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Plasmid Mediated Chromate Resistance in Bacteria Isolated from Industrial Waste

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Abstract: In order to study the genetic basis and mechanism of chromate resistance in chromate resistant bacteria, conjugal transfer as well as curing of plasmids in these bacteria and its effect on chromium uptake are being investigated. From the effluent of Shafiq Tannery, Kasur, Pakistan, four bacterial strains STCr-1, STCr-2, STCr-3 and STCr-4 which could endure 40 mg mL^{-1} of potassium chromate in nutrient agar medium were isolated. All of them were gram negative, aerobic and motile rods. One strain STCr-1 was identified as *Ochrobactrum* species by 16S rRNA gene sequence homology. Each strain harboured a single conjugative plasmid, which conferred resistance to chromate. Maximum plasmid transfer was recorded after 24 h of mating except for plasmid residing in STCr-3 (pSH1322), which transferred maximally after 16 h of mating. The plasmids harboring STCr-1 (pSH1320), STCr-3 (pSH1322) and STCr-4 (pSH1323) manifested highest transfer frequency at donor:recipient ratio 1, while plasmid resident of STCr-2 (pSH1321) preferred donor:recipient ratio 5. Transfer frequency of plasmids pSH1320 and pSH1322 was maximal at 28°C and that of pSH1321 and pSH1323 at 37°C. Optimum pH for plasmid transfer was 8 for these plasmids except pSH1320, which opted for pH 6 or 7. Curing of chromate resistant plasmids from these strains was achieved with SDS at high temperature and curing of plasmid was associated with the loss of chromate resistance phenotype. A comparison of Cr uptake by the parental strains and their cured derivatives revealed that plasmids in these strains express high level resistance to chromate by exerting stringent control on the accumulation/uptake of Cr.

Key words: Chromate-resistance, curing, conjugation, Cr-uptake

INTRODUCTION

The extensive industrial usage of chromium compounds and subsequent release of effluents in the environment contaminated the ecosystem. The chromium discharge from industries like metal finishing industry, petroleum refining, leather tanning, iron and steel industries, textile manufacturing and paper industry elevated its concentration in aquifers and ground water. The discharged effluents from these industries have been found to contain high concentrations of this metal[1]. In spite of its crucial role in biological life, above critical level chromium is known to have toxic, mutagenic, carcinogenic and genotoxic effects[2,3]. Biotoxic effects of chromium are valence dependent. Hexavalent chromium is highly soluble and carcinogenic whereas the trivalent form is less soluble and less toxic. Phytotoxic effects manifested by increased concentration of Cr, are associated with increased uptake and bioaccumulation of chromium, which affect animals and humans via food chain.

It is not only exigent to extract the toxic chromium from effluents before discharging in the environment but also to detoxify the contaminated lands and aquifers *in situ*/*ex situ*. The routine methods for treatment of chromium pollution generally involve the chemical reduction of Cr^{6+} to Cr^{3+} and subsequent precipitation of less soluble Cr^{3+} at or near neutral pH. These require high inputs of energy or expensive chemicals. Hence more practical and economical methods are being explored[4]. Bacterial potential for enzymatic reduction of Cr^{6+} to Cr^{3+}[5-8] offer an alternative candidate for treatment of contaminated sources. The isolation of Cr-resistant bacteria and evaluation of their Cr-detoxification/removal potentials are primary steps in developing bioremediation processes. Isolation of chromium resistant bacteria has been reported by many workers[6,8,9]. Chromate resistance in bacteria is either chromosomal or plasmid determined[10,11]. Plasmid borne chromate resistance has been found in many bacteria[12-14] which result from decreased net accumulation of chromium by the resistant cells[10,13].

Attempts are being made for the isolation of chromate resistant bacteria from industrial waste. In order to study the genetic basis and mechanism of chromate resistance

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in these bacteria, conjugal transfers as well as curing of plasmids in these bacteria and its effect on chromium uptake are being investigated.

