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Full Length Research Paper

The effect of a continuous mercury stress on mercury reducing community of some characterized bacterial strains

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Active resistance to the mercuric ion is widely distributed in environmental microbes and results from the action of mercuric reductase. Five mercury resistant bacteria: *Escherichia coli* Z1*, E. coli* Z3*, Pseudomonas putida* Z2*, Serratia marcescens* Z4 **and *Xanthomonas* **sp. Z5 were isolated and identified from sludge sample. The presence of mercury resistance determinants was screened by polymerase chain reaction (PCR) using *merA*-specific primers. Based on the analysis of *merA* amplicons, high similarity was recorded between the *merA* region of the strains *P. putida Z2* and *Xanthomonas* sp. Z5 with those of Tn*5053*; while the *merA* of *E. coli* Z1 was analogous to those of Tn*21.* In case of the bacterial strains *E. coli* Z3 and *S. marcescens* Z4 a great matching was obtained between their *merA* and those of Tn*5036*. The effect of mercury stress upon the structure of mercury reducing biofilm at the species level and the type of mercury resistance determinants was studied in a continuous bioreactor. Community analysis suggested that the bacterial strain *E. coli* Z3 containing Tn*5036*-like determinant is well adapted strain that tolerated elevated levels of mercury whereas the other strains showed a less fitness under these extreme conditions.**

Key words: Mercury resistant bacteria, mercuric reductase gene, polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP), mercury stress.

INTRODUCTION

The removal of widespread industrial and agricultural heavy metal contamination is considered a challenge for environment management. Microorganisms in contaminated environment have developed resistance to mercury and are playing a major role in natural decontamination (Cursino et al., 1999). The detoxification of mercury by mercury resistant bacteria offers a potential cheaper and safer alternative to conventional methods; moreover, some mercury resistant bacteria can not only detoxify mercury but also remove other metals such as cadmium and lead (De et al., 2008).

Resistance against mercury has been identified in a wide range of Gram-negative and Gram-positive bacteria in natural and mercury contaminated environments and it is often found on plasmids or other mobile genetic elements such as transposons (Osborn et al., 1997; Narita et al., 2004). Mercury resistance mechanism is based on a group of genes located in a mercury resistant operons which allows bacteria to reduce the toxic Hg(II) into volatile metallic mercury Hg(0) through its enzymatic reduction (Summers, 1986; Brown et al., 1991; Misra, 1992; Barkay et al., 2003); these operons contain genes encoding the functional proteins for regulation (*merR*), transport (*merT, merP*) and reduction (*merA*) in addition to some accessory genes *(merC, merF* and *merB*) (Ji and Silver, 1995; Nies, 1999). The bioremediation of mercury from synthetic solution or wastewater via volatilization using natural or immobilized mercury resistant bacterial cells has been described by several investigators (Brunke et al., 1993; von Canstein et al., 1999, 2002; Dzair et al., 2004; Wagner-Dobler et al., 2000).

Microbial biodiversity has become a research subject for understanding engineered ecosystems. Several studies have reported the importance of measuring the microbial diversity in laboratory bioreactors in order to understand the relationship between the composition of

Table 1. Synthetic oligonucleotide primers used in this study.

Primer	Sequence 5′-3′	Amplified region	Size	References
PA	AGAGTTTGATCCTGGCTCAG	Conserved region of 16S rDNA gene	500 bp	Lane et al., 1985
530r	GTATTACCGCGGCTGCTG			
F3	GGGGGCACCTCAGAAAACGGA	IR - *merT* region of Tn*21*-like operon	730 bp	Essa et al., 2003
R4	GGAATCGCGCAGACCTCACCT			
KI	GGGGTCGTCTCAGAATTCGG	IR - *merR* region of Tn*5036*-like operon	350 bp	Essa et al., 2003
KII	GACAAGCCCTATGGCAGCAT			
MI3	GGAGTCGCCTCAGAAAACG	IR - *merR* region of Tn*5053*-like operon	500 bp	Essa et al., 2003
MI2	TACGGAGTCAAGCGATATGGA			
MRS1	ACCATCGGCGGCACCTGCG	*merA* region of Hg^r operons	1300 bp	Glendinning, 2000
MRS2	AAGGTCTG**S***GCCGC**R***AGCTTC			

S* = C+G, R* = A+G.

the microbial community and operational parameters (Liu et al., 1997; Boon et al., 2002). It is well established that toxic effects of heavy metals are highly selective in microbes; such selective targeting of specific enzymatic systems and pathways suggests that certain members of the microbial community would be more sensitive to heavy metal exposure than others, depending on the sensitivity of their critical metabolic pathways (Fulladosa et al., 2005; Sobolev and Begonia, 2008).

