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Isolation and Characterization of Chromium-Resistant Bacteria from Tannery Effluents

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Chromium (Cr), a transition metal, is one of the major sources of environmental pollution. It is discharged into the environment through the disposal of wastes from industries like leather tanning, metallurgical and metal finishing, textiles and ceramics, pigment and wood preservatives, photographic sensitizer manufacturing, etc. (Komori *et al.* 1990; Desh and Gupta 1991). In the environment chromium occurs mainly in trivalent and hexavalent forms. The hexavalent chromium (Cr^{6+}) compounds are comparatively much more toxic than those of trivalent chromium (Cr^{3+}) (Ishibashi *et al.* 1990). The reason for such toxicity appears to be due to its rapid permeability through biological membranes and subsequent interaction with intracellular proteins and nucleic acids (Horitsu *et al.* 1978).

The tanning industry, which commonly utilizes "chrome liquor" in the tanning process, discharges the effluents into the environment containing chrome salts in excess of the maximum permissible limits (Khasim and Nanda Kumar 1989; Thiagragan 1992). Sludge deposition from such effluents, therefore, provides a natural environment for enrichment of chromium-resistant bacteria. Chromium-resistant microorganisms from such chromium-contaminated sediments have been isolated by several investigators (Horitsu *et* al. 1978; Luli *et al.* 1983; Losi and Frankenberger 1993).

The present study was an attempt to evaluate the status of chromium-resistant bacteria in the tannery effluent sediments of Calcutta-based tanning industries.

MATERIALS AND METHODS

Tannery-effluent sediments were collected from 3 different sites of the drainage system of local tanning industries in Calcutta, West Bengal (India). Samples collected in sterilized glass containers were brought to the laboratory for microbiological and chemical analyses.

For the isolation and enumeration of bacteria, samples were serially diluted in sterile distilled water and plated in Cr-free and Cr^{6+}- supplemented Peptone Yeast Extract (PYE) agar medium. Separately sterilized chromium solution (K_2CrO_4) was added to the medium at a concentration of 12.5 and 25.0 µg of Cr^{6+}/mL. Plates were incubated at 30-35°C for 3-7days

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and the total numbers of bacteria were determined from the colony forming units (cfu). Colonies differing in morphology were isolated in pure form and maintained on PYE agar slants.

The isolates were tested for their resistance to chromate by growth in PYE agar plates containing various concentrations of Cr^{6+} (25, 62.5. 125 & 250 µg/mL.) as K_2CrO_4. The Cr^{6+}- incorporated plates were streaked with freshly grown culture of the isolates and incubated at 30-35°C for 3-7 days. Growth of the isolates was determined visually as positive or negative. Degree of resistance of selected isolates were also evaluated in PYE broth containing 25, 125 & 250 µg of Cr^{6+}/mL. Growth of the isolates in PYE broth was determined by measuring the optical density at 540 nm using the uninoculated broth as the blank. Relative growth of the isolates was expressed as the percentage of those obtained in untreated control which was taken as 100%.

The resistance of selected isolates against other chromium salts and heavy metals was also tested in the PYE broth. The other chromium salts and metals that were tested include the following : $CrCl_3.6H_20$, $K_2Cr_2O_7$, CrO_3, $HgCl_2$, $CuCl_2.2H_20$, $NiCl_2.6H_2O$. $CdSO_4.8H_2O$, $PbNO_3$, $MnSO_4.H_2O$ and $CoCl_2.6H_20$. The relative resistance of the isolates was determined from the percent inhibition of growth over the control. Growth was also determined by measuring the optical density at 540 nm.

