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Biosorbing Potentials of *Pseudomonas aeruginosa* SFP1 to Combat Cr(VI) Stress in *Cicer Arietinum* Seedlings

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ABSTRACT

Hexavalent chromium among metal pollutants is a major threat due to its mutagenic and carcinogenic impacts. Considering these, bacterial strain SFP1 was isolated from metal polluted soil (identified as *Pseudomonas aeruginosa* using 16SrRNA gene sequencing) showed significant tolerance to Cr (VI) and displayed chromium reducing ability under variable environmental conditions. The dried biomass of SFP1 adsorbed chromium maximally at pH 6 and 30±2^{0}C which decreased consistently with increase in Cr concentration. The values obtained for chromium sorption bystrain SFP1 using both Langmuir (R^2=0.992)and Freundlich isotherms (R^2=0.999) were strongly and positively correlated. The surface functional groups of dried biomass detected by Fourier transform infrared (FTIR) spectroscopy were amino, carboxyl, hydroxyl, and carbonyl groups.Also SEM-EDX revealed significant deposition of Cr and modification of bacterial cells after Cr(VI) exposure. The chickpea seeds primed with SFP1 strain displayed enhanced germination compared with metal treated but uninoculated plants. The present study suggests that the bacteria removes chromium efficiently and hence, could be used for the management of industrial wastes and other environmental contaminants.

1. Introduction:

Among heavy metals, chromium has been described as a priority pollutant by US EPA and is considered carcinogen. Among different oxidation states ,the trivalent chromium (Cr(III) and hexavalent chromium (Cr(VI) has been reported to be approximately 100 times more toxic [1] and 1000 times more mutagenic than Cr(III) [2]. Conventionally, different physico-chemical methods like precipitation, electrochemical treatment and ion exchange have been used to remediate metal polluted environment. However, these methods are expensive, environmentally unfriendly and produce residues which are even more toxic than the parent metals. Due to these problems, the bioremediation approach especially the use of microorganisms is regarded as an inexpensive and environmentally safe strategy that offers the possibility to destroy toxic chromium to harmless forms [3,4]. Biosorption involving bacteria is an efficient method employed for the removal of Cr (VI) from industrial effluents [5] which decrease the concentration of chromium ions in solution.Realizing the chromium threat and importance of microbes in toxicity abatement on the other hand, the present study was aimed at identifying bacteria capable of biosorbing chromium under different conditions and used to assses the bioremediation potential using chickpea (*Cicer arietinum* L.)

2. Materials and methods

2.1 Isolation and screening of chromium tolerant bacteria

The bacterial strain SFP1 was isolated from rhizosphere grown in metal polluted fields of Unnao (26°32'25.0"N 80°29'14.3"E),UP India. The ability of bacterial strains to grow under increasing concentrations of chromium was tested both on solid agar plate and in liquid culture medium. For this, bacterial strains were aseptically streaked on nutrient agar plates supplemented with 100–2000 µg/ml potassium dichromate, and checked for growth after incubation at 30±2^{0}C for 48 h. The bacterial strain SFP1 exhibiting the highest tolerance to Cr (VI) was selected for further studies. Chromium containing NB (0-100µg/ml) was inoculated with overnight grown cultures and incubated at 30±2^{0}C for 4 days under continuous shaking (120 rpm) in a rotary shaker. Further SEM micrographs of both untreated and Cr(VI) treated cells was observed for morphological alterations while EDX analysis was carried to determine the metal deposition.

2.2 Molecular identification and phylogenetic tree of metal tolerant strain SFP1

The chromium tolerant strain SFP1 was selected and characterised by standard morphological and biochemical methods [6]. The nucleotide sequence of strain SFP1 was analysed commercially by Macrogen Inc,, Seoul, South Korea using the 16S rRNA genes) involving universal primers, 785F (GGATTAGATACCCTGGTA) and 907R (CCGTCAATTCMTTTRAGTTT).The sequence (859 bp) so obtained were analyzed by adopting BLASTn tool (http://www.ncbi.nlm.nih.gov/BLAST) to accurately identify and match the sequence of isolates with the nearest neighbour sequence obtainable at the NCBI database. All the sequence were aligned using Clustal W and the aligned data was used for phylogenetic analysis using MEGA7 by neighbour-joining method with 1000 boot strap replicates.

