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Original Research Article

Evaluation of Cr (VI) remediation potential of Eichornia sp in conjunction with chromium-resistant bacterial strains

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Abstract

Purpose: *Evaluation of Cr (VI) removal by indigenous chromium resistant bacterial strains alone and in combination with Eichornia sp.*
Methods: *Three chromium resistant bacterial strains S-4 Ochrobactrum grignonense, SF-5 Bacillus sp. and S-6 Ochrobactrum pseudogrignonenses were isolated from industrial effluent. The isolated chromium-resistant bacterial strains were subjected to heavy-metal resistance profiling. Cr (VI) reduction was evaluated in mobilized as well as immobilized forms. The phytoremediation potential of Eichornia sp. in conjunction with chromium resistant bacterial isolates was also determined. Fourier transform infra-red (FTIR) spectroscopy was performed to rule out the involvement of various functional groups in the binding activity of Cr (VI).*
Results: *Three bacterial strains resisted up to 1000 µg/mL of potassium dichromate (K_2CrO_4). Bacterial strains S-4, SF-5 and S-6 showed significant Cr (VI) removal in mobilized state (84.93, 85.85 and 83.97 % respectively) compared with immobilized state (41.27, 37.99, 37.96 %) at an initial concentration of 500 µg/mL of chromate. Bacterial strains caused reduction in chromate uptake in inoculated plants relative to control plants. FTIR spectra revealed significant changes in the absorption peaks, reflecting the binding of Cr (VI) ions with bacterial cell surface under stress conditions.*
Conclusion: *The selected isolates tested possess the ability to remove Cr (VI) synergistically with Eichornia sp.*

Keywords: *Phytoremediation, Heavy-metal, Pollution, Chromium-resistant bacteria*

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INTRODUCTION

Chromium (Cr) is one of the toxic heavy metals often found in the environment due to industrial and man-made activities. This metal is widely used in leather tanning and textile industries. Chemically chromium exists in +2 to +6 valence states [1]. Hexavalent chromium is mutagenic, carcinogenic and 10-100 times more potent than trivalent form [2]. Cr (VI) can be removed from the environment by reduction through biological or physicochemical methods. The physic-chemical methods include precipitation of chemicals through evaporation, filtration through membranes, ion exchange resins and reverse osmosis [3]. These expensive methodologies generate toxic by-products, so ecologically cost effective methods are required to remediate hexavalent chromium pollution. Bacterial reduction of Cr (VI) to Cr (III) serves as a better technology to remediate contaminated soils and ground waters [4]. Many bacterial strains that were isolated from polluted environment such as *Brevibacterium sp. Bacillus pumilis, Cellulosi-microbium cellulans* and *Exiguobacterium sp.*

reported to reduce toxicity of hexavalent chromium [5,6].

Microorganisms transform heavy metals by various mechanisms like metal absorption, metal accumulation and metal resistance. These organisms can be engineered to decrease Cr (VI) pollution [7]. In order to protect the bacterial cell from harsh chemicals, the immobilization technique proves to be effective. Bacterial cells attached with support matrix could tolerate higher concentration of Cr (VI) bringing about easy separation of cells from treated liquids. Various natural supportive materials like agar, sodium alginate and activated carbon along with artificial matrices such as polyacrylamide, and polyvinyl alcohol are used to attain the immobilization of cells. The selection of support matrix is a key factor in using an immobilized biocatalyst [8]. Water hyacinth (*Eichornia)* is an aquatic weed used to study deleterious effect of heavy-metal contaminants because of its biosorption capacity [9]. The present research work was carried out to evaluate the chromium remediation potential of indigenous Cr (VI) resistant bacteria alone as well as in conjunction with *Eichornia* sp.

EXPERIMENTAL

Isolation of bacterial strains

Bacterial strains were isolated from the waste water of Ittehad Chemical Industries, Kala Shah Kaku, Great Trunk road, Pakistan by serial dilution method on L-agar plate amended with Cr (VI) stress (500 µg/mL of $K_2Cr_2O_4$). Isolated colonies were streaked and purified after 24-48 hours of incubation.