To determine the antibiotic sensitivity of the bacterial isolates, antibiotic-impregnated discs (Dia. 6mm, Hi-media) were placed on freshly prepared lawns of each isolate on nutrient agar plates. The plates were incubated at 30-35°C for 24 hr. The diameter of the inhibition zones was measured to the nearest mm and the isolates were classified as resistant (R), intermediate (I) and susceptible (S) following the standard antibiotic disk sensitivity testing method (DIFCO Manual 10th ed. DIFCO Laboratories Inc.) Discs containing the following antibiotics were tested : chlortetracycline (30 µg). rifampicin (5µg), neomycin (30µg), streptomycin (10 µg), bacitracin (10 U), cycloserine (200 µg), vancomycin (30 µg), ampicillin (l0 µg), chloramphenicol (30µg), gentamycin (10 µg), penicillin-G (10 U) and erythromycin (15 µg).

The total chromium content of the air-dried sediment samples was determined by atomic absorption spectroscopy (in Varian Spectra AA 20 plus) following digestion of samples in a hot HNO_3: HCl (1:3) mixture (Luli *et al.* 1983).

RESULTS AND DISCUSSION

Total chromium, pH, and the microbial content of the samples are shown in Table 1. The total Cr content of the contaminated sediments varied from 20.02 to 35.55 mg/g: the pH ranged between 8.3-8.6. The bacterial population (cfu) in 3 different sites did not show any significant variation and was in the order of 2.15-2.77 x 10^6 cfu/g of sediment. The count of bacteria on PYE agar containing Cr^{6+} decreased with increasing concentrations of Cr. This may have been due to the inability of sensitive organisms to grow on Cr-supplemented plates. Similar declines in bacterial populations (cfu) of the Cr-contaminated sediments were also reported by Luli *et al.* (1983) and Losi and Frankenberger (1993). Forty-five bacterial colonies differing in morphology were isolated in pure form fromCr^{6+}- incorporated plates and were subjected to assessment for relative Cr-resistance.

Table 1. **Physical and microbiological characteristics of tannery waste sediments.**

Sample	Physical character		Microbiological character		
	pH	Total chromium (mg/g)	Microorganisms (cfu x 10^6/g)		
			PYE	PYE+A	PYE+B
NT-1	8.5	35.55	2.15	1.85	1.70
SC-2	8.4	34.23	2.77	1,88	1.25
FT-3	8.6	20.02	2.37	1,70	1.40

Values are presented as mean of triplicates.

A = Cr^{6+}, 12.5 µg/mL.; B= Cr^{6+}, 25.0 µg/mL

Table 2. **Chromium tolerance of selected bacterial isolates.**

Isolate	Incubation period, hr	Relative Growth, % Chromium concentration (µg/mL.)		
		25	125	250
TEM-22	24	62.66	22.60	10.80
TEM-41	54	47.70	30.60	10.70
TEM-61	30	79.20	45.80	35.80
TEM-93	24	61.80	09.86	07.89
TEM-111	24	60.40	13.30	10.40
TEM-212	24	70.40	21.50	11.36
TEM-213	30	63.60	08.86	06.25
TEM-214	24	32.50	15.20	08.14
TEM-216	12	87.50	40.00	18.25
TEM-253	30	81.80	58.18	40.00

All values represent the average of triplicates.

For chromate-resistance, all 45 isolates were screened primarily on chromate-supplementcd solid media and the results are shown in Figure 1. Only 10 (22.22%) isolates were resistant to 250 µg/mL of Cr^{6+}. Tolerance of these selected isolates to different concentrations of Cr^{6+} in liquid medium are shown in Table 2.

Based on their performance in different concentrations of Cr^{6+} isolates TEM-61, TEM-216 and TEM-253 were found to be promising, showing 18-40% of growth at a concentration of 250 µg/mL. of Cr^{6+}. Patterns of growth of all three isolates in chromate-containing medium are shown in Figure 2. Growth of the isolates at the lowest concentration (25 µg/mL.) was comparable to that of the control which declined at higher concentrations irrespective of isolates. Optimum growth of isolate TEM-61, however, was delayed at higher Cr concentrations. In the present study no attempt was made to determine the mechanism of such tolerance. Chromate-tolerance mechanisms in bacteria, however, have been reported to include reduction, methylation, precipitation at the cell surface, blocking cellular uptake by altering the uptake pathway, and removal from the cytoplasm by 'efflux pumps' (Ishibashi *et a1.* 1990; Shuttleworth and Richard 1993; Lovley and Phillips 1994).

