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Chromium(VI) Resistance and Extracellular Polysaccharide (EPS) Synthesis by *Pseudomonas*, *Stenotrophomonas* and *Methylobacterium* Strains

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Microorganisms have great value in research, for multiple purposes during the last several decades. Resistance of various strains of microorganisms against chromium and the production of the extracellular polysaccharide (EPS) had been experimented and reveals the fact that the most of the strains (*Pseudomonas*, *Stenotrophomonas*, *Methylobacterium*) which produced high concentration of EPS shown resistance to Cr(VI). The most resistant *Methylobacterium mesophilium* MU141 (435 mg/L) secreted higher EPS than the rest of the strain. The toxic effect of Cr(VI) on cell viability and total cell protein of *M. mesophilium* MU 141 was treated by different concentration of Cr(VI), ranged from 15 and 35 ppm for 24 h. Hence, concentration of Cr(VI) can affect the EPS production at certain spectrum. Therefore, the result discloses the correlation between Cr(VI) resistance and EPS production among *Pseudomonas*, *Stenotrophomonas*, *Methylobacterium* strains.

KEY WORDS: Chromium (VI) toxicity; Extracellular Polysaccharide; Methylobacterium; Pseudomonas; Stenotrophomonas

1. Introduction

Toxic heavy metals that cause environmental contamination are of increasing economic, public health and environmental significant.[1)] Chromium is a heavy metal of particular importance. It is extensively used in industry, which creates contamination to both aquatic (fresh water and marine water) as well as terrestrial life. In general Cr(VI) is considered to be of greatest concern due to studies in mammalian systems that show it to be carcigenic and toxic.[2)] Several studies have found that metals influence microorganisms by adversely affecting their growth, morphology, and biochemical activities, resulting in decreased biomass.[3,4)] Despite these toxic stresses, most of the microorganisms have evolved metal resistance, detoxification mechanisms, including volatilization, extra cellular precipitation and exclusion, intracellular sequestration and off course membrane-associated metal pumps.[3,5)]

Microbial based metal remediation realize on the ability of some microorganisms to resist and detoxify metals. Metals, like all elements, are not biodegradable and can only be transformed from one chemical state to another.[6)] In laboratory studies, Cr(VI) is actively transported into cells and then intracellularly reduced and effluxed as Cr (III) *via* the ChrA system.[7)] In *Pseudomonas* spp., Cr (III) then accumulates on the cell wall and outer membrane.[8)] Similar processes have been documented for other bacterial species and toxic metals.[9–12)] Recently, surface complexation models have been invoked to describe the adsorption of metals onto individual functional group sites on the bacterial cell wall.[13–15)]

Microbial polysaccharides forms thick layer outside the cell wall, called extra cellular polysaccharides.[16)] There is tremendous structurally diversification among EPS with unique properties.[17)] Biosynthesis of EPS is a most prominent feature of several bacteria that also offers a protective barrier to cell against environmental stresses, heavy metal stress could be one such.[18)] Also, bacterial-produced exopolymer plays a crucial role in the metal biosorption process. In addition, exopolymer is important in the formation and maintenance of bacterial biofilms.[19,20)] The role of exopolymer in biofilm production is to mediate attachment of bacteria to surfaces and to aid in the formation of the complex biofilm structure.[20,21)] Biofilm exopolymer is composed of many biogenic components; however, possibly the most significant one is extracellular polysaccharide or EPS.[22)]

It is interesting to see interaction of heavy metal with microorganisms. Metal cations bind to cell surface polymers through several of mechanisms, such as cation exchange, complexation, coordination and precipitation reactions.[23)] The absorption mechanisms vary with the metal and the bacterial strain. The effects of acid, the concentrations of available nutrients and oxygen on bacterial surfaces are important for the sorption of metals by bacteria.[15,24)] Metal adsorption on the bacterial EPS vary widely in specificity and

affinity depending on the constituents of individual polymers. EPS polymers bind with metals highly efficiently and have been shown to enhance bacterial metal absorption by several orders of magnitude.[25)]