MATERIALS AND METHODS

Isolation and characterization of chromate resistant bacteria: An effluent sample was collected from Shafiq Tannery, Kasur, Pakistan, in a sterile screw capped glass bottle. Chromate resistant bacteria were isolated on nutrient agar[15] plates containing 100 µg mL^{-1} of potassium chromate at 37°C. The chromate resistant isolates were purified and then exposed gradually to higher levels of chromate salt. Four bacterial strains STCr-1, STCr-2, STCr-3 and STCr-4 which could endure 40 mg mL^{-1} of potassium chromate in nutrient agar were subjected to morphological and biochemical characterization[15]. The taxonomic identity of the strain STCr-1 was ascertained by 16S rRNA gene sequencing. A part of the 16S rRNA gene was amplified and the extension product was then sequenced on an automated DNA sequencer (Applied Biosystems) and the data were compared with known sequences using BLAST. The bacterial strains were also checked for resistance against the various metallic salts (100 µg mL^{-1} of $BaCl_2$, $CdCl_2$, $CoCl_2$, $CuSO_4$, $FeCl_3$, $HgCl_2$, $MnSO_4$, $NiCl_2$, $Pb(NO_3)_2$ and $ZnSO_4$) and antibiotics (Ampicillin, Ap -300 µg mL^{-1}, Cefradine, Cdn-100 µg mL^{-1}, Cefadroxil, Cdx -100 µg mL^{-1}, Chloramphenicol, Cm -5 µg mL^{-1}, Cyprofloxacin, Cf -100 µg mL^{-1}, Doxycilline hyclate, Dc -100 µg mL^{-1}, kanamycin, Km -50 µg mL^{-1}, Streptomycin, Sm -500 µg mL^{-1} and tetracycline, Tc -20 µg mL^{-1}).

Plasmid screening, conjugation and transformation: The ability of a characteristic to be transferred from one bacterium to another provides a good presumptive evidence of plasmid involvement and can be used as a mean of identifying a plasmid conferred property[16]. To probe the possibility of presence of chromium resistance marker on the plasmids residing in these strains and also to explore the ability of these plasmids to mobilize their own transfer, conjugation experiments were performed by using *E. coli* strain as recipient. The plasmid harbouring property of these strains was ascertained by gel electrophoresis of the total cell lysate[17] and conjugation experiments were performed by using broth mating technique[18]. Selection of transconjugants was made on double selective plates (1000 µg mL^{-1} Sm +20 mg mL^{-1} K_2CrO_4) at 37°C after 2-3 days of incubation. *E. coli* K12 strain CSR603 (*recA1 phr-1* derivative of AB1886 (*thr-1 leu-6 lacY1 galK2 ara-14 xyl-5 mtl-1 proA2 his-2 str-31 tsx-33 sup-37 uvxA6*; source D. Rupp)) was used as recipient in all conjugation experiments. Both donor as well as recipient strains alone were also plated on double selective plates and incubated to serve as control. The effects of some environmental factors (mating time, donor:recipient ratios, temperature and pH) on the transfer frequencies of the plasmids were also investigated[19,20]. Transfer frequency was determined as the number of transconjugants obtained per initial number of recipients. Initial recipient density was calculated by plating dilutions of recipient on Sm supplemented plates. All transfer frequencies were transformed to logarithmic values. The plasmids were isolated by the method of Brinboim and Doly[21]. Transformation experiments were performed as described by Thomas[22] by using *E. coli* K12 strain MV10 (C600 Δ *trpE5* (*thr-1 leu-6 thi-1 lacY1 supE44 tonA21 trpE5*); source D. R. Helinski) as host. The selection of the transformants was made on LB-agar plates[15] supplemented with 1 mg and 10 mg chromate salt mL^{-1} at 37°C.

Curing of plasmids: For the curing of chromate resistant plasmids from these bacterial strains, four curing agents, i.e., high temperature (45°C), ethidium bromide, sodium dodecyl sulphate and trimethoprim[15,16] were used. The chromate sensitive colonies obtained were checked for the loss of plasmid by total cell lysate method[17].

Determination of chromium: Chromium uptake by growing cells was determined at 20 µg mL^{-1} of chromate by the parental strains and their cured derivatives. The cells were grown in nutrient broth supplemented with desired concentration of chromate at 37°C with 150 rpm. After 24 h the bacterial cells were collected, washed and then total Cr was determined by digesting samples and reoxidising any Cr^{3+} to Cr^{6+} by treating with $KMnO_4$ followed by spectrophotometric determination for Cr^{6+} by diphenylcarbazide method[23] in bacterial cells and cell wash water.

