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GRAM-NEGATIVE BACTERIA FROM INDUSTRIAL WASTES CONFERRING PLEOTROPIC METAL AND ANTIBIOTIC RESISTANCE

ANJUM NASIM SABRI, MARRYUM ABBAS AND SHAHIDA HASNAIN

Department of Botany, University of the Punjab, Quaid-e-Azam Campus Lahore-54590, Pakistan

Abstract: Ten isolates, MA-1 to MA-10, were isolated from the effluents of ICI Industry and Nalla Dek near Sheikhupura. They exhibited resistance to cadmium, chromium, mercury, lead, zinc, iron, nickle, copper, manganese, cobalt and molybdenium. They all tolerate salts of Cr, Ni and Co (50μg ml^{-1}), Cu (100μg ml^{-1}), Mn and Fe (150μg ml^{-1}), Pb (1000μg ml^{-1}) and Mo (7000μg ml^{-1}) in the medium. They tolerate kanamycin, chloramphenicol and ampicillin in the medium. These isolates differ morphologically, physiologically and biochemically from one another. But all of them had catalase and oxidase enzymes. They could reduce nitrate, hydrolyse gelatin and produce acid from arabinose. MA-5 could be affiliated with Pseudomonadaceae. MA-1, MA-2, MA-6, MA-7, and MA-9 belong to Gram-negative facultative anaerobic rods. MA-3, MA-4, MA-8, and MA-10 shared maximum characters with family Bacillaceae. Different strains favour different pH values (6-9) for maximal growth. Gel electrophoresis of total cell lysate revealed presence of plasmid only in MA-6 and MA-8. Conjugation experiments exhibited that plasmid residing in MA-6 was conjugative and confer resistace to molybdenum.

Key words: Metal resistant bacteria, antibiotics resistance bacteria, bacteria from industrial effluent.

INTRODUCTION

The industrially polluted aquatic environment is frequently contaminated by jeopardous heavy metals. The toxic elements, after a part of life cycle of soil, enters the biotic strata and affects its biological activities (Capone *et al.*, 1983). The heavy metals show menacing effects by binding with essential functional groups, replacing the essential metal ion or modifying the active confirmation of biological molecules (Collins and Stotzky, 1989; Guzzo *et al.*, 1991). In addition, metal ions and their complexes have potential to cause genetic damage and has carcinogenic effects (Abbott, 1985). Their long half-lives and accumulation in tissues aggravate the danger.

Bacteria can tolerate heavy metal stresses due to prosence of cellular mechanisms of combating the toxic effects. These mechanisms include gene amplification (Beach and Palmiter, 1981), enhanced transcription of metallothionein gene (Hildebrand *et al.*, 1982), cadmium efflux (Burke and Pfister, 1986), alteration in cell wall and plasma membrane complex (Grindle, 1984), deposition of material in walls (Brown and Smith, 1976), release of bacterial exudates (Birch and Bachofen, 1990). Metal resistant bacteria are important as an index of pollution as well as clearing agents for heavy metals from the environment. In the present work isolation of bacteria, which confer pleotropic metal resistance, is being described.

MATERIALS AND METHODS

For isolation of metal resistant bacteria, two samples from ICI Industry Sheikhupura, one from inlet effluents (odourless, colourless with black suspended solid particles, pH 2.45), other from outlet effluent (odourless, colourless with blackish suspended particles and pH 8.46) and one sample from Nallah Dek (with yellowish suspended particles, pungent smell and pH 3.11) were collected in sterilized glass bottles. Water sample (50μl) was plated onto nutrient-agar plates supplemented with 25μg ml^{-1} of each $CdCl^2$, CrO^3, $NiCl^2$, $CoCl^2$, $CuSO^4$, $MnSO^4$, $FeCl^3$, $HgCl^2$, $Pb(NO^3)^2$, $ZnSO^4$, and $NaMoO^4$ for the selection of Cd, Cr, Ni, Co, Cu, Mn, Fe, Hg, Pb, Zn and Mo resistance, respectively. The isolates were purified and were taken to elevated levels. Ten strains, that could deter salts of Cr, Ni and Co (50μg ml^{-1}), Cu (100μg ml^{-1}), Mn and Fe (150μg ml^{-1}), Pb (1mg ml^{-1}) and Mo (7mg ml^{-1}) in the medium are the subject of this study. They were designated as MA-1, MA-2, MA-3, MA-4, MA-5, MA-6, MA-7, MA-8, MA-9 and MA-10. Ensuing Gerhardt *et al.* (1981) the isolates were characterized morphologically, physiologically and biochemically. Additional twenty biochemical and cytochrome oxidase tests were performed by using QTS-20 and Co-strips, obtained from DESTO Laboratories, Karachi. Spore forming ability was corroborated by the method of Moir (1981). They were also checked for thier sensitivity behaviour against antibiotics, ampicillin (Ap), kanamycin (Km), tetracycline (Tc), chloramphenicol (Cm) and streptomycin (Sm).

