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Inducible Tolerance to Heavy Metals in Air Borne Bacteria

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Abstract: Airborne bacteria were isolated from three polluted sites around Karachi. Among 30 isolates CMG 921 (*Proteus mirabilis*), 922 (*Pseudomonas putida*), 923 (*Pseudomonas pseudomallei*) and 924 (*Yersinia aldovae*) were selected and studied. All these strains belonged to Gaddani and were resistant to multiple metals indicating the high level of pollution in Gaddani. These strains were sensitive to most of the antibiotics except ampicillin. It was found that the metal resistant did not involve the outer membrane permeability however the resistance to copper and nickel was inducible. A single band of plasmid DNA of 2 kb was observed in CMG 921 and CMG 922 which have shown the same level of metal resistance. This has suggested that there is a horizontal transfer of plasmid pAN10 from one strain to another and perhaps the metal resistant determinants present on this plasmid.

Key words: Bacteria, plasmids, induction, metal tolerance and airborne

Introduction

Air is a mixture of different gases surrounding any celestial object (such as the Earth). Beside gases air also has particles which are of biological origin. For clean healthy environment clean air is the basic need i.e. quality of air should be high. Air quality is determined by a broad scale monitoring of atmosphere. Less quality air or polluted air directly affects human health. Epidemiological studies indicated that industrial air pollution has cumulative effect on susceptible people. Many types of industrial emissions produce, irritation of eyes, nose and throat, dryness of mucous membrane, headache, dizziness and comma. The reckless industrialization has already produced extraordinary levels of pollution and made some areas simply uninhabitable. The growth, behavior and life history of an organism is therefore influenced by the environment in which they live. When ever air is being polluted two factors are mainly responsible; Automobiles and Industrial waste. Automobiles emit cocktail of nitrogen oxides, carbon monoxide, volatile organic compounds, particulates, heavy metals and sulphur dioxide (Farmer, 1997). Industrial waste also discharge toxic compounds such as aromatic compounds, chlorinated hydrocarbons, oils, gases and heavy metals (Rizvi, 1993). Karachi with a population of around twelve million people and is the major seaport and a centre of industries. Karachi has over 5,00,000 vehicles and industries both of these emit and discharge toxic chemicals however thermal power plants are the worst polluters.

Presence of heavy metals in the atmosphere also effects the quality of air. The total aerial burden of metals presumed to originate both indirectly from wind blown contaminated soils and industrial waste and directly from dusts, smokes and car exhausts include lead, copper, nickel, cadmium, zinc (Goodman and Roberts, 1971).

Bacteria are in the front line when it comes to coping with pollutants in the environment especially with heavy metals. Many bacteria require essential metals such as Ni, Mn, Zn, Cu, Fe, Co etc. in trace amount for their growth however these essential metals become toxic if present in large amounts.

Heavy metals present in the environment are also deleterious for human health and are responsible for dangerous diseases. For example nickel is responsible for induced chromosomal aberrations (Umeda and Nishimura, 1979) and for causing bronchitis and allergies (McDonagh *et al.*, 1992). Cadmium is an occupational and environmental contaminant which after absorption is efficiently retained by human body and is

accumulated throughout life (Bernard and Lauwerys, 1984). Cadmium is responsible for disorders like anemia, aborted fetus, neonatal death, heart problem (Kjellstrom, 1985) renal tubular dysfunction, disturbances of the calcium homeostasis (Staessen *et al.*, 1992). Lead interferes with the activity of enzymes and effects many organs and systems such as kidneys and central nervous system (Bornemann and Colburn, 1985).

The present study was conducted to isolate, characterize the air borne bacteria of the polluted environments and to study the relation ship of pollutants and bacterial genetic characters.

Materials and Methods

Sampling sites: Samples were collected from polluted areas of Karachi which include Empress Market, Gadani and Tanneries at Korangi sector 7A.

Media preparation: Nutrient agar and tris-gluconate agar were used as a solid media. Nutrient agar was prepared by adding agar agar (Oxoid) to nutrient broth (Oxoid) in distilled water. Tris-gluconate broth prepared as described by Maniatis *et al.* (1982), nutrient broth and Luria-Bertani broth (LB) were used as liquid media.

Sample collection: Samples were collected by Settle plate method (Russell *et al.*, 1984). Prepared nutrient agar plates were opened in the above mentioned locations from 1-2 m above ground level for about 30-60 minutes. These plates were incubated at 37°C for 24- 48 hours.

