Heavy Metals Induced Modulations in Growth, Physiology, Cellular Viability, and Biofilm Formation of an Identified Bacterial Isolate

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ABSTRACT: The release of untreated tannery effluents comprising biotoxic heavy metal (HM) compounds into the ecosystem is one of our society’s most serious environmental and health issues. After discharge, HM-containing industrial effluents reach agricultural soils and thus negatively affect the soil microbial diversity. Considering these, we assessed the effect of HMs on identified soil beneficial bacteria. Here, the effects of four heavy metals (HMs), viz., chromium (Cr), cadmium (Cd), nickel (Ni), and lead (Pb), on cellular growth, physiology, cell permeability, and biofilm formation of Enterobacter cloacae MC9 (accession no.: MT672587) were evaluated. HMs in a concentration range of 25–200 μg mL⁻¹ were used throughout the study. Among HMs, Cd in general had the maximum detrimental effect on bacterial physiology. With increasing concentrations of HMs, bacterial activities consistently decreased. For instance, 200 μg Cr mL⁻¹ concentration greatly and significantly (p ≤ 0.05) reduced the synthesis of indole-3-acetic acid (IAA) by 70% over control. Furthermore, 200 μg mL⁻¹ Cd maximally and significantly (p ≤ 0.05) reduced the synthesis of 2,3-dihydroxybenzoic acid (2,3-DHBA), salicylic acid (SA), 1-aminocyclopropane 1-carboxylate (ACC) deaminase, and extra polymeric substances (EPSs) of strain MC9 was completely inhibited at 150, 175, and 200 μg mL⁻¹ concentrations of Cr and Cd. The confocal laser scanning microscopic (CLSM) analysis of HM-treated bacterial cells showed an increased number of red-colored dead cells as the concentration of HMs increased from 25 to 200 μg mL⁻¹. Likewise, the biofilm formation ability of strain MC9 was maximally (p ≤ 0.05) inhibited at higher concentrations of Cd. In summary, the present investigation undoubtedly suggests that E. cloacae strain MC9 recovered from the HM-contaminated rhizosphere endowed with multiple activities could play an important role in agricultural practices to augment crop productivity in soils contaminated with HMs. Also, there is an urgent need to control the direct discharge of industrial waste into running water to minimize heavy metal pollution. Furthermore, before the application of HMs in agricultural fields, their appropriate field dosages must be carefully monitored.

INTRODUCTION

The primary sources of environmental contamination that contribute large quantities of radioactive metals to the atmosphere including soils are industrial waste, tanning plants, sewage waste, and many other metal discharging industries. Biologically nondegradable heavy metals that remain in the atmosphere pose a significant threat to biological entities including plants, microbial population, fertility, soil microorganisms, and their associated activities. These issues desperately need to be resolved to sustain soil quality and crop productivity at the same time. Considering these, a number of microorganisms belonging to the plant growth-promoting rhizobacteria (PGPR) community and comprising the distinctive properties of heavy metal tolerance and plant growth promotion are reported. Soil beneficial microbes that have the ability to resist either one or multiple metals reduce toxic effects of heavy metals by a variety of mechanistic processes such as exclusion, extracellular and intracellular sequestration, and conversion of toxic forms of HMs into less toxic forms.

According to the World Health Organization (WHO), cadmium (Cd), chromium (Cr), cobalt (Co), copper (Cu), lead (Pb), nickel (Ni), mercury (Hg), and zinc (Zn) are the
most dangerous metals. Chromium, among HMs, is a product of chrome plating, wood manufacturing, metal chelating, and many other industries. It is one of the most toxic metals among the most popular heavy metal pollutants and it affects both plants and microbes negatively and adversely. Of the nine oxidation states of chromium, hexavalent chromium \([\text{Cr(VI)}]\) is extremely stable and highly toxic to microbes as well as agricultural crops. Generally, Cr(VI) is typically inhibitory because of (i) cell membrane permeability (ii) ability to interact with macromolecules (DNA and protein), and (iii) cell water solubility.

A number of reports are available on the toxic effect of heavy metals on soil beneficial microbes and their associated activities. In this context, Thomas and Benov investigated the mechanistic behavior of Cd on *Escherichia coli* after heavy metal exposure. They reported that higher concentrations of Cd damage the DNA, denature the protein, and inhibit the process of cell division and transcription in bacterial cells. The growth and microbial activities of nitrate-reducing *Pseudomonas stutzeri* RCH2 strain were negatively influenced when bacterial cells were treated with Cr(VI). Similarly, in other studies, it has been reported that Cu disrupted the cellular function, inhibited enzymatic activities, retarded growth and cellular viability, and induced oxidative stress in numerous soil bacterial species. The biochemical constituents, enzymatic activities, lipid content, and cell membrane of a soil isolate were severely affected by single and mixed exposure of Ni and Cr. Similarly, two HMs, Cd and Pb, inhibited the bacterial growth, denatured the nucleic acids and proteins, and disrupted the enzymes and caused damage to the membrane.

Considering these threatening problems associated with bacteria, the current study was carried out to (i) determine the MICs of four HMs, viz., Cr, Cd, Pb, and Ni, toward the bacterial isolates recovered from the rhizosphere, (ii) assess the effect of heavy metals on growth, viability, and cellular permeability of *Enterobacter cloacae* strain MC9, (iii) evaluate the impact of HMs on growth-regulating substances such as indole-3-acetic acid and phenolate siderophores: SA and 2,3-dihydroxybenzoic acid (2,3-DHBA) synthesized by *E. cloacae*, (iv) determine the influence of HMs on ACC deaminase enzyme activity and extra polymeric substances (EPSs) released by *E. cloacae* MC9, (v) evaluate the P solubilization potential of *E. cloacae* in the presence of HMs, and (vi) observe the effect of HMs on the biofilm formation ability of *E. cloacae* MC9.