The aim of this study is the use of PCR-based techniques targeting the *merA* gene that codes for mercuric reductase in order to explore the functional diversity of a mercury reducing community under continuous mercury stress.

MATERIALS AND METHODS

Isolation and purification of mercury resistance bacterial strains

Luria Bertani (LB) broth supplemented with 10 µg/ml $HgCl_2$ was inoculated with sludge sample obtained from the Zenein Waste Water Treatment Plant (ZWWTP) localized in the Giza Governorate, Egypt and incubated at 30°C on shaking incubator (200 rpm) for 48 h followed by pour plate method on LB agar medium. A single bacterial colony was aseptically picked up and transferred onto a fresh medium with a streaking technique and incubated for 24 h at 30°C. Transferring was repeated until obtaining a pure bacteria culture and the isolated colonies were plated on LB agar plates supplemented with 20 µg/ml $HgCl_2$. Bacterial colonies which showed better growth on $HgCl_2$ plates were taken and streaked in the LB agar slants and stored.

Total DNA and plasmid preparation

The total bacterial DNA was prepared according to the method of Goldberg and Ohman (1984), the small scale purification of plasmid DNA was performed by the modified alkaline method of Le Gouill et al. (1994).

PCR amplification of DNA encoding the 16S rRNA gene

Amplification of the 16S rDNA gene was carried out by using primer pair pA/530r (Table 1). The PCR mixture was prepared as the following; 10 µl (10x) PCR buffer, 3 µl (50 mM) $MgCl_2$, 1 µl (20 pmole/µl) of each primer, 1 µl (10 mM) dNTPs mixture, 0.5 µl (2.5U) Taq DNA polymerase, 2 µl total DNA extract, and the volume is completed to 100 µl by SDH_2O. PCR were carried out for 35 cycles under the following conditions: denaturation step at 94°C for 40 s, annealing step at 55°C for 1 min, extension step at 72°C for 2 min and final extension at 72°C for 10 min. An aliquot of the PCR products (10 µl) was mixed with 2 µl of DNA loading buffer and analyzed by electrophoresis (15 V/cm, 60 min) on 0.7% horizontal agarose gel in TBE buffer containing 0.5 µg/ml ethidium bromide, then visualized on an UV transilluminator.

PCR for amplification of *merA* region

The purified plasmid DNA of the mercury resistant strains was used as a template in PCR by using MRS1/MRS2 primers (Table 1) to amplify the *merA* region. The PCR mixture was prepared as described above and PCR were carried out for 35 cycles under the following conditions: denaturation step at 94°C for 40 s, annealing step at 57°C for 1 min, extension step at 72°C for 2 min and final extension at 72°C for 10 min. The PCR products were analyzed as mentioned above.

Purification of the PCR products and nucleotide sequence analysis

Aqueous PCR products were purified by using a QIAquick PCR purification kit as described by the manufacturer's instructions. The purified PCR products were sequenced using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kits with Ampli Taq DNA polymerase (CliniLab, Egypt). The sequence data were analysed by BLASTN search at the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov) to identify the most similar sequences.

PCR-RFLP Pattern

According to the DNA sequence and the restriction map of the *merA* regions that were amplified from the bacterial isolates (Restriction Site Analyzer and Map Generator, www.algosome.com), the restriction enzymes: *Psh*AI, *Acc*I and *Eco*01091 (GibcoBRL, Life Technologies) were chosen to digest the *merA* amplicons (Table 2). The reaction was set up as follows;

Table 2. The restriction enzymes: *Psh*AI, *Acc*I and *Eco*01091 were used for the RFLP analysis of *merA* amplicons based on their DNA sequence.

