J. BIOL. ENVIRON. SCI.,
2012, 6(18), 233-242

Original Research Article

Bio-Monitoring of Heavy Metal Resistance in *Pseudomonas* and *Pseudomonas* Related Genus

Özgür CEYLAN[1] and Aysel Uğur[2*]

[1] Apiculture Program, Ula Ali Kocman Vocational School, Mugla University, 48640, Ula, Mugla, TURKEY

[2] Department of Basic Sciences, Section of Medical Biology, Faculty of Dentistry, Gazi University, 06500, Emek, Ankara, TURKEY

Received: 15.05.2012; Accepted: 10.09.2012; Available Online: 25.01.2013

ABSTRACT

The aim of present study was to determine the level of heavy metal resistance patterns and to determine if there is a relationship between heavy metal resistance and plasmid presence. From 28 identified strains, 39.3% corresponded to *Stenotrophomonas maltophilia,* 17.9% to *Chryseomonas luteola,* 14.3% to *Pseudomonas fluorescens,* 10.7% to *Pseudomonas putida,* 7.1% to *Sphingomonas paucimobilis,* 7.1% to *Methylobacterium mesophilicum* and 3.6% *Pseudomonas stutzeri.* The resistance of these Gram-negative bacteria to 8 heavy metals, was investigated by agar dilution method. Most isolates showed tolerance to different concentrations of heavy metals, and minimal inhibition concentrations ranged from 0,005 $mmol^{-1}$-20 $mmol^{-1}$. All strains displayed high resistance to zinc and lead (100% and 96,4% respectively) and high susceptibility to silver, cobalt and mercury (92.9%, 92.9% and 96.4% respectively). *M. mesophilicum* strains were determined as the most resistant strains to studied heavy metals. Isolated bacteria were tested for the presence of plasmids using the modified hot alkaline lysis method. The study also demonstrated that about 17.8% of isolated bacteria carried 0.89-21.59 kb sized plasmids and metal resistance profiles of bacteria carrying the same plasmids were similar. This study reveals the heavy metal resistance profiles of non-aeruginosa Pseudomonas species and other related species and the association between the occurrence of plasmids and the resistance to heavy metals.

Key Words: Metal resistance, Plasmid profili, *Pseudomonas* spp., *Stenotrophomonas, Chryseomonas, Sphingomonas, Methylobacterium*

Pseudomonas ve *Pseudomonas* İlişkili Cinslerin Ağır Metal Dirençliliklerinin Biyolojik İzlenmesi

ÖZET

Bu çalışmanın amacı ağır metal dirençliliğinin düzeyinin belirlenmesi ve eğer varsa ağır metal dirençliliği ile plazmid varlığı arasındaki ilişkinin ortaya çıkarılmasıdır. İdentifikasyonu yapılmış 28 suşun %39.3'ünün Stenotrophomonas maltophilia, %17.9'unun Chryseomonas luteola, %14.3'ünün Pseudomonas fluorescens, %10.7'sinin Pseudomonas putida, %7.1'inin Sphingomonas paucimobilis, % 7.1'inin Methylobacterium mesophilicum ve %3.6'sının Pseudomonas stutzeri olduğu bulunmuştur. Bu Gram-negatif bakterilerin 8 ağır metale karşı dirençlilikleri agar dilüsyon metodu kullanılarak ortaya çıkarılmıştır. Çoğu izolat ağır metallere karşı farklı konsantrasyonlarda tolerans göstermiş ve minimum inhibisyon konsantrasyonları 0,005 mmol-1-20 mmol-1 aralığında tespit edilmiştir. Bütün suşlar çinko(%100) ve kurşun(%96.4)'a yüksek oranda direnç gösterirken, gümüş(%92.9), kobalt(%92.9) ve civa(%96.4)'ya karşı yüksek oranda duyarlı bulunmuştur. Methylobacterium mesophilicum suşları çalışmada kullanılan ağır metallere karşı en dirençli suşlar olarak tespit edilmiştir. Bakterilerde plazmid varlığı modifiye edilmiş hot alkalin lizis metodu kullanılarak ortaya çıkarılmıştır. Çalışmada bakterilerin %17.8'inin 0.89-21.59 kb. aralığında plazmidler taşıdığı ve aynı plazmidleri taşıyan bakterilerin benzer metal dirençliliği profillerine sahip oldukları görülmüştür. Bu çalışma non-aeruginosa Pseudomonas türlerinin ve diğer Pseudomonas ilişkili türlerin ağır metal dirençlilik profillerini ve bu ağır metal dirençliliği ile plazmid varlığı arasındaki ilişkiyi ortaya koymaktadır.

Anahtar Kelimeler: Metal Dirençliliği, Plazmid profili, *Pseudomonas* spp., *Stenotrophomonas, Chryseomonas, Sphingomonas, Methylobacterium*

INTRODUCTION

Pollution due to heavy metal toxicity is an ever-increasing problem in the developing nations. Heavy metals are major pollutants in marine, ground, industrial and even treated wastewater (Valdman et al. 2001). Presence of high concentration of toxic heavy metals in wastewater directly leads to both contamination of receiving water bodies and deleterious impact on aquatic life (Moten and Rehman 1998). Use of such polluted water for

* Corresponding author: ayselugur@hotmail.com

consumption and other purposes can bring severe problems to human health. At higher concentration, heavy metals form toxic complex compounds in the cell that are too dangerous for any biological functions (Rajbanshi 2008).

