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Heavy Metal Resistant of *E. coli* Isolated from Wastewater Sites in Assiut City, Egypt

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Abstract Twelve isolates of *E. coli* were isolated from wastewater of El-Malah canal located in Assiut, Egypt and were checked for their heavy metal tolerance. One isolate has tested for its multiple metal resistances and found to be plasmid mediated with molecular weights 27 and 65 kb for hexa- and trivalent chromium. It was identified as *E. coli* ASU 7. Its minimal inhibitory concentration (MIC) for Cu^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Cr^{6+}, Cr^{3+}, Cd^{2+} and Pb^{2+} were 1.57, 2.55, 1.7, 9.17, 0.48, 7.69, 4.4 and 3.1 mM, respectively. Growth kinetics was determined under Cr^{6+} and Cr^{3+} stress. SDS-PAGE of protein profile shows that 10 ppm (0.19 mM) of Cr^{6+} induces new protein with molecular weight 23 kDa.

Keywords Heavy metal resistance · *E. coli* · Antibiotics resistance · Wastewater

Heavy metals are major toxicant found in industrial wastewater and may adversely affect the biological treatment of wastewater. Its specific weight is more than 5.0 g/cm^3 (Volesky 1990; Bishop 2002). Heavy metal cations play an important role in many biochemical reactions due to its ability to form complex compounds (Neis 1999; Rossbach et al. 2000). Some heavy metals (Co, Cu, Fe, Mn, Ni and Zn) are essential, serve as micronutrients and are used to stabilize molecules through electrostatic interactions; as components of various enzymes; and regulation of osmotic pressure (Bruins et al. 2000). They function as catalysts for biochemical reactions, are stabilizers of protein structures and bacterial cell walls (Bruins et al. 2000). The growth requirements for essential heavy metals are normally very low. Many other metals have no biological role (e.g. Ag, Al, Cd, Au, Pb and Hg), are nonessential (Bruins et al. 2000) and potentially toxic to living organisms, especially microorganisms. Toxicity of nonessential metals occurs through the displacement of essential metals from their native binding sites or through ligand interactions alterations in the conformational structure of nucleic acids and proteins or interference with oxidative phosphorylation and osmotic balance (Poole and Gadd 1989; Bruins et al. 2000). In addition, at high levels, both essential and nonessential metals can damage cell membranes, alter enzyme specificity, disrupt cellular functions, bind with greater affinity to thiol-containing groups and oxygen sites than do essential metals and damage the structure of DNA (Bruins et al. 2000; Hughes and Poole 1989; Hussein et al. 2005). Microbial survival in polluted soils depends on intrinsic biochemical and structural properties, physiological, and/or genetic adaptation including morphological changes of cells, as well as environmental modifications of metal speciation (Ehrlich 1997; Wuertz and Mergeay 1997). 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 et al. 2001; Wuertz and Mergeay 1997). Chromium (Cr) is an essential trace element for all living organisms. The valence state of Cr ranges from −2 to +6, but only the trivalent and the hexavalent forms of chromium appear to be of significance, and only the trivalent form appears to be essential. Trivalent chromium is necessary for fat and glucose metabolism and proper functioning of insulin (Thacker et al. 2007). The trivalent is mobile, while hexavalent is

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easily soluble and 100-fold more toxic than trivalent chromium. Hexavalent chromium has been recognized as one of the most dangerous environmental pollutants due to its ability to cause mutations, irritation, corrosion of the skin and respiratory tract to most microorganisms; it also causes lung carcinoma in humans (Liu et al. 2006; Bhinde et al. 1996; Ganguli and Tripathi 2002). Microorganisms can play an important role in removal of hexavalent chromium from the polluted sites (Thacker et al. 2007).

The objective of this study is to determine heavy metals and antibiotic resistance of *E. coli*, checking for its plasmid-encoded metal-tolerance, MIC, growth parameters and SDS-PAGE under hexa- and trivalent chromium stress.