Morphological and biochemical characterization

Bacterial strains (S-4, SF-5, and S-6) were examined for their colony morphology, cell morphology, gram nature, spore forming ability and motility. Various biochemical tests (Catalase, cytochrome oxidase, methyl red, gelatin hydrolysis, nitrate reduction) were performed to characterize the biochemical nature of the bacterial strains.

Evaluation of heavy metal-resistant profile

Different heavy-metals like Copper sulphate ($CuSO_4$), potassium chromate (K_2CrO_4), mercuric chloride ($HgCl_2$), cadmium chloride ($CdCl_2$) were selected to check the multiple resistances of the isolates to other heavy-metals. For this purpose, 10 % stock solutions were prepared. The required amount of stock solution of each heavy-metal was added in L-agar media prepared separately just before pouring under sterile conditions. All the strains were streaked on these heavy metal supplemented plates and incubated at 37 ° C for 24 - 48 h.

Estimation of Cr (VI) reduction potential

Bacterial strains were grown aerobically in L-broth for 24 hours at 37 ^{o}C. Cells were harvested and re-suspended in normal saline. Bacterial population was adjusted to same optical density at 600 nm. Sodium alginate was used to transform the bacterial strains into beads [2]. Beads were transferred to L-Broth containing 500 µg/mL of Cr (VI) at pH 7.0 and incubated at 37 ^{o} C for 24, 48, and 72 hours at 150 rpm. After incubation, reduction of Cr (VI) by mobilized and immobilized bacterial strains was monitored by using the diphenyl carbazide method [10].

Evaluation of Cr (VI) removal by *Eichornia*

For the evaluation of Cr (VI) removal by hydrophytes, *Eichornia sp.* and industrial effluent both were collected from the industrial sites near Arohi Waste Drainage, Kasoor. These plants were kept under sunlight. To find out the rate of Cr (VI) removal and Cr (VI) uptake, experiment was designed in two phases. In phase one, evaluation of the Cr (VI) removal and uptake was determined after seven days of inoculation, and in second phase Cr (VI) removal and uptake was evaluated after 14 days of inoculation at two initial concentrations of hexavalent chromium (100 and 300 µg/mL).

For the estimation of chromium uptake, the *Eichornia* plants were grown in natural environment. Two sets of pots were prepared. Each set consists of three pots for each strain and fourth one taken as control. Each pot contained one plant provided with Hoagland nutrient solution [11]. One set of pot was maintained at 100 µg/mL of chromium, and the second was amended with 300 µg/mL of Cr (VI). Each pot was inoculated (2 ml-OD, 0.2 at 600 nm) with respective strain except control. The pots were kept in sunlight 6-8 hours for 7 days in phase 1 while; plants of phase 2 were kept in sunlight for 14 days for the same time period. After specific time of incubation (7 and 14 days), the plants were harvested and oven-dried for 24 - 48 h. The dried plant material was then digested, and estimation of chromium was done by following method of Humphries [12]. Estimation of chromium (VI) removal was done by following the method of Rand *et al* [10].

Fourier transforms infrared (FTIR) spectroscopy

For FTIR analysis; bacterial strain (SF-5) was grown overnight in L-Broth under stress (1000 µg/mL of K_2CrO_4) and in non-stress condition at 37 ^oC for 24 - 48 h. After incubation, cultures were centrifuged at 100,000 rpm for 10 minutes and pellets were obtained. The pellets were dried and processed with potassium bromide (KBr) and analyzed by FTIR The peak shifts were observed and recorded.

16S rRNA gene sequencing of bacterial strains

Three bacterial strains (S-4, SF-5 and S-6) were sent to the Macrogen Inc. Seoul; Korea, for 16S rRNA gene sequencing. Partial 16S rRNA gene sequence was determined by Macrogen using genomic DNA provided to them. The primers were chosen for the wide coverage of both eubacteria and archaea, encompassed variable regions, V5 and V6 of the 16S rRNA gene. Sequences were analyzed using Ribosomal Database Project, and a phylogenetic tree was constructed using a weighted neighbor-joining tree-building algorithm therein [13].

Statistical analysis

All experimental work was performed in triplicate. For statistical analysis of the data, SPSS personal computer statistical package (version 16, SPSS Inc, Chicago) was used. $P < 0.05$ was considered statistically significant.

