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Effect of Ecological Factors on Conjugal Transfer of Chromium-Resistant Plasmid in *Escherichia coli* Isolated from Tannery Effluent

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Abstract

The influence of total organic carbon (TOC), pH, and mating temperature on transfer of chromium-resistant plasmid between *Escherichia coli* strains in terms of variation in the number of transconjugants formed and variation in transfer frequency was investigated. In vitro transfer was studied in five chromate-tolerant *E. coli* strains isolated from tannery effluent using *E. coli* K12 J62 (Nal^r Lac^-) as a recipient. Conjugal transfer of different selection markers was observed in three strains. The study was carried out in sterile wastewater. A gradual decrease was observed both in the number of transconjugants and in transfer frequencies as the concentration of TOC in the mating medium descended from 10,095 to 1.2 mg of C/L, obtaining the maximum values with a TOC concentration of 10,095 mg of C/L. The number of transconjugants and the transfer frequency were maximum at 30°C. However, neither the transfer frequency nor the transconjugant number varied significantly in the range of pHs assayed. The strains were also found resistant to different heavy metals and antibiotics. Curing of these strains resulted in loss of one or more resistance markers indicating the plasmid-borne resistance. It is inferred that plasmid transfer by conjugation occurs in wastewater bodies within a wide range of conditions.

Index Entries: Chromium; *Escherichia coli*; R-plasmid; conjugation; wastewaters; total organic carbon; mating temperature.

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Introduction

Hexavalent chromium compounds have been employed in a wide variety of commercial processes, and unregulated disposal of the chromium containing effluent of these units at developing and developed countries has led to the contamination of soil, sediment, and surface and groundwaters *(1,2)*. An elevated concentration of metals in the environment has caused wide range of impacts on microbes, plants, animals, as well as the human population *(2)*.

Bacterial resistance to heavy metals in the polluted environment is widespread. Studies regarding the impact of environmental pollution and other factors on bacterial strains harboring chromium-resistant plasmid are attracting considerable interest. Such metal-tolerant bacteria are also resistant to antibiotics, which may be a desirable characteristic for an organism to have in a natural environment of mixed population *(3)*. This resistance also provides the selective advantage necessary for maintaining multiple resistance *(4)*. Such resistance may enable the organism to be better adapted to a particular niche in the environment *(5,6)*.

The amount of wastewater discharged increases daily in both urban and rural environments. The greater survival of these strains may be owing to the fact that in some cases the resistance to antibiotics seems to be associated with resistance to environmental factors such as light, temperature, pH, nutrient status, and presence of different heavy metals *(7,8)*. Several studies revealed that plasmid-bearing strains have greater chances of survival in such environments *(5,9,10)*. The same has also been reported for *Escherichia coli* strains *(11)*. Among natural communities, the development of metal and antibiotic resistance could be greatly enhanced by the horizontal dispersal of genetic information. Conjugation has been suggested to play an important role in the spreading of genes in the natural environment and in establishing new genetic traits in diverse environments *(12,13)*. Numerous investigators have demonstrated the transfer capacity of plasmids through conjugation, in both in vitro *(8,14,15)* and *in situ* experiments using diverse microsomes *(16–18)*. Apart from R-factors, bacteria may also harbor other plasmids such as those that transmit enteropathogenicity among *E. coli*. Thus, the presence of pathogenic *E. coli* in the environment, particularly in water bodies, is a situation that requires the highest attention. The environmental spread of such metal-resistant bacteria may have a greater effect on public health *(19)*. Large amounts of contaminated tannery effluents reaching the surface and eventually groundwater sources have further aggravated the situation.

The possibility of transfer is essential to carrying out studies on the influence of ecologic factors of the system in which the process of transfer frequency and transconjugant formation take place. In this way, the effects of temperature, pH, mating time, concentrations of nutrients, and density of parent cells on plasmid transfer have been studied in epilithic bacteria *(20–23)*. However, biotic and abiotic factors affecting transfer in aquatic

systems between free-living bacteria are rarely investigated *(8,24)*. The present study, therefore, was aimed at ascertaining the survival and propagation of chromium-resistant, free-living tannery isolates along with the influence of total organic carbon (TOC), pH, and temperature on stability and transmissibility of chromium-resistant plasmid. In vitro transfer studies were carried out in sterile wastewater of Pipra Ghat and Sahid Smarak stations (Lucknow, India). The influence was evaluated in terms of the variation in transfer frequency observed.

Materials and Methods

Chemicals

The heavy metals employed were salts of $MnCl_2$ and As_2O_3 obtained from E. Merck and $CuSO_4$, $ZnSO_4$, $Co(NO_3)_2$, $NiCl_2$, $CdCl_2$, and $HgCl_2$ from Qualigens. Their purity levels were 99.85, 99.95, 99.98, 99.93, 99.89, 99.91, 99.96, and 99.94, respectively.