***merA* ampilcons**	***Psh*AI**	***Acc*I**	***Eco*01091**
Tn*21*-like operon	Single cut	Do not cut	Do not cut
Tn*5036*-like operon	Do not cut	Do not cut	Single cut
Tn*5053*-like operon	Do not cut	Single cut	Do not cut

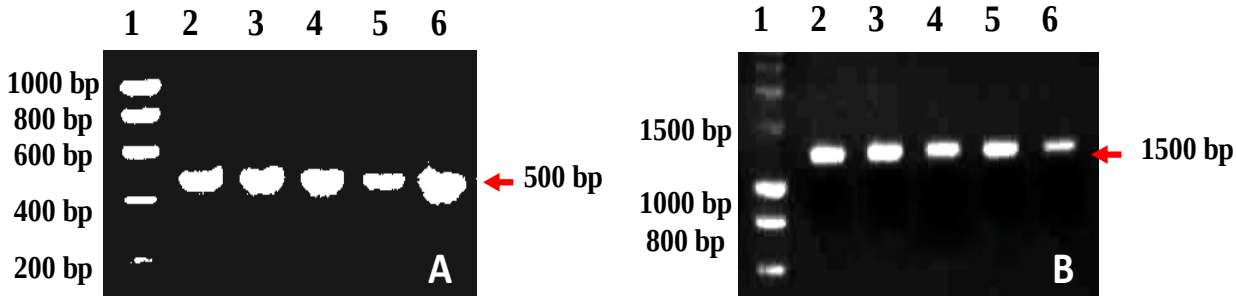

Figure 1. Gel electrophoresis of PCR products of: A) the partial 16S rDNA gene of the bacterial isolates Z1 (lane 2), Z2 (lane 3), Z3 (lane 4), Z4 (lane 5) and Z5 (lane 6), B) the *merA* gene from plasmid DNA of *Escherichia coli* Z1 *(*lane 2)*, Escherichia coli* Z3 (lane 3)*, Pseudomonas putida* Z2 (lane 4)*, Xanthomonas* sp. Z5 (lane 5), *Serratia marcescens* Z4 (lane 6). Lane 1 in both figures contains Hyperladder I marker.

1.5 µg PCR product, 5 µl restriction enzyme, 10 µl (10x) restriction enzyme buffer, and the volume was completed up to 100µL by sterile distilled water. The reaction was incubated at 37°C for 1 h. After inactivation (65°C for 20 min), the reaction mixture was mixed with 0.2 volume of DNA loading buffer and analyzed by electrophoresis.

PCR with primers specific for the different *mer* determinants

According to the obtained DNA sequence of *merA* region, some primers were used to discriminate between the different *mer* operons (Table 1). The purified plasmid DNA of the mercury resistant strains was used as a template in PCR by using the following primers E3/E4 to identify the Tn*5075* operon, MI3/MI2 to identify the Tn*5053* operon and KI/KII to identify the Tn*5036* operon. The PCR mixture was prepared as described above. PCR were carried out for 35 cycles under the following conditions; denaturation step at 94°C for 40 s, annealing step at 56°C for 1 min, extension step at 72°C for 2 min and final extension at 72°C for 10 min. The PCR products were analyzed as mentioned above.

Bioreactor setup and operation

The mercury resistance bacterial isolates from the sludge sample, which contains different mercury resistant determinants, were grown individually in LB broth supplemented with 10 µg/ml $HgCl_2$ at 37°C on a shaking incubator at 200 rpm for 24 h. A 25 ml of each culture were mixed together and used as an inoculum for the bioreactor which contains about 1.5 L LB broth. The bioreactor was maintained under aerobic conditions by pumping in filter-sterilized air, 37°C for 30 days. The LB broth supplemented with $HgCl_2$ (10 to 60 µg/ml) was flowed through the bioreactor at 100 ml/h and the bacterial growth was monitored by measuring the protein content. Moreover, the DNA extracted from the effluent of the bioreactor during the operating period was subjected to PCR by using specific primers for the different determinants (Table 1). At the end of the experiment (30 days), the community composition at the strain level was analyzed by 16S ribosomal DNA analysis. At the same time, the RFLP technique was used to profile the type of the mercury resistant determinants based on their *merA* genes. The protein content was used to follow the bacterial growth under different $HgCl_2$ concentrations. Samples from the effluent of the bioreactor were centrifuged for 10 min (10,000 rpm), and cell pellets were re-suspended in 500 µl of NaOH (0.5 M) and lyzed for 1 h. The protein content was estimated according to the method of Lowry et al. (1951).