For genetic analysis, bacteria were screened for the presence of plasmid by total cell lysate method (Thomas, 1984). To characterize plasmid, conjugation experiments were performed (Willetts, 1984). MA-12 (Tc^{r20}, Mo^{s1000}) was used as a recipient.

RESULTS AND DISCUSSION

Bacterial growth obtained, was purified and subjected to elevated levels, MA-1, MA-4, and MA-5 were isolated from sample 1, while isolates MA-6, MA-7, MA-9 and MA-10 were obtained from sample 2. MA-3 and MA-8 were from sample 3. All isolates could tolerate salts of Cr, Ni and Co (50μg ml^{-1}), Cu (100μg ml^{-1}), Mn and Fe (150μg ml^{-1}), Pb (1mg ml^{-1}) and Mo (7mg ml^{-1}). MA-1, MA-2, MA-3, MA-4, MA-9 and MA-10 could also resist 100μg ml^{-1} of $ZnSO_4$, while remaining four isolates (MA-5, MA-6, MA-7 and MA-8) could tolerate only 25μg ml^{-1} of this metallic salt. Only MA-9 could bear Cd and Hg salts (50μg ml^{-1}) in the medium, other strains were sensitive to these metallic salts. Both Gram-negative (Mergeays *et al.*, 1985; Nies *et al.*, 1989; Malik *et al.*, 1991; Hasnain and Sabri, 1991, 1992) and Gram-positive (Burke and Pfister, 1986; Mahler *et al.*, 1986) metal resistant bacteria have been reported previously. Multivariant resistance determining *Alcaligenes eutropus* (Ni, Hg, Co, Zn, Cd -- Mergeay *et al.*, 1985), *Staphylococcus aureus* (Cd, Zn -- Perry and Silver, 1982), *Bacillus* (Cd, Hg, -- Mahler *et al.*, 1986), (Cu, Ni, Sn, Mn, Ba -- Hasnain and Sabri, 1991), *Thiobacillus thiooxidans* (Cd, Zn -- Sakamoto *et al.*, 1989) members of Bacillaceae and Vibrionaceae (Ni, Cu, Co, Mn, Sn, Zn, Fe -- Malik *et al.*, 1991; Hasnain and Sabri, 1992) were described by different workers. None of them was qouted to confer resistance to Cr, Pb and Mo metallic salts. Chromium resistant *Pseudomonas* species (Ohtake *et al.*, 1987; Horitsu *et al.*, 1983, 1987) have not been described conferring resistance to other heavy metals. In *P. flourescence* genetic