RESULTS

Isolation of bacterial strains

Three bacterial strains (S-4, SF-5, and S-6) were selected for further experiments because of their high Cr (VI) resistivity. These strains resisted up to 1000 µg/mL of potassium dichromate (K_2CrO_4).

Morphological and biochemical characteristics

Morphological characterization revealed that S-4 and S-6 were Gram-negative rods, non-spore former and motile whereas SF-5 was Gram-positive rods, spore former, and motile. All selected strains showed positive results for catalase, oxidase, nitrate reduction and gelatin hydrolysis while negative results for MR.

Heavy metal resistance profile

All selected bacterial strains were able to resist the concentration of 1000 µg/mL of potassium chromate (K_2CrO_4), which is the maximum tolerable range. Mercuric chloride ($HgCl_2$) proved to be toxic as all bacterial strains were sensitive to this heavy metal. Bacterial strains S-4 and S-6 could tolerate up to 200 µg/mL of $CdCl_2$ except SF-5, which could not resist cadmium chloride even at low concentration (10 µg/mL).

Cr (VI) reduction potential in mobilized and immobilized states of the bacterial strains

At an initial concentration of 500 µg/mL of Cr (VI), bacterial strain S-6 exhibited maximum reduction potential (91.77 %) in the mobilized form after 24 hours of incubation. However, it gradually increased (92.54 %) after 48 hours of incubation, but showed a sudden decrease (83.97 %) after 72 hours. SF-5 exhibited higher Cr (VI) reduction (85 %) in mobilized form after 72 hours of incubation. S-4 showed 90.3 % Cr (VI) reduction in the mobilized and 47.96 % in the immobilized form after 48 hours of incubation (Table 1).

Cr (VI) removal in Phase 1

For phase 1, all the plants were incubated for 07 days only. Plants inoculated with S-4 (60.47 and 76.04 %), SF-5 (43.95 and 78.6 %) and S-6 (45.42 and 84.6 %) showed effective Cr (VI) removal as compared to un-inoculated plants (3.2 and 43.4 % respectively) at 100 and 300 µg/mL of K_2CrO_4 (Table 2).

Table 1: Cr (VI) reduction potential of mobile and immobile chromium resistant bacteria

Strain	Reduction potential (%)					
	Mobile bacteria			***Immobile bacteria***		
	24 h	***48 h***	***72 h***	***24 h***	***48 h***	***72 h***
S-4	70.28±0.37 (a)	90.3±0.67 (e)	84.93±0.65 (d)	29.43±0.48 (a)	47.96±0.69 (e)	41.27±0.38 (d)
SF-5	31.33±1.07 (e)	78.44±0.31 (e)	85.85±0.37 (d)	31.81±0.52 (b)	49.58±0.60 (f)	37.99±0.33 (c)
S-6	91.77±0.53 (a)	92.54±0.69 (c)	83.97±0.54 (d)	33.16±1.09 (ab)	51.71±0.59 (ef)	37.96±0.31(c)

(±SEM, n = 3, In each column, figures followed by different letter (s) in parenthesis indicate significant difference by Duncan's multiple range test (p <0.05)

Cr (VI) Uptake

At an initial concentration of 100 µg/mL of K_2CrO_4, plants inoculated with bacterial strain S-4 (0.109 Cr mg/g) and SF-5 showed maximum Cr (VI) uptake (0.109 Cr mg/g and 0.11 Cr mg/g, respectively). As an initial concentration of K_2CrO_4 was raised up to 300 µg/mL, again maximum Cr (VI) uptake was also exhibited by SF-5 (2.30 Cr mg/g), and S-4 (1.803 Cr mg/g) as shown in Figure 1.