Antibiotics

Sensitivity to antibiotics was determined by the disk diffusion method *(23)*. Disks containing the following antibiotics were tested: 10 µg/disk of gentamycin, 50 µg/disk of polymixin-B, 30 µg/disk of chloramphenicol, 30 µg/disk of kanamycin, 30 µg/disk of tetracycline, 10 µg/disk of bacitracin, 25 µg/disk of streptomycin, 25 µg/disk of ampicillin, 100 µg/disk of carbencillin, 30 µg/disk of nalidixic acid, and 30 µg/disk of cephaloridine.

Site Description of Wastewater Bodies for In Vitro Studies

In vitro transfer studies were carried out in wastewater collected from Pipra Ghat and Sahid Smarak stations (Lucknow) during the monsoon season (July–August 2000). The wastewaters were collected in sterile, glass, stoppered sampling bottles. The water samples were filter sterilized before using as a mating medium.

Pipra Ghat

Pipra Ghat is situated downstream from a paper mill and the nala's draining Lucknow domestic sewage. The levels of TOC, chemical oxygen demand (COD), biological oxygen demand (BOD), dissolved solids, and suspended solids were 0.8 mg of C/L, 25.3, 13.2, 228.0, and 8.0 mg/L, respectively. The concentrations of heavy metals detected were 0.08 mg/L of nickel, 0.06 mg/L of manganese, 0.02 mg/L of copper, 0.11 mg/L of zinc, 0.03 mg/L of lead, and 0.09 mg/L of chromium.

Sahid Smarak

Sahid Smarak is 2 km from the station after the river has been polluted by sewage and domestic wastewater from old Lucknow and a little downstream from a brewery effluent discharge. The levels of TOC, COD, BOD, dissolved solids, and suspended solids were 1.3 mg of C/L, 23.6, 12.65,

268.0, and 28.0 mg/L, respectively. The concentrations of heavy metals detected were 0.1 mg/L of nickel, 0.06 mg/L of manganese, 0.03 mg/L of copper, 0.09 mg/L of zinc, 0.05 mg/L of lead, 0.09 mg/L of chromium, and 0.07 mg/L of cadmium.

Sampling and Isolation of E. coli

Samples of tannery effluent were collected from Common Effluent Treatment Plant (Unnao, India) in sterile glass bottles, transported on ice to the laboratory, and processed within 6–8 h of collection. Thermotolerant *E. coli* were isolated from the positive fecal coliform test samples by using the most probable number (MPN) method *(25)*. The MPN test was slightly modified by adding Cr^{6+} (50 μg/mL) for primary isolation of chromium-resistant thermotolerant coliforms. The bacterial isolates were further purified by repeated streaking on several chromium-amended MacConkey agar plates. Organisms were identified according to the modified scheme of Chattopadhyay and Basu *(26)*. Strains were serotyped at the National *Salmonella* and *Escherichia* Research Centre, Kasauli, India.

Minimal Inhibitory Concentration of Chromium

The minimal inhibitory concentration (MIC) of chromium was determined by the agar dilution method *(27,28)*. MacConkey agar plates supplemented with different concentrations of Cr^{6+} (50–250 μg/mL) were inoculated aseptically by exponentially growing culture of bacterial isolates. The plates were incubated for 36–48 h at 37°C; growth of the isolates indicated positive tolerance.

Metal Tolerance and Antibiotic Susceptibility Test

Chromium-resistant thermotolerant *E. coli* were tested for their tolerance to different heavy metals. The freshly grown broth culture of the isolates was inoculated aseptically on MacConkey agar plates supplemented individually with other heavy metals. The metal ion concentration tested ranged from 25 to 200 μg/mL.

Growth Curve Determination

Bacterial growth curves were determined for each species, grown in 100 mL of wastewaters amended with 5% glucose. Inoculated flasks were incubated under shaking conditions (150 rpm for 48 h) at 37°C. The optical density (OD) was measured at 660 nm after every 5-h interval by taking out a 5-mL aliquot aseptically. The absorbance was plotted against the time interval for every individual strain to obtain their growth curves.

Conjugal Transfer Assays

Standard Mating Conditions

The *E. coli* strains were tested for their ability to transfer their resistances to recipient strain *E. coli* K12 J62 (lac^-, pro^-, his^-, trp^-, Nal^r) *(29)*.

Cultures (0.1 mL) grown overnight were inoculated into 10 mL of peptone water (Hi-Media), incubated for 6 h, and then 0.1 mL each of donor and recipient cultures was mixed and incubated for 18 h at 30°C for conjugation. Transconjugants were selected on MacConkey agar plates containing nalidixic acid with appropriate antibiotic/heavy metal by spreading the dilutions of the mixed culture. Dilutions of the mixed culture were also plated on MacConkey agar plates containing an appropriate concentration of the respective antibiotic to enumerate the donors. The rates of plasmid transfer (transfer frequency) were expressed as the number of transconjugants formed per donor:

$$\text{Rate of transfer} = \frac{\text{Number of transconjugants}}{\text{Number of donor}}$$

Modified Mating Conditions

Factors affecting transfer were tested in laboratory simulations. To assess the influence of TOC on R-plasmid transfer, the wastewater of Pipra Ghat and Sahid Smarak was used as a mating medium. In addition, the same study was performed in the wastewaters supplemented with varying concentrations of glucose (2–6%). The amount of TOC in the mating medium was analyzed using a Maihack Defor infrared analyzer (model Tocor 2; Westinghouse, Hamburg, Germany).