RESULTS AND DISCUSSION

Tanneries are one of those industries, which are blamed for environmental pollution[1,24]. The most important component of pollutants from tanneries is Cr and according to Khalil *et al.*[25] effluents only from Kasur, Pakistan, includes about 300 kg day^{-1} of Cr, which led to the deterioration of aquifers and irrigated land. Hence it is imperative to extract/detoxify Cr before the discharge of effluents. Bacterial interaction with metals offer an alternative candidate for detoxification of heavy metals and metal resistant bacteria can be utilized for this purpose. Four bacterial strains STCr-1, STCr-2, STCr-3

and STCr-4 exhibiting a very high level of resistance to chromate i.e., 40 mg mL^{-1} of potassium chromate in nutrient agar were isolated from the effluents of Shafiq Tannery, Kasur, Pakistan. This resistance level is much higher than for the strains reported by other workers[6-9]. All the strains were gram-ve motile aerobic rods and possessed cytochrome oxidase and catalase enzymes. They showed positive results for ONPG (weak positive) and gelatin hydrolysis. All of them gave negative results for spore formation, methyl red, denitrification, sodium citrate, sodium malonate, arginine dihydrolase, ornithine decarboxylase, H_2S production, urea hydrolysis, tryptophan deaminase, indole and acetoin tests. They were able to produce acid from rhamnose but were unable to produce acid from glucose, maltose, sucrose, mannitol, sorbitol and inositol. They, however, showed variable results for nitrate reduction, starch hydrolysis, lysine decarboxylase and acid from arabinose. Nitrate was not reduced by STCr-2 while starch could not be hydrolysed by STCr-1. Positive test for lysine decarboxylase was shown only by STCr-4 and acid from arabinose was produced (weakly) by STCr-3 and STCr-4. Based on the 16S rRNA gene sequence homology, the strain STCr-1 showed 98% identity with an *Ochrobactrum* sp. Chromate resistant *Ochrobactrum* strains have been reported previously but they are resistant to quite lower concentration of chromate[26-27].

Gel electrophoresis screening of total cell lysates revealed that in each strain a single plasmid was present. Plasmids residing in STCr-1, STCr-2, STCr-3 and STCr-4 were respectively designated as pSH1320, pSH1321, pSH1322 and pSH1323. A number of transconjugants were scored clearly demonstrating that plasmids residing in STCr-1 (pSH1320), STCr-2 (pSH1321), STCr-3 (pSH1322) and STCr-4 (pSH1323) confer resistance to Cr and are also transferable. In all the cases the transconjugants were able to grow in medium containing 40 mg mL^{-1} of chromate salt. The genotype of the transconjugants was checked which confirmed that they were recipient strains containing the donor plasmids. Plasmid transfer in nature could bring about exchange of DNA cassettes even among unrelated bacterial population, which might lead to speedy evolution of bacteria in nature. Horizontal dissemination of genes conferring resistance to toxic pollutants may play an important role in the adaptation of bacteria to toxic contaminants in the environment. Both biotic and abiotic factors affect plasmid transfer, hence effects of different factors on plasmid transfer were investigated. In three cases (pSH1320, pSH1321, pSH1323) plasmid transfer could be accomplished within 2 h of mating, whereas plasmid pSH1322 required 16 h of mating for transfer (Fig. 1a). Maximal plasmid transfer was after 16 (pSH1322) or 24 h (pSH1320, pSH1321, pSH1323) of mating. Our previous work with other chromium resistant bacterial strains (isolated from effluent of paint industry) exhibited maximum plasmid transfer from 4-8 h of mating requiring at least two hours of mating for plasmid transfer[20]. Another strain from effluent of tanneries needed 16 h of mating for plasmid transfer and 24 h for maximal transfer[28]. It seems that bacteria from common source have some common attribute for their plasmid transfer. Donor to recipient ratio 1 (pSH1320, pSH1322, pSH1323) or 5 (pSH1321) yielded maximal plasmid transfer (Fig. 1b) and with the increase in donor per recipient cell decrease in transfer frequency was observed. Donor to recipient ratio 1[28,29], 0.4-30[30], 1-4[20], 10[29] for maximal plasmid transfer has been reported by various workers. Temperature and pH are important environmental factors which in addition to affecting the bacterial growth, also affect plasmid transfer[19,30]. The highest transfer frequency of plasmids pSH1320 and pSH1322 was observed at 28°C while the other two plasmids transferred maximally at 37°C (Fig. 2a). No transconjugants were scored at 45°C. Many metal resistant plasmids transfer between 20°C and 37°C[19,20,28,30]. The optimum pH for plasmid transfer varies between pH 6.5 and 8.5[19,30] hence