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 environmental 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 (Ünaldı et al. 2003).

Microbes apply various types of resistance mechanisms in response to heavy metals (Bruins et al. 2000, Nies 2003). These mechanisms may be encoded by chromosomal genes, however, most resistance systems appear to be associated with plasmids (Cervantes and Gutierrez-Corona 1994, Wuertz and Mergeay 1997). The incidence of plasmid-bearing strains is higher in polluted sites than in the unpolluted zone (Hada and Sizemore 1981).

Resistance to heavy metals is observed in a wide variety of bacteria, especially in gram negative bacteria (Poole 2002), such as *Pseudomonas, Alcaligenes, Ralstonia* and *Burkholderia* (Wuertz and Mergeay 1997, Malik and Jaiswal 2000, Kozdro'j and Van Elsas 2001, Ellis et al. 2003, Ünaldı et al. 2003, Akinbowale et al. 2007, Zolgharnein et al. 2007, Matyar et al. 2008, Singh et al. 2010). Bacteria of the genus *Pseudomonas* are well-studied and are of great interest not only because of their high resistance to heavy metals and other toxic substances, but also for their simple nutritional requirements and rapid growth on standard laboratory media (Pardo et al. 2003).

In the last decade, numerous studies have reported on the heavy metal resistance of *Pseudomonas aeruginosa* obtained from different heavy metal polluted environments (Filali et al. 2000, Malik and Jaiswal 2000, Kozdro'j and Van Elsas 2001, Raja et al. 2006, Akinbowale et al. 2007, El-Sayed et al. 2008, Raja and Selvam 2009). Therefore, it has been considered as a water quality indicator organism (Kozdro'j and Van Elsas 2001) and as a good candidate for heavy metal removal from polluted sites (Malik and Jaiswal 2000). Surprisingly, little information is available regarding the pattern of heavy metal resistance of the non-aeruginosa *Pseudomonas* species and *Pseudomonas* related genera such as *Chryseomonas, Stenothrophomonas, Sphingomonas* and *Methylobacterium* species. On the other side, there are very few studies showing the relationship between these species heavy metal resistance patterns and the presence of plasmid.

In the present study, we evidenced the tolerance of the strains that belongs to non-aeruginosa *Pseudomonas* species and *Chryseomonas, Stenothrophomonas, Sphingomonas* and *Methylobacterium* species to various toxic metal as nickel, zinc, lead, cobalt, copper, chrome, mercury and silver. This study also revealed the relationship between the presence of metal resistance and plasmids.

MATERIALS AND METHODS

Bacterial strains and growth conditions

In this study, 40 strains which are thought to belong to *Pseudomonas* and *Pseudomonas* related genera were taken from Mugla University culture collection (MU) (Laboratory of Microbiology, Faculty of Arts and Sciences, University of Mugla, Turkey). All strains were cultured in nutrient broth (NB) (Difco) at 30.0 ± 0.1ºC.

Phenotypic characterization

All strains were biochemically identified by conventional tests (Collins et al. 1995) followed by use of API 20 NE system (BioMerieux, Marcy l'Etoile, France). The results were obtained in duplicate and analysed employing the Apilab Plus Software (BioMe´rieux). Test for pigment production were performed on King B medium (Merck) (King et al. 1954). Growth on selective CFC medium (Oxoid) was checked for all strains. Oxidase and catalase tests were performed on Nutrient agar (NA) (Difco). Methyl Green DNase Agar (Difco) was used for DNase test. Finally, enzymatic tests were performed on enzymatic strips (API ZYM, BioMerieux).

Heavy metal resistance

Heavy metal resistance of all isolates were determined by agar dilution method (Washington and Sutter 1980). Plates containing 20 mL of one-half strength NA and different concentrations of metal were poured on day of experiments. Concentrations for metals with tested $NiCl_2.6H_2O$, $ZnSO_4.7H2O$, $Pb(CH_3COO)_2.3H_2O$, $CoCl_2.6H_2O$, $CuSO_4.5H_2O$, $K_2Cr_2O_7$, $AgNO_3$, and $HgCl_2$ were as follows (in mmol l^{-1}): 0.005; 0.01; 0.05; 0.1; 0.5; 1; 2.5; 5; 10; 20 and 40, respectively. Plates were dried at 37 ºC for 30 min and inoculated with 0.1 ml from exponentially grown cultures. Plates were incubated at 37 ºC for 2 days. Plates containing media with no added metal were inoculated in the same manner to serve as controls. MICs were determined as lowest concentrations of metal ion preventing growth. To define metal resistance, strains not inhibited by 1 mmol l^{-1} $NiCl_2$, $ZnSO_4$, $Pb(CH_3COO)_2$, $CoCl_2$, $CuSO_4$, $K_2Cr_2O_7$, $AgNO_3$ and 0.1 mmol l^{-1} $HgCl_2$ were regarded as resistant.

On the other hand, it is well known that there are no currently acceptable concentrations of metal ions that can be used to distinguish metal-resistant from metal-sensitive bacteria. However, concentrations used in this study were employed in similar studies reported on heterotrophic bacteria in which testing media utilized nutrient agar (Thompson and Wattling 1983, Sadhukhan et al. 1997). Control strains used for all assays included *P.aeruginosa* ATCC 27853 and *P.aeruginosa* ATCC 29212. All tests were carried out in duplicate.