Materials and Methods

Different polluted water samples were collected in sterile glass bottles from different sites in El-Malah canal, Assiut city (Egypt); it is far about 3 km from the main River Nile, in the west site of Assiut city which exposed to domestic sewage disposal and industrial effluent. Temperature, pH and Electrical conductivity were determined by water checker model Horiba U-10 and according to (Elith and Garwood 2001).

E. coli was enumerated from wastewater samples by Most Probable Number (MPN) techniques using lactose broth medium (MacFaddin 1985). The positive tubes were confirmed on Eosin Methylene Blue Agar (E.M.B). Twelve isolates were selected randomly from E.M.B, purified and were preserved on nutrient agar for further studies.

In order to minimize the complexation of heavy metals, the isolates were grown in tris minimal broth medium (Mergeay 1995). Tolerance to heavy metals was determined by an agar dilution method (Washington and Sutter 1981). The plates containing 20 mL of media of above medium at different concentrations (1–12 mM) of heavy metals studied Cd^{2+}, Cr^{6+}, Cr^{3+}, Pb^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+}. The plates inoculated with cultures and incubated at 37°C for 2 days. In the case of lead toxicity test, the amount of sodium β-glycerophosphate was reduced to 0.02 g/L, omitting tris buffer and adjusting pH to 6.0 to limit metal precipitations. Among the isolates, isolate ASU 7 showed high resistance to heavy metals. Isolate have primarily been identified by phenotypic characterization according to (Bergey's manual 2005).

Minimal inhibitory concentration (MIC) for different heavy metals was registered on agar plates of tris minimal medium using agar dilution methods and confirmed on broth tris minimal medium, for broth medium a 5 mL of tris minimal medium containing different concentration of heavy metals salts $CuSO_4 \cdot 5H_2O$, $Co(NO_3)_2 \cdot 6H_2O$, $Ni(NO_3)_2 \cdot 6H_2O$, K_2CrO_4, $Cr(NO_3)_2 \cdot 9H_2O$, $(CHCOO)_2Zn \cdot 2H_2O$, $Cd(NO_3)_2 \cdot 2H_2O$ and $Pb(NO_3)_2$ inoculated with 200 µL of an 18 h old culture of the studied bacterial strains *E. coli* ASU 7 (resistant) and *E. coli* DH5α (sensitive) incubated at 37°C for 2 days. The lowest concentration of heavy metals that completely preventing growth known as MIC (Yilmaz 2003).

The susceptibility of *E. coli* ASU 7 to various antibiotics was tested by the disk diffusion method (Baur et al. 1966). The following antibiotics discs were used: (N) Neomycin (5 mg/L), (AK) Amikacin (300 µg/L), (OFX) Ofloxacin (5 mg/L), (CIP) Ciprofloxacin (5 mg/L), (CN) Gentamicin (30 mg/L), (CAZ) Cefatazidime (30 mg/L), (NOR) Norfloxacin (10 mg/L) and (VA) Vancomycin (30 mg/L).

The plasmids were isolated from *E. coli* ASU 7 by the modified miniprep method (Birnboimm and Doly 1979). The isolated plasmids were characterized by agarose gel electrophoresis according to the standard procedure of (Sambrook et al. 1989).

To confirm the plasmid-encoded tolerance to the metals studied, competent cells of *E. coli* DH5α, sensitive to heavy metals, were transformed with respective plasmids using the standard chemical method (Sambrook et al. 1989). The suspensions (100 µL) of transformed *E. coli* DH5α were plated on tris minimal media supplemented with 0.38 or 3.85 mM of hexa- or trivalent chromium. Plasmid DNA of transformant and nontransformant one of *E. coli* DH5α were compared with a resistant one by using agarose gel electrophoresis.

The growth rate and growth parameters of resistant strain of *E. coli* ASU 7 and sensitive one of *E. coli* DH5α to hexa- and trivalent chromium were determined according to (Filali et al. 2000; Ghosh et al. 1997).

