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Plasmid-Encoded Heavy Metal Resistance in *Pseudomonas* sp.

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Heavy metal pollution is a principle source of environmental contamination. Heavy metals are toxic to living organisms. While some have no known beneficial biological function, others have essential roles in physiological reactions (Roane and Kellogg, 1996). For example, copper is an essential trace element that is utilized in a number of oxygenases and electron transport proteins, but it is also a highly toxic heavy metal, against which all organisms must protect themselves (Brown et al., 1992). Despite the fact that heavy metals are acutely toxic to microbes, there are metal-resistant bacteria. The toxic effects of heavy metals immediately upon introduction to enviromental samples have been documented for a broad array of microbial processes. Long term exposure to metals imposes a selection pressure that favors the proliferation of microbes that are tolerant/resistant to this stress.

Some microbial strains possess genetic determinants that confer resistance. In bacteria, these determinants are often found on plasmids, which has faciliated their study at the molecular level (Cervantes et al., 1994). Plasmid-determined resistance to toxic metal ions has been demonstrated for many bacterial species and is a useful selectable marker for these DNA molecules.

Studying metal ion resistance gives us important insights into enviromental processes and provides an understanding of basic living processes (Nies and Silver, 1995). This is the first study on Maximum Tolerable Concentration (MTC) of some heavy metals in *Pseudomonas* sp. that were identified in the Sofulu garbage dump in Adana, Turkey. Because *Pseudomonas* sp. had several chromosomal and plasmid-encoded heavy metal resistance, we found interesting to study *Pseudomonas* sp. The present study was aimed to determine the resistance level of *Pseudomanas* sp. strains isolated from soil and water samples to heavy metals and to assess the genetic transfer of their native plasmids to *Escherichia coli* AB3505.

MATERIALS AND METHODS

Soil samples were collected from a garbage dump near Sofulu village in Adana. Water samples were taken from a pool near the same place. Soil slurries were made and both soil and water samples were serially diluted, then plated onto GSP

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Agar; starch (soluble) 20 g/L, Na-L-Glutamate 10 g/L, $MgSO_4$ 0.5 g/L, KH_2PO_4 2 g/L, Phenol red 0.36 g/L, agar 12 g/L pH:7.2 (Kielwein, 1969). After the incubation at 30°C for 24 hr, colonies which have red precipitation zone around the colony were selected and identified in our laboratory following Bergey's Manual of Determinative Bacteriology (1994). *Escherichia coli* AB3505 (plasmid free and metal sensitive) was obtained from the Ç.Ü. Microbiology Laboratory culture collection and strains were grown at 37°C in Luria-Bertani (LB) medium; Tryptone 10 g/L, Yeast extract 5g/L and NaCI 5 g/L (Gerhardt et al., 1994).

For testing heavy metal resistance, the cells were streaked onto TY Agar plates (Tryptone 5 g/L, Yeast extract 5 g/L, NaCI 5 g/L, Na-gluconate 2 g/L) containing various concentrations of $CuSO_4$, $NiCl_2$, $CdCl_2$ and $K_2Cr_2O_7$ and incubated at 30°C for 2 days The maximum tolerable concentration (MTC) of heavy metal was designated as the highest concentration of heavy metal which allows confluent growth after 2 days (Schmidt and Schlegel, 1994).

Ethidium Bromide was used to eliminate the plasmids from the strains, and also heat treatment was applied as a second control (Mergeay et al., 1985). Plasmid DNA was extracted from the strains by a minipreparation method (Dillon, 1985) and separated by agarose gel electrophoresis through a horizontal slab gel of 0.8% agarose submersed in TBE (Tris-HCI, boric acid, EDTA) runnig buffer at 70 V for 2 hr. DNA bands were stained with ethidium bromide and visualized on a UV transilluminator (Sambrook et al., 1989). Plasmid DNA was transformed into chemically competent *Escherichia coli* AB3505 (Dillon, 1985).

RESULTS AND DISCUSSION

Fifty *Pseudomonas* sp. strains (30 strains from soil samples, 20 strains from water samples) were selected and identified, and their heavy metal MTC was determined (Tables I and II). All isolates were found to be least tolerant to chromium with MTC between 1-1.7 mM (Table I and Table II). 32% of the strains possessed tolerance to 6 mM copper, 14 % to 9 mM nickel, 28 % to 9 mM cadmium and 42% to 1.7 mM chromium. Strain WP19, isolated from water samples, tolerates relatively high concentrations of copper (21 mM), nickel (18 mM), chromium (1.7 mM) and cadmium (5 mM). Strain WP17 tolerates copper (7 mM), nickel (7 mM), cadmium (10 mM) and chromium (1.5 mM). Strain WP8 tolerates copper (7 mM), nickel (9 mM), cadmium (7 mM) and chromium (1.5 mM). To determine if the resistance gene is encoded by a plasmid, we attempted to eliminate plasmids from the all strains with ethidium bromide and heat. Among the resistant strains used in this study, WP19, WP17 and WP8 were found to have plasmid resistance to copper and nickel ions. One strain (WP19) which had the highest tolerance to copper was selected for further studies. This strain was resistant to concentrations of copper up to 21 mM. Susceptible strains, SP21, WP21 and WP22, had tolerated the maximum 2 mM $NiCl_2$ on TY agar plates. The concentration of 2 mM $CuSO_4$ was tolerated by the susceptible strain SP 23