Plasmid profiling

Plasmid DNA was prepared according to a previously described modification of hot alkaline lysis procedure (Kieser 1984, Foght et al. 1996). Agarose gel 0.7% (wt/vol) was prepared and 12 µl of DNA preparation was loaded into each well. Electrophoresis was conducted for 4 h at 90 V and gel was stained with 0.5 µg/mL ethidium bromide. A plasmid DNA band was observed with 3 UV transilluminator (model LMS-203, UVP, Inc., Upland, CA, USA) and photographed with a Polaroid MP4 camera equipped with red filter and type 667 Polaroid film. Approximate sizes of plasmids were calculated from logarithmic plots against reference plasmids of DNA loader supercoiled (SIGMA D-5292). Plasmid profiles that differed by at least one plasmid band were regarded as different plasmid profiles.

RESULTS

In this study, 40 isolates which are taken from Mugla University culture collection, were studied. All the isolates were characterized by phenotypical characteristics, namely gram staining, colony typing, oxidase and catalase reaction, DNase and pigment production and growth on selective CFC medium. According to results of these tests, 28 isolates determined to belong to non-fermenting Gram negative bacilli and work was carried on with these isolates. After preliminary identification of the isolates were subjected by means of the API 20NE system. From 28 identified strains, 39.3% corresponded to *Stenotrophomonas maltophilia*, 17.9% to *Chryseomonas luteola*, 14.3% to *Pseudomonas fluorescens*, 10.7% to *Pseudomonas putida*, 7.1% to *Sphingomonas paucimobilis*, 7.1% to *Methylobacterium mesophilicum* and 3.6% *Pseudomonas stutzeri* (Table 1). API ZYM enzymatic strips were carried out in order to support the identifications (Table 2).

Table 1. Number of species of Gram negative bacteria.

Genus	Number of isolates	%
Stenotrophomonas maltophilia	11	39.3
Chryseomonas luteola	5	17.9
Pseudomonas fluorescens	4	14.3
Pseudomonas putida	3	10.7
Sphingomonas paucimobilis	2	7.1
Methylobacterium mesophilicum	2	7.1
Pseudomonas stutzeri	1	3.6
Total	28	100

Table 2. Identifications and characteristics that differentiate groups of Gram negative strains.

	Reaction[a] of the following Gram negative strains						
Characteristics	*S.maltophilia* (11)	*C. luteola* (5)	*P. fluorescens* (4)	*P.putida* (3)	*P.stutzeri* (1)	*S.paucimobilis* (2)	*M.mesophilicum* (2)
Classical tests							
Colony type	S	S	S	S	R	S	S
Oxidase reaction	-	-	+	+	+	+	+
Catalase reaction	+	+	+	+	+	+	+
DNase production	+	+	+	+	-	-	-
Production of pigment	-	-	-	-	-	+	+
API 20 NE							
Indol production on tryptophan	-	-	-	-	-	-	-
Glucose acidification	-	+	-	-	-	-	-
Arginine dihydrolase	-	-	+	+	-	-	-
Urease	-	-	-	-	-	-	+
Esculin hydrolisis	+	+	-	-	-	+	-
Gelatin hydrolisis	+	-	d	-	-	-	-
β–Galactosidase	+	+	-	-	-	+	-
D-Glucose	+	+	+	+	+	+	-
L-Arabinose	-	+	+	+	-	d	d
D-Mannose	+	+	+	-	-	d	-
D-Mannitol	-	+	+	-	+	-	-
N-Acetyl-D-glucosamine	+	+	+	-	+	d	-
Maltose	+	+	d	-	+	+	-
Gluconate	-	+	+	+	+	-	-
Caprate	D	-	+	+	+	-	-
Adipate	-	-	d	-	-	-	-
L-Malate	+	+	+	+	+	-	+
Citrate	+	+	+	+	+	-	-
Phenylacetate	-	+	d	+	-	-	-
API ZYM							
Alkaline phosphatase	+	+	-	-	+	+	d
Esterase(C4)	+	+	+	+	+	+	+
Esterase Lipase(C8)	+	-	+	+	-	+	+
Lipase (C14)	-	-	-	-	-	-	-
Leucine arylamidase	+	+	+	+	+	+	+
Valine arylamidase	+	+	d	-	-	+	-
Cystine arylamidase	-	+	-	-	-	-	-
Trypsin	+	+	+	+	+	d	+
α-chmotrypsin	-	-	d	+	-	d	-
Acid phosphotase	+	+	+	+	-	+	d
Naphtol-AS-phosphohydrolase	+	+	+	+	-	+	+
α-galactosidase	D	-	-	-	-	-	-
β-galactosidase	+	+	-	-	-	+	-
β-glucuronidase	-	-	-	-	-	-	-
α-glucosidase	+	+	-	-	-	+	-
β-glucosidase	+	-	-	-	-	-	-
N-acetyl-β glucosidase	-	d	-	-	-	d	-
α-mannosidase	-	-	-	-	-	-	-
α-fucosidase	-	-	-	-	-	-	-

[a] +, 65% or more the total, -, 35% or less the total; d, 36 to 64% the total.