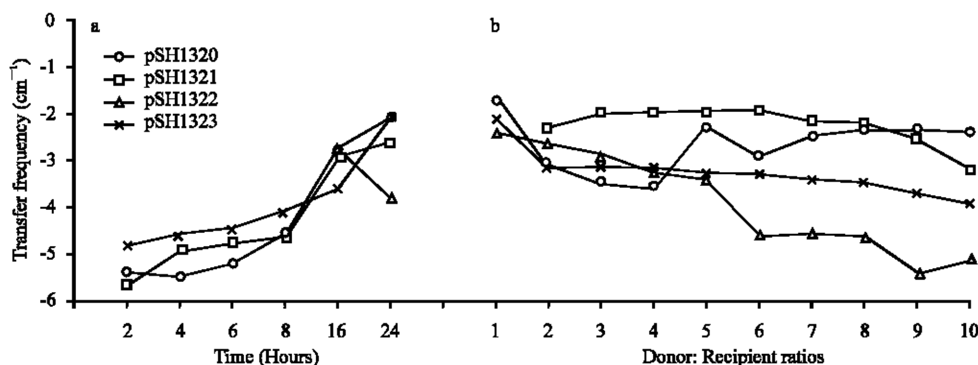

Fig. 1: Effect of (a) varying mating time and (b) donor:recipient ratios on transfer frequencies of plasmids residing in chromate resistant bacteria to *E. coli*.

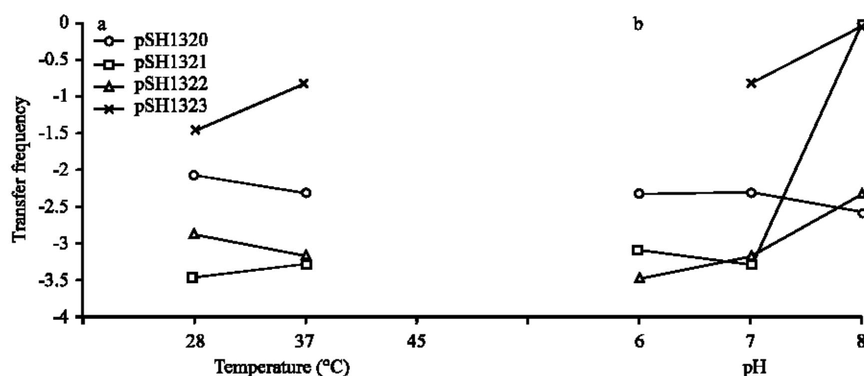

Fig. 2: Effect of (a) varying temperatures, (b) varying pHs on transfer frequencies of plasmids residing in chromate resistant bacteria to *E. coli*.

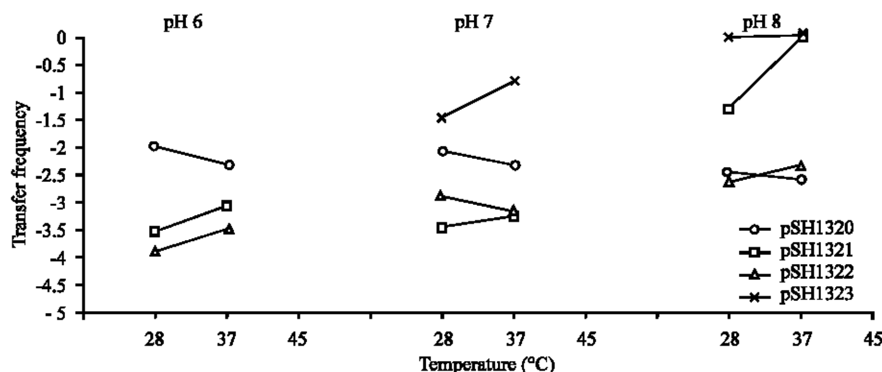

Fig. 3: Effect of varying temperatures in combination with pHs on transfer frequencies of plasmids residing in chromate resistant bacteria to *E. coli*.