Table 1. MTC of the *Pseudomonas* isolates from soil samples

Strains	$CuSO_4$ mM	$NiCl_2$ mM	$CdCl_2$ mM	$K_2Cr_2O_7$ mM	Strains	$CuSO_4$ mM	$NiCl_2$ mM	$CdCl_2$ mM	$K_2Cr_2O_7$ mM
SP 1	13	4	4	1	SP 16	15	9	9	1.7
SP 2	13	7	9	1.5	SP 17	3	4	5	1
SP 3	17	4	9	1.7	SP 18	13	4	4	<1
SP 4	7	4	9	1.7	SP 19	15	9	3	1
SP 5	7	4	10	1	SP 20	5	5	7	1
SP 6	7	4	4	1	SP 21	6	2	4	1
SP 7	6	2	5	1	SP 22	6	4	4	1
SP 8	6	8	4	1.5	SP 23	2	5	4	1.7
SP 9	16	5	8	1.7	SP 24	6	5	9	1
SP 10	12	8	9	1	SP 25	2	5	5	<1
SP 11	9	5	4	1.7	SP 26	3	4	4	1
SP 12	9	9	4	1.5	SP 27	3	4	9	<1
SP 13	12	8	5	1.7	SP 28	6	4	8	1
SP 14	16	7	5	1.7	SP 29	6	4	9	1
SP 15	15	9	5	1.7	SP 30	6	4	5	1

SP: Soil *Pseudomonas* - n: 3 (replication number of experiment)

Table 2. MTC of the *Pseudomonas* isolates from water samples

Strains	$CuSO_4$ mM	$NiCl_2$ mM	$CdCl_2$ mM	$K_2Cr_2O_7$ mM	Strains	$CuSO_4$ mM	$NiCl_2$ mM	$CdCl_2$ mM	$K_2Cr_2O_7$ mM
WP1	17	2	9	1.7	WP12	6	7	10	1.7
WP2	12	4	9	1.7	WP14	13	5	5	1.7
WP3	12	7	7	<1	WP15	14	5	5	1.7
WP4	17	8	9	1.7	WP16	6	8	8	1.5
WP5	6	9	5	1.7	WP17	7	7	10	1.5
WP6	12	7	7	1.5	WP18	9	20	7	1.5
WP7	12	2	9	1.7	WP19	21	18	5	1.7
WP8	7	9	7	1.7	WP20	6	9	9	1.7
WP10	6	7	7	1.5	WP21	6	2	9	1.7
WP11	6	4	7	1.5	WP22	6	2	7	1.5

WP: Water *Pseudomonas* - n: 3 (replication number of experiment)

Plasmid DNA from WP19 was purified by mini prep isolation. Strain WP19 had 4 plasmids of approximetely 20.8, 19.6, 8 and 4.7 kb, respectively (Fig 1-Lane 3). All the plasmids were supercoiled.

Then we attempted to transfer the plasmid into *Escherichia coli* AB3505. Plasmid DNA was transformed into competent *Escherichia coli* AB3505. Transformant strains were streaked onto GSP agar plates containing $CuSO_4$, at concentrations ranging from 5 mM to 14 mM, $NiCl_2$ (6 to 14 mM), $CdCl_2$ (5 to 10 mM) and $K_2Cr_2O_7$ (1 to 2 mM). Confluent growth after 2 days was observed on plates contaning $\leq$10 mM $CuSO_4$ and on plates containing $\leq$12 mM $NiCl_2$. Plates containing $K_2Cr_2O_7$ and $CdCl_2$ did not show any bacteria growth (Fig 2).