### RESULTS AND DISCUSSION

#### Heavy Metal Tolerance and Strain Identification

A total of 15 bacterial isolates were retrieved from the chili rhizosphere grown in metal-polluted areas. The ability of the bacterial isolates to withstand various metals (Cd, Cr, Pb, and Ni) was also tested. Among them, strain MC9 exhibited maximum tolerance to Cd (2000/800), Cr (1600/600), Pb (1800/1000), and Ni (1200/400) when cultured on solid (agar plates)/liquid (broth) nutrient medium (Table 1), respectively. Generally, the tolerance for heavy metals in nutrient agar plates was generally higher than that for nutrient broth (NB). The strain’s higher heavy metal resistance with increasing nutrient agar may be attributed to the polymeric aspect of the solid agar medium-limiting metal supply, as suggested by other workers. On the other hand, HMs may have become readily accessible to bacteria due to their greater solubility in aqueous conditions (nutrient broth). Whatever the cause, high levels of heavy metals reduce bacterial population, likely due to metal ion surface binding and degradation of membrane functions.

Table 1. Minimum Inhibitory Concentration (MIC) of Heavy Metals

| bacterial isolates | Cd NA/NB | Cr NA/NB | Pb NA/NB | Ni NA/NB |
|--------------------|----------|----------|----------|----------|
| MC1                | 1600/400 | 1000/400 | 1600/600 | 1000/400 |
| MC2                | 1200/300 | 800/200  | 1200/300 | 1200/300 |
| MC3                | 1000/200 | 800/200  | 1200/400 | 1000/200 |
| MC4                | 1800/400 | 1000/300 | 1200/300 | 1200/300 |
| MC5                | 2000/500 | 1200/400 | 1200/300 | 1000/200 |
| MC6                | 1000/200 | 1000/400 | 1000/200 | 600/100  |
| MC7                | 1600/400 | 1200/400 | 1000/200 | 1200/300 |
| MC8                | 1600/400 | 1200/300 | 1600/400 | 1000/200 |
| MC9                | 2000/800 | 1600/600 | 1800/1000| 1200/400 |
| MC10               | 1200/400 | 1000/200 | 1000/200 | 1200/300 |
| MC11               | 1000/200 | 1200/300 | 1000/200 | 1000/200 |
| MC12               | 1600/400 | 1400/400 | 1200/300 | 1200/300 |
| MC13               | 1800/600 | 1800/1000| 1200/300 | 1000/200 |
| MC14               | 1200/300 | 1200/300 | 1000/200 | 800/200  |
| MC15               | 1200/400 | 1000/200 | 800/100  | 800/100  |

Each value is a mean (mean ± standard deviation, SD) of three independent replicates.

Furthermore, bacterial resistance/tolerance to one or more metals may be due to a variety of mechanisms developed within bacteria, including metal efflux through ATPases, complexation of different intracellular materials, metal binding to bacterial cell envelopes, or reduction of toxic metal types to less toxic forms. In a comparable research investigation, greater amounts of manganese and other metals were tolerated by Gram-negative *Rhizobium halophytocola* strain RT7 and Gram-positive *Bacillus circulans* insulated from industrial soils. Similarly, *Pantoea agglomerans* was shown to withstand Cd up to 3000 g mL\(^{-1}\), while *Enterobacter asburiae* was found to tolerate 2000 g mL\(^{-1}\) in nutrient broth medium. Furthermore, strain MC9 was identified due to its strong multimetal tolerance and capacity. Moreover, the strain exhibited variable biochemical reactions (Table 2). On the basis of various biochemical tests, strain MC9 was identified as *Enterobacter* sp. at the genus level and *E. cloacae* (GenBank accession no.: MT672587) by 16S rDNA sequence analysis. Figure 1 shows a phylogenetic tree (built with MEGA 6.0’s neighbor-joining algorithm) of various bacteria and isolate MC9, with sequencing data submitted in NCBI GenBank.

#### Bioactive Molecules Synthesized by *E. cloacae* MC9 were Negatively Affected by HMs

Here, strain MC9 was discovered to have high PGP potential as well as high tolerance for multiple metals. Therefore, the impact of HMs on bioactive molecules synthesized by *E. cloacae* MC9 was studied. Indole-3-Acetic Acid (IAA) and Siderophore Production. Under controlled conditions (in the absence of HMs), *E. cloacae* MC9 produced a considerable amount of IAA that, however, significantly \((p \leq 0.05)\) decreased with increasing doses of HMs from 25 to 200 μg mL\(^{-1}\). In general, the higher concentration, i.e., 200 μg mL\(^{-1}\), of each HM poses the most vigorous negative effect and the minimum amount of IAA synthesis was recorded at this concentration. Among them, 200 μg Cr mL\(^{-1}\) had the maximum toxic effect where it reduced the IAA production activity of MC9 by 70% over untreated control.

