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MANGANESE RESISTANT BACTERIUM FROM POLLUTED WATER: SOME ENVIRONMENTAL FACTORS INFLUENCING CONJUGAL TRANSFER OF Mn^{2+}-RESISTANT PLASMID

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Abstract: A manganese resistant strain AnMn-1 was examined, which could prevent the toxic action of Mn^{2+} up to 2000 μg/ml in the medium. It was Gram-negative, pleomorphic, motile with high convex, undulate and circular colonies. It favoured neutral to alkaline media and was sensitive to Co^{2+}, Hg^{2+}, Cm and Sm but could tolerate Ni^{2+}, Zn^{2+}, Ba^{2+}, Sn^{2+}, Fe^{3+}, Cu^{2+}, Ap, Tc and Km in the medium. It was facultative anaerobic, spore-former and had catalase, oxidase and urease enzymes. It had the ability to produce acid from arabinose, rhamnose and glucose. It could hydrolyse urea, decarboxylase lysine and ornithine. Single plasmid was present in it. Conjugation experiments under different set of environmental conditions like time (0-24hrs), pH (6.5-8.5), temperature (25°, 28°, 32°, 37°) and donor to recipient ratio (1 to 10) revealed that maximum transfer frequency was found at 37°C when pH of the medium was 8 and also at donor to recipient ratio 10. It shared maximum characters with Gram-negative *Bacillus*.

Key words: Mn^{2+} resistant bacteria, plasmid, conjugal transfer

INTRODUCTION

Heavy metal burdened industrial effluents pose serious problems to biological life since many of them are toxic with long decay time. Majority of these metals exist in insoluble forms in the industrial wastes (Vallee and Ulmer, 1972; Moore and Ramammoorthey, 1984). One of these metals is manganese, traces of which are essential for plant and animal life. It activates certain enzymes such as decarboxylase, dehydrogenase and oxidase (Bidwell, 1979); cotrols nitrogen metabolism and assimilation reaction in plants (Bidwell, 1979). It also has a structural role in chloroplast membrane system and in the photosynthetic split of H_2O (Salisbary and Ross, 1986). Nevertheless, excess of manganese is harmful to living organisms. Under low redox potential manganese is reduced which is more soluble in acidic pH. Reduced manganese absorbs at the root surface and cause adverse effect on plant growth (Jeffrey and Helm, 1987). Increased amount of Mn^{2+} (0.1-1.0mM) in combination with reduced pH effect more significantly the content of chlorophyll a, carotene and ultimately the biomass production (Kummerova and Buresova, 1989a,b). Excess of manganese also causes iron deficiencies (Brady, 1984) and destruction of IAA (Devlin and Witham, 1986)

In animals surplus amount of manganese is pneumotoxic (Richard and Morris, 1989), and in rat kidney it blocks the induction of hemo-oxygenase (Drummond and Kappas, 1989). Excess amount of manganese stimulates lysozyme production in *Micrococcus lysodektious* and cause lysis of cells (Sack, 1981). It also effects the transport system of microbes (Perry and Silver, 1982). Furthermore Cd^{2+} and Mn^{2+} are the competitive ihibitors of each other (Perry and Silver, 1982; Nies and Silver, 1989). Metal resistant bacteria can detoxify the adverse effects of metals (Wood and

Wang, 1985). For the said reasons the present work deals with the isolation and characterization of metal resistant bacteria from industrial wastes. Effects of some environmental factors on conjugal transfer of Mn-resistant plasmid, present in this strain, have also been discussed.