The effect of pH on mating was studied in wastewater amended with 3 and 5% of glucose with their pH ranging between 6.5 and 8.0. Similarly, the effect of temperature on transfer frequency using peptone water as the mating medium was studied. The range of temperature assayed was 10–37°C.

Curing of R-plasmid

The loss of R-plasmid was attempted in transconjugants after growth in wastewaters. Curing was done using acridine orange (20 µg/mL). A tube containing 10 mL of wastewater (with added 5% glucose) was supplemented with the curing agent, inoculated with 0.1 mL of overnight broth culture, and incubated at 37°C for 24 h. Appropriate dilutions of the culture were plated on nutrient agar to obtain single-copy isolates after 24 h of incubation. Resulting colonies were tested for loss of plasmid on MacConkey agar plates incorporated with the appropriate antibiotic/metal ion. In the control experiment, peptone water was used for the growth of wild strains and the method just described was followed.

Results

Isolation, Identification, and MIC Determination

Three chromium-resistant thermotolerant coliforms were isolated from the tannery effluent and were identified as *E. coli*. The MIC of Cr^{6+} in strains 1, 2, and 3 were 100, 225, and 200 µg/mL, respectively (Table 1).

Table 1
Resistance Pattern of Chromate-Tolerant Pathogenic *E. coli*

Strain no.	Serotype	Resistance pattern: Cr^{6+} (μg/mL)	Resistance pattern: Other markers
1	04-UPEC	100	Cu^{2+}, Cd^{2+}, As^{3+}, Hg^{2+}, bacitracin, cephaloridine
2	04-UPEC	225	Cu^{2+}, Cd^{2+}, As^{3+}, bacitracin, cotrimazole
3	Untypeable	200	Zn^{2+}, Cu^{2+}, Cd^{2+}, As^{3+}, Ni^{2+}, Co^{2+}, bacitracin

Table 2
TOC Content of Indigenous Pipra Ghat and Sahid Smarak Wastewater and with Varying Concentrations of Glucose (1–6%) Added to It

Sample type	TOC concentration (mg of C/L): Pipra Ghat	TOC concentration (mg of C/L): Sahid Smarak
Indigenous (I)	0.6	1.3
I + 1% glucose	1.2	2.7
I + 2% glucose	9.3	13.59
I + 3% glucose	187.0	264.0
I + 4% glucose	1531	2019.75
I + 5% glucose	4049	4832
I + 6% glucose	8375	10,095

Resistance to Selection Markers and Serotyping of Chromate-Tolerant E. coli

The resistance to different heavy metals and antibiotics among chromate-tolerant *E. coli* is shown in Table 1. The strains were further characterized and grouped under different serotypes (Table 1). The results revealed that strains 1 and 2 belong to serotype 04, which represents uropathogenic: *E. coli* (04-UPEC). Both strains were found resistant to bacitracin. Additionally, strain 1 was resistant to cephaloridine, whereas strain 2 was resistant to cotrimazole. Among heavy metals, both strains were tolerant to Cu^{2+}, Cd^{2+}, and As^{3+}. Strain 1 was also found resistant to mercury. Strain 3 was serologically untypeable. In addition to bacitracin resistance, the organism was resistant to a battery of heavy metals: Cu^{2+}, Cd^{2+}, As^{3+}, Zn^{2+}, Ni^{2+}, and Co^{2+}.

TOC Concentration in Wastewaters

The TOC concentrations in the indigenous Pipra Ghat and Sahid Smarak wastewaters were 0.8 and 1.3 mg of C/L, respectively. The TOC concentration of both the wastewaters amended with varying glucose concentration (1–6% [w/v]) is shown in Table 2. The amount of TOC ranged

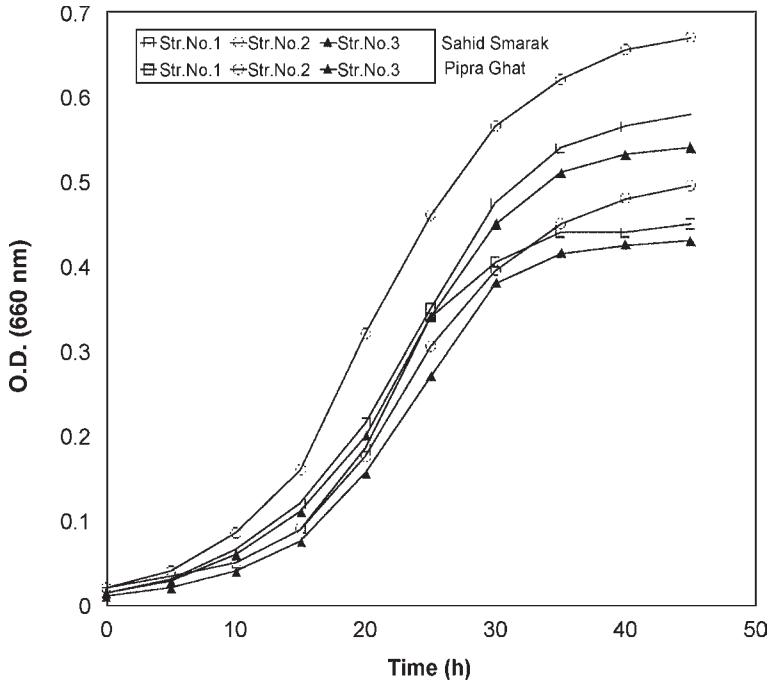

Fig. 1. Survival of chromium-resistant pathogenic *E. coli* in wastewater (TOC: 4049–4832).

from 1.2 to 8375 and 2.7 to 10,095 mg of C/L, respectively, in Pipra Ghat and Sahid Smarak wastewater amended with glucose.