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The IAA production was significantly reduced at different metal concentrations and decreased in the order Cr > Cd > Pb > Ni. The decrease in IAA development at higher concentrations of HMs may be attributed to bacterial cells’ slower growth and altered physiological behavior. The mechanism behind this was that HMs may influence the cell’s metabolism by binding to amino sulfides and thus impede bacterial cell metabolic activity and minimize phytohormone secretion. The secretion of IAA, the most important auxin (phytohormone), is the characteristic feature of the majority of soil beneficial microorganisms. It plays a crucial role in a number of physiological and metabolic activities such as root initiation, development of an embryo, formation of leaves and fruits, etc. IAA is synthesized mainly from tryptophan through multiple enzymatic pathways by many different genera of soil PGPR including *Bacillus*, *Rhizobium*, *Azotobacter*, *Pseudomonas*, *Enterobacter*, *Burkholderia*, etc. In line with these results, phytohormone synthesized by *Bradyrhizobium japonicum* was declined in the presence of different concentrations of heavy metals.

Another essential active biomolecule that sequesters iron within soil and indirectly aids plant growth is siderophore, a low-molecular-weight iron-chelating compound. They form stable complexes with heavy metals and increase the soluble metal concentration. Thus, it helps to alleviate the stresses imposed on plants by heavy metals in soil. As a result, the siderophore producing ability of *E. cloacae* was tested further by growing them both in liquid medium and CAS agar plates added with different metal concentrations. The size of the orange-colored siderophore zone (halo) decreased normally.

**Table 2. Microbiological, Morphological, and Biochemical Features of Isolated Strain *E. cloacae* MC9**

| features              | *E. cloacae* MC9 strain |
|-----------------------|-------------------------|
| morphological features|                         |
| Gram’s reaction       | −ve                     |
| configuration         | round                   |
| margin                | entire                  |
| surface               | smooth                  |
| pigmentation          | white to creamy         |
| opacity               | translucent             |
| shape                 | short rods              |
| cultural characteristics|                        |
| optimum temperature   | 28 ± 2 °C               |
| biochemical reactions |                         |
| citrate utilization   | yes                     |
| indole reaction       | no                      |
| methyl red            | no                      |
| nitrate reduction     | yes                     |
| oxidase               | no                      |
| Voges–Proskauer       | yes                     |
| carbohydrate utilization |            |
| dextrose              | yes                     |
| lactose               | yes                     |
| mannitol              | no                      |
| sucrose               | yes                     |
| urea hydrolysis       | no                      |
| starch hydrolysis     | yes                     |
| gelatin hydrolysis    | yes                     |

(Figure 2, panel A). The IAA production was significantly reduced at different metal concentrations and decreased in the order Cr > Cd > Pb > Ni. The decrease in IAA development at higher concentrations of HMs may be attributed to bacterial cells’ slower growth and altered physiological behavior. The mechanism behind this was that HMs may influence the cell’s metabolism by binding to amino sulfides and thus impede bacterial cell metabolic activity and minimize phytohormone secretion. The secretion of IAA, the most important auxin (phytohormone), is the characteristic feature of the majority of soil beneficial microorganisms. It plays a crucial role in a number of physiological and metabolic activities such as root initiation, development of an embryo, formation of leaves and fruits, etc. IAA is synthesized mainly from tryptophan through multiple enzymatic pathways by many different genera of soil PGPR including *Bacillus*, *Rhizobium*, *Azotobacter*, *Pseudomonas*, *Enterobacter*, *Burkholderia*, etc. In line with these results, phytohormone synthesized by *Bradyrhizobium japonicum* was declined in the presence of different concentrations of heavy metals.

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with gradually increasing concentrations of Cd, Cr, Pb, and Ni. Here, strain MC9 synthesized substantial amounts of phenolate-type siderophores: SA (58.6 ± 3.0 μg mL⁻¹) and 2,3-DHBA (35.6 ± 1.5 μg mL⁻¹), which, however, decreased as the concentrations of HMs increased. Among HMs, 200 μgCd mL⁻¹ had a highly inhibitory effect and it reduced the synthesis of salicylic acid and 2,3-DHBA by 80 and 81%, respectively, compared to untreated control (Figure 2, panels B and C). The adsorption of HMs on the microbial cell surface, which influences ion transfer, is one potential cause for decreased bacterial siderophores. The ability of E. cloacae to produce siderophores suggests that it may be used as a biocontrol agent to inhibit the growth of phytopathogens that damage crops, thus indirectly promoting plant growth. In a similar experiment, Cr among different HMs showed the maximum toxic effect on the siderophore production activity of a soil bacterium Pseudomonas aeruginosa strain SFP1.²⁸

**ACC Deaminase, Extracellular Polymeric Substances (EPSs), HCN, and Ammonia.** Many PGPR secrete ACC deaminase, which indirectly promotes plant growth by lowering the extremely high levels of ethylene produced in plants when they are growing in a stressed climate. Here, when grown on DF medium amended with/without different concentrations of HMs, E. cloacae MC9 showed a positive response toward the ACC deaminase activity. It was observed that strain MC9 showed that ACC deaminase in DF medium was maximally produced in the absence of any metals relative to those treated with different metal rates. Also, ACC deaminase activity steadily decreased as the concentration of metal was increased. For instance, 200 μgCd mL⁻¹ reduced the ACC deaminase activity of strain MC9 by 77% compared to untreated control (51.3 ± 4.6 μM α-ketobutyrate mg⁻¹ protein h⁻¹; Figure 3, panel A). Among HMs used, Cd had the maximum toxic effect on the bacterial synthesis of ACC deaminase. The order of toxicity of the heavy metals was Cd > Cr > Pb > Ni. Likewise, in a similar study, the ACC deaminase activity of Enterobacter sp. PR14 was adversely affected by different heavy metals.²⁹ Furthermore, the ACC deaminase activity of two strains of Bacillus (Bacillus gibsonii and Bacillus xiamenensis) was modulated when bacterial cells were cultured in liquid medium amended with different rates of HMs.³⁰