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2.3 Biosorption efficiency

To assess the biosorbing potential of the selected bacterial strain, biomass of SFP1 was produced by growing bacterial culture in nutrient broth (pH 7) at30± 2^{0}C for 24 h. Cells were harvested by centrifugation (at 8000rpm) for 20 min. Cell pellets were washed three times with distilled water and dry biomass was prepared by overnight vacuum drying (at 90^{0}C). The sorption of chromium by dried biomass of SFP1 was determined by batch equilibrium method [7]. For this, metal stock solution was prepared by adding appropriate amount of $K_2Cr_2O_7$ in 100 ml of double distilled water. All set of experiments were conducted employing fixed volume (100 ml) of single metal ion solution in a 250 ml Erlenmeyer flask. Bacterial biomass was exposed to metal solutions for 96h on an orbital shaking incubator (Remi, India) at 160 r/min. Biomass was separated by centrifugation at 8000rpm for 15 min. and the supernatant was analysed for residual metal concentration by flame atomic absorption spectrophotometer (Model: GBC 932B Plus).

2.3.1 Optimisation of biosorbing efficiency of strain SFP1

Furthermore, the conditions that influenced the metal removal efficiency by the bacterial strain such as pH (2, 4, 6, 8 and 10), contact time (12h, 24h, 48h, 72h, 96h, 120h) and initial concentration (25, 50, 100, 200, 400 µg/ml) were also studied.

2.3.2 Biosorption isotherms

Biosorption process using batch technique necessitates an understanding of the relationships between metal ions and biosorbent. The biosorption experiments were performed taking into account using the following equation:Metal adsorbed % = $\frac{(C_o-C_f)}{C_o}\times 100$; where C_0 and C_f represent initial and final Cr(VI) concentration. The biosorption capacity was estimated as-$Q=\frac{(C_o-C_f)}{M}\times V$ where Q indicates the extent of adsorbed metal ion onto the biomass surface at equilibrium (mg/l), M signifies the amount of biomass in the suspension (L), and V is the volume of the suspension (L). The Langmuir model considers monolayer accumulation of adsorbents on homogenous biosorbent surface and can be expressed as $Q=\frac{Q_{max}bC_f}{1+bC_f}$. The binding constant ($Q_{max}$,) and the sorbent capacity (b) are estimated by plotting $1/Q$ against C_f[8]. The model simulations for Cr(VI) with the calculated values of K_L and b are given in Fig 5 respectively. The Freundlich isotherm is an experimental model that highlights the adsorption intensity of the sorbent to the biosorbent. The isotherm is applied to depict the reversible adsorption among the adsorbing species and does not consider the monolayer formation. The Freundlich isotherm model can be represented as-$\log Q = \log K_F + (1/n)\log C_f$. A plot of logQ versus $\log C_f$ gives a straight line with slope $b_F = 1/n$ and intercept K_F.

2.3.3Separation factor (Sf) and Surface coverage (Ø)

The shape of the isotherm can be used to predict whether adsorption system is favourable or unfavourable in a batch adsorption system. Accordingly, the important aspect of Langmuir isotherm was stated in terms of dimensionless constant called the separation factor as $S_F=\frac{1}{(1+bC_0)}$. Surface coverage (Ø) denotes the number of adsorption sites used divided by number of adsorption sites available. The adsorption behaviour of the metal ions on the biomass was determined by the formula $Ø=\frac{bC_0}{1+bC_0}$.

2.4 Determination of Functional group moieties involved in Cr(VI) biosorption

For FTIR analysis, thebacterial biomass unexposed (control) and exposed to Cr (VI) at concentration of 100 mg/l was obtained by centrifugation (at 8000 rpm) for 15 min. at 4°C was driedat 80^{0}C. The harvested biomass was washed, dried in oven at 40ºCand crushed to powder form after biosorption of chromium ions under the same conditions. A 2.5 mg dried bacterial biomass was mixed and ground with 75 mg of KBr in an agate mortar and was immediately analysed with a spectrophotometer in the range of 400–4000 cm^{-1} with a resolution of 5 cm^{-1} using a FTIR Spectrometer (Thermo Nicolet, Nexus 670).

