 plasmid. Lane 1 = BC15 plasmid DNA; Lane 2 = SDS cured 1; Lane 3 = SDS cured 2; Lane 4 = Ethidium bromide (EtBr) cured; Lane 5 = Acridine orange (AO) cured; Lane 6 = Novobiocin (NB) cured; Lane 7 = Temperature at 40 °C and Lane 8 = λ Hind III digest molecular weight marker

Table 1: Antibiotic sensitivity of *P. aeruginosa* BC15

Antibiotics ($\mu\text{g}/\text{disc}$)	BC15 (wild type)	BC15 (cured)	Transformant	DH5 α
Ampicillin (10)	R	S	R	S
Tetracycline (10)	R	S	S	S
Chloramphenicol (30)	R	S	S	S
Streptomycin (10)	R	S	S	S
Kanamycin (30)	R	S	S	S
Erythromycin (50)	R	S	S	S

Note: R-resistant; S-sensitive

and morphology of the colonies (Rosas, 1983). The antibiotic resistance pattern of wild type, cured, transformant and *E. coli* DH5 α were described in Table 1. This pattern revealed that wild type BC15 and transformant were resistant to ampicillin. On the other hand, cured and *E. coli* DH5 α were sensitive to ampicillin. From this data, the genes encoding resistance to ampicillin are located on the plasmid pBC15. Furthermore, wild type BC15 was resistant to tetracycline, chloramphenicol, streptomycin, kanamycin and erythromycin. But cured strain was sensitive to these antibiotics indicating the possible plasmid borne nature of genes encoding resistance to these antibiotics. However, transformant did not develop resistance to these antibiotics in spite of acquiring the plasmid pBC15. This suggested that loss of antibiotic resistance phenotype in cured strain may be either because of mutation as a result of incubation in the presence of the curing or genes encoding resistance to the above antibiotics are not transferred to *E. coli*. Chromosome and plasmid encoded metal resistance genetic systems have been studied in *Pseudomonas*, as well as in related bacteria (Cervantes, 1996). *Pseudomonas* species resistant to cadmium up to 1.2

mM maximum tolerable concentration was isolated from heavy metal contaminated soil (Roane and Kellogg, 1996). *P. aeruginosa* BC15 resistant to cadmium up to 6 mM in LB agar plates (data not shown). The growth studies of *P. aeruginosa* BC15 at different concentrations of metals cadmium, chromium, nickel and lead in LB medium is shown in Figs. 4 a, b, c, and d. The measurements of the culture incubated for 30 h were in good agreement with bacterial resistance for each metal. When strains were grown in cadmium supplemented media, wild type and cured type exhibited maximum peak value, but no growth was observed from transformant and *E. coli* DH5 α . In the presence of nickel, wild type and transformant exhibited maximum peak values. Growth of *P. aeruginosa* BC15 in the presence of chromium wild type and cured were exhibited maximal peak value compared to transformant and *E. coli* DH5 α . The growth of all strains in the presence of lead indicates that there is no change in the growth pattern. From the results, it is cleared that wild type and transformant were resistant to nickel, in contrast the cured derivative and *E. coli* DH5 α were sensitive to nickel. The above results indicate that the plasmid can replicate in *E. coli* DH5 α for nickel