In Vitro Growth of Chromium-Tolerant Pathogenic E. coli *in Wastewater*

Chromate-tolerant pathogenic *E. coli* were studied for their survival and propagation in wastewater collected at Pipra Ghat and Sahid Smarak. Their pattern of growth is given in Fig. 1. The wastewaters were amended with 5% glucose in order to support the growth of the organisms. It was hence noted that the indigenous TOCs of Pipra Ghat and Sahid Smarak wastewaters were 0.8 and 1.3 mg of C/L, respectively (Table 2), which did not support the growth of organisms under study. Overall, in wastewater collected at Sahid Smarak, the growth of test organism was observed at a higher rate compared with the growth in Pipra Ghat wastewater. This very well correlates with the level of TOC because the water samples at Sahid Smarak had almost twice the level of TOC as found in Pipra Ghat wastewater. The results of the study clearly indicated that wastewater supports the growth of chromium-resistant organisms. Among the strains, no. 2 showed a maximum growth compared with the other two strains in wastewater collected from both stations. This clearly indicates that the strains when grown in Sahid Smarak wastewater attained their log phase after 8–10 h of growth and was prolonged up to 35 h, whereas Pipra Ghat wastewa-

Table 3
Influence of TOC on Plasmid Transfer in Pipra Ghat Wastewater

Resistance marker tested	Transfer frequency of resistance markers in varying TOC content (mg of C/L)				
	9.3	187.0	1531	4049	8375
Strain no. 1					
Cr^{6+}	3.0×10^{-2}	7.56×10^{-2}	12.29×10^{-2}	19.37×10^{-2}	31.12×10^{-2}
Cu^{2+}	1.2×10^{-2}	4×10^{-2}	9.67×10^{-2}	15.0×10^{-2}	26.17×10^{-2}
As^{3+}	2.5×10^{-2}	5.18×10^{-2}	11×10^{-2}	17.88×10^{-2}	32.5×10^{-2}
Cephaloridine	3.1×10^{-2}	7.69×10^{-2}	14.8×10^{-2}	23.12×10^{-2}	38×10^{-2}
Bacitracin	2.9×10^{-2}	6.05×10^{-2}	14×10^{-2}	20.67×10^{-2}	30.95×10^{-2}
Strain no. 2					
Cr^{6+}	3.8×10^{-2}	7.92×10^{-2}	12.36×10^{-2}	21.0×10^{-2}	39.06×10^{-2}
Cu^{2+}	1.52×10^{-2}	3.75×10^{-2}	8.91×10^{-2}	15.7×10^{-2}	27.17×10^{-2}
Cd^{2+}	1.48×10^{-2}	3.5×10^{-2}	7.0×10^{-2}	12.37×10^{-2}	21.75×10^{-2}
Bacitracin	2.0×10^{-2}	5.65×10^{-2}	10.84×10^{-2}	17.12×10^{-2}	30.0×10^{-2}
Strain no. 3					
Cr^{6+}	2.5×10^{-2}	5.39×10^{-2}	9.95×10^{-2}	15.0×10^{-2}	24.9×10^{-2}
Zn^{2+}	1.8×10^{-2}	4.14×10^{-2}	11.2×10^{-2}	19×10^{-2}	31.0×10^{-2}
Cu^{2+}	1.2×10^{-2}	2.3×10^{-2}	4.87×10^{-2}	7.92×10^{-2}	14.09×10^{-2}
Cd^{2+}	2.1×10^{-2}	4.6×10^{-2}	8.79×10^{-2}	11.57×10^{-2}	21×10^{-2}
Ni^{2+}	1.15×10^{-2}	3.0×10^{-2}	6.95×10^{-2}	9.83×10^{-2}	19.20×10^{-2}
Bacitracin	1.48×10^{-2}	3.95×10^{-2}	6.12×10^{-2}	8.96×10^{-2}	17.4×10^{-2}

ter the log phase appeared after 15 h of growth and prolonged up to 30 h except for strain 2, which reached stationary phase after 35 h. A markedly longer lag phase, reduced growth rate, and lower cell density were observed for all three strains when grown in Pipra Ghat wastewater as compared with Sahid Smarak wastewater. In strain 2, the lag phase was shortest, whereas in strain 3 an extended lag phase was observed for both Pipra Ghat and Sahid Smarak samples.

