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Effect of Cr^{6+} Stress on Photosynthetic Pigments and Certain Physiological Processes in the Cyanobacterium *Anacystis nidulans* and Its Chromium Resistant Strain

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Abstract A MNNG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) induced chromium resistant strain (Cr^rI8) of unicellular cyanobacterium *Anacystis nidulans* has been isolated and characterized. The resistant strain could grow (although restricted to 50% of control) in chromium concentration (180 μM) lethal to the wild-type. Sublethal (160 μM) concentration of Cr^{6+} significantly reduced (13– 37.5%) all the photosynthetic pigments of *A. nidulans* with maximum reduction in phycoerythrin followed by Chl *a*. Pigments of *A. nidulans* were drastically decreased in lethal concentration of Cr^{6+} with maximum reduction in phycoerythrin (75%) and allophycocyanin (67.5%). Resistant strain Cr^rI8 resisted toxic effects of sublethal and lethal concentrations of Cr^{6+} on photosynthetic pigments as revealed by less decrease in pigments as compared to *A. nidulans*. Effect of Cr^{6+} stress was also studied on nitrogen assimilation and phosphate uptake. Sublethal concentration of Cr^{6+} drastically reduced (71.5%) nitrate uptake by *A. nidulans* while a decrease of 29% was observed in strain Cr^rI8. Short (2 day) exposure of *A. nidulans* and its resistant strain Cr^rI8 to Cr^{6+} did not affect nitrate reductase and glutamine synthetase (transferase), whereas longer (10 day) exposure to Cr^{6+} lowered activities of both enzymes in *A. nidulans* but not significantly in the strain Cr^rI8. Ammonium uptake by both strains was not affected by Cr^{6+}. Thus, Cr^{6+} affected photosynthetic pigments, nitrogen assimilation, and phosphate uptake of *A. nidulans*, while strain Cr^rI8 was able to resist toxic effects of the metal. Advantages of using strain Cr^rI8 for bioremediation purposes have been evaluated by studying Cr^{6+} removal from the solution. Resistant strain Cr^rI8 was able to remove 33% more Cr^{6+} than *A. nidulans* and thus it can prove to be a good candidate for bioremediation of Cr^{6+} from polluted waters.

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Heavy metals enter the aquatic environment from both natural and anthropogenic sources. Anthropogenic input of heavy metals into the environment far exceeds input from natural sources [23]. Although some metals are required by microorganisms in trace amounts for their various metabolic processes [25], high concentrations of all metals, including those essential for growth and metabolism, exert toxic effects on the metabolic machinery of the organisms. The effects may be at cellular, community, population, or ecosystem levels of organization. Algae, being an ubiquitous component of the water ecosystems, besides other biota are obviously affected by the presence of these metal ions. The effects of heavy metal pollution on metabolic processes of algae have been reviewed [25, 26, 42].

The occurrence of some algae, including cyanobacteria, in metal contaminated aquatic bodies may lead us to conclude that these organisms are able to resist/tolerate metal toxicity. Algae are able to tolerate certain concentrations of heavy metals either at the uptake level or at cellular level. After uptake, heavy metals may be excluded from the cells or may be detoxified intracellularly [6, 33]. The intracellular metal detoxification mechanisms include binding with polyphosphate granules [8], binding with peptides and proteins [6], and binding and precipitation within the cytoplasm/vacuole [30]. These mechanisms may be physiological and biological adaptations or may have become an intrinsic property of the organisms after their prolonged exposure to varying degrees of metals. Thus, these resistant/tolerant algal species may be used as indicators and as scavengers of heavy metal pollution.

The toxicity of heavy metals to various metabolic processes has been investigated by using either laboratory organisms or those species growing naturally in metal contaminated waters. Though their potential as bioscavengers is being visualized, no attempts have been made to raise metal resistant strains in the laboratory and to evaluate their potential as bioscavengers. During the present study, besides raising and characterizing a Cr^{6+} resistant strain of a unicellular cyanobacterium *Anacystis nidulans* with reference to photosynthetic pigments, nitrogen assimilation, and phosphate uptake, advantages of using this strain for bioremediation purpose have been evaluated.

MATERIALS AND METHODS

Organism and Culture Conditions

Anacystis nidulans is an isolate of our laboratory from Sutlej river running near Ludhiana city of Punjab State of India. Pure and axenic cultures of the organism were propagated in 250-ml glass Erlenmeyer flasks containing nitrate-enriched (KNO_3; 5 mM) modified Chu-10 medium [32]. Cultures were incubated at 28±2°C in a culture room and illuminated for 14 h a day with cool white fluorescent lights giving a radiant flux of 9.8 W/m^2 on the surface of glass vessels. The cultures were hand-shaken at least twice daily.