Considering the biological and agronomic significance of extracellular polymeric compounds produced by a variety of soil bacteria, the impact of HMs in the study on EPS released by E. cloacae was assessed. Here, strain MC9 produced a significant quantity of EPS in the absence of HMs, which, however, decreased with increasing concentrations of Cr, Cd, Pb, and Ni. For instance, Cr at 200 μg mL⁻¹ reduced the EPS production activity of E. cloacae by 50% when compared with untreated control (74.1 ± 5.1 μg mL⁻¹). Among HMs, the higher concentration of Cd had the maximum toxic effect on the EPS synthesizing ability of strain MC9 where it reduced the EPS production by 50% when compared with untreated control (74.1 ± 5.1 μg mL⁻¹). Among HMs, the order of toxicity of the heavy metals was Cd > Cr > Pb > Ni. Likewise, in a similar study, the ACC deaminase activity of Enterobacter sp. PR14 was adversely affected by different heavy metals.²⁹ Furthermore, the ACC deaminase activity of two strains of Bacillus (Bacillus gibsonii and Bacillus xiamenensis) was modulated when bacterial cells were cultured in liquid medium amended with different rates of HMs.³⁰

**Figure 2.** Effect of different doses of HMs on indole-3-acetic acid (A) and siderophore production: salicylic acid (B) and 2,3-dihydroxybenzoic acid (C) synthesized by E. cloacae strain MC9. In this figure, the line diagram and histograms represent the mean values of three replicates (n = 3). Corresponding error bars represent the standard deviation (SD) of three replicates (SD, n = 3). Means followed by similar alphabets are significantly different from each other according to Duncan’s multiple range test (DMRT).
Bacillus. Cyanogenesis in various rhizospheres are separated from other HCN-sensitive bacterial populations to produce HCN with competitive and selective advantages. Similarly, it has been shown that rhizosphere bacteria in contaminated soil restrict hydrogen cyanide and NH₃. Under stressful environmental conditions, rhizobacterial strains produce less HCN and ammonia, which might be related to the impairment of different metabolic processes.

P Solubilization under HM Stress. The solubilization into its soluble form of the insoluble form of inorganic phosphate (Pi) is a trait of soil microbes in different ambient conditions. By developing bacterial strain in liquid PKV medium, the qualitative and quantitative phosphate solubilization ability of E. cloacae was evaluated under variable concentrations of HMs. Here, in the present investigation, it was noticed that the quantity of P solubilization in liquid broth decreases as the concentration of HMs increases from 25 to 200 μg mL⁻¹. However, a higher concentration (200 μg mL⁻¹) of Cd, Cr, Pb, or Ni had the most notable and pronounced toxic impact. For instance, after 2 days of incubation, at 200 μg mL⁻¹ concentration, Cd, Cr, Pb, and Ni decreased the P solubilization efficiency of E. cloacae MC9 by 81% (decreased from 292 to 53 μg mL⁻¹), 85% (292–44 μg mL⁻¹), 75% (from 292 to 71 μg mL⁻¹), and 57% (from 292 to 123 μg mL⁻¹), respectively, over control (292 μg mL⁻¹, Table 4). The biggest negative effect for PSA was seen in the following order when comparing the average levels of HMs: Cr > Cd > Pb > Ni (Table 4). A substantial reduction in the pH value of inoculated and HM-treated PKV liquid broth was also observed at various intervals. Many workers have suggested the pH reduction in soils, coupled with their capacity to produce organic low molecular weights such as citric acid, oxalic acid, malic acid, acetic acid, succinic acid, maleic acid, gluconic, 2-ketogluconic acid, etc. The variations in the pH values of the liquid media under HM stress reflect the decrease in the P-solubilizing potential of strain MC9. These shifts/changes might reduce the secretion of organic acids or detoxify HM byproducts; in fact, the pH of heavy metal-amended liquid media can be increased for both reasons. The chemical-dependent inhibitions in the P solubilization capacity of a soil beneficial isolate Azotobacter vinelandii, as well as a reduction in the pH of liquid broth, have recently been described, which are similar to the current observation.

Colony-Forming Units (CFUs) Counts under HM Stress. In this study, the number of CFU mL⁻¹ of E. cloacae strain MC9 decreased consistently with an increase in doses of Cd, Cr, Pb, and Ni from 25 to 200 μg mL⁻¹ (Figure 4, panels A–D). The lower concentrations of HMs pose a lesser effect, and the higher one had the maximum notable effect on cellular viability. For instance, at 150–200 μg mL⁻¹ concentrations, Cd and Cr completely reduced the cell viability. As a result, the growth inhibition reported in this study might be related to metal ion transport/uptake across membranes. The loss of bacterial cell respiration is one of the most common causes of bacterial cell viability decrease. The interaction of HMs with components of the bacterial plasma membrane has resulted in respiratory inhibition, resulting in a reduction in cell viability. Similar to this, the increasing concentration of the chemical compound has been reported to decrease the cellular permeability (in the form of CFU count mL⁻¹) of two soil isolates, viz., Pseudomonas putida and Pseudomonas fluorescens, under in vitro studies.
### Table 3. Effect of Different Concentrations of Heavy Metals on Plant Growth Regulating Substances Synthesized by *E. cloacae* Strain MC9