The status of resistance against eight different heavy metals i.e. $NiCl_2$, $ZnSO_4$, $Pb(CH_3COO)_2$, $CoCl_2$, $CuSO_4$, $K_2Cr_2O_7$, $HgCl_2$ and $AgNO_3$ was investigated. Heavy metal resistance rates against all the bacteria in the study are as follows; $ZnSO_4$, 100%; $Pb(CH_3COO)_2$, 96.4%; Cu SO_4, 60.7%; $NiCl_2$, 32.1%; $CoCl_2$, $K_2Cr_2O_7$ and $AgNO_3$, 7.1%. All strains showed uniform tolerance of $ZnSO_4$ and $Pb(CH_3COO)_2$. $CoCl_2$ and $AgNO_3$ resistance was only detected in *M.mesophilicum* (Table 3).

Among the tested bacterial strains, *S. maltophilia* (formerly *Pseudomonas maltophilia*) was the most frequently detected microorganism. The present study indicated that frequencies of resistance in *S. maltophilia* strains to each metal ion tested were as follows: $ZnSO_4$, $Pb(CH_3COO)_2$ and $CuSO_4$, 100%, $NiCl_2$, 72.7%. All *S.maltophilia* strains were sensitive to $CoCl_2$, $K_2Cr_2O_7$, $AgNO_3$ and $HgCl_2$ (Table 3).

Table 3. Metal resistance patterns and plasmid profiles of isolated strains .

Strains	Resistance patterns	No of plasmids	Molecular weight
P.fluorescens			
MU 66	Zn,Pb	0	-
MU 75	Zn,Pb	0	-
MU 87	Zn,Pb	2	21.59,12.68
MU 97	Zn,Pb	0	-
P. putida			
MU 73	Zn,Pb,Cu	0	-
MU 83	Zn,Pb,Cu	0	-
MU 139	Zn,Pb	0	-
P.stutzeri			
MU 70	Zn,Pb,Cr	0	-
C. luteola			
MU 18	Zn,Pb,Cu	0	-
MU 56	Zn,Pb	0	-
MU 58	Zn,Pb	0	-
MU 65	Zn,Pb	0	-
MU 96	Zn,Pb,Cu	0	-
S.maltophilia			
MU 23	Zn,Pb,Cu	0	-
MU 25	Ni,Zn,Pb,Cu	0	-
MU 52	Ni,Zn,Pb,Cu	5	13.86,6.24,5.71,2.35,0.89
MU 53	Ni,Zn,Pb,Cu	0	-
MU 63	Ni,Zn,Pb,Cu	2	6.24,2.57
MU 64	Ni,Zn,Pb,Cu	0	-
MU 69	Ni,Zn,Pb,Cu	1	13.86
MU 94	Zn,Pb,Cu	0	-
MU 99	Zn,Pb,Cu	0	-
MU 136	Ni,Zn,Pb,Cu,Cr	0	-
MU 137	Ni,Zn,Pb,Cu	0	-
S.paucimobilis			
MU 67	Zn	0	-
MU 145	Zn,Pb	0	-
M. mesophilicum			
MU 140	Ni,Zn,Pb,Co,Cu,Hg,Ag	0	-
MU 141	Zn,Pb,Co,Cu,Cr,Ag	2	21.59,11.61

In this study, *C. luteola* was determined as a second prevalent species. While all *C.luteola* strains were resistant to $ZnSO_4$ and $Pb(CH_3COO)_2$, two of them were resistant to $CuSO_4$ additionally.

Pseudomonas strains were resistant to $ZnSO_4$ and $Pb(CH_3COO)_2$. In addition, two *P.putida* (MU 73 and MU 83) were resistant to $CuSO_4$ and P.stutzeri MU 70 was resistant to $K_2Cr_2O_7$. Trends in heavy metal toxicity was in order of Hg=Ag=Co=Ni>Cr>Cu>Pb=Zn for non-aeruginosa *Pseudomonas* spp. (Table 4).

Table 4. MIC values against the metals of isolated strains.

	Metal Ions							
	Ni	Zn	Pb	Co	Cu	Cr	Hg	Ag
Strains	Minimum Inhibition Concentrations ($mmol^{-1}$)							
P.fluorescens								
MU 66	0.5	1	10	0.05	0.1	0.5	0.005	0.005
MU 75	0.1	1	5	0.05	0.1	0.1	0.005	0.005
MU 87	0.5	5	2.5	0.1	0.5	0.5	0.05	0.05
MU 97	0.5	2.5	2.5	0.1	0.5	0.5	0.05	0.01
P. putida								
MU 73	0.5	20	2.5	0.25	1	0.5	0.01	0.01
MU 83	0.5	20	2.5	0.25	1	0.5	0.01	0.05
MU 139	0.5	5	2.5	0.25	0.25	0.25	0.01	0.1
P.stutzeri								
MU 70	0.5	1	5	0.1	0.5	1	0.005	0.005
C. luteola								
MU 18	0.5	10	2.5	0.25	1	0.25	0.01	0.01
MU 56	0.5	20	5	0.1	0.5	0.5	0.01	0.005
MU 58	0.5	20	5	0.1	0.5	0.5	0.01	0.005
MU 65	0.1	10	5	0.1	0.5	0.5	0.005	0.005
MU 96	0.5	20	2.5	0.5	1	0.5	0.05	0.01
S.maltophilia								
MU 23	0.5	10	5	0.1	1	0.5	0.005	0.005
MU 25	1	20	10	0.1	1	0.5	0.01	0.005
MU 52	1	20	5	0.1	1	0.5	0.01	0.05
MU 53	1	20	5	0.1	1	0.5	0.01	0.005
MU 63	1	20	5	0.1	1	0.5	0.01	0.005
MU 64	1	20	10	0.1	1	0.5	0.005	0.005
MU 69	1	20	5	0.1	1	0.5	0.01	0.005
MU 94	0.5	20	2.5	0.25	1	0.5	0.05	0.05
MU 99	0.5	10	2.5	0.5	2.5	0.5	0.05	0.1
MU 136	1	20	5	0.1	1	1	0.01	0.005
MU 137	1	20	5	0.1	1	0.5	0.01	0.005
S.paucimobilis								
MU 67	0.5	0.5	0.5	0.1	0.5	0.5	0.005	0.005
MU 145	0.5	10	5	0.1	0.5	0.1	0.005	0.005
M. mesophilicum								
MU 140	2.5	20	10	2.5	1	0.25	0.1	1
MU 141	0.5	20	5	2.5	2.5	2.5	0.05	2.5