RESULTS AND DISCUSSION

Isolation and characterization of mercury resistant bacteria

The mercury resistant bacterial strains designated Z1, Z2, Z3, Z4 and Z were isolated from the sludge sample (ZWWTP) and were identified by partial 16S ribosomal DNA technique (Figure 1A). The purified PCR products were sequenced and databank compared. The isolates Z1 and Z3 were identified as *Escherichia coli* (99.6 and 99.9% identity, respectively), isolate Z2 was identified as *Pseudomonas putida* (96.7% identity), isolate Z4 was

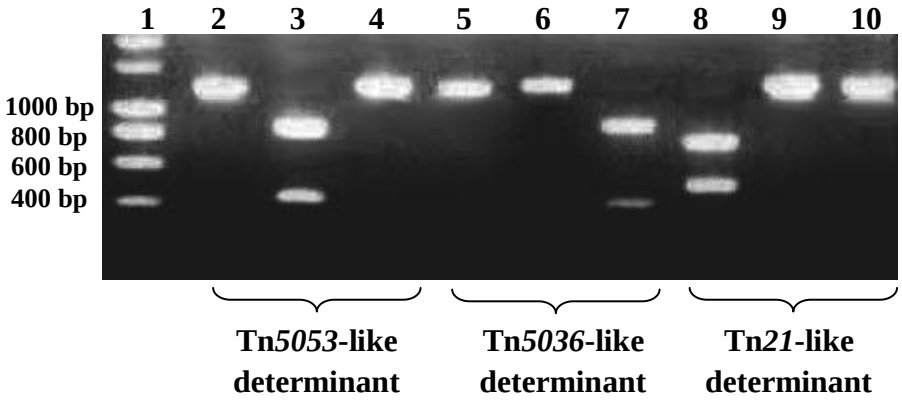

Figure 2. Gel electrophoresis of RFLP pattern of *merA* amplicons digested with *Eco*01091, *Acc*I and *Psh*AI. *Xanthomonas* sp. Z5 containing Tn*5053*-like determinant is represented in lanes (2 to 4), *S.marcescens* Z4 containing Tn*5036*-like determinant is represented in lanes (5 to 7) and *E. coli Z*1 containing Tn*21*-like determinant is represented in lanes (8 to 10). Digestion by *Eco*01091 is represented in lanes (4, 7 and 10), *Acc*I in lanes (3, 6 and 9) and *Psh*AI in lanes (2, 5 and 8). Lane 1 contains Hyperladder I marker.

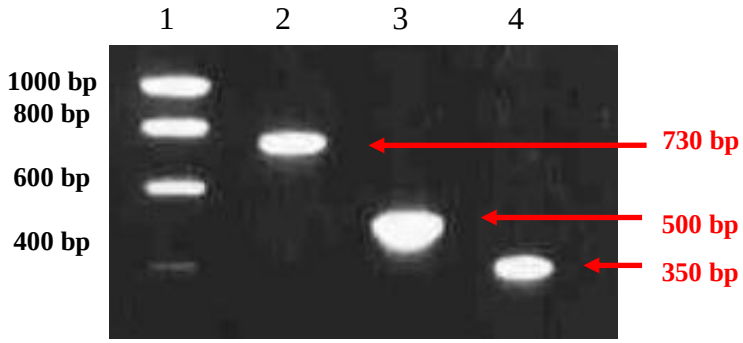

Figure 3. Gel electrophoresis of PCR products from plasmid DNA of *E. coli Z*1 containing Tn*21*-like determinant by using primers E3/E4 (lane 2), *Xanthomonas* sp. Z5 containing Tn*5053*-like determinant by using primers MI3/MI2 (lane 3) and *S. marcescens* Z4 containing Tn*5036*-like determinant by using primers KI/KII (lane 4). Lane 1 contains the hyperladder I marker.

identified as *Serratia marcescens* (99.7% identity), and isolate Z5 was identified as *Xanthomonas* sp. (97.8% identity).