The protein patterns were analyzed using SDS-PAGE according to (Laemmli 1970). The LabImage 1D (2006) program was used in molecular weight determination of plasmid DNA and protein.

Results and Discussion

The temperature, pH and Electrical conductivity ranged from 23.7 to 27°C, 7.9 to 8.12 and 0.34 to 0.35 ms/cm, respectively. The concentration of Cr expressed in mg/L and ranged from 0.38 to 0.88 mg/L. It was noted that the obtained results exceeded the safe limit of WHO (2005) it is 0.05 mg/L of chromium.

MPN of this study indicated that *E. coli* were present in very substantial numbers in the samples of wastewater investigated. It was ranged from 3,500 to 5,400 colonies/100 mL at different sites of El-Malah canal, Assiut city (Egypt). Twelve isolates were selected randomly on (EMB), purified and were preserved on nutrient agar for further studies.

The purified isolates of *E. coli* were tested for tolerance against essential heavy metals (Cu^{2+}, Co^{2+}, Ni^{2+}, Cr^{6+}, Cr^{3+}, Zn^{2+} and nonessential ones (Cd^{2+} and Pb^{2+}) at concentrations 0.09–12 mM. The percentages of the tolerated isolates to various concentrations of heavy metals ion are shown in Table 1. The results indicated that most of isolates grew well at low concentrations of heavy metals and their numbers gradually decreased as the concentration increased. For essential heavy metals studied all the isolates were able to grow at concentration 0.09 mM, but hexvalent chromium was considered the most toxic heavy metals since above concentration 0.2 mM, no isolates were grew. For cadmium all isolates tolerated to concentration 2 mM and drastically decreased by 67.7% at 4 mM. In other wise lead is consider as a toxic heavy metals since 84.4% of the isolates were inhibited by 2 mM and there's no isolate can grew above this concentration. These results differ from that reported by (Hassen et al. 1998) they stated that three strains of *Pseudomonas aeruginosa* (S6, S7 and S8) isolated from natural polluted environments on nutrient agar were tolerant to $CuSO_4$, $Cr_2(SO_4)_3$, $CoSO_4$ corresponding to 1.6, 1.2 and 0.8 mM, respectively of metal ions concentrations. The order of toxicity of heavy metals towards the isolates of *E. coli* are $Cr^{6+} > Cu^{2+} > Co^{2+} > Pb^{2+} > Ni^{2+} > Cr^{3+} > Cd^{2+} > Zn^{2+}$ Table 1; so the chromium is more toxic than Zn^{2+}. This result are in agreement with (Yilmaz 2003) who found the order of toxicity to *Bacillus* EB1 are $Cd^{2+} = Co^{2+} > Cu^{2+} > Ni^{2+} > Zn^{2+} > Mn^{2+}$. Among the isolates, *E. coli* ASU 7 showed high resistance to the above mentioned metals it resistance 0.38, 5.77 and 2.41 mM for Cr^{6+}, Cr^{3+} and Pb^{2+}, respectively.

The isolate *E. coli* ASU 7 was identified according to (Bergey's manual 2005), it was characterized by: single or in pair's rod-shaped motile, gram-negative, nonspore forming. It gave positive reactions with the following tests: indole test, methyl red, catalase test, while it gave negative reactions with oxidase test, citrate test, hydrogen sulfide and voges-proskauerar, The obtained results show that the latter isolate identified as *E. coli* ASU 7 based on phenotypic methods.

