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COBALT - RESISTANT PSEUDOMONADS FROM INDUSTRIAL EFFLUENT

SAMINA YASMIN AND SHAHIDA HASNAIN

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

Abstract: Three cobalt resistant bacteria were isolated from the industrial waste water and were designated as SCo-1, SCo-2 SCo-3. They could resist $CoCl_2$ upto 300ug/ml. Colonies of these isolates were circular with entire margins and convex elevation. Colour of Colonies were off white (SCo-1), light brown (SCo-2) and creamy (SCo-3). They had motile rod shaped cells. All were aerobic, had oxidase and catalase enzymes. They were non spore former and were able to produce pigments. All of them shared almost all biochemical characters. On the basis of morphological and biochemical characters these isolates could be affiliated to family pseudomonadaceae. These strains showed optimum pH range 6-8.5 for their growth. They could also bear Zn, Sn, Mn, Cd and Pb (200ug/ml) in the medium. All isolates showed resistance to ampicillin (300ug/ml) and chloramphenicol (5ug/ml), while they were sensitive to streptomycin, kanamycin and tetracycline. Gel electrophoresis of total cell lysates of these bacteria revealed that only SCo-1 harbour a single plasmid. No transconjugant on $CoCl_2$ + Sm plates were attained. However the yield of large number of transformants on plates amended with $CoCl_2$ revealed that cobalt resistance in SCo-1 was encoded by plasmid.

Key words: Pseudomonad strains, cobalt-resistent bacteria, heavy-metal resistant bacteria

INTRODUCTION

With the increase in industrialization, heavy metal laden effluents increased tremendously which may interfere with man's use of his environment. The biological effect of heavy metals including cobalt is potentially perilous to human beings and other organization (Brady, 1984). Cobalt which belong to first series of transition metals is carcinogenic (Sunderman, 1977), mutagenic (Abbott, 1985) and bioaccumulating in organism (Abbott, 1985). Its excessive dosage in animals and human beings is toxic and is cause of polycythaema (Bidwell, 1979), Excess of cobalt in plants is also toxic and may cause zinc and phosphorous defficiency, and also reduces biomass production (Wallace, 1989). It completely blocks oxidation and growth of *Thiobacillus ferroxidans* (Sugio *et al*., 1989). Cobalt is essential for nitrogen fixing symbiotic bacteria and actinomycetes (Jafferey, 1987).

Bacteria exhibit a number of metabolism dependent and independent processes for the uptake and accumulation of heavy metals (Gadd, 1990) and transformation of organic and inorganic compounds to innocuous form (Francis, 1990) by, i, oxidation and reduction of metals; ii, changes in pH and Eh which affect the ionic state of metal; iii, solubilization and leaching of certain elements by alkylation and chelation; iv, immobilization leading to the formation of stable minerals; and v, remineralization of metals. At present we are desired to isolate metal resistant bacteria from polluted water. Present study deals with the isolation of cobalt-resistant bacteria and their characterization with respect to growth, morphology, physiology, biochemistry and genetics.

MATERIALS AND METHODS

Samples from industrial waste water of Kasoor, which was dark green in colour and had pungent smell with pH 8, was brought in the laboratory. A 50μl of it was plated on nutrient agar plates as well as on media described by Nies *et al*., (1987), containing 25μg/ml of $CoCl_2$. Bacterial growth was observed at 37 °C after 24 hours of incubation. Different colonies from bacterial growth were picked, purified and then were taken to higher concentration of $CoCl_2$. Only three strains (SCo-1, SCo-2, SCo-3) which could resist $CoCl_2$ upto 300μg/ml were used for further studies. These isolates were characterized morphologically, physiologically, biochemically (Gerhardt *et al*., 1981) as well as genetically. Additional 21 biochemical tests were performed using QTS-20 (Quick test strips) and CO (cytochrome oxidase) strips obtained from DESTO Laboratories, Karachi. pH range of these isolates was checked. The strains were also screened for the resistance to other metals (zinc sulphate, stannous chloride, manganese sulphate, cadmium chloride, lead accetate) and antibiotics (ampicillin, tetracycline, kanamycin, streptomycin, chloramphenicol). By the gel electrophoresis of total cell lysate (Thomas, 1984) isolates were screened for the presence of plasmids. Plasmid DNA was extracted by the method of Smith and Thomas (1983). Genetic bases of cobalt resistance was determined by conjugation and transformation experiments. For conjugation *E. coli* strain CSR603 (*recAl pharI* derivatives of AB1886 (*thr-l leu-6 lacY1 galK2 ara-14 xyl-5 mtl-1 proA2 his-4 str-31 tsx-33 sup-37 uvxA6)* was used as recipient. Both broth and plate mating techniques were performed (Willetts, 1984). Transconjugants were selected on plates containing 300◊g/ml $CoCl_2$ and 500μg/ml streptomycin. For transformation *E. coli* K-12 strain MV10 [C600 ∆ *trpE5 (thr-1, leu-6, thi-1 lacY1 supE44 tonA21 trpE5)* was used. MV10 was made competent by the method described by Thomas (1981). Transformats were selected on nutrient agar plates supplemented with 300μg/ml of $CoCl_2$. Liquid cultures were grown in L. broth and bacterial growth was at 37°C.