The purified plasmid DNA of each mercury resistant strain was screened by PCR for *merA* genes. Results in Figure 1B demonstrated the presence of the *merA* gene (approximately 1300 bp) in the mercury resistant isolates. The purified PCR products were sequenced and databank compared; the *merA* region of *E. coli* Z1 strain showed a high similarity to those of Tn*21* (99%, Liebert et al., 1999). In case of *P. putida* Z2 and *Xanthomonas* sp. Z5 the amplified *merA* region recorded the highest identity to those from Tn*5053* (96 and 98%, Kholodii et al., 1995) while, the amplified merA region of *E. coli* Z3 and *S. marcescens* Z4, showed the highest identity to those from Tn5036 (96 and 99%, Yurieva et al., 1997).

According to the DNA sequence and the restriction map of the *merA* region of the different determinants (Restriction Site Analyzer and Map Generator, www.algosome.com), some restriction enzymes were used to digest the *merA* amplicons (Table 2) resulting in a specific RFLP pattern (Figure 2). *Psh*AI digested the *merA* of Tn*21*-like determinant into two fragments (760 to 480 bp), *Acc*I digested the *merA* of Tn*5053*-like determinant into two fragments (880 to 360 bp) whereas *Eco*01091 digested the *merA* of Tn*5036*-like determinant into two fragments (790 to 450 bp); moreover, PCR with specific primers (Table 1) was used to confirm the type of these determinants in the bacterial strains. Data in Figure 3 showed that PCR amplicons were obtained by using primers E3/E4 with Tn*21*-like determinant (730 bp), MI3/MI2 with Tn*5053*-like determinant (500 bp), and