Influence of TOC on R-Plasmid Transfer

The results corresponding to the influence of TOC concentration in the mating medium on the transfer frequencies are shown in Tables 3 and 4. The TOC concentrations in Pipra Ghat and Sahid Smarak samples when used as a mating medium descended from 8375 to 1.2 and 10,095 to 2.7 mg of C/L, respectively. In general, a gradual decrease was observed both in the number of transconjugants and in transfer frequencies as the concentration of TOC in the mating medium decreased, obtaining maximum values with TOC concentrations of 8375 and 10,095 mg of C/L for Pipra Ghat and Sahid Smarak samples, respectively. There was less variation in the transfer frequency calculated when the TOC concentration in the mating medium varied from 4049 to 1.2 and 4832 to 2.7 mg of C/L; the values in this range of TOC concentration were comparatively lower than those obtained

Table 4
Influence of TOC on Plasmid Transfer in Sahid Smarak Wastewater

Resistance marker tested	Transfer frequency of resistance markers in varying TOC content (mg of C/L)				
	13.59	264.0	2019.75	4832.0	10,095
Strain no. 1					
Cr^{6+}	3.85×10^{-2}	7.75×10^{-2}	13.36×10^{-2}	25.12×10^{-2}	40.0×10^{-2}
Cu^{2+}	1.52×10^{-2}	3.18×10^{-2}	8.56×10^{-2}	19.5×10^{-2}	34.5×10^{-2}
As^{3+}	3.1×10^{-2}	8.67×10^{-2}	14.09×10^{-2}	25.89×10^{-2}	43.02×10^{-2}
Cephaloridine	4.45×10^{-2}	9.97×10^{-2}	20.63×10^{-2}	29.0×10^{-2}	42.0×10^{-2}
Bacitracin	3.46×10^{-2}	9.0×10^{-2}	20.0×10^{-2}	31.95×10^{-2}	45.44×10^{-2}
Strain no. 2					
Cr^{6+}	4.26×10^{-2}	9.3×10^{-2}	17.8×10^{-2}	28.09×10^{-2}	45.12×10^{-2}
Cu^{2+}	2.0×10^{-2}	6.95×10^{-2}	11.83×10^{-2}	20.47×10^{-2}	39.31×10^{-2}
Cd^{2+}	3.1×10^{-2}	7.29×10^{-2}	13.78×10^{-2}	29.14×10^{-2}	48.07×10^{-2}
Bacitracin	2.48×10^{-2}	7.06×10^{-2}	12.16×10^{-2}	25.0×10^{-2}	38.63×10^{-2}
Strain no. 3					
Cr^{6+}	2.89×10^{-2}	6.76×10^{-2}	15.0×10^{-2}	23.17×10^{-2}	38.95×10^{-2}
Zn^{2+}	1.96×10^{-2}	4.29×10^{-2}	9.41×10^{-2}	16.0×10^{-2}	31.0×10^{-2}
Cu^{2+}	1.0×10^{-2}	2.07×10^{-2}	6.45×10^{-2}	12.37×10^{-2}	26.19×10^{-2}
Cd^{2+}	2.5×10^{-2}	5.2×10^{-2}	9.95×10^{-2}	15.4×10^{-2}	27.97×10^{-2}
Ni^{2+}	1.77×10^{-2}	4.0×10^{-2}	8.72×10^{-2}	13.06×10^{-2}	21.75×10^{-2}
Bacitracin	2.02×10^{-2}	4.63×10^{-2}	9.76×10^{-2}	14.82×10^{-2}	23.0×10^{-2}

when the TOC concentrations were 8375 and 10,095 mg of C/L for Pipra Ghat and Sahid Smarak samples, respectively.

In transconjugants of strain 1, resistance to chromium was increased from 3×10^{-2} to 31.12×10^{-2} in Pipra Ghat wastewater with a gradual increase in TOC concentration from 9.3 to 8375 mg of C/L. A similar trend was observed for Sahid Smarak wastewater. In all the three strains studied, a transfer trend of resistance to copper and bacitracin was found similar to chromium. In strains 2 and 3, the transfer of resistance to cadmium was also detected. Additionally, in strain 3, resistance to zinc and nickel was transferred. Resistance to arsenic and cephaloridine was transferred in strain 1.

Influence of pH on R-Plasmid Transfer

The pH values assayed were 6.5, 7.0, 7.5, and 8.0. When mating was carried out in the varying concentrations of TOC, the variation in pH gave rise to no significant difference in the transfer frequency values for different resistance markers (Tables 5 and 6). In other words, pH was not found to affect the transfer of R-plasmid in wastewaters.