The minimum inhibitory concentration of the resistant strain *E. coli* ASU 7 and sensitive one *E. coli* DH5α to heavy metals were registered in tris minimal broth and on solid media are shown in Table 2. The values for resistance and sensitive one in liquid media are 1.57, 2.55, 1.7, 9.17, 0.48, 7.69, 4.4 and 2.8; 1.2, 1.9, 1.7, 1.5, 0.19, 1.33, 1.9 and

Table 1 Percentage of tolerated isolates of *E. coli* at different concentrations of heavy metals

	% of tolerance (mM)											
	0.09%	0.2%	0.5%	0.8%	1%	2%	3%	4%	6%	8%	10%	12%
Cu^{2+}	100	100	58	8.3	8.3	0	0	0	0	0	0	0
Co^{2+}	100	100	100	75	75	8.3	0	0	0	0	0	0
Ni^{2+}	100	100	100	25	25	16.6	8.3	8.3	0	0	0	0
Cr^{6+}	100	75	0	0	0	0	0	0	0	0	0	0
Cr^{3+}	100	100	100	83.3	83.3	25	25	16.6	0	0	0	0
Zn^{2+}	100	100	100	100	100	91.6	91.6	91.6	91.6	33.3	3.33	8.3
Cd^{2+}	100	100	100	100	100	100	91.6	33.3	8.3	0	0	0
Pb^{2+}	100	100	75	16.6	41.6	16.6	0	0	0	0	0	0

Table 2 MIC for different heavy metals and antibiotics resistance of *E. coli* ASU 7

Metal	MIC (mM) in tris minimal broth medium		MIC (mM) in tris minimal solid medium		Antibiotics	Resistance
	Strain (ASU 7)	*E. coli* DH5α	Strain (ASU 7)	*E. coli* DH5α		
Copper	1.57	1.2	2.33	1.57	Neomycin (5 mg/L)	S
Cobalt	2.55	1.9	3.4	2.2	Amikacin (300μg/L)	S
Nickel	1.7	1.7	2.55	2.1	Ofloxacin (5 mg/L)	R
Zinc	9.17	1.5	10.7	2	Ciprofloxacin (5 mg/L)	S
Cr^{6+}	0.48	0.19	0.57	0.28	Gentamicin (30 mg/L)	S
Cr^{3+}	7.69	1.33	8.6	1.7	Cefatazidime (30 mg/L)	R
Cadmium	4.4	1.9	5.3	2.66	Norfloxacin (10 mg/L)	S
Lead	2.8	0.96	3.1	1.92	Vancomycin (30 mg/L)	R

Abbreviations: MIC: minimum inhibitory concentrations and defined as the lowest concentration of metal that completely preventing growth; R: resistance; S: sensitive

0.96 mM, for Cu^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Cr^{6+}, Cr^{3+}, Cd^{2+} and Pb^{2+}, respectively. The MIC on solid medium for the metals studied was higher than in liquid medium and ranged from 0.57 to 10.7 mM on solid medium while in the broth from 0.48 to 7.69 mM. The MIC on solid medium was higher than in broth where the condition of diffusion, complexation and availability of metals were different from those observed on solid medium. This suggests that organic matter component on the latter forming complexes with heavy metals (Pb^{2+}, Cd^{2+} and Zn^{2+}); thereby reducing the concentration of free metal ion. This observation is in agreement with (Hassen et al. 1998; Mergeay 1995; Babich and Stotzky 1977). *E. coli* ASU 7 was able to grow at high concentration of heavy metals in liquid media which might be important for the capacity of bacterium to survive in different sources of pollution with elevated heavy metal levels. Our results show high tolerance and are in agreement with (Ünaldi et al. 2003) they isolated strains of *Pseudomonas* on GSP agar medium from soil treated with industrial wastewater with MIC 4, 7 and 6 mM for nickel, cadmium and cupper, respectively. While (Piotrowska-seget et al. 2005) isolated *Pseudomonas gladioli* from soil polluted with heavy metals on nutrient agar and found MIC are 10, 3, 0.1 and 3 mM for Zn^{2+}, Cd^{2+}, Pb^{2+} and Cu^{2+}, respectively.