Isolation and identification of bacteria: Morphologically different colonies were picked stained and streaked on nutrient agar and incubated for 24 hours. After several restreaking, pure colonies were picked and maintained on nutrient agar slants at 4°C. They were also checked on tris minimal media. Bacterial isolates were identified on the basis of colonial morphology, cellular morphology, Gram's reaction and using API-kits.

Mtc of tolerance to heavy metal and resistance

antibiotics: In order to find out the maximum tolerable concentration (MTC) of heavy metals and antibiotics, cultures were streaked tris minimal media plates containing different concentration of heavy metal salts. These heavy metal salts included CuSO₄, CrO₃, NiCl₂, MnSO₄, CdCl₃, COCl₂ and ZnSO₄. The concentration of

metal salts used were 0.5-8.0 mM. The resistance and sensitivity against different antibiotics like chloramphenicol, tetracycline, kanamycin, streptomycin and ampicillin was studied on nutrient agar plates. Antibiotic solutions were prepared as described by Maniatis *et al.* (1982) and stored at -20°C. The concentrations of antibiotics used were 25 µg-500 µg. The cultures were streaked on nutrient agar plates having antibiotics and growth was observed after 24-48 hours of incubation.

Study of growth curve: Growth curves were observed by measuring the optical density of the cultures at 600 nm with spectrophotometer (photic-100). 100 µl pre-cultures were inoculated in fresh nutrient broth. Cultures were then incubated at 37°C in a shaker incubator (80 rpm) and optical density was taken at 1 hour interval.

Study of induction curve: To find out whether metal resistance was inducible or not, induction curves were studied in tris minimal media and nutrient broth by measuring the absorbance of the cultures at 600 nm with spectrophotometer (photic-100).

Study of cell permeability: To determine whether the resistance mechanism of the bacterial strains to metals was due to impermeability of outer membrane or not, the permeability of the outer membrane was increased as described by Leiva (1966).

Isolation of plasmid dna: Plasmid DNA of the selected strains was isolated by the method of Kado and Liu (1981). Pre-cultures were prepared by inoculating the bacterial strains in LB for over night incubation at 37°C. 2 × 1.5 ml pre-culture was taken in eppendorf tubes and centrifuged for 6 minutes at 14,000 rpm. The supernatant was discarded and the pellets were used for plasmid isolation.

Agarose gel electrophoresis: Electrophoresis of plasmid DNA was performed in horizontal 0.7% agarose slab gel using TAE running buffer. 5 µg/ml ethidium bromide was added in gel and buffer for staining. 10 µl of plasmid DNA in each sample was loaded in the well with 2 µl bromo-phenol dye and Lambda DNA cut with Hind III was used as a marker. Gel was run for 2 hours at 60 Volts and observed under UV transilluminator with a 320 nm UV light source. Photograph was taken by using Polaroid camera.

Results

All the isolates which showed tolerance to heavy metals and antibiotics belong to Gadani and were coded as CMG 921, 922, 923 and 924 these were studied in detail. These bacterial strains were identified on the basis of their colonial morphology (Table 1), cellular morphology gram reaction (Table 2) and API system. All four strains were found to be Gram-negative.

CMG 921 and 922 were resistant to 1 mM copper while CMG 923 and 924 were tolerant to 2 mM nickel and were found to be sensitive to cadmium chloride and lead acetate but all the four strains were to 8 mM MnSO₄ (Fig. 1a).

None of the strains were highly resistant to commonly used antibiotics. CMG 921 and 922 were resistant to 125 mg ampicillin while CMG 923 and 924 were sensitive to all the tested antibiotics (Fig. 1b).