In recent years microorganisms have been used for the production of valuable polysaccharide for various industrial applications.[26)] Some of these are xanthan, gellan, welan gums, and dextrans, have been commercially used in food, pharmaceuticals industries and in oil well drilling.[17,27)] A number of lactic acid bacteria are known to produce EPSs that can be beneficial for the texture of dairy products. Generally, they may replace polysaccharides used in the food industry as thickeners, stabilizers, emulsifiers, bodying agents, foam enhancers and gelling agents.[28)]

The present investigation is aimed to determine correlation between metal resistance and EPS production by some *Pseudomonas*, *Stenotrophomonas* and *Methylobacterium* strains. It is also aimed to investigate the toxic effect of Cr(VI) concentrations on EPS production, viability and total protein concentrations by *Pseudomonas*, *Stenotrophomonas*, and *Methylobacterium* strains.

2. Experimental

Thirteen *Pseudomonas*, two *Stenotrophomonas*, and one *Methylobacterium* strains were obtained from the Culture Collection of the University of Mugla. The species, codes of the strains and original habitats are listed in **Table 1**.

2.1. Bacterial Culture

Bacteria were cultured in Nutrient Broth (NB) (Difco) at 30±0.1°C for 18–24 h. The bacterial cultures were maintained in Nutrient Agar (NA) (Difco) slants at 4°C and used as stock cultures.

2.2. Isolation of EPS

Bacterial EPS was isolated as described by Cérantola *et al.*[29)] Each strain was grown on Pseudomonas Agar P (Difco) medium supplemented with 2% (w/v) glycerol for 3 d at the appropriate temperature (30°C or 37°C). Agar plate cultures were then washed with saline (0.9% NaCl solutions) using a glass rod and the resulting suspensions [Optical density (OD) at 600 nm, 22±0.05] were stirred with glass beads to detach EPS associated with the bacterial cells. Cells were then removed by centrifugation at 10 000×g for 30 min at 4°C. The resulting supernatants were precipitated overnight at 4°C with six volumes of 95% ethyl alcohol (EtOH). Precipitated EPS was recovered by centrifugation, and the EtOH precipitation step was repeated. After centrifugation (12 000×g for 30 min at 4°C), pellets were dissolved in distilled water. Total EPS (expressed as mg/L) was estimated in each sample by the phenol-sulfuric method using glucose as the standard.[30)] All experiments were performed in duplicate.

2.3. Cr(VI) Resistance

Cr(VI) resistance of *Pseudomonas*, *Stenotrophomonas*, and *Methylobacterium* strains was determined by the agar dilution method.[31)] Solutions of different metal concentrations were prepared by dissolving $K_2Cr_2O_7$ salt (Merck) in distilled water to reach metal concentrations of 10, 20, 30, 40, 50 and 60 ppm. Cr(VI) solutions were sterilized by filtration with a 0.2 μm pore size filter. Plates containing 20 mL of one-half strength Pseudomonas Agar P Medium [supplemented with 2% (w/v) glycerol] with different Cr(VI) concentrations were inoculated with 100 μL of overnight culture of *Pseudomonas*, *Stenotrophomonas*, or *Methylobacterium* and incubated at 37°C for 48 h. Also, plates containing medium lacking Cr(VI) were inoculated in the same manner to serve as controls. Cr(VI) resistance was evaluated by comparison with the control.

2.4. Viability, Total Protein and EPS Production by *M. mesophilicum* MU141

The viability, total protein, and EPS production by *M. mesophilicum* MU141 were determined in the presence of 15 or 35 ppm Cr(VI) concentrations. *M. mesophilicum* MU141 was selected on the basis of its resistance to Cr(VI).