Loss of R-Plasmid in Transconjugants

The loss of R-plasmid in transconjugants after growth in wastewater was studied in all three strains. The comparison of R-plasmid curing in

Table 5
Influence of pH on Plasmid Transfer in Pipra Ghat Wastewater

Resistance marker tested	Transfer frequency (10^{-2}) in varying TOC content (mg of C/L)							
	187.0				4049			
	pH 6.5	7.0	7.5	8.0	pH 6.5	7.0	7.5	8.0
Strain no. 1								
Cr^{6+}	7.29	7.42	7.8	8.0	19.21	18.5	19.17	18.63
Cu^{2+}	4.14	3.5	3.95	4.06	14.8	15.22	14.09	15.57
As^{3+}	5.79	6.24	6.87	6.48	16.95	16.83	17.31	17.74
Cephaloridine	8.41	7.0	8.75	8.91	22.83	24.2	23.87	24.0
Bacitracin	6.95	7.98	7.36	6.65	21.0	22.15	21.75	21.83
Strain no. 2								
Cr^{6+}	6.95	7.92	7.83	6.18	20.0	21.75	21.95	20.02
Cu^{2+}	3.87	4.18	4.92	4.67	18.0	17.09	18.88	18.20
Cd^{2+}	4.65	5.48	5.37	4.0	15.8	14.69	14.5	15.12
Bacitracin	6.95	5.9	5.57	5.29	16.14	16.8	17.91	17.88
Strain no. 3								
Cr^{6+}	4.81	5.79	5.73	4.11	17.88	17.37	16.7	17.0
Zn^{2+}	4.69	4.71	3.0	3.91	19.30	20.0	19.06	20.47
Cu^{2+}	3.75	3.1	3.8	3.95	6.87	6.95	7.90	7.56
Cd^{2+}	5.28	4.81	4.39	4.87	11.0	11.24	12.31	11.5
Ni^{2+}	2.9	2.37	2.11	3.0	9.68	10.37	10.88	9.94
Bacitracin	4.18	4.77	4.0	3.25	9.54	8.71	9.92	9.0

Table 6
Influence of pH on Plasmid Transfer in Sahid Smarak Wastewater

Resistance marker tested	Transfer frequency (10^{-2}) in varying TOC content (mg of C/L)							
	264.0				4832			
	pH 6.5	7.0	7.5	8.0	pH 6.5	7.0	7.5	8.0
Strain no. 1								
Cr^{6+}	8.56	8.0	8.92	7.99	26.19	26.75	26.97	26.28
Cu^{2+}	2.21	2.96	3.48	2.02	18.0	18.37	19.42	18.72
As^{3+}	8.97	7.73	8.24	8.16	23.55	23.71	23.2	24.11
Cephaloridine	9.68	9.45	9.0	9.52	27.97	27.14	28.0	27.26
Bacitracin	8.63	7.29	7.74	8.0	29.31	29.85	29.26	29.52
Strain no. 2								
Cr^{6+}	9.47	10.82	9.07	9.63	27.97	27.55	27.02	27.8
Cu^{2+}	5.48	5.26	5.22	6.61	21.48	21.75	21.99	21.0
Cd^{2+}	7.26	6.45	7.89	7.68	28.41	28.72	29.75	28.14
Bacitracin	6.44	6.02	6.96	6.31	26.17	26.4	26.0	26.98
Strain no. 3								
Cr^{6+}	6.43	5.95	5.06	5.27	23.12	23.63	23.0	22.95
Zn^{2+}	4.25	3.46	3.78	3.77	18.36	18.5	17.89	17.90
Cu^{2+}	2.48	1.52	2.0	1.98	10.97	11.16	10.54	11.45
Cd^{2+}	4.16	4.29	5.0	4.0	16.43	15.72	16.5	15.83
Ni^{2+}	5.18	5.32	6.03	6.0	13.11	13.78	13.44	13.0
Bacitracin	5.26	5.95	5.89	5.21	15.63	14.16	15.25	15.79

Table 7
Loss of R-Plasmid in Transconjugants After In Vitro Survival in Wastewater

	Curing (%)								
	1			2			3		
Marker tested	Wild	Pipra Ghat	Sahid Smarak	Wild	Pipra Ghat	Sahid Smarak	Wild	Pipra Ghat	Sahid Smarak
Cr^{6+}	87.09	92.47	91.57	91.57	100	89.17	89.13	94.73	84.50
Zn^{2+}	—	—	—	—	—	—	13.04	75.0	34.40
Cu^{2+}	34.40	84.61	81.05	Not cured	Not cured	Not cured	80.60	64.28	87.55
Ni^{2+}	—	—	—	—	—	—	85.86	72.72	94.73
Cd^{2+}	—	—	—	83.15	86.20	97.84	Not cured	Not cured	Not cured
As^{3+}	Not cured	Not cured	Not cured	—	—	—	—	—	—
Bacitracin	88.17	97.55	93.47	87.36	93.10	96.77	92.39	95.78	90.32
Cephaloridine	83.87	96.73	89.36	—	—	—	—	—	—