Growth curves of these bacterial strains were studied in

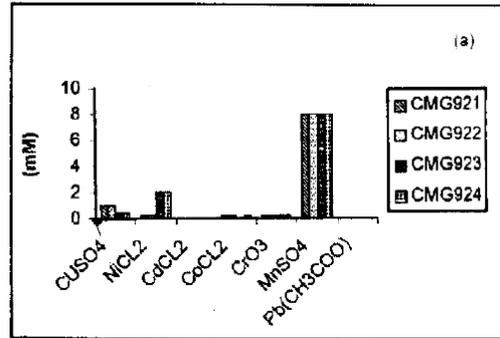


Fig. 1a: Study of heavy metal resistance among air borne bacterial strains

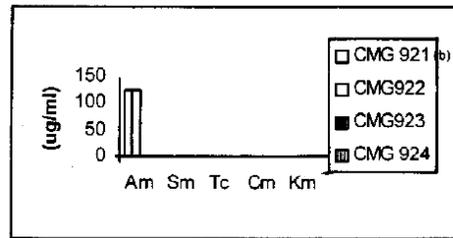


Fig. 1b: Study of antibiotic resistance among air borne bacterial strains

nutrient broth and tris broth Results have shown that all four bacterial strains grew well as compared to tris media CMG 921 spent 12 and 10 hours in log phase in nutrient broth and tris broth respectively after which it enters in stationary phase (Fig. 2a). CMG 922 enter in log phase with in one hour of transfer in to fresh nutrient broth and tris and spent 4 hours in this phase in both media and then enter into the stationary phase (Fig. 2b). CMG 923 spent 6 hours in log phase in both media and then enter into stationary phase (Fig. 2c). CMG 924 spent 4 hours in log phase in both media (Fig. 2d).

To find out whether tolerance to heavy metals such as Cu, Ni and Mn was inducible or not. Induction curves were studied. CMG 921 showed extended lag phase in the presence of CuSO₄ and MnSO₄ in tris media (Fig. 3a and 4a). CMG 922 also followed the same and showed the extended lag phase when metals are present in tris media (Fig. 3b). CMG 923 showed extended lag phase with Ni (Fig. 5a) where as CMG 924 also showed long lag phase with Ni and Mn The results suggested that the tolerance to CuSO₄ and Mn was inducible in CMG 921 and 922 however tolerance to NiCl₂ was inducible in CMG 923 and CMG 924 and in addition CMG 923 also showed inducible tolerance to MnCl₂ (Fig 5a, b, c).

To find out whether metal tolerance was due to impermeability of the outer membrane or not the permeability was altered with EDTA and the results showed that the metal tolerance was not due to impermeability of the cell membrane (Table 3) CMG 921 and CMG 922 showed one band of plasmid DNA (Fig. 6) on the agarose gel having approximately same molecular weight. The molecular weight was calculated as described by Schaffer and Sederoff (1981) and it was found to be 2 kb.

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Table 1: Colonial morphology of Air-borne bacteria

Code	Identification	Shape	Size	Color	Opacity	Elevation	Surface
CMG 921	<i>Proteus mirabilis</i>	Round	Large	Dirty white	Opaque	Convex	Mucoid
CMG922	<i>Ps. purida</i>	Round	Large	Dirty White	Opaque	Convex	Dry
CMG923	<i>Ps. Pseudomalli</i>	Round	Large	White Translu	-cent	Convex	Mucoid
CMG 924	<i>Yersina aldovae</i>	Round	Pin Headed	Translu-cent	Translu-cent Convex	Convex	Mucoid

Table 2: Cellular morphology of Air borne bacteria

Codes	Gram reaction	Shape	Arrangement
CMG921	Negative	Road	Scattered
CMG922	Negative	Road	Scattered
CMG923	Negative	Road	Scattered
CMG924	Negative	Road	Scattered

Table 3 : Assessment of cell permeability of the bacterial isolates

Codes	IRIS								N.A							
	Control		Mn (4 mM)		Cu (0.5 mM)		Ni (1 mM)		Control		Mn (4mM)		Cu (0.5 mM)		Ni (1 mM)	
	UT	T	UT	T	UT	T	UT	T	UT	T	UT	T	UT	T	UT	T
921	0	0	4	+	+	+	+	+	+	+	+	+	+	+	+	+
922	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
923	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+
924	+	+	+	+	+	+	+	+	+	+	+	+	+	0	+	+

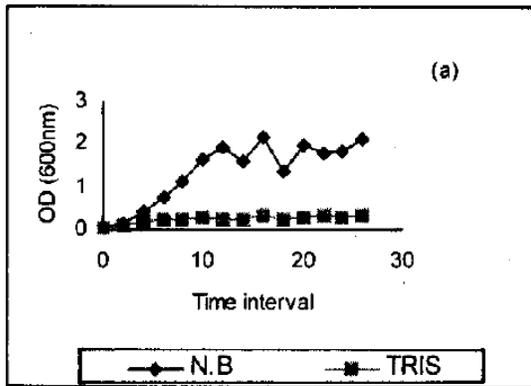


Fig. 2a: Optical densities of growth curve of CMG 921 in Nutrient broth (N.B) and Tris minimal media