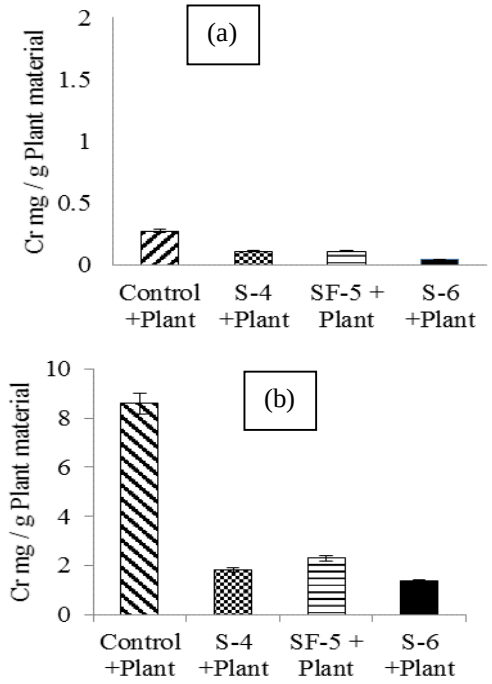

Figure 1: Cr (VI) uptake at (a) 100 and (b) 300 µg/mL of Cr (VI) when *Eichornia* were grown with bacteria for 7 days (Phase I experiment)

Cr (VI) removal in Phase II

All inoculated plants exhibited the significant Cr (VI) removal (43.1 - 75.3 %) as compared to un-inoculated plants (3.14 and 1.68 %) at an initial K_2CrO_4 concentration of 100 and 300 µg/mL. Among inoculated plants, SF-5 showed significant Cr (VI) removal of 54.3 % at 100 µg/mL of K_2CrO_4, and S-6 removed 75.3 % of Cr (VI) at 300 µg/mL (Table 2).

Cr (VI) uptake

Maximum Cr (VI) uptake was shown by *Eichornia* plants inoculated with bacterial strain S-6 (3.74 Cr mg/g) at 100 µg/mL of Cr (VI). At higher initial concentration of K_2CrO_4 (300 µg/mL), chromate uptake was also enhanced. Plants inoculated with strains S-6 and S-4, exhibited approximately same uptake values (~5.98 Cr mg/g) and plants inoculated with SF-5 exhibited 4.56 mg/g Cr (VI) uptake value as shown in Figure 2.

Fourier transform infrared (FTIR) spectra

FTIR spectroscopy is one of the famous techniques used to determine the role of various functional groups involved in the binding with the metal ions. The FTIR spectrum was monitored in the range of 500 to 4000 cm^{-1} (wave number). The shifts in peaks in the region of 1100 cm^{-1}, 1500 - 1750 cm^{-1} were observed. Shifts in peaks in the range of 1100 - 1000 cm^{-1} were due to C-O bonding. The infrared shift of 1650 - 1550 cm^{-1} was characteristic of amide I and II bonding attachment. The peak shifts were shown by SF-5 under Cr (VI) stress and non-stress conditions from 1092.45 to 1081.622 cm^{-1}, 1528.7 to 1520.4 cm^{-1} and 1554.15 to 1560.13 cm^{-1}. The shifts were shown due to stretching of C-O bonding. The peak shifts of 1644.35 to 1637.56 cm^{-1}, 1644.3 to 1695.41 cm^{-1} and 1733.2 to 1730.1 cm^{-1} were also observed. These shifts were due to stretching of the N-H bonding (Figure 3).

16SrRNA gene sequencing

Bacterial strain S-4 showed 96 % homology with *Ochrobactrum sp.* whereas, SF-5 exhibited 99 %

Table 2: Cr (VI) removal at 100, and 300 µg/mL concentrations of Cr (VI) by *Eichhornia* alone and in combination with bacteria

Bacterial strain		Cr (VI) removal (%)	
		100 µg / mL	***300 µg / mL***
Phase I	Control	03.2±0.02 (a)	43.4±0.87 (b)
	S-4	60.47±0.78 (c)	76.04±1.21 (d)
	SF-5	43.95±0.32 (b)	78.6±0.54 (e)
	S-6	45.42±0.82 (b)	84.6±0.56 (d)
Phase II	S-4	43.1±0.59 (a)	59.7±0.54 (a)
	SF-5	54.3±0.70 (c)	37.9±0.34 (e)
	S-6	40.1±0.62 (bc)	75.3±1.11 (f)
	Control	3.14±0.30 (d)	1.68±0.04 (b)

(±SEM, n = 3, In each column, figures followed by different letter (s) in parenthesis indicate significant difference by Duncan's multiple range test (P<0.05)