MATERIALS AND METHODS

From the effluents of Shan Ghee which was odourless, colourless, with oily gradients and pH 7, a manganese-resistant bacterial strain was isolated. Fifty μl of sample water was plated onto nutrient-agar plates containing 25μg/ml of $MnSO_4$ (having 9.09μg/ml of Mn^{2+}). Bacterial growth was observed within 24 hours at 37^{O}C. Purified strain, which was designated as AnMn-1 was taken progressively to higher levels of $MnSO_4$ in the medium. AnMn-1 was characterized morphologically, physiologically as well as biochemically (Gerhardt *et al.*, 1981). Twenty biochemical and the cytochrome oxidase tests were performed by using QTS-20 (20 Quick Test Strips for Bacterial Characterization) and CO-strips (Cytochrome oxidase strips), respectively (DESTO Laboratories, Karachi). Spore forming ability was authenticated by the method of Moir (1981). AnMn-1 was also checked for the resistance against antibiotics Ap (ampicillin), Km (kanamycin), Sm (streptomycin), Cm (chloramphenicol), and Tc (tetracycline); (300, 50, 500, 5 and 25μg/ml, respectively) and other metallic salts *i.e.* $ZnSO_4$ (250μg/ml), $NiCl_2$, $CoCl_2$ and $HgCl_2$ (25μg/ml), $FeCl_3$ (50μg/ml), $CuSO_4$ (200μg/ml), $SnCl_2$ (600μg/ml), $BaCl_2$ (200μg/ml) and $CdCl_2$ (50μg/ml). For the detection of plasmid total cell lysate method (Thomas, 1984) was used. For characterization of plasmid, broth mating technique of Willetts (1984) was followed and conjugation experiments were performed using *E. coli* K12 strain CSR603 (*recA1 phr1* derivative of AB1886 (*thr-1 leu-6 lacY1 galK2 ara-14 xyl-5 mtl-1 proA2 his-4 str-31 tsx33 sup37 uvxA6*) as recipient. Transconjugants were selected on media containing 2000μg/ml $MnSO_4$ and 500μg/ml streptomycin. For determining the effect of environmental factors on the transfer frequency, time (0-24 hrs), pH (6.5-8.5), temperature (25, 28, 32, 37^{O}C) and donor to recipient ratio (1-10) were taken into consideration. For studying transfer frequency, AnMn-1 (donor) and CSR603 (recipient) were grown overnight in 5ml of L. broth with continuous agitation (150 rpm) at 37^{O}C. Initial recipient density was determined by plating dilutions (10^{3}, 10^{4}, 10^{5}) on nutrient-agar containing 500μg/ml of Sm. Afterwards the mating mixture was diluted and spread on double inhibitor-supplemented plates. Colonies obtained on double inhibitor-supplemented (Mn+Sm) agar at 37^{O}C were scored as transconjugants. Frequency of transfer was calculated as the number of presumptive transconjugants per initial amount or number of recipients.

RESULTS AND DISCUSSION

Strains purified at conceentration of 2mg/ml $MnSO_4$, was characterized morphologically, physiologically as well as biochemically. Single colonies obtained after 24 hours of incubation were used for study. They were opaque, creamy-yellow, circular, undulate with high convex elevation, ranging in size from 3-3.5mm. The cells were motile, pleomorphic, and Gram-negative. When isolate was taken to elevated levels of $MnSO_4$the growth rate declined which clearly indicates that cellular growth was inhibited by extremes of environmental metallic toxicity. In addition to metallic