While one of the *S.paucimobilis* strains was resistant to $ZnSO_4$ and $Pb(CH_3COO)_2$, the other one was resistant only $ZnSO_4$. In this study, *S. paucimobilis* strains were sensitive to the other six heavy metals. *M.mesophilicum* strains were the most resistant bacteria against heavy metals in this study. While *M.mesophilicum* MU 141 was sensitive to $NiCl_2$ and $HgCl_2$, *M.mesophilicum* MU 140 was sensitive to only $K_2Cr_2O_7$ (Table 4).

During the evaluation of plasmid profile, it was recorded that 5 strains harbored plasmids, illustrating different plasmid profiles; while the remaining 23 strains carried no detectable plasmids. The majority of strains

that contained plasmid were *S.maltophilia* strains and three strains of eleven *S.maltophilia* contained plasmids with different molecular weights. Among these strains, MU 52 contained five plasmids with molecular weights of 0.89, 2.35, 5.71, 6.24 and 13.86 kb, respectively. MU 63 contained two plasmids with molecular weights of 2.57 and 6.24 kb, respectively, while MU 69 contained one plasmid with molecular weight of 13.86 kb. These three strains of *S.maltophilia* show different plasmid profiles; results regarding heavy metal susceptibility and plasmid profile are shown in Table 3.

On the other hand, *P.fluorescens* MU 87 contained two plasmids with molecular weights of 12.68 and 21.59 kb, while *M.mesophilicum* MU 141 contained two plasmids with molecular weights of 11.61 and 21.59 kb, respectively (Table 3).

DISCUSSION

This study was carried out *S. maltophilia, C. luteola, S. paucimobilis, M. mesophilicum* and members of the *Pseudomonas* spp. (*P. fluorescens, P. putida* and *P. stutzeri*) that are cause significant clinical and environmental problems. And also revealed correlating heavy metal resistance and plasmid presence.

S.maltophilia has recently emerged as an important nosocomial pathogen (Al-Jasser 2006). In severely ill patients, *S.maltophilia* causes a wide range of infections such as bacteremia, pulmonary infections, urinary tract infections, wound infections, meningitidis and endocarditis (Denton and Kerr 1998, Micozzi et al. 2000, Koseoglu et al. 2004). Treatment of invasive infections caused by this organism is difficult as the bacterium is frequently resistant to a wide range of commonly used antimicrobials (Al-Jasser 2006). *S maltophilia* exhibits high-level intrinsic resistance to a variety of structurally unrelated antibiotics, including β-lactams, quinolones, aminoglycosides, tetracycline, disinfectants, and heavy metals (Alonso and Martinez 1997, Zhang et al. 2000).
In the present study, resistance to three heavy metals (Zn, Pb, Cu) was investigated for all *S.maltophilia* strains. Holmes et al. (2009) reported the *S.maltophilia* O2 appeared to be much more resitant to Hg(II), Cd(II), Zn(II), Cu(II), Au(III), and Cr(VI) than the other study strain (*Enterobacter* sp. YSU). While, In this work the *S.maltophilia* O2 MIC for Zn and Cu was 5 mM, our MIC results were 10-20 mM and 1-2.5 mM, respectively. Our strains appeared to be much sensitive to Cr, Hg and Ag than their *S.maltophilia* O2. Pages et al. (2008) in their study found that *S.maltophilia* Sm777 tolerated up to 0.05 mM Hg(II), 5 mM Cu(II), 0.02 mM Ag(I), and 5 mM Pb(II). These concentrations are higher than our results except as Pb(II).

C.luteola has only rarely been reported as a human bacterial pathogen. It has been shown that this organism in particular affects patients with health and indwelling disorders. Most reported cases showed septicemia, meningitidis, endocarditis, or peritonitis (Chihab et al. 2004). In our study, all *C.luteola* strains showed uniform resistance to Zn and Pb. In another study, *C.luteola* is reported to be the most resistant to Pb and Cd in Egypt. In contrast to our results, Özdemir and Baysal 2004 reported that their *C.luteola* strains had been Cr resistant.Two strains of *C.luteola* strains were observed lead and zinc resistance as well as copper resistance.