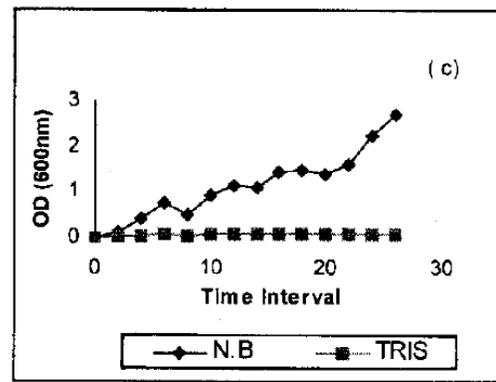


Fig. 2c: Optical densities of growth curve of CMG 923 in Nutrient broth (N.B) and Tris minimal media

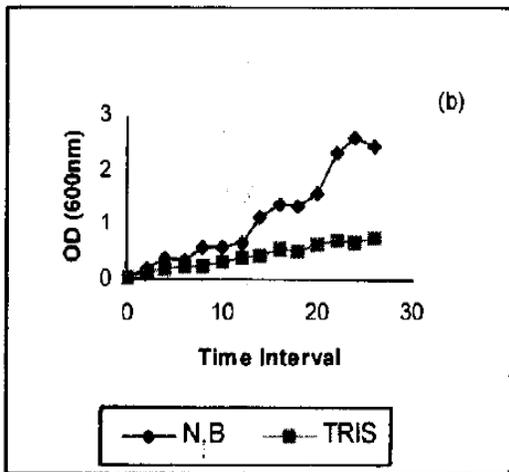


Fig. 2b: Optical densities of growth curve of CMG 922 in Nutrient broth (N.B) and Tris minimal media

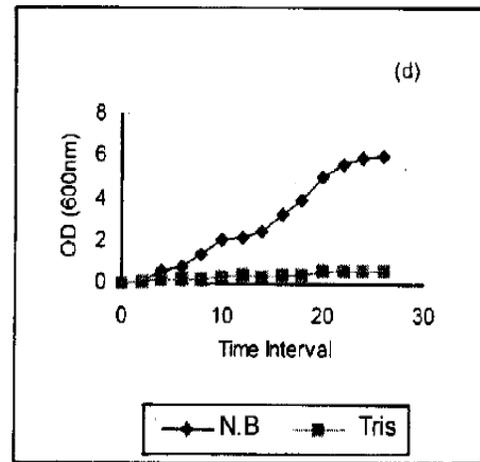


Fig. 2d: Optical densities of growth curve of CMG 924 in Nutrient broth (N.B) and Tris minimal media