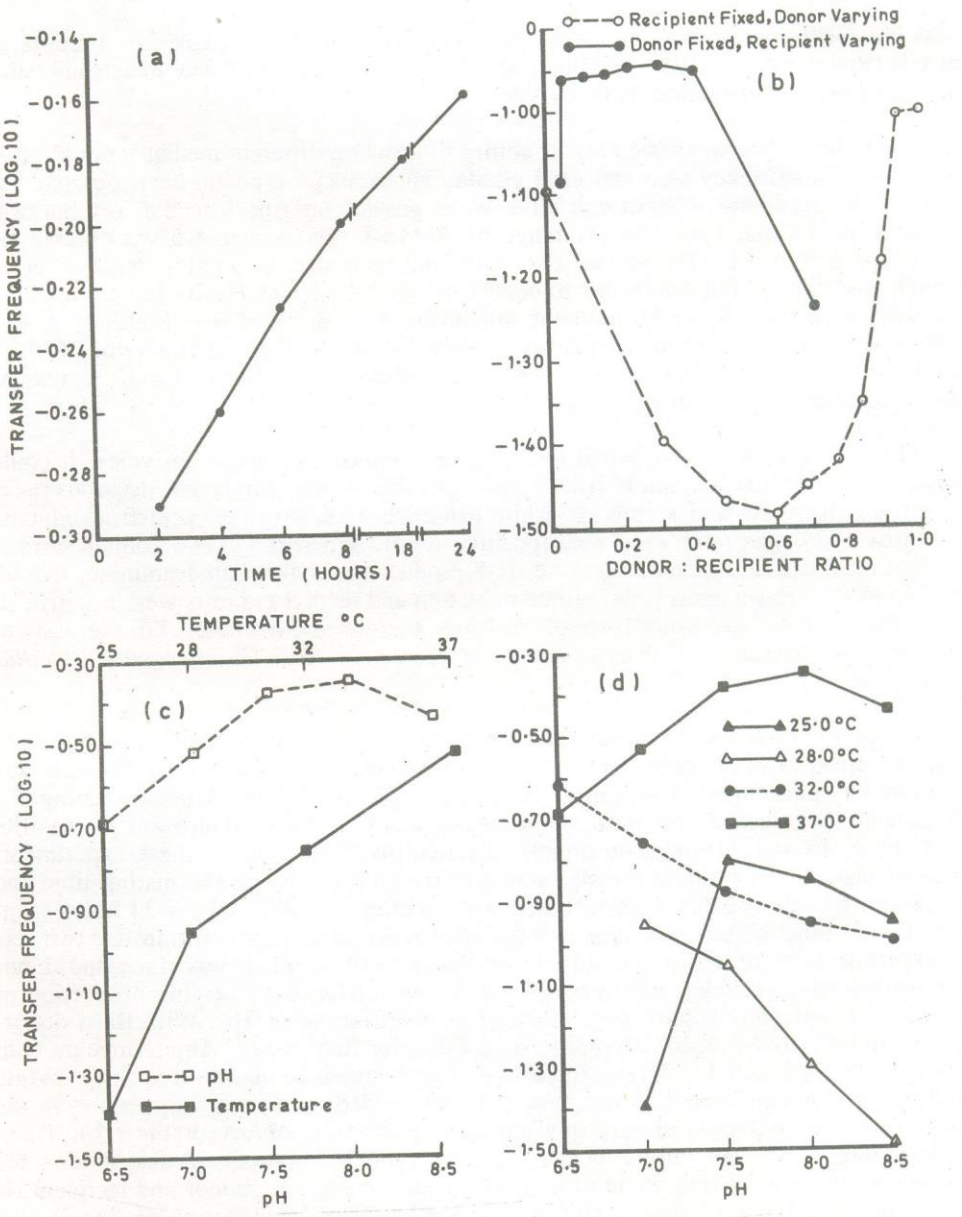

Fig. 1. Factors influencing conjugal transfer of Mn-resistant plasmid from AnMn-1 to *E. coli* strain CsR603. (a) Time, (b) Donor to recipient ratio, (c) Temperature and pH, (d) Combined effect of varying temperature and pH.

salts (in media) other environmental factors also effect the resistance of bacteria to metals (Breteler *et al.*, 1981; Giblin *et al.*, 1983). Resistance to heavy metals may also be due to genetic adaptation (Brock. 1978).

When the isolate was tested for its ability to grow on different media, it could grow on L-agar, MacConkey agar and gave gummy appearance on potato dextrose agar. As regards the antibiotic resistance it gave weak growth on Ap, Km and Tc, but was sensitive to Sm and Cm. The pH range of AnMn-1 was from 6-8.5 with maximum growth at 6.5 to 7.0. The isolate also conferred resistance to $ZnSO_4$, $NiCl_2$, $FeCl_3$, $CuSO_4$ and $SnCl_2$, but could not tolerate $CoCl_2$, $CdCl_2$, and $HgCl_2$ in the medium. Growth of bacteria depends upon the composition of the medium (Mergeay *et al.*, 1985). Cd^{2+} and Mn^{2+} are competitive co-rival of each other. 0.2μM Km of Cd^{2+} hinder Mn^{2+} transport (Perry and Silver, 1982). Sensitivity of AnMn-1 to Cd^{2+} may be due to inhibitory effects of manganese.