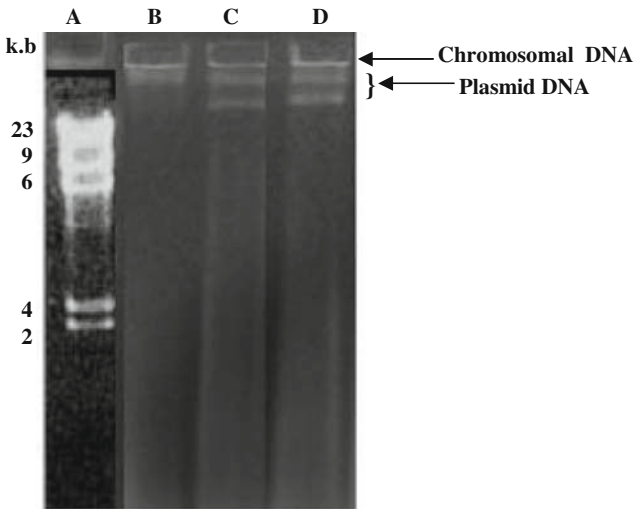

Fig. 1 Plasmid transformation lane A lambda (DNA) marker cleaved with *Hind* II, lane B sensitive strain of *E. coli* DHα5, lane C is resistance strain of *E. coli* ASU 7 and lane D transformed strain of *E. coli* ASU 7

Microorganisms resistant to antibiotics and metals appear to be the result of exposure to metal-contaminated environments that cause coincidental selection for resistance factors for both (Spain 2003). The resistances to antibiotic for *E. coli* ASU 7 are shown in Table 2. It was found that it was resistant to the following antibiotics Cefatazidime, Vancomycin, Ofloxacin but Sensitive to Amikacin, Ciprofloxacin, Neomycin, Norfloxacin and Gentamicin. Many investigators have reported that metal resistance

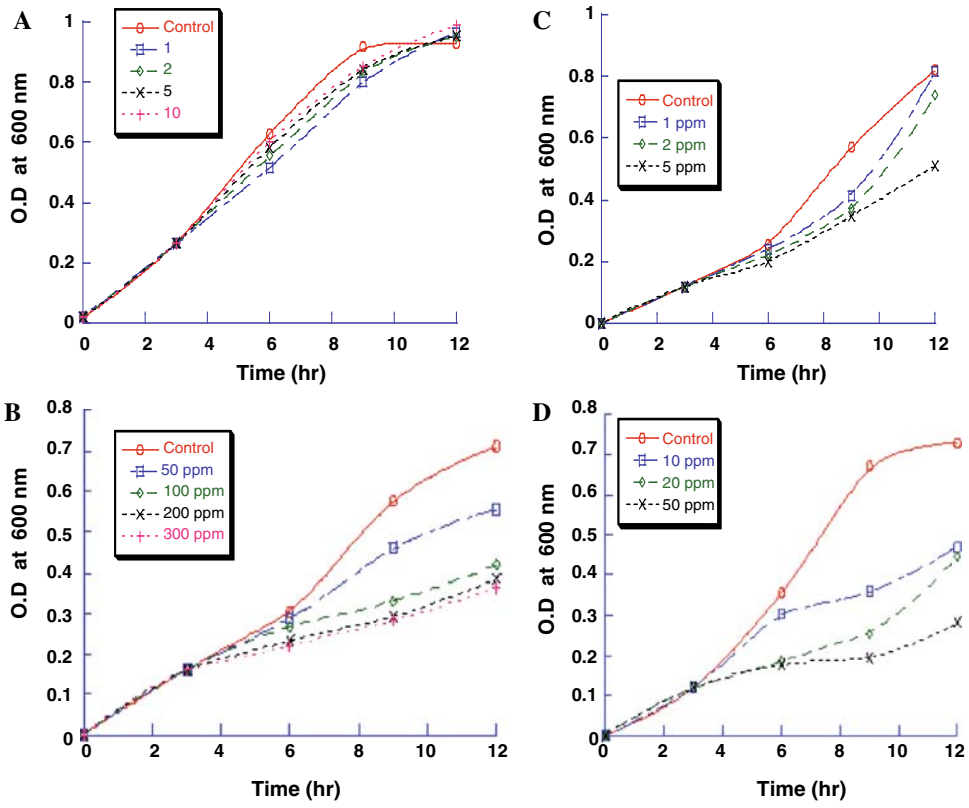

Fig. 2 The growth curves of resistant strain *E. coli* ASU 7 to Cr^{6+} (**A**), Cr^{3+} (**B**) and sensitive strain *E. coli* DHα5 to Cr^{6+} (**C**), Cr^{3+} (**D**) at different concentration grown in tris minimal medium at 37°C with agitation at 150 rpm for 12 h. Heavy metals were added after 3 h of incubations