determinant of resistance was plasmid coded (Ohtake *et al.*, 1987) Only *Alcaligenes eutrophus* which determine resistance to chromate also confer resistance to cobalt (Nies *et al.*, 1989), *Citrobacter* (Macaskie and Dean, 1987) and Pseudomonads (Hasnain *et al.*, 1993) have also been described. The isolates described here are unique in the sense that they also confer resistance to Cr, Pb and Mo. In the previous reports these resistances were not described in the same strain. The growth media, especially those that contain casein amino acids bound heavy metals and reduce the concentration of free ions, affects the bacterial growth (Ramamoorthy and Kushner, 1975) and the bacterial growth depends upon the kinds, forms and concentration of the salts in the medium (Onishi *et al.*, 1984; Hughus and Poole, 1991). These isolates could also tolerate antibiotics Ap (300 μg ml^{-1}), Cm (5 μg ml^{-1}), Sm (100 μg ml^{-1}) and Km (50 μg ml^{-1}) in the medium. They exhibited different behaviour for Tc resistance. MA-1, MA-2, MA-6 and MA-8 were sensitive to Tc (20 μg ml^{-1}) while rest of them could deter it in the medium. All strains were sensitive to Sm (500 μg ml^{-1}). These results are in accord with our previous results (Hasnain and Sabri, 1991, 1992) in that, in addition to conferring resistance to metals they exhibited multiple resistances to antibiotics. Ahmad and Yadava (1988) described Hg-resistant strains, majority of which showed either single or multiple antibiotic resistances whereas according to Mergeay *et al.* (1985) heavy metal resistant bacteria do not show antibiotic resistance.

Table I. Colony and cell morphology of metal resistant bacteria

Isolates	Visual color	Elevation	Form	Margin	Size (mm)	Cell shape	Cell Size (μm)
MA-1	Creamy	Convex papillate	Rhizodial	Entire	1.0-3.00	Pleomorphic rods	
MA-2	Ochre	Umbonate	Circular	Entire	1.7-2.5	Pleomorphic rods	
MA-3	Creamy	Raised	Rhizodial	Filamentous	2.5-7	Rods	2x10
MA-4	Ochre	Raised	Myceloid	Erose	1.4-3.3	Rods	0.5x3.0
MA-5	Bright yellow	Pulvinate	Circular	Entire	1.7-2.0	Cocci	1
MA-6	Orange	Raised	Circular	Entire	2.0-2.5	Rods	1.0x2.5
MA-7	Cream	Raised	Circular	Slightly undulate	2.0-2.5	Rods	1.5-3.0
MA-8	Cream	Convex papillate	Circular	Entire	4.5-4.7	Rods	0.5x2.0
MA-9	Cream	Flat	Circular	Entire	1.5-1.8	Rods	1.0x2.0
MA-10	Cream	Low convex	Filamentous	Filamentous	2.5-2.8	Pleomorphic	

Table II. Biochemical characterization of metal resistant bacteria

Tests	Isolates									
	MA-1	MA-2	MA-3	MA-4	MA-5	MA-6	MA-7	MA-8	MA-9	MA-10
Gram-staining	-	-	-	-	-	-	-	-	-	-
Sproe-staining	-	-	+	+	-	-	-	+	-	+
Urease	-	-	-	-	-	-	-	+	-	-
Catalase	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+
Methyl red	-	-	-	-	-	-	-	-	-	+
Nitrification	+	+	+	+	+	+	+	+	+	+
Denitrification	-	-	-	+	+	+	-	-	+	-
OF test	+	+	+	+	+	+	+	+	+	+
Pigment test	-	-	-	-	+	-	-	-	-	-
ONPG	-	++	++	-	-	-	-	++	-	-
Sodium citrate	-	-	-	-	-	-	-	-	-	-
Sodium malonate	-	-	-	-	-	-	-	-	-	-
Lysine decarboxylase	-	-	-	-	-	-	-	-	-	-
Arginine dihydrolase	-	-	-	-	-	-	-	-	-	-
Ornithine decarboxylase	-	-	-	-	-	-	-	-	-	-
H^2S production	-	-	-	-	-	-	-	-	-	-
Urea hydrolysis	-	-	-	-	-	-	-	-	-	-
Tryptophan deaminase	-	-	-	-	-	-	-	-	-	-
Indole	-	-	-	-	-	-	-	-	-	-
Acetoin	-	-	-	-	-	++	-	-	-	+
Gelatin hydrolysis	++	++	++	++	++	++	+	++	++	++
Acid from glucose	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	++	++	++	++	++	++	++	++	++	++
Acid from maltose	-	-	-	-	-	-	-	-	-	-
Acid from sucrose	-	-	-	-	-	-	-	-	-	-
Acid from mannitol	-	-	-	-	-	-	-	-	-	-
Acid from arabinose	++	++	++	++	++	++	++	++	++	++
Acid from rhamnose	-	-	-	-	-	-	-	-	-	-
Acid from sorbitol	-	-	-	-	-	-	-	-	-	-
Acid from inositol	-	-	-	-	-	-	-	-	-	-