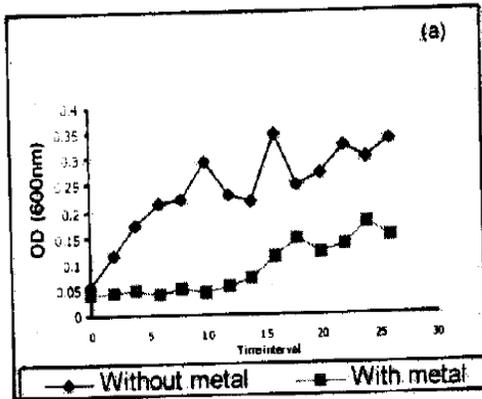


Fig 3a: Study of induction curve of CMG 921 in presence of copper salt

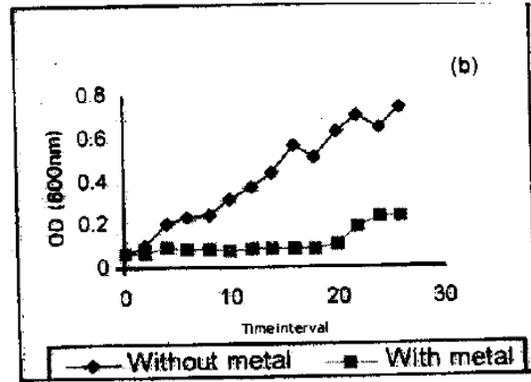


Fig. 4b: Study of induction curve of CMG 922 in presence of Manganese Sulphate

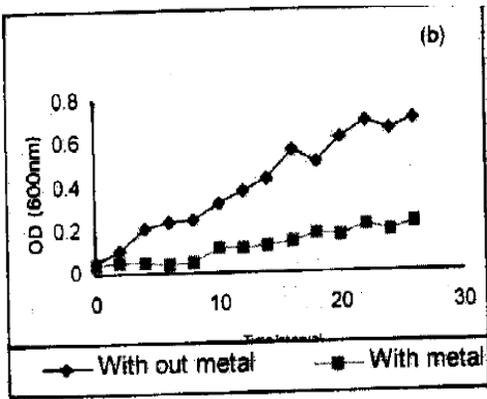


Fig. 3b: Study of induction curve of CMG 922 in presence of copper salt

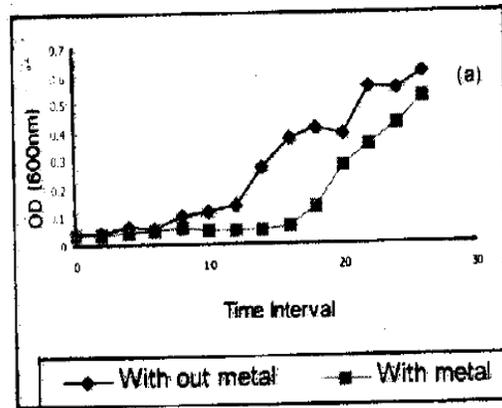


Fig. 5a: Study of induction curve of CMG 923 in presence of Nickle chloride

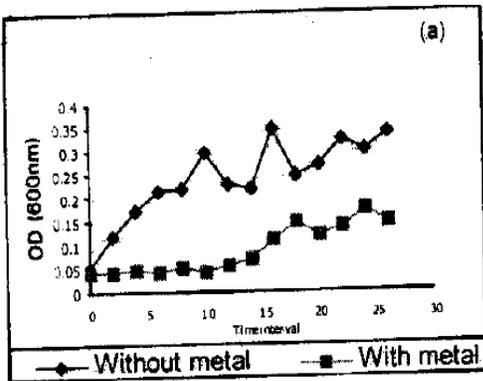


Fig. 4a: Study of induction curve of CMG 9.21 in presence of Manganese Sulphate.

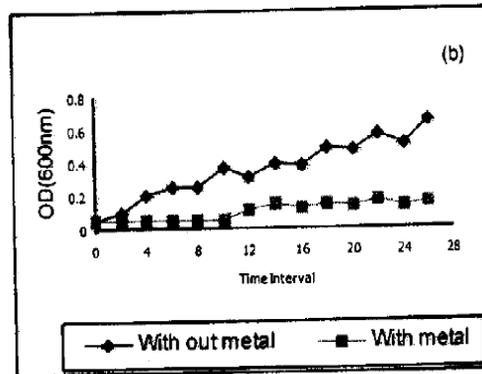


Fig. 5b: Study of induction curve of CMG 924 in presence of Nickle chloride

CMG 923 and 924 did not show any band of plasmid DNA by the method used.

Discussion

Most of the research of air borne bacteria had been conducted about aerosolization of bacteria and effect of

osmoprotectant and resuscitation agents (Marthi and Lighthart, 1990; Marthi *et al.*, 1991). These studies concentrated on collection and death mechanism of airborne bacteria with less investigation about their correlation with pollutants. Mancinelli and Shulls (1978) have shown that there is a definite relation ship

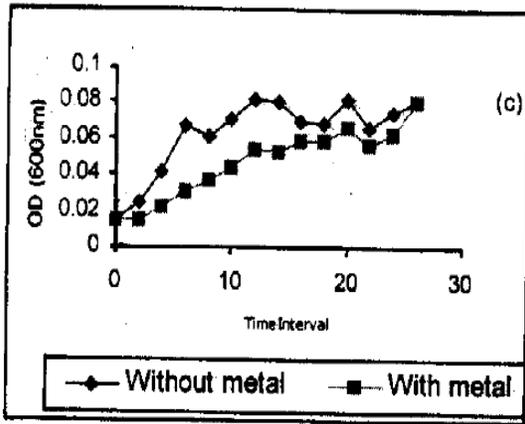


Fig. 5c: Study of induction curve of CMG 923 in presence Manganese Sulphate.

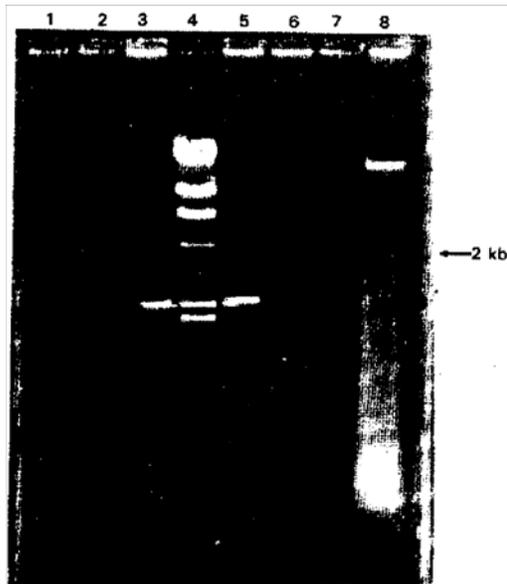


Fig. 6: Agarose gel electrophoresis of extra chromosomal DNA from CMG 921, 922, 923 and 924. Plasmid isolated from CMG 921 (Lane 3) and CMG 922 (Lane 5)

between bacteria and pollution. Some of these relationships are positive i.e., encourage bacterial viability and some are negative i.e., inhibit bacterial viability.