environmental isolates are resistant to a wide array of antibiotics and there are correlation between heavy metal resistance and antibiotic resistance (Bezverbnaya et al. 2005; Pal et al. 2004; Soltan 2001; Dhakephalker et al. 1994; Hassen et al. 1998; Ramteke 1997; Sabry et al. 1997).

The agarose gel electrophoreses pattern of the resistant strains *E. coli* ASU 7 shows that it have two plasmid with molecular weights 27 and 65 kb. To determine whether the resistance markers on *E. coli* ASU7 on plasmid DNA, The transformation was carried on a recipient cell of *E. coli* DH5α. By comparing the growth results, it was found that the transformant strain of *E. coli* DH5α can grow on tris minimal agar containing 0.38 or 3.85 mM of hexa- or trivalent chromium, respectively while the nontransformed cannot. The plasmid composition of viable transformants and original isolate were confirmed by agarose gel electrophoresis. The results showed that the transformation of the plasmids was successful Fig 1.

Metal-tolerant bacteria have evolved various resistance and detoxification mechanisms (Ledrich et al. 2005). The resistance mechanisms are chromosomally encoded or, more often, plasmids of different size and showing conjugative capabilities are carriers of metal-resistance genes (Piotrowska-Seget et al. 2005). In this study, *E. coli* ASU 7contain two plasmids with molecular weights 27 and 65 kb which responsible for metal resistance as confirmed by transformation experiments. These results indicated that the plasmid can replicate in *E. coli* DH5α and that it possesses the genetic information necessary for the expression of resistance of hexa- or trivalent chromium. The results are in agreement with (Piotrowska-Seget et al. 2005; Ünaldi et al. 2003).

The growth curve of tolerant strain *E. coli* ASU 7 and *E. coli* DHα5 to different concentrations of hexa- and trivalent chromium were shown in Fig. 2A–D. The heavy metals are added after 3 h of incubations. It was noted that both heavy metals causes a reduction in growth depending on metal type and toxicity as the concentration increased compared with control. This closely agrees with many investigators (Hussein et al. 2005; Pal et al. 2004; Yilmaz 2003; Filali et al. 2000; Sharma et al. 2000; Hassen et al. 1998).

The growth parameters on *E. coli* ASU 7 and *E. coli* DHα5 are shown in Table 3. In general the results depict

Table 3 Growth parameters of resistant strain of *E. coli* ASU 7 and *E. coli* DHα5 at different concentration of nickel and lead

Heavy metals conc. (ppm)	T_λ(h)	T_α(h)	a	K (h)	T (h)
ASU7 K_2CrO_4					
Control	3	12	6.14	0.14	4.95
1	3	12	6.147	0.14	4.92
2	3	12	6.16	0.142	4.87
5	3	12	6.16	0.142	4.85
10	3	12	6.2	0.146	4.72
$CrCl_3$					
Control	3	12	3.55	0.162	4.27
50	3	12	3.32	0.136	5.09
100	3	12	3.04	0.105	6.6
200	3	12	2.96	0.096	7.22
300	3	12	2.86	0.088	7.87
DHα5 K_2CrO_4					
Control	3	12	6.016	0.214	3.23
1	3	12	6.01	0.213	3.25
2	3	12	5.91	0.202	3.43
5	3	12	5.54	0.161	4.3
$CrCl_3$					
Control	3	12	5.89	0.2	3.46
10	3	12	5.45	0.151	4.59
20	3	12	5.4	0.146	4.74
50	3	12	4.94	0.09	7.37