| Treatment | Concentrations (μg mL⁻¹) | FeCl₃ Test | Siderophore Production (%) | Zone Size on CAS Agar (mm) | NH₃ Production | HCN Production |
|-----------|---------------------------|------------|---------------------------|---------------------------|----------------|----------------|
| Cd        | 25                        | +          | 264                       | 4.2                       | 292            | 4.5            |
|           | 50                        | +          | 234                       | 4.2                       | 231            | 4.1            |
|           | 100                       | +          | 171                       | 4.2                       | 153            | 4.1            |
|           | 150                       | +          | 121                       | 4.2                       | 145            | 4.1            |
|           | 200                       | +          | 53.0                      | 4.2                       | 165            | 4.1            |
| Cr        | 25                        | +          | 239                       | 4.2                       | 214            | 4.1            |
|           | 50                        | +          | 221                       | 4.2                       | 192            | 4.1            |
|           | 100                       | +          | 163                       | 4.2                       | 140            | 4.1            |
|           | 150                       | +          | 102                       | 4.2                       | 112            | 4.1            |
|           | 200                       | +          | 44.0                      | 4.2                       | 12.0           | 4.1            |
| Pb        | 25                        | +          | 236                       | 4.2                       | 214            | 4.1            |
|           | 50                        | +          | 221                       | 4.2                       | 192            | 4.1            |
|           | 100                       | +          | 163                       | 4.2                       | 140            | 4.1            |
|           | 150                       | +          | 102                       | 4.2                       | 112            | 4.1            |
|           | 200                       | +          | 44.0                      | 4.2                       | 12.0           | 4.1            |
| Ni        | 25                        | +          | 238                       | 4.2                       | 214            | 4.1            |
|           | 50                        | +          | 221                       | 4.2                       | 192            | 4.1            |
|           | 100                       | +          | 163                       | 4.2                       | 140            | 4.1            |
|           | 150                       | +          | 102                       | 4.2                       | 112            | 4.1            |
|           | 200                       | +          | 44.0                      | 4.2                       | 12.0           | 4.1            |
| Control   |                           | +          | 292                       | 4.2                       | 292            | 4.2            |

*Each value is a mean (mean ± SD) of three independent replicates. Symbols + and − represent the positive and negative reactions, respectively.*

### Table 4. Effect of Different Concentrations of HMs on the Phosphate Solubilizing Activity of *E. cloacae* Strain MC9 Cultured in Liquid PKV Medium and Change in the pH of Liquid Broth at Different Growth Incubations

| HMs | Concentrations (μg mL⁻¹) | P Solubilization (μg mL⁻¹) | Day 2 pH | Day 4 pH | Day 6 pH | Day 8 pH | Day 10 pH | 25081 |
|-----|--------------------------|---------------------------|--------|--------|--------|--------|--------|-------|
| Cd  | 25                       | 265                       | 5.5    | 270    | 5.3    | 280    | 5.2    | 287   | 5.1    |
|     | 50                       | 234                       | 5.6    | 241    | 5.3    | 247    | 5.3    | 263   | 5.2    |
|     | 100                      | 171                       | 5.8    | 178    | 5.4    | 180    | 5.4    | 187   | 5.4    |
|     | 150                      | 121                       | 5.9    | 125    | 5.7    | 132    | 5.5    | 134   | 5.6    |
|     | 200                      | 53.0                      | 5.9    | 55.0   | 6.0    | 58.0   | 6.0    | 61.0  | 6.0    |
| mean|                          | 168.8                     | 5.74   | 173.5  | 5.5    | 179.4  | 5.48   | 186.4 | 5.46   |
| Cr  | 25                       | 239                       | 5.3    | 245    | 5.4    | 250    | 5.2    | 252   | 5.4    |
|     | 50                       | 221                       | 5.4    | 224    | 5.5    | 227    | 5.4    | 231   | 5.5    |
|     | 100                      | 163                       | 5.5    | 166    | 5.6    | 171    | 5.5    | 173   | 5.6    |
|     | 150                      | 102                       | 5.6    | 107    | 5.7    | 112    | 5.7    | 115   | 6.0    |
|     | 200                      | 44.0                      | 5.8    | 47.0   | 5.9    | 52.0   | 5.9    | 54.0  | 6.0    |
| mean|                          | 153.8                     | 5.5    | 157.8  | 5.6    | 162.4  | 5.5    | 165   | 5.7    |
| Pb  | 25                       | 236                       | 5.3    | 240    | 5.3    | 246    | 5.6    | 252   | 5.7    |
|     | 50                       | 221                       | 5.5    | 225    | 5.5    | 231    | 5.8    | 234   | 5.8    |
|     | 100                      | 189                       | 5.6    | 194    | 5.6    | 200    | 5.9    | 203   | 5.9    |
|     | 150                      | 161                       | 5.8    | 165    | 5.8    | 171    | 6.0    | 173   | 6.0    |
|     | 200                      | 71.0                      | 5.8    | 77.0   | 6.0    | 81.0   | 6.1    | 84.0  | 6.1    |
| mean|                          | 175.6                     | 5.6    | 180.2  | 5.6    | 185.5  | 5.8    | 189.2 | 5.9    |
| Ni  | 25                       | 238                       | 5.2    | 243    | 5.5    | 247    | 5.5    | 249   | 5.9    |
|     | 50                       | 214                       | 5.5    | 217    | 5.7    | 221    | 5.8    | 227   | 5.9    |
|     | 100                      | 179                       | 5.6    | 184    | 5.8    | 192    | 5.9    | 200   | 6.0    |
|     | 150                      | 156                       | 5.7    | 159    | 5.9    | 166    | 6.0    | 167   | 6.1    |
|     | 200                      | 123                       | 5.8    | 127    | 6.0    | 132    | 6.0    | 136   | 6.2    |
| mean|                          | 182                       | 5.5    | 186    | 5.7    | 191.6  | 5.8    | 195.8 | 6.02   |
| control (without HMs) | 0                        | 292                       | 4.2    | 299    | 3.6    | 312    | 3.5    | 331   | 3.4    |