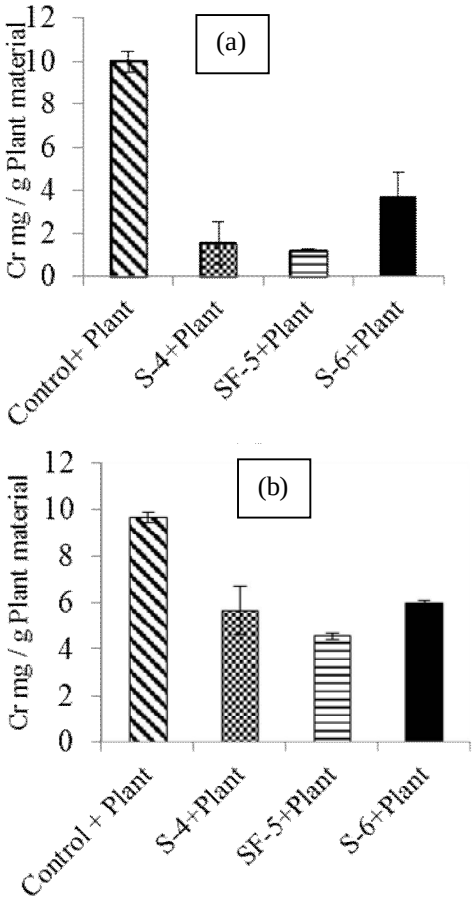

Figure 2: Cr (VI) uptake at 100 and 300 µg / mL of Cr (VI) concentration when *Eichornia* sp. were grown with bacteria for 14 days (Phase II experiment)

similarity with *Bacillus sp*. S-6 was showing 97 % sequence resemblance to *Ochrobactrum grignonenses* strain. The 16S rRNA gene sequence was submitted to NCBI GenBank with the accession number KP299171 (*Bacillus sp.* SF-5) and KR095626 (*Ochrobactrum* sp. S-4) and KR095627 (*Ochrobactrum grignonense* strain S-6).

DISCUSSION

Present work was focused on reduction of hexavalent chromium by indigenous bacteria along with aquatic plant (*Eichornia*). Bacterial strains isolated from Ittehad Chemicals drainage effluent, showed resistance to high Cr (VI) stress (0.5 mg/L – 1 mg/L). Three bacterial strains were selected and named as S-4 (*Ochrobactrum* spp., KR095626), SF-5 (*Bacillus subtilis,* KP299171) and S-6 (*Ochrobactrum grignonense*, KR095627). Bacterial strain S-4 and SF-5 with Gram positive behavior showed less Cr (VI) reduction, while S-6 showed higher chromate reduction with gram negative morphology. Faisal and Hasnain in 2005 reported, gram negative bacteria were commonly found to reduce more hexavalent chromium than gram positive species due to presence of lipopolysaccharides, lipoproteins and phospholipids in the outer membrane [14].

Bannerji *et al* reported *Rhodococcus erythropolis* was capable to grow at 30 mg/L, while our isolated bacterial strains were capable to tolerate 1 mg/L of K_2CrO_4 [15]. In the immobilized state when the isolates were transformed in to beads with sodium alginate, that enhanced the reduction of Cr (VI). In mobilized state, the bacterial strains first got adapted to stress conditions, and then they started reduction [16].