- negative; + positive; ++ strong positive

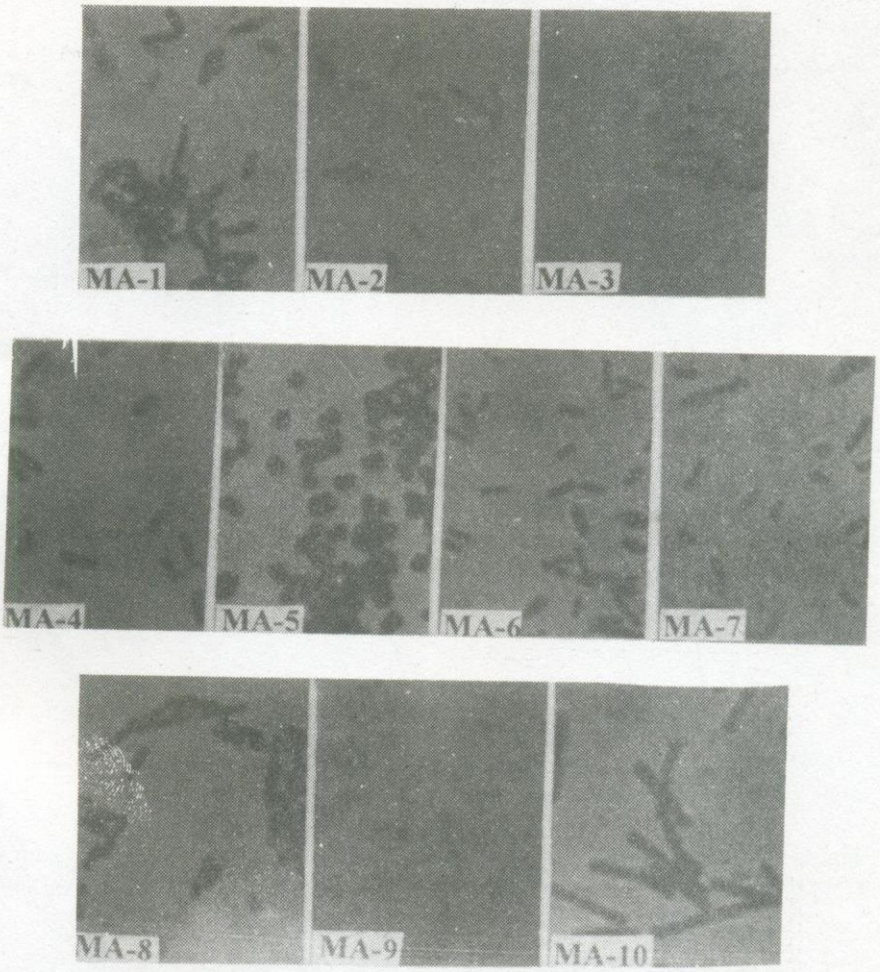

Fig. 1. Isolates from industrial effluents, MA-1, MA-2 and MA-10 pleomorphic Gram-negative rods; MA-3, MA-4, MA-6, MA-7, MA-8 and MA-9 Gram-negative rods; MA-5 Gram-negative Cocci.