we used pH 6, 7 and 8 for mating. The plasmid pSH1320 transferred maximally at pH 6 or 7 while the three remaining plasmids transferred maximally at pH 8 (Fig. 2b). Plasmids pSH1320, pSH1321 and pSH1322 could transfer at all three pHs, but pSH1323 did not yield any transconjugants at acidic pH (pH 6) (Fig. 2b). pH along with temperature had synergistic effects on plasmid transfer (Fig. 3). Plasmids pSH1321, pSH1322 and pSH1323 preferred 37°C and alkaline pH (pH 8) for their maximal transfer whereas for plasmid pSH1320 highest transfer frequency was observed at 28°C with acidic to neutral pH. Acidic pH (pH 6) inhibited the transfer of pSH1323 at all temperatures and high temperature (45°C) hindered the transfer of all plasmids at all pHs (Fig. 3). Synergistic effects of pH and temperature on plasmid transfer have been reported by other workers[19,30]. The pH and temperature optima of three plasmids (pSH1321, pSH1322, pSH1323) preferred alkaline pH at 37°C for their transfer which is comparable with growth conditions of bacterial strains harboring these plasmids. This specific study on other Cr-resistant strains also demonstrates that optimum pH and temperature for plasmid transfer is related with pH and temperature optima for bacterial growth[20].

Transformation experiments were also performed to characterize the plasmids residing in these Cr-resistant strains. But no transformants were observed on plates supplemented by any of the chromate concentrations. Transformation experiments were repeated three times but were not successful. The Cr-resistant plasmid pLHB1 from *P. fluorescens* also could not be transformed to strains of *E. coli* and *P. putida*[31]. Negative results in transformation might be either due to the failure of the plasmid DNA to enter into the recipient cells or due to the failure of the plasmid to replicate in the recipient cells[32].

The curing of a plasmid from a bacterial culture is the best way to substantiate the relationship between a genetic trait and carriage of the specific plasmid by the culture[15]. High temperature (45°C), ethidium bromide (0.05 to 0.2 mg mL^{-1}), sodium dodecyl sulphate (0.1 to 2%) and trimethoprim (0.02 to 0.20 mg mL^{-1}) were used to cure chromate resistant plasmids from the chromate resistant strains but not even a single chromate sensitive derivative was obtained in any case. Then a loopful of bacterial growth was resuspended with 100 μL of SDS (4.5%) and incubated at 45°C. After 15 min the contents were plated onto simple nutrient agar plates. The bacterial colonies

Table 1: Comparison of metal and antibiotic resistance pattern of chromate resistant parental strains and their cured derivatives

	Resistances of		Resistances lost with curing
	Parental strains	Cured derivatives	
STC-1	Ba, Co, Cu, Fe, Mn, Ni, Pb, Zn, Cdn, Cdx, Cm, Km.	Ba, Cu, Fe, Mn, Ni, Pb,	Co, Zn, Cdn, Cdx, Cm, Km
STC-2	Ba, Co, Cu, Fe, Mn, Ni, Pb, Zn, Ap, Cdn, Cdx, Km.	Ba, Cu, Fe, Mn, Ni, Pb,	Co, Zn, Ap, Cdn, Cdx, Km.
STC-3	Ba, Co, Cu, Fe, Mn, Ni, Pb, Zn, Cdn, Cdx, Cm, Km, Tc	Ba, Cu, Fe, Mn, Ni, Pb,	Co, Zn, Cdn, Cdx, Cm, Km, Tc
STC-4	Ba, Co, Cu, Fe, Mn, Ni, Pb, Zn, Cdn, Cdx, Cm, Km, Tc	Ba, Cu, Fe, Mn, Ni, Pb,	Co, Zn, Cdn, Cdx, Cm, Km, Tc