2.5 Germination assay

Seeds of chickpea (var. avrodhi) were surface sterilized both uninoculated and inoculated were placed on soft agar (0.8%)were amended with different concentrations (25-400 ug/ml) of chromium and growth parameters were measured at 7 days after sowing (DAS). Uninoculated and untreated seeds soaked in water served as control.

2.6 Statistical analysis of data

Each experiment was performed in triplicates to avoid any discrepancy of the experimemtal results and repeated under the same conditions to assure reproducibility of the data. Further, One way ANOVA and Duncan multiple range test on experimental data were evaluated by the computer software package, SPSS 17 (SPSS Inc., Chicago, USA).

3. Results and Discussion

3.1 Bacterial characterisation, 16SrRNA gene sequencing and phylogenetic tree construction

The bacterial strain SFP1 recovered from metal polluted rhizosphere tested positive for citrate utilization, nitrate reduction, and oxidase test, and hydrolyzed starch and gelatin. The strain however, showed variable potential to utilise different carbohydrates (data not shown). Based on morphological and biochemical parameters, the bacterial strain SFP1 was presumptively identified to genus level as *Pseudomonas*. The nucleotide sequence of 16S rRNA of SFP1 obtained was approximately 859 bp in size which was identified as *P. aeruginosa* later on submitted to GenBank (accession number KU522247. A phylogenetic tree constructed by MEGA7 software, based on 16S rRNA partial gene sequence is presented in Fig. 1(a).

3.2 Chromium tolerance and bacterial growth under chromium stress

The bacteria strains recovered from the metal polluted soil were screened for their ability to tolerate toxic levels of hexavalent chromium. The strain SFP1 grew well on agar plates amended with 800 mg/l of potassium dichromate. Furthermore, chromium tolerance was proved by growing strain SFP1 in NB medium treated with different concentration of Cr (VI). Strain SFP1 continued to grow well until 200 mg/l of Cr (VI), but the bacterial population decreased thereafter, with prolonged lag period of 8 h.The reduction in growth, however, at higher concentrations of Cr (VI) could possibly be due to altered genetic composition and metabolic and physiological responses of bacteria under stress. Also SEM images revealed significant alteratins in cell surface compared to untreated cells while the EDX shows deposition of Cr at cell surface (Fig 1(b)).The reduction in growth, however, at higher concentrations of Cr(VI) could possibly be due to altered genetic composition and metabolic and physiological responses of bacteria under stress [9, 10].

3.3 Biosorption studies and optimisation studies

In the present study, the biosorption efficiency of *P. aeruginosa* SFP1 was investigated.Strain SFP1 showed the sorption of chromium under different conditions of pH, temperature and initial Cr (VI) concentration. It was observed that an increase in the initial metal concentration resulted in a gradual decrease in the percentage removal of chromium.For example,it was 89.48% at 25 mg/l while it was 89.02% at 50 mg/l initial Cr(VI) concentration. The rate of biosorption was influenced by (i) competition among increasing chromium molecules for the available binding sites on the bacterial surface and (ii) the restriction of movement of chromium ions due to changes in charge distribution at high Cr(VI) concentrations [11, 12]. Among different pH tested, the maximum removal of chromium occurred at acidic pH for the biosorbent and the sorption capacity increased constantly from pH 2 to 6.0 which decreased drastically thereafter. The maximum biosorption however, occurred at pH 6 (90.5%). As the pH of the solution affects different species of chromium in the solution [13, 14] and the ionization states of the functional groups present on the biosorbent. Also, the decrease in chromium removal rate with the increase in pH beyond pH 6 might be due to the osmotic changes and hydrolyzing effect [15]. The maximum biosorption was observed at 120 hr (89%) and it was observed that metal uptake increased in contact time from 12h to 96h however, till 120h there was no significant increase in the biosorption attaining an equilibrium position.The rapid adsorption in the initial hours of exposure followed by equilibrium supports the metal adsorption models that involves adsorption and desorption that depends on residence time between the adsorbing species [16]. The Cr(VI) adsorbed by the bacterial strain reduces the toxic levels of the Cr (VI) which might possibly be due to its conversion to less toxic Cr (III) or after adsorption it accumulates inside the bacterial cell.