In order to study the genetic characters of the air borne bacterial strains isolated from polluted environment these were checked against five different metal salts i.e., CuSO_4 , MnSO_4 , NiCl_2 , CoCl_2 , CrO_3 and antibiotics Km, Sm, Amp, Cm and Tc (Fig. 1a, b). These were CMG 921, 922, 923 and 924, identified as *Proteus mirabilis*, *Pseudomonas putida*, *Pseudomonas pseudomallei* and *Yersinia aldovae* respectively. All of these strains belonged to the same sampling sites i.e. Gadani. Their multiple metal resistance character suggested the presence of heavy metals such as Mn, Ni, Cu etc. in the atmosphere of Gadani. Metal resistant bacteria are usually isolated from highly metal polluted environment (Ahmed and Raihan, 1997).

The resistance to copper and Manganese in CMG 921 (*Proteus mirabilis*) and CMG 922 (*Pseudomonas putida*) was found to be inducible, as both the strains showed extended lag phase (Fig. 3a, b, 4a, b respectively). Similar results had been reported earlier with *Acinetobacter* sp. (Ahmed *et al.*, 1997). Karin *et al.* (1984) had also reported the inducible resistance to copper and demonstrated that it involved a low molecular weight binding protein which is copper chelating. CMG 923 (*Pseudomonas pseudomallei*) and 924 (*Yersinia aldovae*) were tolerant to Ni upto 2 Mm. Nickel is normally required in trace amount by bacteria and is a component of many enzymes (Hausinger, 1987). These strains were also found to have inducible resistance to nickel. Similar type of results have been reported in *Bacillus japonicum* (Siddiqui and Schlegel, 1987) and in *E. coli* (Ahmed and Raihan, 1997).

All the four strains were resistant to Manganese. Manganese resistance was also found to be inducible in CMG 921 (*Proteus mirabilis*), 922 (*Pseudomonas putida*) and 924 (*Yersinia aldovae*) while in CMG 923 (*Pseudomonas pseudomallei*), it was constitutive (Fig. 4) (Siddiqui *et al.*, 1988).

One of the tolerance mechanism to heavy metals is impermeability of outer membrane. This results showed that metal tolerance was not due to impermeability of cell membrane.

A single band of Plasmid DNA pAN 10 was found in CMG 921 (*Proteus mirabilis*) and 922 (*Pseudomonas putida*) each having molecular weight 2 kb (Fig. 4). As CMG921 and 922 have same genetic markers (Cu^+ Mn^+ and Amp^*) and have shown similar induction mechanisms for tolerance of Cu and Mn it is suggested, that these markers might be present on the same plasmid pAN10. It appears that it is the same plasmid in both the isolates i.e. CMG 921 and 922 (*Proteus mirabilis* and *Pseudomonas putida*). Perhaps this plasmid has been transferred from one strain to another by conjugation. Plasmid mediated metal and antibiotic tolerance have been reported in many bacteria by many workers such as *Alcaligenes eutrophus* (Diels and Mergeay, 1990; Mergeay *et al.*, 1985). Nickel resistant determinant has been reported was found on plasmid (Siddiqui and Schlegel, 1987; Raihan, 1997) but resistant determinant plasmids have also been reported on chromosomes in some bacterial strains (Kaur *et al.*, 1990; Mattsby-Baltzer *et al.*, 1989; Stoppel *et al.*, 1995). CMG 923 and 924 showed no plasmid DNA by this method which suggest that Ni resistant might be present on chromosome.

As global pollution increases, developed countries are seeking novel solutions to the ever increasing problems of industrial waste cleanup and disposal of hazardous wastes. There is a need to reduce the levels of harmful inorganic compounds by a number of physical, chemical or biological procedures. As no information was available on the airborne bacteria of Karachi environment, present study was aimed has provided some information about air pollution of Karachi suburb.

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