Table 2: Chromium uptake as accumulated, loosely bound and total Cr sorption by different chromate resistant strains and their cured derivatives after growth in nutrient broth containing 20 μg mL^{-1} of chromate salt for 24 h at 37°C

	Chromium uptake (μg chromium g^{-1} fresh weight)		
Strains	Accumulated	Loosely bound	Total sorption
STCr-1	31.78±1.37	14.75±0.72	46.53±4.01
STCr-1-cured	144.76±7.92	120.47±6.48	265.23±15.58
STCr-2	27.50±1.36	20.83±0.93	48.33±3.41
STCr-2-cured	137.62±8.35	130.48±7.40	268.10±17.08
STCr-3	28.57±1.58	23.10±1.25	52.38±3.72
STCr-3-cured	251.00±11.64	170.00±7.92	421.00±20.78
STCr-4	34.16±1.74	34.16±1.43	68.32±4.07
STCr-4-cured	206.66±9.39	134.00±6.16	340.66±16.47

appearing after 24 h of incubation were screened for chromate sensitivity and plasmid loss. The consequent loss of chromate resistance phenotype with the loss of plasmid in all the bacterial strains confirmed that chromate resistance in these strains is conferred by plasmids. The cured strains could bear upto 20 μg mL^{-1} of chromate salt rather than 40 mg mL^{-1} in rich medium. The low level resistance exhibited by cured derivatives suggests that chromate resistance in the bacterial strains is determined both by plasmid and chromosome. Both plasmid encoded and chromosomally controlled chromium resistances have been described[10]. After establishing that major resistance to chromate in these strains is controlled by plasmids our interest was to ascribe other resistant markers on these plasmids. Hence the parental strains and their cured derivatives were screened for metal and antibiotic resistances. Cured derivatives appeared to be sensitive to Co (all strains), Zn (all strains), Ap (STCr-2), Cefradine (all strains), Cefadroxil (all strains), Cm (STCr-1, STCr-3, STCr-4), Km (all strains) and Tc (STCr-3 and STCr-4) (Table 1). These results reflect that resistance to Co (all strains), Zn (all strains), Ap (STCr-2), Cefradine (all strains), Cefadroxil (all strains), Cm (STCr-1, STCr-3, STCr-4), Km (all strains) and Tc (STCr-3, STCr-4) might also be present on these plasmids. Plasmid encoded multiple metal and antibiotic resistances have been reported by other workers[13,33-35]. From treatment standpoint multiple metal and antibiotic resistances are important which help bacteria in adjusting the variety of toxic contaminants.

Having successfully demonstrated that high level resistance to chromate in these strains was determined by plasmids we analyzed the uptake of Cr by bacterial strains and their cured derivatives to understand the mechanism of chromate resistance in these strains. The parental strains and their cured derivatives were grown in the presence of 20 μg mL^{-1} K_2CrO_4. Loosely bound with cells (in washing) and accumulated Cr in the cells were determined. Curing of plasmid in each strain resulted in substantial increase in Cr-uptake (both accumulated and loosely bound Cr) (Table 2). Maximum increase was manifested in STCr-3 (loss of plasmid pSH1322). The accumulated fraction was more relative to loosely bound one in all the cured derivatives. In parental strains the total sorbed Cr was high in STCr-4 and lowest in STCr-1 (Table 2). A comparison of parental strains and their cured derivatives exhibited that with the loss of plasmid Cr-sorption (accumulated and loosely bound) of bacterial strains increased 5 (STCr-4) - 8 (STCr-3) fold (Table 2). These results thus reflect that high level plasmid mediated chromate resistance in these strains is due to decreased Cr uptake/accumulation by the resistant cells. Nevertheless the level of reduction by different strains varied. Plasmid determined chromate resistance in *A. eutrophus*[13] and *P. aeruginosa*[12] also resulted from reduced accumulation of Cr. The further work at molecular level might explain the extent to which these plasmids/strains differ from one another in exhibiting the resistance to chromate.

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