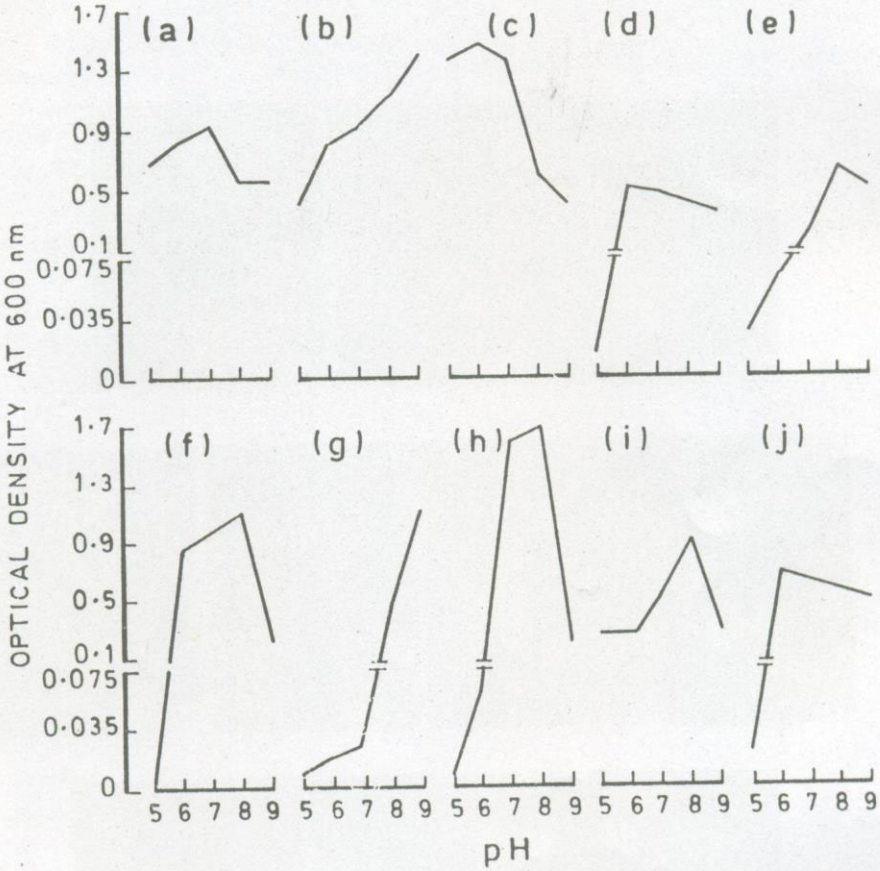

FIG. 2

Fig. 2. Effects of different pH (5, 6, 7, 8 and 9) on the growth of bacteria isolated from the industrial effluents.

For morphological characterization 24 hour old streaked colonies were used. Morphologically colonies differ in colour, shape, size and elevation (Table I). All cells were Gram-negative and motile but vary in shape and size (Table I, Fig. 1). As the concentration of metallic salts increased in the medium the bacterial growth as well as the cell size decreased. Which might be due to reduced cellular growth and metabolic activity under heavy metal stresses (Jonas *et al.*, 1984). All strains were able to grow on L-agar, nutrient agar but not on MacConkey's agar. Only MA-1 and MA-3 could grow on MacConkey's agar. All strains were facultative anaerobe and produce acid from both glucose and mannitol, only MA-10 showed gas production as well. MA-3, MA-4, MA-6 and MA-10 were spore former. Many Cd and Hg-resistant isolates are

spore former (Mahler *et al.*, 1986). All have catalase and oxidase enzyme. Only MA-5 could produce orange pigment on king's medium, while MA-2, MA-4, MA-7, MA-8 and MA-10 produce brownish pigments on media supplemented with salt of manganese. They share many biochemical characters, but differ from one another for some characters (Table II). On the basis of these biochemical characters MA-5 could be affiliated with Pseudomonadaceae, but this strain could grow anaerobically. The *P. nautica* (a denitrifying bacteria) isolated by Bonin *et al.*, (1989) could also grow anaerobically. This isolate MA-5 could resist very high concentration of Mo in the medium. Molybdenum co-transported quantitatively more in glucose, sucrose and mannitol growth cultures (Baxi and Modi, 1988) and its uptake is also dependent upon the metabolizable carbon source. Inhibition of glycolysis also inhibits molybdenum uptake (Baxi and Modi, 1988). Anaerobic breakdown of glucose and mannitol by this strain might be related to the Mo-resistance property of this isolate. Endospore forming, rod shaped MA-3, MA-4, MA-8 and MA-10 could be assigned to family Bacillaceae (Krieg and Holt, 1984). MA-1, MA-2, MA-6, MA-7 and MA-9 belong to Gram-negative, facultative anaerobic rods of Bergey's Manual (Kreig and Holt, 1984). Their further affiliation remained uncertain.