*Each value is a mean (mean ± SD) of three independent replicates.*
Biofilm Formation of MC9 Strain was Negatively Influenced by HMs. The impact of various concentrations of HMs on the % biofilm development of E. cloacae strain MC9 was found in a dose-dependent manner in this study. The inhibition of biofilm formation by Cd, Cr, Pb, and Ni (Figure 5, panels A−E) against strain MC9 was statically (p ≤ 0.05) significant. Higher concentrations of HMs showed the most detrimental influence on the capacity of strain MC9 to produce biofilms. For example, strain MC9’s biofilm-forming capacity was substantially decreased at 200 μg mL⁻¹ concentration of Cd, Cr, Pb, and Ni by 73, 64, 51, and 42%, respectively, compared to untreated control (Figure 5, panel G). Biofilms are complex mixtures of glycocalyx matrices created by bacterial species as a result of cross-talk between quorum sensing molecules, which may also encourage the development of other virulence factors.48-49 Here, the test HMs inhibited the formation of bacterial biofilms. Disrupting/inhibiting the bacterial cells can be caused by cell damage/injury, which leads to intracellular content leakage/flow, thereby preventing the production of additional polymeric substances and others causing pathogenicity.50 The inhibitory impact of HMs on the bacterial biofilm might also be caused by water channel disruption across the biofilm present for nutrient delivery.

In addition, chemical compounds can directly diffuse/disrupt the EPS layer and give an antibiotic effect. Similar to our study, the biofilm formation ability of Bacillus subtilis 1JN2 was inhibited following the exposure of Cd51 as reported in a study conducted by Yang et al.51

HMs Affect the Membrane Permeability of Bacterial Cells. Here, the extent of cellular damage produced by HMs to the cells of E. cloacae MC9 was assessed for cellular membranous permeability, which was deliberately detected when bacterial cells were stained with a fluorescent dye propidium iodide (PI). For this, strain MC9 was grown in media supplemented with escalating dosages of test HMs (25−200 μg mL⁻¹), stained with red-emitting fluorescence DNA binding dye PI (excited at 532 nm), and pictures were captured using a confocal laser scanning microscope (CLSM). Microscopic analysis of HM-treated cells of E. cloacae demonstrated an increase in the number of dead/injured cells in the form of red-colored short rods. (Figure 6, panels B−E) compared to untreated control cells (Figure 6, panel A). Furthermore, the number of dead cells was counted and it was observed that the higher concentration of each heavy metal caused an increase in the number of dead cells (Figure 6, panels a−d). Cell membranes of bacteria function as selectively permeable barriers for a range of chemicals; nevertheless, when permeability increases, cells may take in excessive amounts of elements in their environment.52 Therefore, toxic substances, such as HMs, enhance intracellular access and have a detrimental effect in the form of reactive oxygen species production.53 The metabolically active cells did not take up the DNA-bound dyes. They may, however, easily attach to the nucleic acids of membrane-damaged cells. Likewise, very recently, Shahid et al.54 reported the increasing number of dead cells of Sinorhizobium saheli after exposure to increasing concentrations of other chemical compounds.

**CONCLUSIONS**

The different levels of HMs clearly demonstrated obvious toxicity to E. cloacae MC9. The decrease in PGP characteristics, growth kinetics, and CFU counts indicates that HMs have inhibitory capability. With increasing concentrations of HMs, there was also a reduction in P solubilization, as well as a
decrease in the pH of the liquid medium and inhibition in the development of bacterial biofilms. HMs also changed the metabolic pathways that led to the production of indole-3-acetic acid, siderophores (salicylic acid and 2,3-DHBA), and ACC deaminase enzymes. Furthermore, as the concentration of each heavy metal increased, the number of dead cells _E. cloacae _MC9 also increased. In conclusion, the current study demands workers’ attention to the fact that before applying HMs in agronomic operations, their appropriate field dosages must be properly understood and carefully monitored.

### EXPERIMENTAL SECTION

**Chemicals Used.** The metal salts of potassium dichromate (K₂Cr₂O₇, CAS No. 7778-50-9, mol. wt. 294.18 g mol⁻¹), cadmium chloride (CdCl₂, CAS No. 7790-78-5, mol. wt. 183.32 g mol⁻¹), lead acetate (Pb(CH₃COO)₂·3H₂O, CAS No. 1335-32-6, mol. wt. 325.29 g mol⁻¹), and nickel chloride (NiCl₂·6H₂O, CAS No. 7791-20-0, mol. wt. 237.69 g mol⁻¹) were procured from Sigma-Aldrich.

**Bacterial Isolation and Heavy Metal Resistance/Sensitivity.** Chili (_Capsicum annum_) rhizosphere soil samples were obtained from agricultural fields that were constantly irrigated with industrial effluents. The soil samples were cleaned, dried, and analyzed for their different physicochemical properties including the presence of heavy metals (Table S). For bacterial isolation, soils were diluted in a series (10⁻¹–10⁻⁷) and spread plated on Pikovskaya (PVK) (g L⁻¹: glucose 10; Ca₃(PO₄)₂ 5; (NH₄)₂SO₄ 0.5; NaCl 0.2; MgSO₄·7H₂O 0.1; KCl 0.1) agar plates and incubated at 28 ± 2 °C for two days. Colonies were chosen and restreaked on the same medium three times to achieve a pure culture and preserved on the same medium. The plate and broth dilution process was used to determine the resistance/sensitivity of bacterial isolates against different heavy metals. For this, agar plates and broths were prepared by adding different concentrations (0–2000 μg mL⁻¹) of HMs, viz., Cr, Cd, Pb, and Ni, and 10 μL of the log-phase bacterial suspension (10⁸ cells mL⁻¹) was spotted/inoculated onto/into solid and liquid media. After inoculation, the plates and tubes were incubated at 28 ± 2 °C for 48 h and bacterial growth was checked.