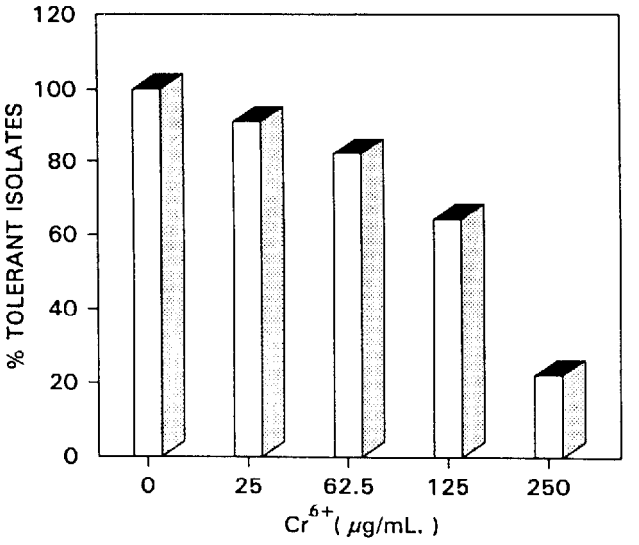

Figure 1. Primary Screening of bacterial isolates for chromate-resistance.

Table 3. Morphological and biochemical characteristics of selected chromium-resistant bacterial isolates.

Character	Response		
	TEM-61	**TEM-216**	**TEM-253**
Gram reaction	+	+	+
Morphology	Cocci, in pairs	Rod, Single & in pairs	Cocci, in pairs
Color	Creamish white	Translucent white	Yellow
Formation of endospore	-	-	-
Motility	+	+	+
Nitrate reduction	-	+	+
Catalase production	+	+	+
Hydrolysis of starch	+	-	+
Hydrolysis of casein	-	-	-
Gelatin liquefaction	+	-	-
Hydrolysis of fat	+	+	+
NaCl tolerance	≥ 16%	≥ 16%	≥ 13%
CARBOHYDRATE UTILIZATION			
Maltose	+	+	+
Glucose	+	+	+
Sucrose	+	+	+
Galactose	+	+	-
Lactose	+	+	-
Mannitol	+	+	+

Table 4. Percent inhibition of growth of selected bacterial isolates by different chromium salts.

Isolate	$CrCl_3$			K_2CrO_4			$K_2Cr_2O_7$			CrO_3		
	25	125	250	25	125	250	25	125	250	25	125	250*
TEM-61	08.50	16.00	42.53	20.80	54.20	64.20	05.30	70.53	99.48	09.10	67.26	99.10
TEM-216	29.10	31.00	36.00	12.50	60.50	81.75	11.37	95.45	98.87	22.92	97.92	98.43
TEM-253	22.00	44.28	52.00	20.20	43.40	60.70	96.20	98.10	29.10	29.10	94.10	97.28

Each value represents mean of triplicates.
* Concentration of Cr, µg/mL.

Table 5. Percent inhibition of growth of selected bacterial isolates by different heavy metals.

Isolate	Cu^{2+}		Ni^{2+}		Co^{2+}		Cd^{2+}		Pb^{2+}		Mn^{2+}		Hg^{2+}	
	50	100	50	100	50	100	50	100	50	100	50	100	50	100
TEM-61	17.65	53.00	29.50	64.70	20.00	58.83	08.83	53.00	62.50	75.00	NI	41.18	71.00	100.00
TEM-216	14.82	18.52	30.00	60.00	55.50	65.00	10.00	55.00	65.00	85.00	05.56	14.82	50.80	88.00
TEM-253	17.25	51.75	74.14	82.76	32.00	52.73	55.18	69.00	93.11	100.00	NI	44.83	80.00	90.00

Each value represents average of triplicates.
* Concentration of metal, µg/mL.; NI = No inhibition.