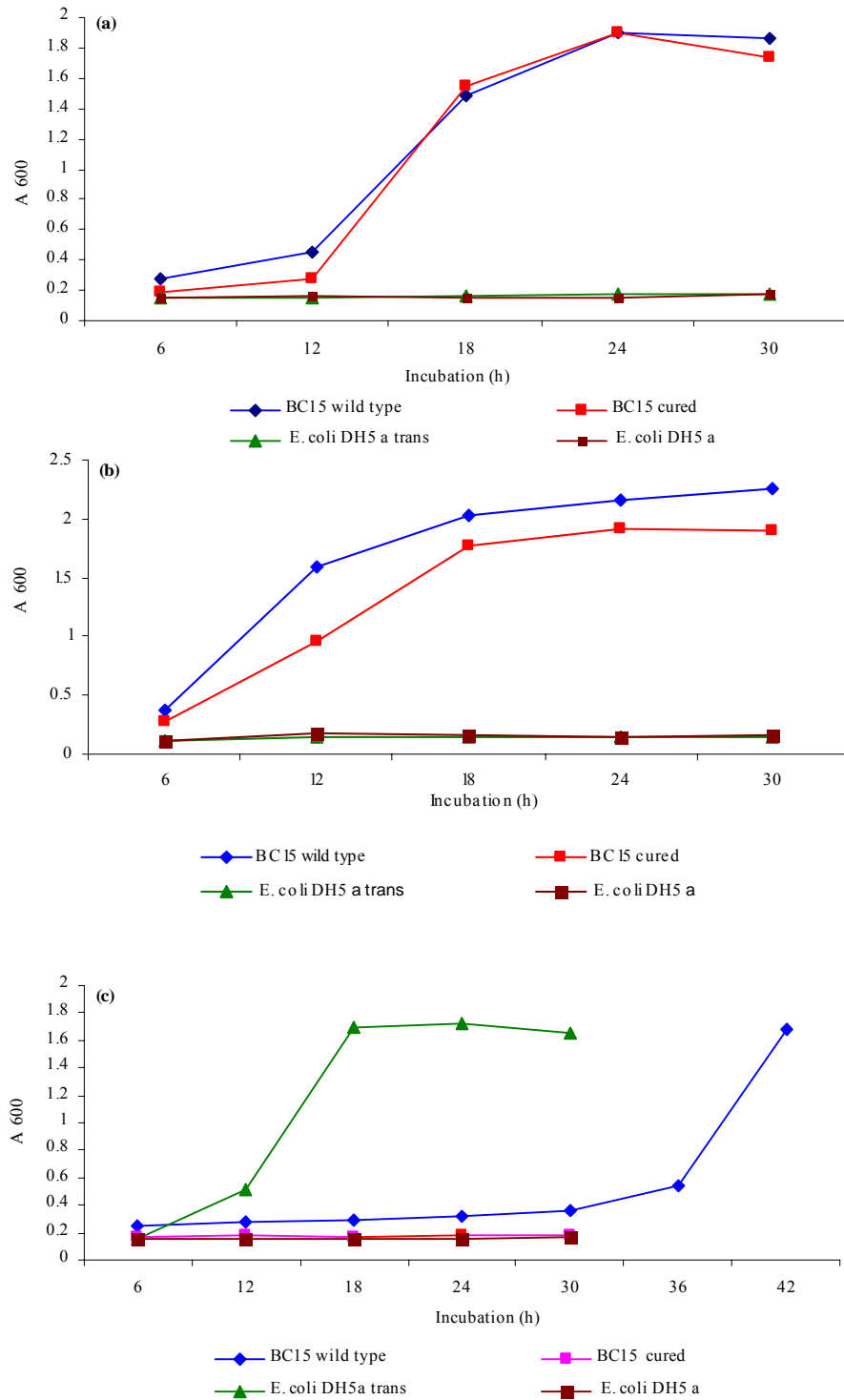


Fig. 4: Growth studies of wild type, cured, transformant and *E. coli* DH5 α in LB medium supplemented with 2 mM cadmium (a), 0.75 mM chromium (b), 2.5 mM nickel (c) 1.75 mM lead and (d) incubated at 37 °C for 30 h