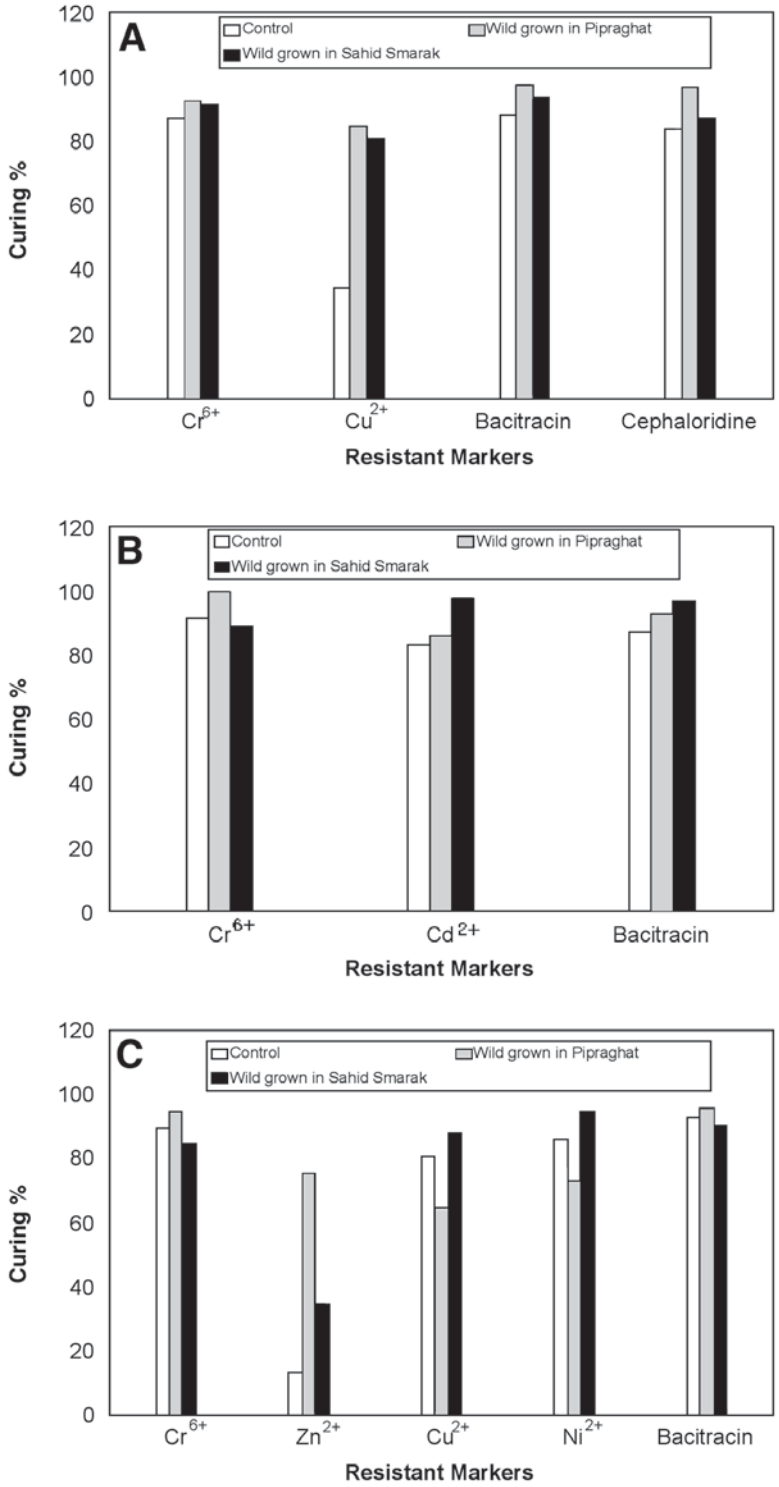

Fig. 2. Comparison of curing of R-plasmid in transconjugant of strain no. 1 **(A)**, no. 2 **(B)**, and no. 3 **(C)**.

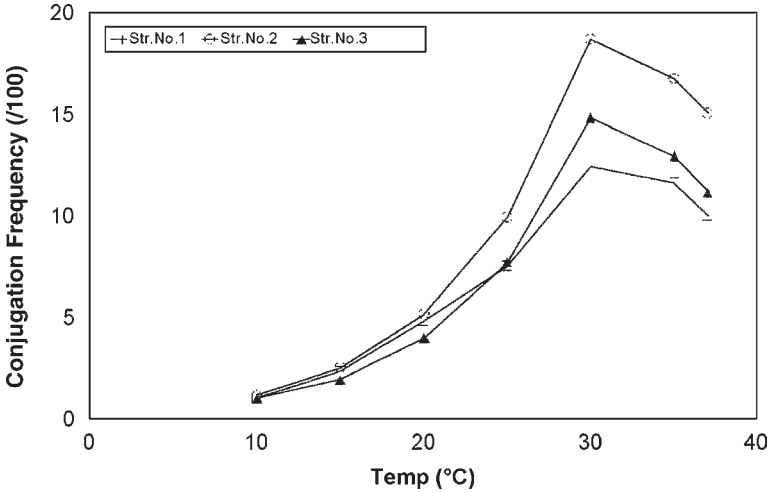

Fig. 3. Influence of mating temperature on R-plasmid transfer.

transconjugants regarding loss of resistance to chromium and other selection markers is depicted in Table 7 and Fig. 2.

Loss of Resistance to Chromium

In strain 2, 100% loss of chromium resistance was observed when the cells were grown in Pipra Ghat wastewater. In strains 1 and 3, chromium resistance was cured in 92.47 and 94.73% cells, respectively, when grown in Pipra Ghat wastewater as compared with Sahid Smarak wastewater.