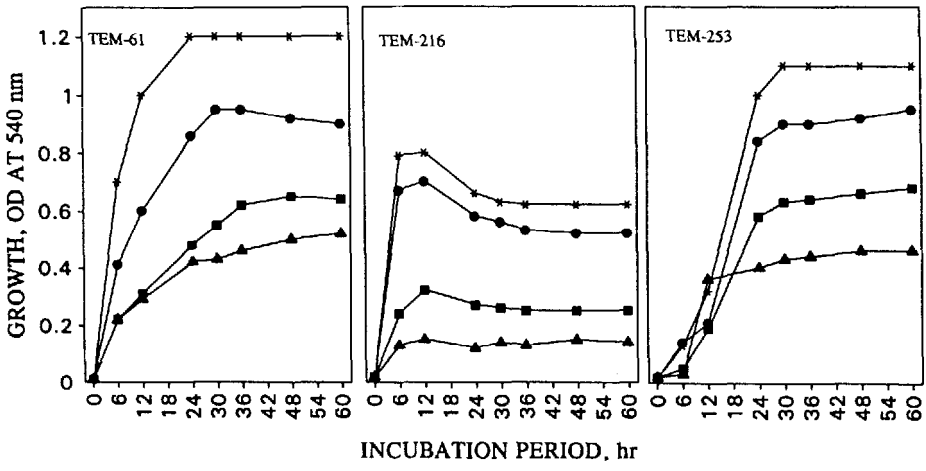

Figure 2. Time course of growth of bacterial isolates TEM-61, TEM-216, & TEM-253 in Cr^{6+} supplemented peptone yeast extract medium (-x-,, control; ●, 25 µg/mL; ■, 125 µg/mL. and ▲, 250 µg/mL.).

Table 6. Antibiotic sensitivity profile of Cr-resistant bacterial Isolates.

Antibiotic Disc (Cone.)	Diameter of inhibition zone, mm		
	Isolate		
	TEM-61	TEM-216	TEM-253
Chlortetracyclin (30 µg)	16.5 (I)	13.0(R)	18.0 (I)
Rifampicin (5 µg)	12.0 (I)	08.0(R)	24.0 (S)
Neomycin (30µg)	23.0(S)	14.0 (I)	15.0 (I)
Streptomycin (10 µg)	27.0 (S)	14.0 (I)	12.0 (I)
Bacitracin (10 U)	10.0 (I)	NI (R)	30.0 (S)
Cycloserine (200 µg)	27.5 (S)	17.0 (I)	26.0 (S)
Vancomycin (30 µg)	24.0 (S)	NI (R)	25.0 (S)
Penicillin-G (1OU)	NI (R)	NI (R)	22.0 (I)
Ampicillin (10 µg)	09.0 (R)	NI (R)	47.0 (S)
Chloramphenicol (30 µg)	20.0 (S)	20.0 (S)	24.0 (S)
Gentamycin (10 µg)	25.0 (S)	16.0 (S)	17.0 (S)
Erythromycin (15 µg)	27.0 (S)	NI (R)	25.0 (S)

NI = No inhibition; Diameter of disc = 6 mm.
Letters in parenthesis indicate sensitivity ; R = Resistance; I = Intermediate; S = Susceptible.

Morphological and physio-biochemical characteristics of the isolates are shown in Table 3. Based on comparison of these character-s with standard descriptions in Bergey's Manual of Determinative Bacteriology (9th ed. 1994), isolates TEM-61 and TEM-253 were identified tentatively as species of *Trichococus* and *Micrococcus,* respectively. Generic identity of isolate TEM-216, a Gram-positive. non-

spore forming rod remained unidentified and needs more detailed studies for confirmation.