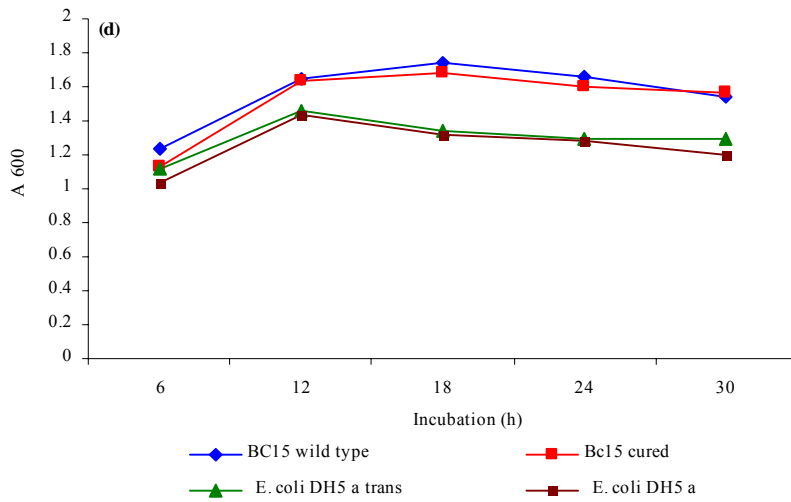


Fig. 4 (continued): Growth studies of wild type, cured, transformant and *E. coli* DH5α in LB medium supplemented with 2 mM cadmium (a), 0.75 mM chromium (b), 2.5 mM nickel (c) 1.75 mM lead and (d) incubated at 37 °C for 30 h

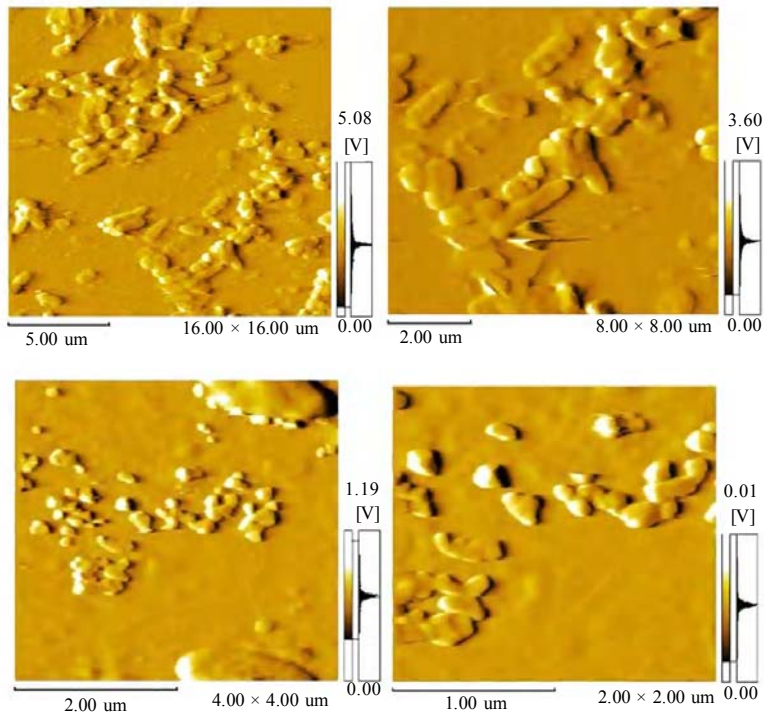


Fig. 5: Atomic force micrographs of *P. aeruginosa* on: (A) wild type; (B) cured type cells

resistance. Therefore, it is evident that genes encoding resistance to ampicillin and nickel were located on plasmid pBC15. Similar result obtained from

Pseudomonas species was isolated from water and soil samples (Unaldi *et al.*, 2003). The wild type BC15 and cured type cultures were imaged with AFM operated

in contact mode liquid conditions (Fig. 5). The wild type BC15 was yellowish brown in colour whereas the colony of the cured strain was white in colour and also smaller in size compared to wild type. Moreover, colonies of the wild type BC15 strain was raised, but the cured strain exhibited flat colonies. A similar finding was observed from a plasmid borne determinant of *Pseudomonas* species that was isolated from leaf of cotton (Saha, 2001). Transformation and curing results of the study suggested that nickel and ampicillin resistance were conferred by plasmid DNA, while cadmium, chromium and lead resistance seems to be encoded by genes of the bacterial chromosome. The development of the metal resistant population in a contaminated soil can result from vertical gene transfer (reproduction), horizontal gene transfer, including transposons and broad host range plasmids and selection pressures on spontaneous mutants due to the presence of heavy metals (Bogdanova, 1998). Transformation of nickel resistance gene suggests that horizontal gene transfer may be an important factor for the development of metal resistant populations in the environment. Indeed, further studies have to be performed to elucidate bioremediation of heavy metals and detoxification processes operating in this system.

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