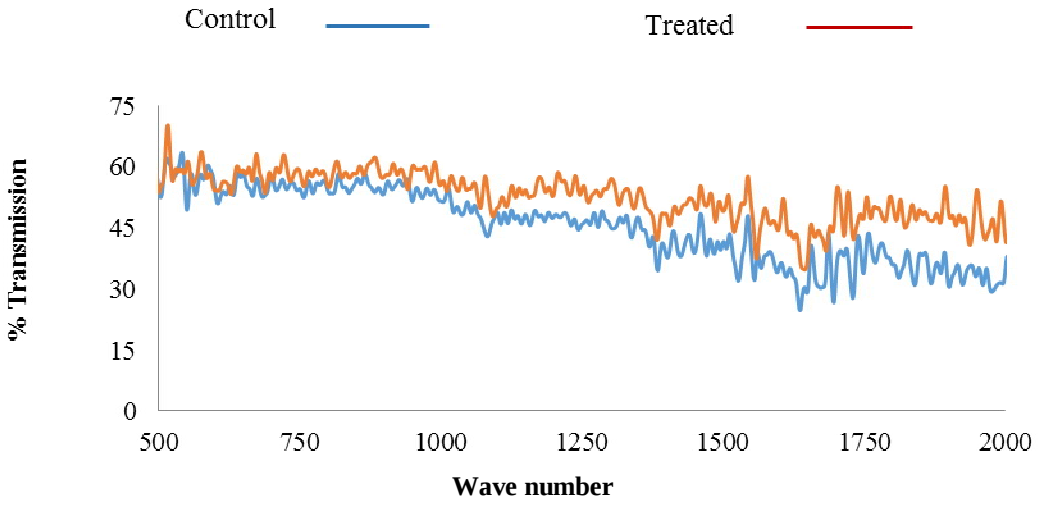

Figure 3: Fourier transform infrared spectroscopy of strain SF-5 grown in the presence (1000 µg/mL) and absence of Cr (VI)

Silva *et al* reported bacteria could efficiently reduce Cr (VI) in mobilized form as compared to immobilized state [2]. Similarly, in the present study Cr (VI) reduction increased gradually in mobilized state with increased time of contact.

Removal of hexavalent chromium by *Eichornia sp.* was more effective in the presence of chromium resistant bacterial strains. Aquatic plants released minerals helping bacteria to survive and in return, bacteria supported plants to grow well [14]. Lytle *et al* stated that the activity of *Eichornia crassipes* at higher concentration was significant as it could uptake and translocate chromium despite of presence of chromium resistant bacterial strains [17]. Similarly, Jaikumar reported that *Eichornia crassipes* decreased the pollution of drainage water lakes by uptake of heavy metals [18]. Present study showed there was rapid decrease in concentration of Cr (VI) due to an initial use of chromium metal by bacteria. Sultan and Hasnain described the Cr (VI) removal by aquatic plant from the waste water even in the absence of bacterial strains due to use of chromate as a metabolite [19]. Mishra *et al* reported that even low concentration of Cr (VI) showed increase in metabolic components of plants producing more chlorophyll a and b, proteins and nitrate reductase enzyme. While at higher levels of Cr (VI), inhibition of plant metabolites was seen and lesser Cr (VI) was taken up by plants [20]. Similarly in current study, the uptake of chromium was more obvious at 100 µg/mL than 300 µg/mL. Camargo *et al* stated that *Pseudomomas flouresence* along with *Eihchornia sp.* caused decrease in chromium content of effluent, whereas high quantity of chlorophyll was seen with increase in biomass of *Eichornia* within 20 days of incubation [21].

The FTIR spectra of SF-5 bacterial strain at 1000 µg/mL in control and Cr (VI) stress were taken to find evidence of involvement of various functional groups present on possible cell and metal ions interactions. The hexavalent chromium was expected to undergo reduction via complexation with carboxyl or amide or hydroxyl moieties [22]. Previously, it was reported the metal cations showed binding to carboxyl, amide and hydroxyl groups of bacterial cell surface in the region of 1100 – 1000 and 1600 - 1750 cm^{-1} [23,24]. Mangaiyarkarasi *et al* reported that under stress shifts in the peaks were observed around 1700 cm^{-1} and suggested the presence of ester group moiety whereas the peaks around 1648 cm^{-1} revealed amide II bonds involvement [24]. 16S rRNA sequencing results showed that strain SF-5 had similarity with *Bacillus* sp. while, S-4 and S-6 revealed homology with *Ochrobactrum sp*. Camargo *et al* also reported several chromium resistant strains belonged to genus *Bacillus* and *Ochrobactrum sp.* [23]. Isolation of Cr (VI) resistant *Ochrobactrum* from metal polluted environments have been reported in the past [12,25].

CONCLUSION

The results indicate that chromium-resistant bacteria, when inoculated with water hyacinth, enhances Cr (VI) removal which has proved to be one of the best bioremediation strategies for detoxification of Cr-polluted zones. The synergistic effect of this plant with chromium-resistant bacteria results in even greater removal of Cr (VI), suggesting the usefulness of this bioremediation tool for reclamation of chromium contaminated areas.

DECLARATIONS

Acknowledgement

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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