AnMn-1 was spore former and had oxidase, catalase and urease enzymes. It could ferment glucose and mannitol. It also gave positive results for lysine decarboxylase, acid from rhamnose and arabinose, while ornithine decarboxylase, acid from glucose and urea hydrolysis tests were weak positive for it. Whereas ONPG, sodium citrate, sodium malonate, arginine dihydrolase, H_2S production, tryptophan deaminase, indole, acetoin (VP), gelatin hydrolysis, nitrate reduction and methyl red tests were negative. It could not produce acid from sorbitol, maltose, sucrose and mannitol. On the basis of biochemical characters it shared maximum characters with Gram-negative *Bacillus* (Krieg and Holt, 1984).

Manganese resistant strain was screened for the presence of plasmid. Only one band was discerned. Conjugation experiments demonstrate that manganese resistance was conferred by plasmid and transconjugants could be obtain after two hours of mating. To investigate the effect of time on the transfer frequency, mating mixture was plated after 2, 4, 6, 8, 18 and 24 hours on double selective plates. Results of these experiments showed that rate of plasmid transfer was directly proportional to the mating time and maximum transconjugants were recorded when mating was allowed for 24 hours (Fig. 1a). To examine the effect of donor to recipient ratio on conjugation transfer, two sets of experiments were performed. In one the quantity of recipient was fixed and donor was varied while in other the amount of donor was fixed with varying proportion of recipient. Contrasting results were obtained in two cases (Fig. 1b). With fixed donor, change in ratio (0.1-0.4, log10) did not affect transfer frequencies. Any change on both sides of this range (0.1 - 0.4) resulted in abrupt decrease in mating frequency. While with fixed recipient, donor to recipient ratio 10 yielded maximum transfer and in the range of 0.3-0.8 (log scale) very low transconjugants were observed (Fig. 1b). These results suggest that not only donor to recipient ratio is important in determining the frequency of conjugal transfer but the nature of the strain, *i.e.*, donor and recipient, is also critical. pH of the donor and recipient strains before and during mating is also important factor in scoring transconjugants.

Different studies on the transfer of drug resistance and metallic compounds have shown optimum pH 6.0 - 7.5 for conjugal mating (Shenderov, 1971; Harada and

Mitsuhashi, 1977; Singleton and Anson, 1983). In the results presented here maximum transconjugants were obtained at pH 8.0 (Fig. 1c). These results demonstrate that plasmid could be transferred between pH 6.5 and 8.5. Beyond both limits of pH no transconjugants were recorded whereas the bacteria could grow at pH 6. Temperature being an important factor of environment also effects the conjugation. Many antibiotic resistant plasmids have been reported to transfer maximally at temperature between 20 - 30°C (Gauthier *et al.*, 1985; Altherr and Kasweck, 1982; Kelly and Reanney, 1984). An increase in the temperature from 27 to 37°C led to a large increase in the transfer frequency of Mn resistant plasmid from AnMn-1 strain (Fig. 1c). Higher optimum temperature for maximum transfer frequency may be attributed to the hot climate from which this strain was isolated. Influence of low temperature on the mating frequency have also been reported by other workers (Harada and Mitsuhashi, 1977; Kelly and Reanney, 1984). Since temperature and pH have coordinated action on plasmid transfer (Singleton and Anson, 1983; Rochelle *et al.*, 1989), the effects of varying pH (6.5 to 8.5) in combination with different temperatures (25, 28, 32, 37°C) on the plasmid transfer were also determined (Fig. 1d). Generally 37°C yielded maximum transconjugants at all pH values (Fig. 1d) when compared with other temperatures at respective pH. At this temperature, up to pH 8.0 progressive augmentation in transfer frequency with the increase in pH value was recorded after which a decrease in conjugal transfer was observed. With low temperature (25, 28°C) no plasmid transfer was observed at pH 6.5. With increasing pH value, 32°C and 25°C caused gradual dismount in transfer frequency of this plasmid. With 28°C maximum conjugal transfer was observed at pH 7.5 but even it was significantly less than that of 37°C at respective pH value. Thus the Mn-resistant plasmid described here favours pH 8 at 37°C for its maximal transfer and acidic pH with low temperature (25, 28°C) hinder its conjugation.

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