Table 1. EPS production and resistance of *Pseudomonas*, *Stenotrophomonas*, and *Methylobacterium* strains against different concentrations of Cr (VI)

			Cell viability					
			Concentrations of Cr(VI) (ppm)					
Strains[a]	EPS values (mg/L)*	Control	10	20	30	40	50	60
Pseudomonas aeruginosa MU187[1]	381±5	+	+	+	+	-	-	-
Pseudomonas aeruginosa MU189[1]	268±3	+	+	+	+	-	-	-
Pseudomonas aeruginosa MU188[1]	270±5	+	+	+	+	-	-	-
Stenotrophomonas maltophilia MU137[2]	232±8	+	+	+	-	-	-	-
Stenotrophomonas maltophilia MU52[2]	232±7	+	+	+	-	-	-	-
Methylobacterium mesophilicum MU141[2]	435±3	+	+	+	+	+	+	+
Pseudomonas luteola MU172[1]	408±7	+	+	+	+	+	-	-
Pseudomonas luteola MU174[1]	395±4	+	+	+	+	-	-	-
Pseudomonas putida MU180[1]	294±4	+	+	+	+	-	-	-
Pseudomonas stutzeri MU194[1]	323±5	+	+	+	+	-	-	-
Pseudomonas putida MU171[1]	181±3	+	+	-	-	-	-	-
Pseudomonas putida MU169[1]	122±6	+	+	-	-	-	-	-
Pseudomonas putida MU181[1]	220±18	+	+	+	-	-	-	-
Pseudomonas mendocina MU195[1]	237±5	+	+	+	-	-	-	-
Pseudomonas stutzeri MU193[1]	136±7	+	+	-	-	-	-	-
Pseudomonas pseudoalcaligene MU196[1]	415±8	+	+	+	+	+	+	-

[1] Isolated from Torba Harbor of Bodrum, Mugla
[2] Isolated from skin of *Anguilla anguilla*
[a] Codes of strains according to the Culture Collection of *Pseudomonas*, *Stenotrophomonas*, and *Methylobacterium* strains of Mugla University
* Values are the means ±SD of dublicate measurements.
+: resistance
-: susceptibility

Total viable counts of *M. mesophilicum* MU141 were determined by a pour plate method using nutrient agar after serial dilution in maximum recovery diluents. Nutrient plates were incubated at 37°C. The toxic effect of Cr(VI) was determined by counting colonies at 2-h intervals for 12 h. Viable cell counts were given as log cfu mL^{-1} (colony formation unit). Total protein concentrations of *M. mesophilicum* MU141 were determined by Bradford assay using a reagent supplied by Amresco (Solon, OH, USA). Bacterial EPS was estimated as described by Cérantola *et al.*[29]

2.5. Effect of Cr(VI) on EPS production by *M. mesophilicum* MU141

Equal biomasses of *M. mesophilicum* MU141 were inoculated onto plates containing Pseudomonas Agar P Medium with different Cr(VI) concentrations (5, 15, 25 or 35 ppm). *M. mesophilicum* MU141 was incubated at 37°C for 48 h. For each Cr(VI) concentration, an equal biomass of cells was collected. EPS was isolated as described by Cérantola *et al.*[29] and total EPS (mg/L) was estimated by the phenol-sulphuric method.[30]

2.6. Experimental Design and Statistical Analysis

The experiment was performed in a completely randomized fashion with five replicates. Each analysis was conducted on two samples from each replicate. Results of each representative experiment were analyzed by ANOVA using Statistica software (Statsoft, Tulsa, Okla), and differences between groups were detected with Dunnett and Tukey grouping tests set at an $\alpha=0.05$ level of significance.

3. Results

3.1. EPS Production

EPS production by *Pseudomonas*, *Stenotrophomonas*, and *Methylobacterium* strains was assessed during their growth in batch culture (Table 1). The range of EPS production by the strains was 122–435 mg/L. Maximum EPS production (435 mg/L) was determined for *M. mesophilicum* MU141 and minimum EPS production was detected for *Pseudomonas putida* MU169 (122 mg/L).