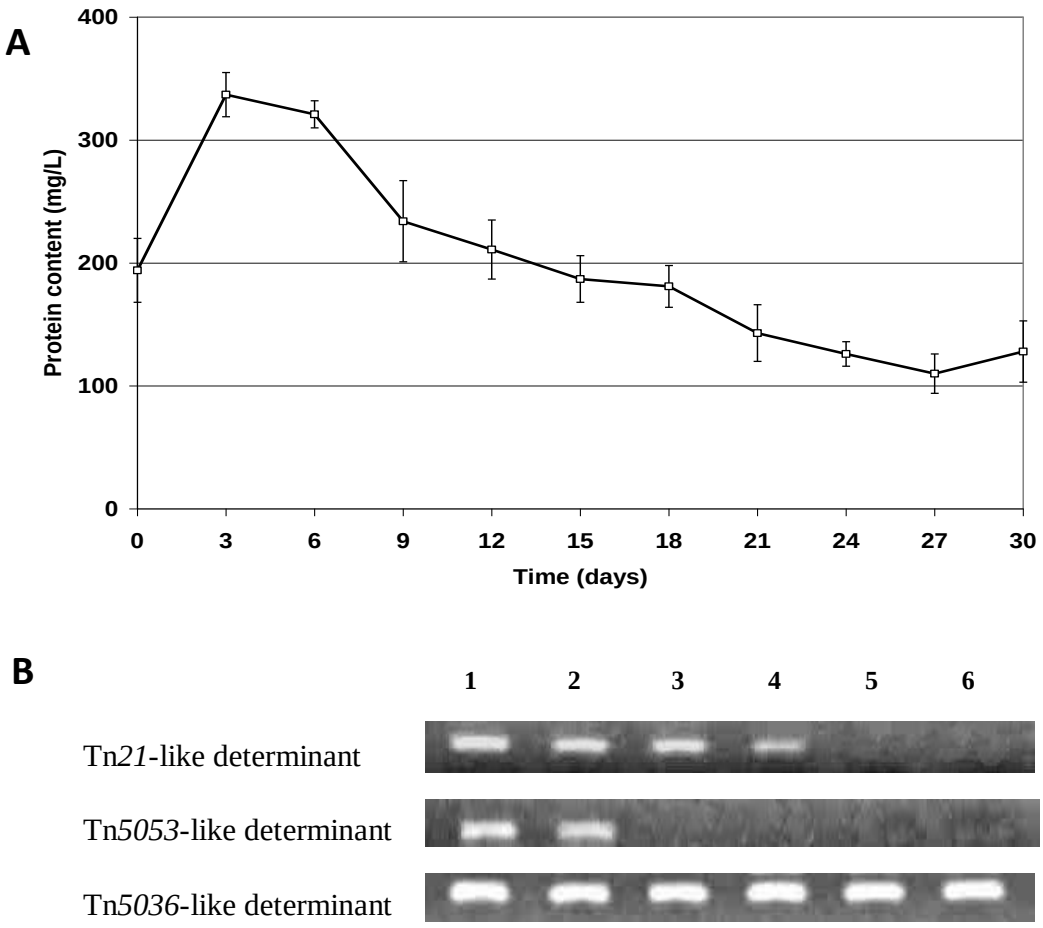

Figure 4. (A) The growth of a mercury resistance bacterial population (expressed as mg/mL protein) consisting of *E. coli* Z1, *E.*Z3, *P. putida* Z2, *Xanthomonas* sp. Z5 and *S. marcescens* Z4 in a continuous aerobic bioreactor for 30 days on LB broth supplemented with different $HgCl_2$ concentrations: 20 µg/mL up to the 3rd day, 30 µg/ml up to the 9th day, 40 µg/ml up to the 15th day, 50 µg/ml up to the 21st day, 60 µg/ml up to the end of the operation.(B) The gel electrophoresis of PCR products from plasmid DNA of the bioreactor effluent obtained at the start of the experiment (lane 1), at 6 days (lane 2), at 12 days (lane 3), at 18 days (lane 4), at 24 days (lane 5), at 30 days (lane 6) by using primers KI/KII for Tn*5036*-like determinant, MI3/MI2 for Tn*5053*-like determinant and E3/E4 for Tn*21*-like determinant. Lane 1 contains Hyperladder I marker.

KI/KII with Tn*5036*-like determinant (350 bp).

Effect of mercury stress on a mercury reducing biofilm

The isolated mercury resistant strains: *E. coli Z1 and Z3, P. putida Z2, Xanthomonas* sp. Z5 and *S. marcescens* Z4, were grown together inside a continuous bioreactor for 30 days under selective continuous mercury stress and the total protein content was monitored as a parameter for the bacterial growth (Figure 4A). At the same time, the DNA extracted from the effluent of the bioreactor upon the pilot plant operation was subjected to PCR by using specific primers for the different mercury resistance determinants. The obtained data (Figure 4B) demonstrated that the Tn*50*53-like determinant disappeared after 18 days (at 40 µg/ml of $HgCl_2$), the Tn*21*-like determinant vanished after 24 days (at 60 µg/ml of $HgCl_2$) whereas the Tn*5036*-like determinant recorded a high tolerance capability under these extreme conditions.

At the end of the experiment (after 30 days), the composition of the mercury resistant community at the strain level was analyzed on the basis of the 16S ribosomal DNA gene showing the presence of only *E. Coli* (Z3), meanwhile the other strains were completely gone. The use of 16S rRNA gene as a marker to study the composition and the dynamics of some bacterial communities has been reported in previous studies