RESULTS AND DISCUSSION

The different colonies obtained from sample were picked and purified on 25μg/ml of $CoCl_2$. These purified isolates were taken to the elevated level of $CoCl_2$. Only three isolates SCo-1, SCo-2 and SCo-3 were able to grow at 300μg/ml of $CoCl_2$. At 400μg/ml no growth was observed. Morphological and biochemical characters of these strains are shown in Table 1 (a+b) and 2, respectively. All isolates had circular, entire and convex colonies, size of which differ with strains (Table 1a). These isolates were Gram-negative and motile rods (Table 1b. Fig.1). They could grow on nutrient agar and L. agar. On Mac-Conkey agar only SCo-3 was able to grow. All strains were aerobic (OF negative), had oxidase and catalase enzymes (Table 2), and gave positive results for methyl red test. None of them could denitrify, had urease enzyme or as spore former. All of them could produce pigments (pinck in SCo-1, brown in SCo-2 and SCo-3) on the King's media (King *et al*., 1954). Results of twenty biochemical tests obtained with QTS-20 strips revealed that SCo-1, SCo-2 and SCo-3 shared all biochemical characters tested, except for lysine decarboxylase test which was negative

in SCo-1 and positive in the rest of two strains. Gelatin hydrolysis, nitrate reduction and acid production from arabinose tests were positive for these strains. For remaining tests these strains gave negative results (Table 2). On the basis of these morphological and biochemical characters these strains were affiliated to the family Pseduomonadaceae, here after will called pseudomonads. Previously cobalt resistant *Alcaligenes* which could bear about 680μg/ml (5mM) $CoCl_2$ in the medium have been described by Mergeay *et al.*, (1985). Although bacterial strains described here could tolerate comparatively less amount of cobalt but cobalt-resistant pseudomonads have not been described previously. Strain of *Alcaligenes* isolated by Mergeay *et al.*, 1985) was obtained from the zinc factory while pseudomonads were attained from the effluent of tennery.

These strains showed optimum growth in the range of pH 6 to 8.5. Screening for resistance to other metals (Zn^{2+} Sn^{2+}, Mn^{2+}, Cd^{2+}, Pb^{2+} and Hg^{2+}) revealed that all strains could tolerate these metals upto 200μg/ml in the medium but were sensitive to Hg^{2+} even at 50μg/ml. The multiple resistance to heavy metals might be due to interrelationship of these metals in cell's metabolism (Nelson and Kennedy, 1971; Novick *et al.*, 1979). Cobalt-resistant *Alcaligenes* have been reported showing resistance to other heavy metals (Mergeay *et al.*, 1985). Pleotropic metal-resistant phenotype of *Alcaligenes* was encoded by plasmids (Mergeay *et al.*, 1985; Nise *et al.*, 1987). Whether multiple metal-resistances conferred by Pseudomonads are determined by plasmid/s remain to be determined. These strains also showed resistance to ampicillin (300μg/ml) and chloramphenicol (5μg/ml). However, they were sensitive to streptomycin (300μg/ml), kanamycin (50μg/ml) and tetracyclin (25μg/ml). Cobalt resistant *Alcaligenes* have not been found associated antibiotic-resistance (Mergeay *et al.*, 1985). Whereas mercury resistant *E. coli* strains showed co-resistances with different antibiotics (Ahmad and Yadava, 1988).