In this study, all *Pseudomonas* spp. were resistant to Zn and Pb. In addition 2 *P.putida* strains were resistant to Cu and *P.stutzeri* was resistant to Cr. These strains were found to be sensitive to Ni, Co, Hg and Ag metals. The incidence of multiple resistance either to metal or antibiotics was observed in the *Pseudomonas* spp. strains. Malik and Jaiswal (2000) reported the frequency of metal resistance in *Pseudomonas* strains isolated from soil to be 80% for Cu, 73.3% for Cd, 71.1% for Cr and Zn, 48.8% for Hg. Similar observation were made by earlier researchers (Bopp et al. 1983, Horitsu et al. 1986, Chaudhary and Kumar 1996). Bopp et al. (1983) isolated a chromate-resistant strain of *P.fluorescens* LB 300 from chromium-contaminated sediment and Horitsu et al. (1986) isolated *P.putida* strain from soil, which exhibited resistance to cadmium at a concentration of 1280µg/ml. Bhagat and Srivastava (1991) isolated some zinc-resistant strains of *P.stutzeri* from industrially polluted area of Delhi (India) which were simultaneously resistant to other heavy metals including Cu, Ni,Cd, Co, Mn and Pb. In our study, *P.stutzeri* has only shown resistance to Zn, Pb and Cr.

Sphingomonas strains were found to be highly susceptible to heavy metals. While one of the *S.paucimobilis* was resistant to zinc, the other one was resistant to zinc and lead. In a similar study in Japan, *S.paucimobilis* KPS01 was reported as highly susceptible to heavy metals (Tada and Inoue 2000). A year later, Tada et al.

(2001) suggested that *S. paucimobilis* KPS01 can be useful for routine monitoring of heavy metals as environmental contaminants, particularly in water sources. Xie et al. (2010) investigated and showed that strain of *S.paucimobilis* DT-T3-03 had high tolerance to heavy metal Zn, and had high resistant ability to many heavy metals such as copper (1.5mM), lead (1.0 mM) and nickel (1.0mM). Similar results for Zn and Pb were found in our study.

In this study, the highest tolerance to heavy metals were also found on *Methylobacterium* strains. These strains were found to highly resistance to heavy metals as Zn, Pb, Co, Cu and Ag. In this study only those strains showed resistance to silver and mercury. One of the strains were resistant to nickel and the other was resistant to chromium. Rajbanshi (2008) reported that most isolates have shown multiple tolerances to both heavy metals and antibiotics. In addition, Rajbansi (2008) has detected a cobalt resistant *Methylobacterium* spp. was only resistant to chloramphenicol and sensitive to tetracycline, ciprofloxacin, cotrimoxazol, gentamicin and ampicillin. So-Yeon and Kyung-Suk (2007) reported that *Methylobacterium* sp. SY-NiR1 showed resistance against multiple heavy metals such as cadmium, chromium, copper, lead, nickel and zinc. In addition study, one *M.mesophilicum* and two *Methylobacterium* sp. strains were reported resistant to Cu, Pb,Cd and Zn. Similar observations were reported for Zn, Ni, Co, Cr, Cd and Cu resistance in *M.mesophilicum* and *M.extorquens* strains (Piotrowska-Seget et al. 2005, Idris et al. 2006). This observation corroborates our results.

Out of the 28 bacterial tested for the presence of plasmids, 5(17.9%) isolates showed plasmid DNA bands on the agarose gel. Among the 5 plasmid containing bacterial strains, 3 strains belonged to *S.maltophilia* , one strain belonged to *P.fluorescens* and one strain belonged to *M.mesophilicum*. *S.maltophilia* MU 52 with highest numbers of plasmids was found resistant to $NiCl_2$, $ZnSO_4$, $Pb(CH_3COO)_2$, and $CuSO_4$ metals. It was also shown that plasmid carrying *S.maltophilia* have same size plasmids as 13.86 and 6.24 kb. However, high-metal multi-resistance was observed in *S.maltophilia* strains carrying slightly same size plasmids. The other two strains that contained plasmid were *P.fluorescens* MU 87 and *M.mesophilicum* MU 141. Among these strains, *P.fluorescens* MU 87 contained two plasmids with molecular weights of 21.59 and 12.68 kb, respectively, while *M.mesophilicum* MU 141 contained two plasmids with molecular weights of 21.59 and 11.61 kb, respectively. These two strains contained the plasmid 21.59 kb in size. Double resistance to Zn and Pb was detected in these strains contained 21.59 kb size plasmid. These results demonstrated that some of these bacteria obtained their heavy metal multi resistance trait through their plasmids. These isolated bacteria demonstrate that resistance to heavy metals and antibiotics by genes present on their plasmids suggests the exertion of selective pressure on such bacteria through contamination with antibiotics and heavy metals in their environment. Antibiotic-heavy metal multi resistance in some similar studies have shown the relationship between the presence of the plasmid (Piotrowska-Seget et al. 2005, Zolgharnein et al. 2007).

As a result, heavy metal resistance in *S.maltophilia, S.paucimobilis, C.luteola, M.mesophilicum* and non-aeruginosa *Pseudomonas* spp.*(P.fluorescens, P.putida* and *P.stutzeri)* were determined and plasmid profiles were obtained, relationship between the plasmid existence and heavy metal resistance was displayed.