The promising isolates were tested for their tolerance to other chromium salts and heavy metals. All three isolates showed a high degree of resistance to trivalent chromium (Cr^{3+}) compared to that of the hexavalent forms (Table 4). Among the other heavy metals tested, Hg^{2+} was the most toxic followed by Pb^{2+}, whereas the isolates showed different degrees of resistance to Mn, Cu, Co, Cd and Ni at 100 µg/mL. (Table 5). Such resistance may be due possibly to exclusion of metal species, production of low molecular weight binding proteins, transformation, bioaccumulation, etc. (Summers 1978; Silver and Misra 1988).

As heavy metal resistance is linked with antibiotic-resistance, the chromate-resistant isolates were tested for their sensitivity to 12 different antibiotics. Isolate TEM-253 appeared to be most susceptible being inhibited by all 12 antibiotics. TEM-61 was resistant to penicillin and ampicillin. Isolate TEM-216 was resistant to as many as 7 antibiotics and showed intermediate to susceptible responses to the rest of the antibiotics (Table 6). This multiple antibiotic-resistance of isolate TEM-216 is also correlated with high degree of resistance to different heavy metals.

From the above results, it is evident that the tannery waste sediments provide an enriched Cr-contaminated environment where from Cr-resistant bacteria could be isolated. Of the 45 isolates, 3 were promising showing resistance to chromium and other heavy metals, although Hg^{2+} and Pb^{2+} were toxic for them. Isolate TEM-216 in particular showed multiple antibiotic-resistance, indicating possible inheritance of plasm id-determined resistance factors. Mechanism(s) of chromate-resistance of these bacteria need to be worked out in detail for their possible utilization as bioremedial tool against Cr toxicity.

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REFERENCES

Desh D, Gupta AK (1991) Hexavalent chromium removal from waste water. Indian J Environ Hlth 33 :297-305

DIFCO Laboratories Inc. (1984) DIFCO manual, 10th ed. DIFCO Laboratories Inc. Detroit, Michigan

Horitsu H, Nishida H, Kato H, Tomoyeda M (1978) Isolation of potassium chromate - tolerant bacterium and chromate uptake by the bacterium. Agric Biol Chem 42:2037-2043

Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST (1994) Bergey's manual of determinative bacteriology, 9th ed. Williams & Wilkins, Baltimore, USA.

Ishibashi Y, Cervantes C, Silver S (1990) Chromium reduction in *Pseudomonas putida*. Appl Environ Microbiol 56:2268-2270

Khasim ID, Nanda Kumar NV (1989) Environmental contamination of chromium in agricultural and animal products near a chromate industry. Bull Environ Contam Toxicol 43:742-746

Komori K, Wang P, Toda K, Ohtake H (1990) A method for removal of toxic chromium using dialysis-sac cultures of' a chromate reducing strain of *Enterobacter cloacae*. Appl Microbiol Biotechnol 33:117-119.

Losi ME, Frankenberger Jr. WT (1994) Chromium resistant microorganisms isolated from evaporation ponds of a metal processing plant. Water Air Soil Pollut 74:405-413

Lovley DR, Phillips EJP (1994) Reduction of chromte by Desulfovibrio vulgaris and its C_3 cytochrome. Appl environ Microbiol 60:726-728.

Luli GW, Joseph WL, William RS, Robert MP (1983) hexavalent chromium resistant bacteria isolated from river sediments. Appl Environ Microbiol 46 :846-854

Shuttleworth KL, Richard FU (1993) Sorption of heavy metals to the filamentous bacterium *Thiothrix* strain Al. Appl Environ Microbiol 59: 1274 - 1282

Silver S, Misra TK (1988) Plasmid-mediated heavy metal resistances. Ann Rev Microbiol 42 : 717 - 743

Summers AC (1978) Microbial transformations of metals. Ann Rev Microbiol 32:637-672

Thiagragan G (1992) In pursuit of better image : Survey of the environment. The Hindu : 143-145