3.2. Cr(VI) Resistance

Evaluation of metal toxicity was based on the agar dilution method for thirteen *Pseudomonas*, two *Stenotrophomonas*, and one *Methylobacterium* strains. Cr(VI) concentrations of 10–60 ppm were examined. The most resistant strains, *M. mesophilicum* MU141, *Pseudomonas pseudoalcaligene* MU196, *Pseudomonas luteola* MU172, could grow at 40–60 ppm Cr(VI) for 48 h. *P. putida* MU169, *Pseudomonas stutzeri* MU193, and *P. putida* MU171 were the most sensitive to Cr(VI), surviving only at the lowest Cr(VI) concentration (10 ppm) after 48 h. Also, it was determined that, higher EPS-producing strains of *Pseudomonas*, *Stenotrophomonas*, and *Methylobacterium* were more tolerant to Cr(VI) (Table 1).

3.3. Viability, Total Protein and EPS Production by *M. mesophilicum* MU141 and Effect of Cr(VI) on EPS Production

Cr(VI) (15–35 ppm) had significant toxic effects on viability, total protein concentrations, and EPS production by *M. mesophilicum* MU141 compared with the control (**Fig. 1**). Cell viability of *M. mesophilicum* MU141 was reduced by 25.4% at 15 ppm and by 39.5% at 35 ppm after 12 h (Fig. 1(a)).

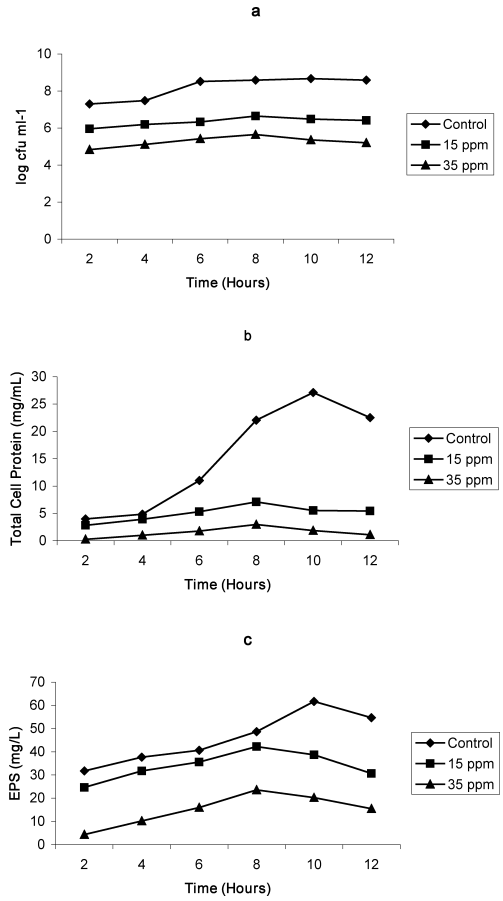

Fig. 1. Toxic effect of Cr(VI) on viability (a), on total cell protein (b) and on EPS production (c) of *M. mesophilicum* MU141.

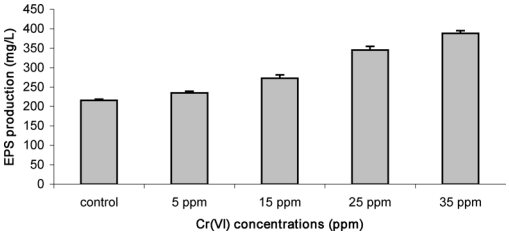

Fig. 2. Effect of Cr(VI) concentrations (5, 15, 25, 35 ppm) on EPS production of *M. mesophilicum* MU141. Statistical analysis was performed using ANOVA. Different concentrations were significantly different by Dunnet and Tukey tests homogeneity of proportions ($P<0.05$). Bars represent standard deviations.

EPS production by *M. mesophilicum* MU141, selected on the basis of its high EPS production and Cr(VI) resistance, was examined in the presence of 5, 15, 25 or 35 ppm Cr(VI) concentrations for determining effect of Cr(VI) on EPS production. Equal biomasses were used to determine EPS production after 48 h. Significant increases in EPS production were observed with increasing Cr(VI) concentrations (ANOVA: $F_{4,5}=133.54$, $P=0.0001$; **Fig. 2**).