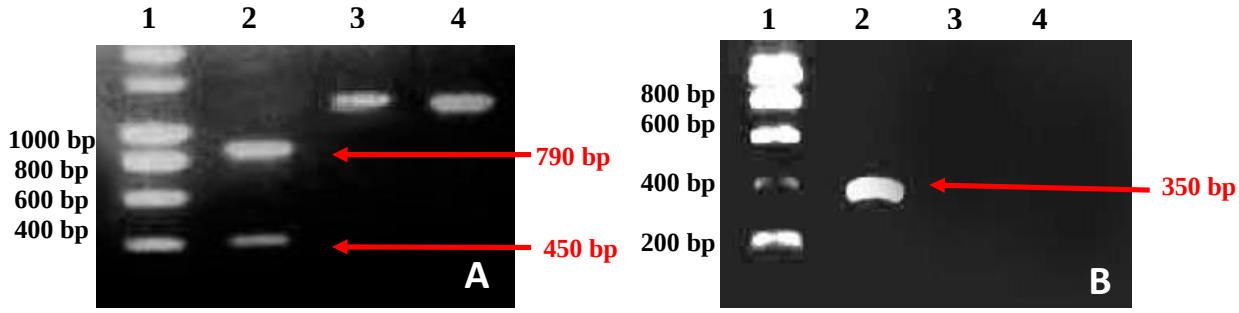

Figure 5. The gel electrophoresis of: A) RFLP pattern of *merA* amplicons obtained from plasmid DNA of the bioreactor effluent at the end of the experiment (30 days) digested with *Eco*01091 (lane 2), *Acc*I (lane 3) and *Psh*AI (lane 4), B) the PCR products from plasmid DNA of the bioreactor effluent obtained after 30 days by using primers KI/KII (lane 2), MI3/MI2 (lane 3) and E3/E4. Lane 1 contains Hyperladder I marker.

(Wagner-Dobler et al., 2000; Saikaly et al., 2005).

Actually, the alteration of a bacterial community in laboratory bioreactors under the influence of the heavy metals stress by targeting of some marker genes especially those responsible for the resistance mechanism will produce more accurate image for the biodiversity of the bacterial population; so the mercury resistant community was analysed to profile the type of their determinant through using RFLP analysis of the obtained *merA* amplicons which showed the presence of Tn*5036*-determinant while the other determinants were not found (Figure 5A). These results were confirmed via subjecting the DNA extracted from the effluent of the bioreactor after 30 days to PCR by using some specific primers for the different *mer* determinants (Figure 5B). A clear DNA band (350 bp) was obtained with primers MR3/MR2 that are specific to Tn*5036*-like determinant whereas no PCR products were obtained by using the primers of the other determinants. These results are compatible with those who used the *merA* gene as a molecular marker to follow the assortment of mercury resistant bacterial population under the pressure of mercury toxicity in aerobic and anaerobic environments (Felske et al., 2003; Simbahan et al., 2005; Sotero-Martins et al., 2008).

This study clarified the presence of a strong selective pressure on the microbial community inside the bioreactor due to the mercury toxicity which led to the predomination of *E. coli* (Z3) containing Tn*5036*-like determinant. These results are in accordance with other studies which showed that the continuous exposure to elevated levels of mercury altered the microbial community and exclusively select bacteria that can cope with such levels (Osborn et al., 1993; Müller et al., 2001; Ramaiah and De, 2003).

The domination of *E. coli* Z3 containing *Tn5036*-like determinant over the other strains under the strong selective pressure exerted by mercury toxicity was attributed to the well adaptation of this strain which might be linked with the type of mercury resistance determinant. This assumption is consistent with the finding of previous studies that correlated the functional diversity and the adaptation ability of some bacterial communities under the influence of mercury with the frequency and the type of mercury resistance operon (Smalla et al., 2006; Chien et al., 2010); moreover, the capability of *E. coli* Z3 to tolerate an elevate level of mercury could be based on the presence of some additional mechanisms beside mercury volatilization that give this organism an extra-tolerance capability to cope with such mercury stress. Agreeing with this hypothesis, Haferburg and Kothe (2007) reported that the adaptation to heavy metal rich environments resulted in microorganisms which show activities for biosorption, bioprecipitation, extracellular sequestration and chelation. Such resistance mechanisms may play a role in transforming the toxic metals into other forms that are not biologically available to the cells. One of these mechanisms is the precipitation of the soluble metal ions away from the cells via its complexation into insoluble metal precipitates via the production of some metabolites (Essa et al., 2006).

Finally, an environment with a raised concentration of heavy metals constitutes a prospective stimulus for toxic metal tolerant bacteria; such polluted environments encourage adaptation for heavy metal resistance and markedly affect on the composition of a bacterial biofilm. The use of *merA* gene, the key enzyme of mercury volatilization, as molecular markers in order to follow the diversity of mercury resistant bacterial population under the pressure of mercury toxicity can provide significant information about the functional alterations of these communities especially in contaminated environments. Despite of the intrinsic role of the different *mer* operons in mercury detoxification process, work is still necessary to illustrate the distribution and diversity of these genetic determinants in the bacterial communities under heavy metals stress in order to employ them for the

bioremediation of these toxic pollutants.

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