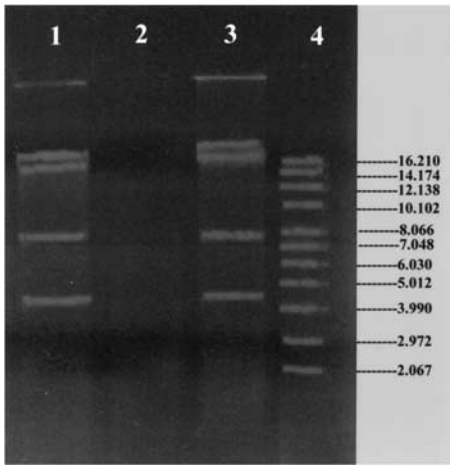

Figure 1. Agarose gel electrophoresis of plasmid DNA of WP19, *Escherichia coli* AB3505 and transformant strains.
lane 1, plasmids of transformed AB 3505 strain; lane 2, plasmids of AB 3505 *E.coli*; lane 3, plasmids of WP19; lane 4, DNA ladder (11 supercoiled fragments)

Copper and nickel resistance was transferred from WP19 to AB3505 at a frequency of approximately $2x10^{-5}$ per recipient cell. The plasmid composition of viable transformants was confirmed by agarose gel electrophoresis of the mixture of plasmids isolated by minipreparation. One *Escherichia coli* AB3505 transformant harbored a plasmid of the same size as that in WP19 (Figure 1-Lane 1). These results indicate that the plasmid can replicate in *Escherichia coli* AB3505 and that it posseses the genetic information necessary for the expression of resistance to copper. Transformation and curing results suggest that nickel and copper were conferred by plasmid DNA, while cadmium and chromium resistance seems to be encoded by genes of the bacterial chromosome.

Several chromosome and plasmid-encoded metal resistance genetic systems have been studied in *Pseudomonas* as well as in related bacteria (Cervantes and Silver, 1996). Cervantes et al. (1991) reported that resistance to toxic heavy metals has been found in bacteria from clinical and environmental origins and the genetic determinant of resistance are frequently located on plasmids or transposons.

Heavy metal resistant microorganisms are thought to naturally occur primarily in metal impacted soil. Roane and Kellog (1996) found resistant *Pseudomonas* (cadmium MTC:1.2 mM) isolates from heavy metal contaminated soil. It has also been reported that nickel resistant bacteria, *Alcaligenes xylosoxidans* 31A isolated from a galvanization tank tolerate relatively high concentrations of nickel (40 mM), cobalt (20 mM), zinc (10 mM), cadmium (1 mM) and copper (1 mM) (Schmidt and Schlegel, 1994). In this study, it was found that cadmium MTC of the WP19 was 5 mM.

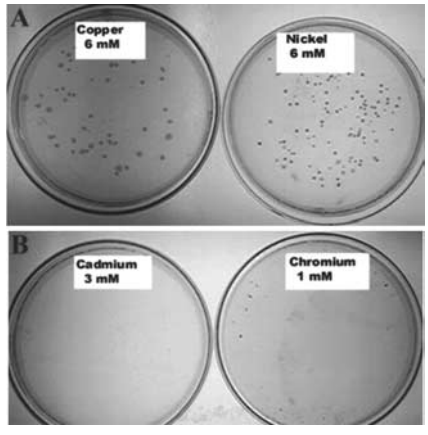

Figure 2. Growth of transformant strains of *Escherichia coli* AB3505 after the transformation in heavy metal containing media. Fig 2A.Confluent growth on plates containing 6 mM $CuSO_4$ and 6 mM $NiCI_2$ after 2 days.Fig 2B No growth on plates containing 3 mM $CdCI_2$ and 1 mM $K_2Cr_2O_7$

It is frequently thought that these resistances arose as a result of human pollution in recent decades. Furthermore, long term exposure to metals imposes a selection pressure that favors the proliferation of microbes which are tolerant/resistant to this stress. The development of the metal-resistant population in a contaminated soil can result from (i) vertical gene transfer (reproduction), (ii) horizontal gene transfer (including transposons and broad host range plasmids), (iii) and selection pressures on spontaneous mutants (due to the presence of metals). Transposable elements carrying mercury resistance genes have been linked to the distribution of this trait in nature (Bogdanova et al., 1998).

Heavy-metal resistant bacteria may be useful in biotechnology. First, adding metal resistance to a microorganism may faciliate a biotechnological process, which may or may not be linked to heavy metals. Second, heavy-metal resistant bacteria may be used for any kind of bio-mining of expensive metals, directly on ores or by recovering metals from effluents of industrial processes. Third, heavy-metal-resistant bacteria may be used for bioremediation of metal-contaminated environments (Nies, 1999). For example, it would be useful in the bioremediation of aromatic compounds in the presence of toxic heavy-metals as co-contaminants.

The transformation of the copper and nickel resistance genes suggests that horizontal gene transfer may be an important factor for the development of metal resistant populations in the enviroment. Future research will include molecular genetic analyses of plasmids in both resistant and sensitive strains.

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