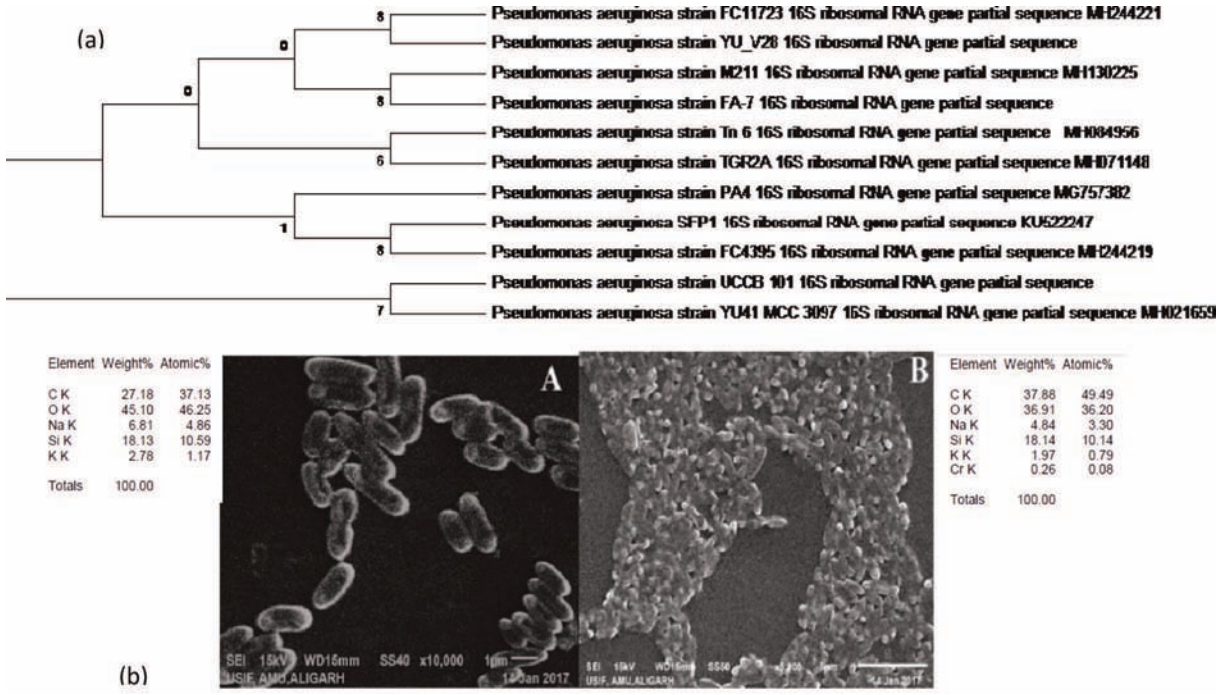

Fig 1.(a) Phylogenetic analysis based on 16S rRNA comparison of 859bp showing relationship between *Pseudomonas aeruginosa* **SFP1** (GenBank accession no. KU522247) and other closely related *P. aeruginosa* strain and values at node represents percentage of 1000 bootstrap replicates. (b) Effect of Chromium concentrations (µg/ml): 0and 200 on growth of *P. aeruginosa* as observed under SEM.