ACKNOWLEDGMENT

This work is a part of Ozgur Ceylan's Master of Science Thesis.

REFERENCES

Akinbowale OL, Peng H, Grant P, Barton MD (2007). Antibiotic and heavy metal resistance in motile aeromonads and pseudomonads from rainbow trout (*Oncorhynchus mykiss*) farms in Australia. Int. J Antimicrob Agents 30, 177–182.

Al-Jasser AM (2006). *Stenotrophomonas maltophilia* resistant to trimethoprim-sulfamethoxazole: an increasing problem. Ann Clin Microbiol Antimicrob 5- 23.

Alonso A, Martínez JL (1997). Multiple antibiotic resistance in *Stenotrophomonas maltophilia*. Antimicrob Agents Chemother 41, 1140-1142.

Bhagat R, Srivastava S (1991). Sequestration of zinc by *Pseudomonas stutzeri*. Paper presented at International Golden Jubilee Symposium of Indian Society of Genetics and Plant Breeding.Delhi (India), 10-12 February.

Bopp HL, Chakrabarty AM, Ehrlich HL (1983). Chromate resistance plasmid in *Pseudomonas fluorescens*. J Bacteriol 155, 1105-1109.

Bruins MR, Kapll S, Oetme FW (2000). Microbial resistance in the environment. Ecotoxicol Environ Safety 45, 198–207.

Cervantes C, Gutierrez-Corona F (1994). Copper resistance mechanisms in bacteria and fungi. FEMS Microbiol Rev 14, 121–138.

Chaudhary P, Kumar R (1996). Association of metal tolerance with multiple antibiotic resistance of enteropathogenic organisms isolated from coastal region of Deltaic Sunderbans. Indian J Med Res 104, 148-151.

Chihab W, Alaoui AS, Amar M (2004). *Chryseomonas luteola* identified as the source of serious infection in a Moroccan University hospital. J Clin Microbiol 42,1837–1839.

Collins CH, Lynes, PM, Grange, JM (1995). *Microbiological Methods* (7th edition). Butter wont-Heinemann Ltd, Britain pp. 175-190.

Denton M, Kerr KG (1998). Microbiological and clinical aspects of infection associated with *Stenotrophomonas maltophilia*. Clin Microbiol Rev 11, 57-80.

Ellis RJ, Morgan P, Weightman AJ, Fry JC (2003). Cultivation dependent approaches for determining bacterial diversity in heavy-metal-contaminated soil. Appl Environ Microbiol 69, 3223–3230.

El-Sayed MS, Rehab MM, Ahmet AS (2008). Behavioral response of resistant and sensitive Pseudomonas aeruginosa S22 isolated from Sohag Governorate, Egypt to cadmium stress. Afr J Biotechnol 7(14), 2375-2385.

Filali BK, Taoufik J, Zeroual Y, Dzairi FZ, Talbi M, Blaghen M. (2000). Wastewater bacterial isolates resistant to heavy metals and antibiotics. Curr Microbiol 41, 151-156.

Foght JM, Westlake D, Johnson WM, Ridgway HF. (1996). Environmental gasoline-utilizing isolates and clinical isolates of *Pseudomonas aeruginosa* are taxonomically indistinguishable by chemotaxonomic and molecular techniques. Microbiol 42, 1333-1340.

Hada HS, Sizemore RK (1981). Incidence of plasmids in marine Vibrio spp. isolated from an oil field in the North Western Gulf of Mexico. Appl Environ Microbiol 41, 199-202.

Holmes A, Vinayak A, Benton C, Esbenshade A, Heinselman C, Frankland D, Kulkarni S, Kurtanich A, Caguiat J (2009). Comparison of Two Multimetal Resistant Bacterial Strains: Enterobacter sp. YSU and *Stenotrophomonas maltophilia* ORO2. Curr Microbiol 59, 526-531.

Horitsu H, Yamamoto K, Wachi S, Kawai K., Fukuch A (1986). Plasmid determined cadmium resistance in *Pseudomonas putida* GAM-1 isolated from soil. J Bacteriol 165, 334-335.

Idris R, Kuffner M, Bodrossy L, Puschenreiter M, Monchy S, Wenzel WW, Sessitsch A (2006). Characterization of Ni-tolerant methylobacteria associated with the hyperaccumulating plant *Thlaspi goesingense* and description of *Methylobacterium goesingense* sp. nov. Syst Appl Microbiol 29, 634–644.

Kieser T. (1984). Factors affecting the isolation of DNA from *Streptomyces lividans* and *Escherichia coli*. Plasmid 12, 19-36.

King EO, Ward MK, Raney DE (1954). Two simple media for the demonstration of phycocyanin and fluorescin. J Lab Clin Med 44, 301–307.

Koseoglu O, Sener B, Gulmez D, Altun B, Gur D (2004). *Stenotrophomonas maltophilia* as a nosocomial pathogen. New Microbiol 27, 273-279.

Kozdro´j J, van Elsas JD (2001). Structural diversity of microorganisms in chemically perturbed soil assessed by molecular and cytochemical approaches. J Microbiol Methods 43, 197-212.

Malik A, Jaiswal R (2000). Metal resistance in *Pseudomonas* strains isolated from soil treated with industrial wastewater. World J Microbiol Biotechnol 16, 177-182.