Loss of Resistance to Other Selection Markers

In strain 1, loss of resistance to copper, bacitracin, and cephaloridine was higher when the transconjugants were grown in wastewater of Pipra Ghat as compared to the cells grown in Sahid Smarak wastewater. In strain 2, loss of resistance to cadmium and bacitracin was observed when the transconjugants were grown in Sahid Smarak wastewater, but no significant difference was observed in curing when compared with that of cells grown in the Pipra Ghat sample. In strain 3, loss of resistance to zinc was observed in a higher number of transconjugants when grown in wastewater of Pipra Ghat as compared with the Sahid Smarak sample. Copper and nickel resistance was cured in 87.55 and 94.73% colonies of thermotolerant coliforms, respectively, when grown in Sahid Smarak wastewater, which is higher than that of Pipra Ghat wastewater. No significant difference was observed in loss of resistance to bacitracin in both the wastewater samples.

Influence of Mating Temperature on Plasmid Transfer

The results corresponding to the effect of temperature on transfer frequency using peptone water as the mating medium are depicted in Fig. 3.

The range of temperature assayed was 10–37°C. The number of transconjugants remained almost constant at 10 and 15°C; thereafter, it increased progressively, reaching a maximum at 30°C. At an incubation temperature >30°C, a gradual decrease in transfer frequency was observed, indicating that the optimum temperature for transfer of chromium resistance was 30°C.

Discussion

The results clearly suggest the profound influence of environmental factors such as level of nutrients and temperature on transfer of R-plasmid in the environment, especially in wastewater. The experiments conducted to determine the effect of carbon availability (TOC) on transfer of R-plasmid indicate that plasmid transfer is greatly influenced. A gradual increase in transfer frequency of chromium was observed with increasing carbon concentration in wastewater. Other investigators have also reported a close correlation between the transfer and availability of nutrients, with frequency increasing along with the nutrient concentration in the medium *(8)*. It was found that plasmid transfer in stream water occurs only when it is amended with dilute nutrient broth *(30)*. Ganguli and Tripathi *(31)* reported that supplementation with a carbon source supported bacterial multiplication in industrial wastewaters. Sandt and Herson *(15)* also found that a relatively large increase in the levels of nutrients in the environment enhanced the frequency of plasmid mobilization. Contrary results were also observed for organisms attached to the surface clearly differentiating carbon incorporation between attached and free-living organisms *(32,33)*. The results suggest that transfer of R-factors was not of a comparable trend for adhered and free-living organisms. This also supports the view of Rochelle et al. *(23)* that physiology is an important determinant in transfer of R-plasmid.

The effect of pH and temperature on plasmid transfer has also been studied by several researchers. The results of our study demonstrated the transfer of R-plasmid at a pH range varying from 6.5 to 8.0; however, variations in pH did not significantly affect the frequency of transfer of R-plasmids. Similar results were also observed by others *(6)*. Several other researchers found maximum transfer frequencies at pH values close to neutral, while acidic pH values were reported to affect the process negatively *(34–36)*. However, contrary to this, it is reported that there is no effect on transfer within a pH range from 5.0 to 8.0 at 25°C *(23)*. Harada and Mitsuhashi *(37)*, while studying the transfer kinetics of *Escherichia-Shigella* systems, showed temperature and pH to be the primary abiotic factors controlling in vitro plasmid transfer. They found that a temperature range of 25–45°C (37°C optimum) and pH range of 5.0–9.0 (7.5 optimum) supported plasmid transfer. With respect to the influence of mating temperature on plasmid transfer, it was noted from the results that both the number of transconjugants and the transfer frequency were found maximum at 30°C, which descended with a decrease in temperature. It is reported that

the transfer of plasmid R1 drd-19 between laboratory strains of *E. coli* was maximum at 37°C, with transfer frequencies decreasing directly with temperature *(8,11)*. Many researchers found that low temperature has a negative effect on transfer exhibiting maximum conjugation frequencies in the range of 20–30°C *(20,21,23,36,38)*. The results of our study also indicate a negative effect of temperature on plasmid transfer since the frequencies detected at low temperatures (10 and 15°C) were significantly lower than those observed at higher temperatures (20, 25, and 30°C). Our results support the observations of other researchers who reported that maximum transfer of R-plasmid (conjugation) takes place at temperatures higher than would normally be expected in temperate aquatic environments *(8)*.

The tannery strains carrying R-plasmid seem to survive well in wastewaters, thus suggesting that these plasmids could provide a reservoir of genes that may be involved in adaptation mechanisms or simply can be advantageous to the survival of bacteria. In addition, in vitro studies revealed the high stability of plasmids in the hosts employed. These plasmids might be transferred to a wide host range, enhancing their environmental dissemination. The conjugative plasmids contain more information than just the resistance markers. Because some plasmids can contribute directly to virulence *(39)* and thermotolerant *E. coli* is a widely distributed potential pathogen, whose pathogenic mechanisms have not been completely elucidated, the high incidence of R-factors among these bacteria may represent a serious health hazard. We conclude that plasmid transfer by conjugation occurs in wastewater bodies within a wide range of environmental as well as nutritional conditions, and, thus, a large quantum of contaminated tannery effluents reaching the surface and eventually groundwater sources may further aggravate the situation.

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