**Morphological, Biochemical Characterization, and Bacterial Identification.** Identification of bacterial isolates at the genus level was carried out using normal morphological and biochemical tests. Gram’s staining, shape, and scale/size of the cell and the formation of the pigment were included in the morphological characterization, while the biochemical experiments included methyl red, Voges–Proskauer, use of citrate, reduction of nitrate, catalase, oxidase, and urease studies, and use of starch and lipid hydrolysis as well as mannitol. The 16S rRNA partial gene sequencing (a commercially available service, provided by Macrogen, Seoul, South Korea) was conducted for molecular characterization of isolate MC9. The resulting DNA sequence was reviewed and compared with the already available sequences of other genera at the NCBI server using the BLASTn tool. A phylogenetic
tree was developed using the nearest neighbor sequence means of MEGA 7.0 software.

**Effect of HMs on Active Biomolecules Secreted by *E. cloacae* MC9.** In the presence and absence of heavy metals, the assay of all plant growth-promoting substances secreted by MC9 was carried out. The concentrations of Cr, Cd, Pb, and Ni were 0, 25, 50, 100, 150, and 200 μg mL⁻¹ throughout the study.

**Assay for Indole-3-Acetic Acid (IAA).** To assess the effect of HMs on IAA secreted by *E. cloacae* MC9 strain, the modified method of Bric et al. was used.⁵⁷ For the assay, 100 μL of bacterial culture was inoculated in 25 mL of Luria Bertani broth (g L⁻¹; tryptone 10, yeast extract 5, NaCl 10, and pH 7.5; HiMedia, Pvt. Ltd., Mumbai, India) supplemented with different concentrations of HMs. Bacteria-inoculated broth was incubated at 28 ± 2 °C in shaking conditions (125 rpm) for 2–3 days.

After completion of incubation, 5.0 mL of bacterial grown culture was centrifuged (10 000g) for 20 min and 2.0 mL of the supernatant was taken out. Further, the supernatant was mixed with 4.0 of the Salkowsky reagent following the addition of few drops of orthophosphoric acid. After addition of reagents, tubes were kept in darkness for 30 min. The developed pink color was read at 530 nm using a UV–vis spectrophotometer (UV-2450, Shimadzu). The IAA synthesized by bacterial strain was estimated.

**Siderophore Production.** To assess the effect of HMs on the siderophore production activity of *E. cloacae* MC9, bacterial cells were cultured in liquid/solid medium amended with varying concentrations of Cd, Cr, Pb, and Ni and assessed.⁵⁸–⁶⁰

**Determination of ACC Deaminase and Exopolysaccharide (EPS).** Enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase secreted by *E. cloacae* MC9 was qualitatively assayed by spot inoculation of 10 μL of overnight grown bacterial culture into DF salt minimal medium. Strain MC9 was inoculated in 10 mL of DF medium amended with different concentrations of Cd, Cr, Pb, and Ni and incubated at 28 ± 2 °C for 24–48 h. The cell pellets were collected by centrifugation (9000 rpm) and washed twice with 0.1 M Tris-HCl (pH 7.5) and resuspended in 2 mL of modified DF medium supplemented with 2 mM ACC. Following this, the culture was again incubated at 28 ± 2 °C for 36–72 h. After incubation, the bacterial cells were harvested by centrifugation.
Table 5. Physicochemical Analysis of the Rhizosphere Soil Sample Irrigated with Industrial Wastewater)

| Parameter          | Wastewater Irrigated Soil |
|--------------------|---------------------------|
| pH                 | 8.04 ± 0.76               |
| EC (μS cm⁻¹)       | 2.54 ± 0.48               |
| Texture            | loamy                     |
| Carbonate (mg kg⁻¹) | 213 ± 15.6                |
| Bicarbonate (mg kg⁻¹)| 176 ± 8.4                 |
| Organic carbon (%) | 1.32 ± 0.28               |
| Chloride (mg kg⁻¹) | 38.7 ± 3.2                |
| Phosphorous (mg kg⁻¹)| 28.6 ± 1.67               |
| Potassium (mg kg⁻¹) | 57.3 ± 7.3                |
| Sulfur (mg kg⁻¹)   | 8.2 ± 0.4                 |
| Cadmium (mg kg⁻¹)  | 4.55 ± 0.1                |
| Chromium (mg kg⁻¹) | 24.5 ± 3.1                |
| Copper (mg kg⁻¹)   | 12.6 ± 0.9                |
| Nickel (mg kg⁻¹)   | 16.3 ± 2.1                |
| Lead (mg kg⁻¹)     | 19.4 ± 1.5                |
| Iron (mg kg⁻¹)     | 7.43 ± 0.65               |
| Manganese (mg kg⁻¹)| 11.04 ± 0.0               |
| Zinc (mg kg⁻¹)     | 6.87 ± 0.42               |

*Each value is a mean (mean ± SD) of three independent replicates.