Matyar F, Kaya A, Dinçer S (2008). Antibacterial agents and heavy metal resistance in Gram-negative bacteria isolated from seawater, shrimp and sediment in Iskenderun Bay, Turkey. Sci Total Environ 407, 279-285.

Micozzi A, Venditti M, Monaco M, Friedrich A, Taglietti F, Santilli S, Martino P (2000). Bacteremia due to *Stenotrophomonas maltophilia* in patients with hematological malignancies. Clin Infect Dis 31, 705-711.

Moten AM, Rehman A (1998). Study on heavy trace metal ions in industrial waste effluents in Pakistan**.** Enviromental-expert.com, article-909.

Nies DH (2003). Efflux-mediated heavy metal resistance in prokaryotes. FEMS Microbiol Rev 27, 313–339.

Ozdemir G, Baysal SH (2004). Chromium and aluminum biosorption on *Chryseomonas luteola* TEM05. Appl Microbiol Biotechnol 64, 599-603.

Pages D, Rose J, Conrod S, Cuine S, Carrier P, Heulin T, Achouak W, Ward N (2008) Heavy metal tolerance in *Stenotrophomonas maltophilia*. PLoS ONE 3, e1539.

Pardo R, Herguedas M, Barrado E, Vega M (2003). Biosorption of cadmium, copper, lead and zinc by inactive biomass of *Pseudomonas putida*. Anal Bioanal Chem 376, 26-32.

Piotrowska-Seget Z, Cycon M, Kozdroj J (2005). Metal-tolerant bacteria occurring in heavily polluted soil and mine spoil. Appl Soil Ecol 28, 237-246.

Poole K. (2002). Outer membranes and efflux: The Path to Multidrug Resistance in Gram- Negative Bacteria. Curr Pharm Biotechnol 3, 77-98.

Raja CE, Anbazhagan K., Selvam GS (2006). Isolation and characterization of metal-resistant *Pseudomonas aeruginosa* strain. World J. Microbiol Biotech 22, 577-585.

Raja CE, Selvam GS (2009). Plasmid profile and curing analysis of *Pseudomonas aeruginosa* as metal resistant. Int J Environ Sci Tech 6(2), 259-266.

Rajbanshi A (2008). Study on heavy metal resistant bacteria in Guheswori sewage treatment plant. Our Nature 6, 52-57.

Sadhukhan PC, Ghosh S, Chaudhuri J, Ghosh DK, Mandal A (1997). Mercury and organomercurial resistance in bacteria isolated from freshwater fish of wetland fisheries around Calcutta. Environ Pollut 97(1–2), 71–78.

Singh V, Chauhan PK, Kanta R, Dhewa T, Kumar V (2010). Isolation and characterization of Pseudomonas resistant to heavy metals contaminants. Inter J Pharm Sci Rev Res 3(2), 164-167.

So-Yeon K, Kyung-Suk C (2007). Characterization of a heavy metal-resistant and plant growth-promoting rhizobacterium, Methylobacterium sp. SY-NiR1 Han'gug mi'saengmul saengmyeong gong haghoeji 35(1), 58-65.

Tada Y, Inoue T (2000). Use of oligotrophic bacteria for the biological monitoring of heavy metals. J Appl Microbiol 88, 154-160.

Tada, Y., Kobata, T., Nakaoka, C. (2001). A simple and easy method for the monitoring of environmental pollutants using oligotrophic bacteria. Lett Appl Microbiol 32, 12-15.

Thompson GA, Wattling RJ (1983). A simple method for the determination of bacterial resistance to metals. Bull Environ Contam Toxicol 31, 705-711.

Ünaldi MN, Korkmaz H, Arikan B, Coral G (2003). Plasmid-Encoded heavy metal resistance in *Pseudomonas* sp. Bull Environ Contam Toxicol 71, 1145-1150.

Valdman E, Erijman L, Pessoa FLP, Leite SGF (2001). Continuous biosorption of copper and zinc by immobilized waste biomass of *Sargassum* sp. Process Biochem 36, 869-873.

Washington JA, Sutter VL (1980). Dilution susceptibility test: agar and macrobroth dilution procedures. In: Lennette, E.H., Balows, A., Hausler, W.J., Truant, J.P., editors. Manual of clinical microbiology. ASM Press, 3rd ed. Washington, D.C., 453–458.

Wuertz S, Mergeay M (1997). The impact of heavy metals on soil microbial communities and their activities, p. 607-639. In: J.D. Elsas, J.T. Trevors, and E.M.H. Wellinngton (eds.), Modern soil microbiology, Marcel Dekker, New York, NY, USA.

Xie X, Fu J, Wang H, Liu J (2010). Heavy metal resistance by two bacteria strains isolated from a copper mine tailing in China. Afr J Biotechnol 9(26), 4056-4066.

Zhang L, Li XZ, Poole K (2000). Multiple antibiotic resistance in *Stenotrophomonas maltophilia*: involvement of a multidrug efflux system. Antimicrob Agents Chemother 44, 287-293.

Zolgharnein H, Azmi MLM, Saad MZ, Mutalib AR, Mohamed CAR (2007). Detection of plasmids in heavy metal resistance bacteria isolated from the Persian Gulf and enclosed industrial areas. Iran. J Biotechnol 5, 232-239.