3.4 Biosorption isotherms and functional group characterisation on cell surface

The metal ions deposited at the bacterial surface including cell wall is generally represented by conventional isotherms [17]. In biosorption studies, the term Freundlich and Langmuir isotherm models representsheterogeneous and homogeneous processes of biosorption, respectively (Fig 2). From the calculated maximum metal uptake (Q) Langmuir isotherm, the achievable Cr uptake was 78.7 mg/gdw. It was observed that the Freundlich isotherm performed better than Langmuir isotherm because oftheir higher r^2. The Freundlich constants K_F (1.15 mg/.gdw) corresponded to the binding capacity (maximum adsorption capacity), and b_F (1.0341) characterises the affinity (adsorption intensity) between the isolates (sorbent) and Cr (sorbate) (Fig. 6). The separation factor (S_f) indicates the shape and nature of biosorption process and principally the separation factor value between 0 and 1 represents favourable isotherm. It has been discovered that the adsorption is irreversible when S_f = 0 while linear adsorption occurs when S_f = 1 [18].Our results in this respect were favourable according to adsorption equation because all values of S_f were >0 and less than 1 at all the tested concentrations of chromium (0.453-0.049). Since, both adsorption of Cr to the biological surface of *P. aeruginosa* and conjugation of Cr to the functional groups present in the cell wall occur simultaneously,it indicated a heterogeneous binding process.While comparing the FTIR spectra of untreated cells (control) with Cr(VI) treated cells, Cr(VI) treated cells showed several distinct, medium and weak bands at different wavelength. From Fig.3, it was evident that the peak appearing before metal loading 3294 cm^{-1} stretched to 3303 cm^{-1}which indicates the presence of amine and hydroxyl group (Park et al. 2005). The peak at 2967 cm^{-1} could be due to the–CH stretch in the biomass[19, 20]. After contacting with the metal solution, -COO shifted from 1403 to 1405 cm^{-1}suggesting the affinity of carboxyl group to metal ions. The shift in1400 cm^{-1} band is due to the vibration of O–H carboxylate ions. The peak around 1243 cm^{-1} shows the presence of carboxyl group. The peak at 1541 and 1458 cm^{-1} reveals the presence of C=O which plays a vital role in the chromium biosorption [21]. Heavy metals tend to change the functional groups on cells surface either by stimulating or suppressing the biomolecules present on cell surface. As a result, the bacterial populations respond differently to metal toxicity. Therefore, it was evident from this study that the spectral differences in cell surface functional groups following adsorption on bacterial biomass might have played an important role in chromium binding and consequently the chromium removal [22, 23].

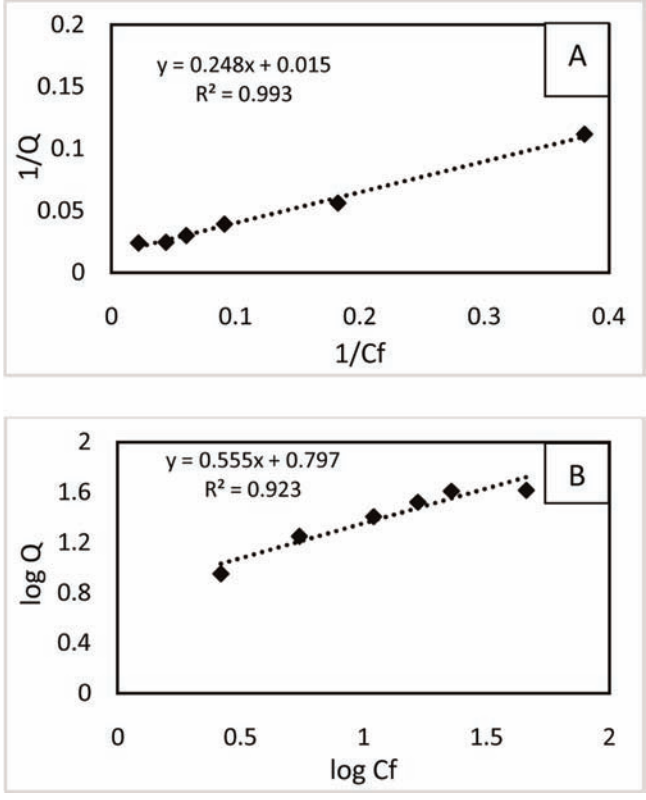

Fig 2. Linearised Langmuir (a) and Freundlich (b) adsorption isotherm for heavy metal ions on biosorbent biomass of *Pseudomonas aeruginosa* SFP1.

3.5 Enhancement of seed germination by metal tolerant strain SFP1

A considerable decline in chickpea growth was observed which further increased with increasing concentration of toxic chromium (Table 1). However, chickpea seeds inoculated with strain SFP1 showed better growth even in the presence of chromium. At 400 µg Cr/ml, the radicle length and shoot length was remarkably reduced each by 85%. In contrast, inoculated plants exhibited improved growth and reduction in radicle length (81.5%) and shoot length (80.7%) was significant at 400 µgCr/ml.