4. Discussion

Bacterial exopolymers have attracted interest as potential metal-binding agents for the detoxification of contaminated waters.[32] There are many reports on metal accumulation by several microorganisms.[33,34] Although hyperproduction of EPSs in response to starvation, dehydration, or antibiotic stress has been reported (especially for *Pseudomonas* strains), the effects of heavy metals on such polymer production are less well studied.[18] Also, there are no conclusive reports on the correlation between EPS production and heavy metal resistance. Some observations for different organisms[35] indicated that extracellular anionic polysaccharide-producing bacteria are less susceptible to heavy metals than the non-producing variants due to reduced free metal ion concentration at the cell surface.[36]

In the present study, different strains grown under the same conditions had significant differences in EPS production. The correlation between Cr(VI) resistance and EPS production was examined for some *Pseudomonas*, *Stenotrophomonas*, and *Methylobacterium* strains. *M. mesophilicum* MU141, *P. pseudoalcaligene* MU196, and *P. luteola* MU172 were the most resistant to Cr(VI) and also produced the highest amounts of EPS. In contrast, *P. putida* MU169, *P. stutzeri* MU193, and *P. putida* MU171 were the most sensitive to Cr(VI) and produced the lowest amounts of EPS. A similar correlation between metal resistance and EPS production was found in a study of *Pseudomonas aeruginosa* BU1 and BU2. EPS production by the Cu-resistant strain *Pseudomonas aeruginosa* BU1 was considerably higher than by its Cu-sensitive counterpart.[18] Likewise, Richau *et al.*[36] reported that EPS-producing strains of *P. aeruginosa* and *Sphingomonas paucimobilis* were more tolerant to sublethal concentrations of copper than were EPS-defective variants. Also, Looijesteijn *et al.*[37] reported that production of EPS by *Lactococcus lactis* increased resistance to copper. Studies of *Enterobacter cloaceae* grown in sea water showed that Cr(VI) exposure resulted in increased EPS production and metal binding to cells and EPS.[38] Many bacteria can reduce chromate under aerobic and anaerobic conditions[39] *via* constitutive chromate reductases.[40,41] Rapid chromate reduction to Cr (III) has been observed for *Pseudomonas* spp. by some authors.[40,42,43] Thus, the Cr(VI) resistance of our strains may result from chromate reduction.

Cr(VI) has toxic effects in many organisms and can inhibit microbial growth.[44] Bacterial growth was estimated by measurement of total cellular protein using the Bradford method.[11,45–47] Cell viability also served as a visual check for contamination.[11] 15 and 35 ppm Cr(VI) concentrations were tested for *M. mesophilicum* MU141 to evaluate toxic effect. The effects of Cr(VI) toxicity were observed in terms of viability, inhibition in total cell protein and EPS production. It was determined that EPS production, viability and cellular protein concentrations of *M. mesophilicum* MU141 were decreased at 15 and 35 ppm Cr(VI) after 8th hour. Singh *et al.*[48] reported that Ni, Cu, and Hg decreased EPS production by *Nostoc spongiaeforme* according to viability.

M. mesophilicum MU141 was selected for investigating the effect of Cr(VI) (5–35 ppm) on EPS production. After 48 h of Cr(VI) exposure, equal biomasses for each Cr(VI) concentration were analyzed. When compared with the control, a correlation was found between EPS production by *M. mesophilicum* MU141 and increasing Cr(VI) concentrations. These results indicated that high Cr(VI) concentrations can increase EPS production by *M. mesophilicum* MU141. Many reports have shown that stress factors such as salt, cold, and UV promote EPS production.[49] The present study indicates that, Cr(VI) may be an important stress factor that positively affects EPS production. Thus, yields of commercially important, microorganism-originated EPS products may be increased by Cr(VI) treatment.

EPS production by different strains of bacteria may be an important criterion for the selection of strains effective in the recovery of heavy metals. Furthermore, *M. mesophilicum* MU141, which produces high amount of EPS, may be favorable for heavy metal bioremediation and industrial applications.

Very little information is available on potential EPS-mediated heavy metal resistance mechanisms or on the effects of heavy metals on EPS production, especially in the case of practical applications. Thus, further research in this area is warranted.

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