(3000g for 5 min) and washed twice with 0.1 M Tris-HCl (pH 7.5). The cell pellets were resuspended in 200 μL of 0.1 M Tris-HCl (pH 8.5) and were tolenuized with 5% toluene (v/v). The cell suspension was incubated with 5 μL of a 0.3 M ACC solution. Fifty microliters of the cell suspension without ACC served as negative control, while 50 μL of 0.1 M Tris-HCl (pH 8.5) with 5 μL of 0.3 M ACC served as blank. The samples were later acidified with 500 μL of 0.56 N HCl and mixed thoroughly. Following this, the cell debris was removed by centrifugation (12 000 g for 5 min). A solution of 400 μL of 0.56 N HCl and 150 μL of DNF (0.1 g of 2,4-dinitrophenyl hydrazine prepared in 100 mL of 2 N HCl) was added to 500 μL of the aliquot of the supernatant and the mixture was again incubated at 28 ± 2 °C for 30 min. After incubation, 1 mL of 2 N NaOH was added to each sample, and absorbance was measured at 540 nm.61

The exopolysaccharides (EPSs) released by strain MC9 were extracted by the method of Mody et al.62 For this, bacterial cultures were grown in nutrient broth supplemented with 5% sucrose and treated with different rates of HMs and incubated for 5 days at 28 ± 2 °C at 120 rpm. Culture broth was centrifuged (8000 rpm min⁻¹) for 20 min and EPS was extracted by mixing chilled acetone (CH₃COCH₃) and the supernatant in a ratio of 3:1. The precipitated EPS so obtained was washed three times alternately with distilled water and acetone and transferred to filter paper and weighed after overnight drying at room temperature.

Cyanogenic Compounds (HCN) and Ammonia Production. For the determination of HCN, E. cloacae MC9 was inoculated on an HCN induction medium (g L⁻¹: tryptic soy broth 30, glycine 4.4, and agar 15) supplemented with varying doses of each heavy metal and incubated at 28 ± 2 °C for 4 days. A disk of Whatman filter paper No. 1 soaked in 0.5% picric acid and 2% Na₂CO₃ was placed under the lid of Petri plates and sealed with a parafilm. After 4 days of incubation at 28 ± 2 °C, the orange-brown color of the paper confirmed the production of HCN.51 The NH₃ production by PGPR strain was grown in peptone water in the absence and presence of Cd, Cr, Pb, and Ni and incubated at 28 ± 2 °C for 4 days. Nessler’s reagent (1.0 mL) was added to each tube and the development of yellow color showed ammonia production.63

Phosphate Solubilization. The impact of HMs on the P solubilization efficiency of strain MC9 was evaluated. For this, bacterial cells were grown in 100 mL of liquid Pikovskaya (PKV) medium added with 0–200 μg mL⁻¹ Cd, Cr, Pb, and Ni. The available P was quantitatively assessed by growing the bacterial cultures in liquid PKV medium, 20–200 μg mL⁻¹ concentrations of Cd, Cr, Pb, and Ni were added individually to 100 mL of PKV broth and inoculated with 1.0 mL of 10⁶ cells mL⁻¹ of bacterial culture and incubated at 28 ± 2 °C with intermittent shaking (at 120 rpm). The available P was measured in the bacterial supernatant on the 2nd, 4th, 6th, 8th, and 10th days after incubation. The change in the pH values of liquid PKV following P solubilization was also recorded.64–66

Determination of Cellular Membrane Injury. To assess the membrane damaging potential of test HMs, E. cloacae MC9 was grown in nutrient broth (NB). Following incubation of 12 h, growing cells were treated with 25–200 μg mL⁻¹ concentrations of Cd, Cr, Pb, and Ni and further incubated for 6 h in shaking conditions (at 120 rpm). After this, the cells were harvested following centrifugation (10 000 rpm for 5 min), and bacterial cell pellets were washed out at least three times with sterile phosphate-buffered saline (PBS) and tagged with a mixture solution of fluorescently labeled DNA binding dyes: propidium iodide (PI) and acridine orange (AO) at a concentration of 50 μM for 20 min at room temperature.66 The cells were carefully and gently washed with PBS before being observed on a glass slide using a confocal laser scanning microscope (Leica TCS, CLSM; Leica Microsystems, Germany), and the number of dead cells was counted.

Bacterial CFU Counts under HMs. For CFU count, 0.1 mL volume of 24 h growing culture was spread out on HM-supplemented nutrient agar plates. The plates were incubated under the abovementioned growth conditions to count the viable cells. The number of colony-forming units (CFU) per milliliter (mL) was transformed to log₁₀ CFU mL⁻¹ and displayed against the HM concentration. The CFU was calculated as

\[
\text{colony forming unit (CFU)} = \frac{\text{number of colonies} \times \text{dilution factor}}{\text{volume plated}}
\]

The number of CFU mL⁻¹ was converted to a logarithmic scale (log CFU mL⁻¹) and plotted as a function of HM concentration (μg mL⁻¹).

Determination of Biofilm Formation. To evaluate the development of biofilms by E. cloacae strain MC9, as mentioned, the absorbance-based crystal violet (CV) technique was used as previously described.65,71,72 In brief, bacterial strains were exposed to 25–200 μg mL⁻¹ concentrations of Cr, Cd, Pb, and Ni.

Statistical Analysis. All of the experiments were performed in triplicate (n = 3) to minimize the experimental errors. Duncan’s multiple range test (DMRT) was used to analyze the data, and the least significant difference (LSD) was computed using Minitab 17 statistical software at a 5% probability level.
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Notes
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