pH is an important factor affecting bacterial growth, bioavailibility, reactivity and complex formation of metals (Hughus and Poole, 1991). For physiological characterization these strains were grown in LB media (Sambrook *et al.*, 1989), adjusted to different pH (5, 6, 7, 8, and 9) levels, at 37°C (150 rpm) for 16 hours. After that the OD of the culture was monitored on spectrophotometer at 600 nm. Results of these strains demonstrate that different isolates prefer different pH levels for their growth. MA-3, MA-4 and MA-10 prefer pH6; MA-1 showed best growth at pH7; MA-5, MA-6, MA-8 and MA-9 showed maximum growth at pH8; while MA-2 and MA-7 favour pH9 for their growth. MA-1, MA-3, MA-6 and MA-8 could grow in pH range 5-8, while pH range for growth of MA-2, MA-4, MA-5, MA-7 and MA-10 was from 6-9. Only MA-9 showed comparatively narrow pH range (7-9) for its growth (Fig. 2). According to Rochelle *et al.* (1989) bacteria prefer environmentally related pH for their growth. It is not true for all the isolates obtained in this study. Only isolates from sample two (MA-2, MA-6, MA-7, MA-9) favours alkaline pH (8, 9) and pH of sample two was 8.46. Two other samples, one and three, were highly acidic (2.45 and 3.11 respectively) but the isolates obtained from these samples favoured pH6 (MA-3, MA-4), 7 (MA-1) or 8 (MA-5, MA-8) for their growth. It appears that these strains could survive at highly acidic pH, but their multiplication was restricted and on obtaining favourable conditions they showed extensive growth. MA-3, MA-4 and MA-8 were spore-formers, it seems that these strains were enduring extremely acidic pH by endospore formation. Further, metal resistances of bacterial strains are pH dependent (Wood and Wang, 1985). Gel electrophoresis of total cell lysate revealed the presence of single plasmid in each of MA-6 and MA-8. Hg, Cd, Cr, Co, Zn, Pb, and Fe has been reported plasmid encoded (Perry and Silver, 1982; Mergeay *et al.*, 1985; Mahler *et al.*, 1986; Nies and Silver, 1989; Nies *et al.*, 1989; Hasnain and Sabri, 1991, 92; Malik *et al.*, 1991). Whereas, few reports also dealt with non-plasmid borne cadmium-resistance (Mahler *et al.*, 1986). To determine whether resistance conferred by MA-6 and MA-8 were transcribed by plasmid/s or chromosome, conjugation experiments were performed. Since many broad host ranged plasmids form Gram-negative bacteria (Old and Primrose, 1985) can promote their transfer. MA-12 (Gram-negative, rod

shaped, Cr^r, Pb^r, Cd^s, Mo^s, Ap^r, Cm^r, Km^s, Tc^r) was used as recipient. Quite reasonable number (520) of transconjugant on Mo and Tc plates, when MA-6 was used as donor, demonstrate that Mo-resistance in this strain was encoded by plasmid. No transconjugants were scored when MA-8 was donor. These results do not necessarily reflect the lack of Mo-marker on this plasmid, it might be due to the operation of some host restriction mechanism. Failure in detecting the plasmid band by the electrophoresis of total cell lysate in the rest of strains do not exclude the possibility of plasmid determinants in the rest of strains. Mega-plasmids (cointegrate plasmids) may not be detected by this method (Thomas, 1984). Both plasmids (Mergeay, 1991) and transposons governed (Diels *et al.*, 1987) resistances to heavy metals have been reported. Multiple antibiotic resistances have been described both in plasmids and transposons (Watson, *et al.*, 1987; Bennett *et al.*, 1988). Whether pleotropic metal as well as antibiotics resistance in the strains described here are encoded by plasmids (cointegrate plasmids), transposons or chromosome separately or some integration between different genetic determinants exist for these resistance, remained to be determined.

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