T_λ: lag period, T_α: the end of the exponential phase, a: a symbiotic level = $\ln(OD/OD_0)$, K: the maximum specific growth rate and T: generation time

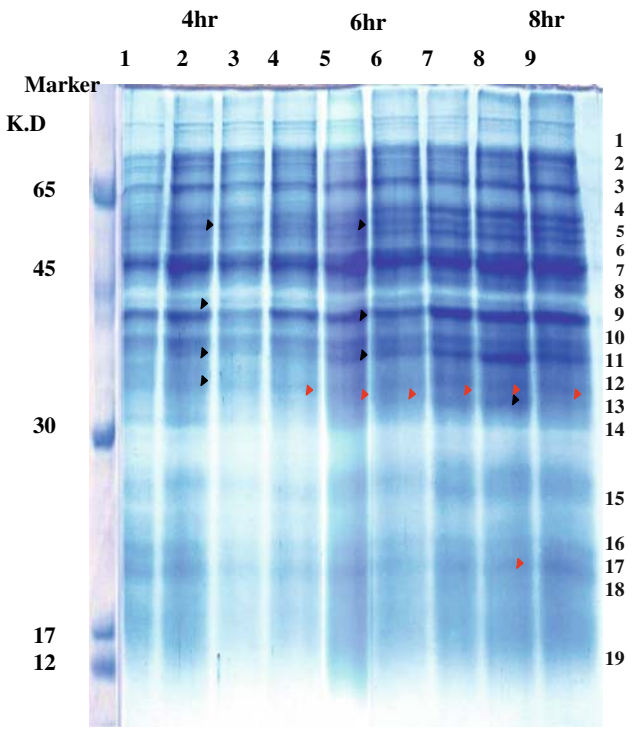

Fig. 3 Soluble protein patterns (coomassie blue stained 12% SDS-PAGE gel) of whole cell lyste of *E. coli* ASU7, 9 lane grown in tris minimal medium containing 0 ppm Cr^{+6} or Cr^{+3} control, 10 ppm Cr^{6+} and 40 ppm Cr^{3+} incubated for 4, 6 and 8 h at 37°C. Lane 1, 4, 7 control (0 ppm Cr^{6+} or Cr^{3+}), lane 2, 5, 8 Cr^{6+} 10 ppm, lane 3, 6, 9 Cr^{3+} 40 ppm after 4, 6, 8 h respectively (►) Red arrowheads point represents new bands that appeared. (▶) Black arrowheads point represents bands that increased in intensity under stress of Cr^{6+}

that the optical density and the maximum specific growth rate (K) decreased while the generation time increased by increasing heavy metals concentration. These results are in agreement with (Ghosh et al. 1997) they stated that growth parameters of *Acidocella* strains resistant to heavy metals have along lag period and long generation time. Either trivalent or hexavalent decreases protein content of the bacterium at high concentrations but in case of *E. coli* ASU 7 the maximum specific growth rate (K) increased while the generation time decreased by increasing the concentration of hexavalent chromium, this phenomena resulted from hexavalent chromium (soluble) reduced to trivalent one (insoluble) so the optical density increased and turbidity appeared.

The inducible response of metal stress (Cr^{6+} and Cr^{3+}) of *E. coli* ASU 7 was studied. The electrophoratic analysis in 12% SDS-PAGE of whole cells lysate protein is summarized in Fig 3. It was obvious that Cr^{6+} induce new protein with molecular weight 23 kDa after 8 h This group of proteins may be responsible for chromate resistance. Our results are in agreement with to that obtained by (Thacker et al. 2007) they reported that protein with molecular weight 30 kDa induced in presence of chromium and this may possibly be associated with resistance of chromate. Similarly (Patel et al. 2006) showed the induction of proteins with molecular weight 48 and 18 kDa proteins which play a role in metal resistance mechanism and induced under nickel stress by a nickel resistant strain of *Pseudomonas fragi*.

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