Metal resistance in bacteria is either plasmid encoded (Laddaga *et al.*, 1987; Silver and Misra, 1988) or it may be chromosomally encoded (Wang *et al.*, 1987). Cobalt resistant bacteria were screened for the presence of plasmid. One band of plasmid was observed in SCo-1 while no band was detected in SCo-2 and SCo-3 by the electrophoresis of total cell lysates. To characterize these strains for the genetic determinant of cobalt resistance, conjugation and transformation experiments were carried out. For conjugation broth and plate mating techniques were used. No transconjugants were obtained on plates amended with $CoCl_2$ and streptomycin, indicating that either cobalt resistance was not encoded by the plasmid or plasmids were non conjugative. Mergeay *et al.* (1985) observed very low conjugation frequency in their strains and it could be enhanced by the helper plasmids, which reflect that *tra* gene in the plasmid/s harbouring *in Alcaligenes* was lacking. The plasmid present in Pseudomonad (SCo-1) my also lack *tra* gene. Another explanation for the failure in obtaining transconjugants might lie in host restriction mechanisms. Other factor which also affect the conjugation are host physiology, type of plasmid present, pH, temperature (Gauthier *et al.*, 1985; Rochelle *et al.*, 1988), growth rate (Herbert and Bhakoo, 1979), mating time, nutrient status and donor to recipient ratio (Beringer, 1974). To confirm whether cobalt resistance in SCo-1 is encoded by plasmid. Plasmid DNA from this strain was extracted and 10μl (0.1μg) of it was transformed to *E. coli*

strain MV10. Before transformation MV10 was checked for cobalt resistance. Transformants were selected on $CoCl_2$ (300μg/ml) plates. With SCo-1 (donor) a large number of transformants were obtained which revealed that the cobalt resistance in SCo-1 was encoded by plasmid. Further stuides at molecular level will exhibit the nature of this plasmid.

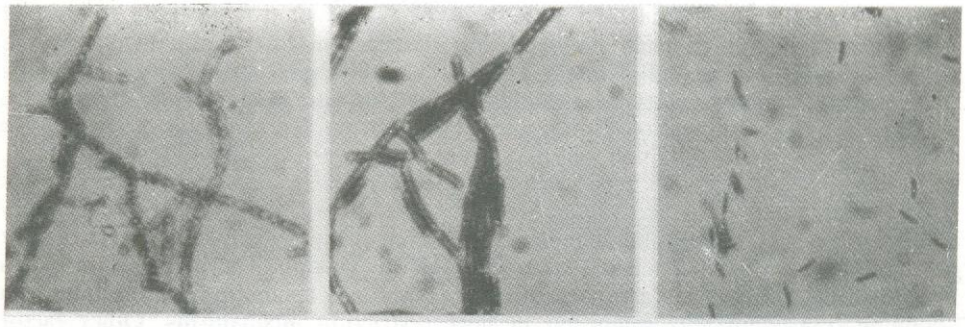
Table-1: Morphological characters of the cobalt-resistant isolates obtained from industrial effluent.

a) colony morphology

Sr. No.	Strains	Visual Colour	Colour under stereomicroscope	Form	Elevation	Margin mm	Colony diameter
1.	SCo-1	Offwhite	Brownish	Circular	Convex	Entire	1.0 - 1.5
2.	SCo-2	Lightbrown	Yellowish	Circular	Convex	Entire	3.0 - 3.5
3.	SCo-3	Creamy	Yellowish	Circular	Convex	Entire	1.0 - 1.5

b) cell morphology

Sr. No.	Strains	Motility	Cell Type	Cell szie (um)	Gram straining	Growth on Mac.Conkey
1.	SCo-1	+	Rods	2.88x1.0	-	-
2.	SCo-2	+	Rods	1.92x.75	-	-
3.	SCo-3	+	Rods	2.88x1.0	-	+



a b c

Figure 1: Cobalt resistant isolates obtained from the industrial effluent of Kasoor. a) SCo-1: b) SCo-2: c) SC0-3

Table-2: Biochemical characters of the cobalt-resistant isolates obtined from industrial effluent.

Tests	SCo-1	SCo-1	SCo-3
Catalase	+	+	+
Oxidase	+	+	+
O.F.	-	-	-
Urease	-	-	-
Denitrification	-	-	-
Tetrazolium (Spors Test)	-	-	-
Methyl red	+	+	+
Pigment Production	+	+	+
ONPG	-	-	-
Sodium citrate	-	-	-
Sodium malonate	-	-	-
Lysine decarboxylase	-	+	+
Arginine dihydolase	-	-	-
Ornithine decarboxylase	-	-	-
H_2S production	-	-	-
Urea hydrolysis	-	-	-
Tryptophane deaminase	-	-	-
Indole	-	-	-
Acetoin (VP)	-	-	-
Celatin 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	-	-	-

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