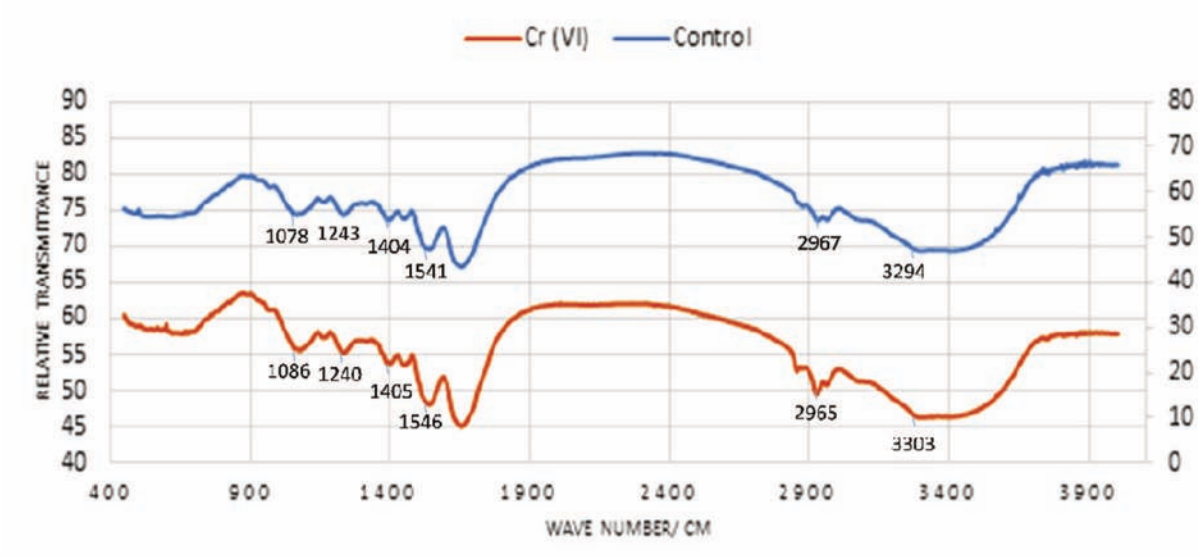

Fig 3. FTIR spectra of *P. aeruginosa* prepared in KBr disks: control and Cr(VI)-treated.

Table 1. Effect of heavy metals on germination attributes of chickpea after 7 days of incubation with and without the presence of metal tolerant SFP1.

Treatment	Dose rate (µg/ml)	Germination percentage		Mean Shoot length (cm)		Mean Radicle length (cm)	
		Uninoculated	Inoculated	Uninoculated	Inoculated	Uninoculated	Inoculated
Control	0	100	100	5.4±0.8a	6.7±1.2a	7.8±0.8a	9.2±0.5a
Chromium	25	98	100	4.7±0.3b	6.5±0.8b	6.5±1.2b	7.2±0.7b
	50	85	90	3.8±1.2c	5.9±1.3b	4.5±1.5c	6.4±0.3b
	100	80	84	2.5±0.4d	4.8±0.7c	3.6±0.5d	5.3±0.5c
	200	60	79	1.7±0.4d	3.7±0.4d	3.0±0.4d	4.6±1.0d
	400	50	63	0.8±0.7f	1.0±0.1e	1.2±0.2e	1.5±0.9e
F value	-	-	-	19.2	18.9	47.3	42.4

This reduction in growth may be due to the toxic effect of chromium leading to inhibition of protein synthesis [24, 25]. Also, it was observed that the seedling growth was enhanced maximally in presence of strain SFP1 at lower concentrations of chromium than at higher concentrations. The growth enhancement by inoculated seeds may be attributed to the both the chromium reducing and biosorbing ability of strain SFP1 which limits the toxic effect of chromium.In agreement to this finding Velez et al [26] also reported the inoculated strains possessing metal tolerant ability promote the growth promotory effect of metal tolerant bacterium on chickpea seedlings even when exposed to chromium stress.

4. Conclusion

The Cr(VI) tolerant Gram negative strainSFP1 possessed significant biosorbing potential and reduced the toxic levels of Cr(VI) under different environmental variables. Also, the chromium tolerant strain enhanced the growth of chickpea even under metal stress. Considering these,*P. aeruginosa* SFP1may be developed to optimize their viability and biological activity under field applications to be efficiently used for bioremediation of chromium contaminated soils. Furthermore techniques to extract the biosorbent after metal removal process has to be exploited to recover the metal from the contaminated sites for successful and effective bioremediation process.

5. Acknowledgements

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