Growth

Growth of the organism and its metal resistant strain in medium, without and with different concentrations (50–220 μM) of Cr^{6+}, was monitored as increase in cell number of the cultures. Cells in the cultures were enumerated manually using a hemocytometer. Average of at least 10 counts was taken. Desired concentrations of Cr^{6+} were obtained by appropriate dilutions of the stock solution of $K_2Cr_2O_7$ in sterilized distilled water.

Estimation of Photosynthetic Pigments

A known volume of culture was washed twice with distilled water by centrifugation at 5,000 ×*g* for 5 min and its cell number was enumerated. Acetone soluble pigments (Chlorophyll *a* and carotenoids) were extracted by suspending the pellet in 80% acetone followed by incubation in dark at 4°C for 8 h and centrifugation. Supernatant was used for spectrophotometric estimation of Chl *a* and carotenoids at 663 nm and 450 nm, respectively. The amounts of pigments were calculated using specific absorption coefficients of 82.04 and 200 for Chl *a* and carotenoids, respectively [20]. Water-soluble pigments were extracted in phosphate buffer (0.2 M; pH 7.0) by freezing and thawing after treatment with lysozyme (Merck Ltd., India). Absorbance of water-soluble pigments was noted at 615, 652, and 565 nm and the relative amounts of phycocyanin (PC), allophycocyanin (APC), and phycoerythrin (PE) were calculated following Bennet and Bogorad [1]. The values were expressed as μg pigment per 10^6 cells.

Equal number of exponentially growing cells (6-day-old) of both *A. nidulans* and resistant strain Cr^rI8 were incubated for 4 days in sublethal (160 μM) and lethal (180 μM) concentrations of Cr^{6+} and changes in photosynthetic pigments were determined.

Isolation of Cr^{6+} Resistant Strains

A total of 5×10^6 cells were treated with LD_{50} (50 μg ml^{-1}) of MNNG (Sigma Chemical Co., St. Louis, MO, U.S.A.) for 10 min and were plated on agar plates supplemented with lethal dose of Cr^{6+}. After 15 days, 40 colonies appeared and 8 healthy colonies were picked up, and numbered and tested for Cr^{6+} resistance. Among 8 clones, strain Cr^rI8 was found to be the best on the basis of growth performance and was chosen for further studies.

Nitrate, Ammonium, and Phosphate Uptake

Uptake of nitrate, ammonium, and phosphate by the organism and metal resistant strain was studied following their depletion from the medium with time. Nitrate and phosphate were determined as described earlier [34] while ammonium was determined by the following method of Solarzano [40].

Cr^{6+} Uptake

Cell density equivalent to 150 μg protein ml^{-1} was inoculated in 25 ml medium with 30 μM Cr^{6+} and incubated under light in the culture room. At desired intervals, known volumes of cells were withdrawn, centrifuged, and the amount of Cr^{6+} present in the medium was determined spectrophotometrically following the method of Vogel [43].

Nitrate Reductase (NR; EC 1.7.99.4) and Glutamine Synthetase (GS; EC 6.3.1.2)

Whole-cell NR activity was measured by the method of Herrero *et al.* [7] and nitrite formed during NR assays was estimated according to Nicholas and Nason [21]. GS (transferase) assay was performed following the method of Shapiro and Stadtman [35].

Protein Estimation

Protein content of the cells was determined following the method of Lowry *et al.* [14]. All data given are the average of three independent experiments.

RESULTS AND DISCUSSION

Cell density equivalent to 3×10^6 cells ml^{-1} of *A. nidulans* and metal resistant strain Cr^rI8 were inoculated in Chu-10 medium supplemented with different concentrations of

Table 1. Effect of sublethal (160 μM) and lethal (180 μM) Cr^{6+} on photosynthetic pigments after 4 days.

Cr^{6+} (μM)	Pigments [μg (10^6 cells)$^{-1}$]									
	A. nidulans					Cr^rI8				
	Chl *a*	Carotenoids	PC	APC	PE	Chl *a*	Carotenoids	PC	APC	PE
Control	0.052± 0 .004	0.041± 0.0035	0.38± 0.004	0.37± 0.003	0.24± 0.002	0.044± 0.004	0.036± 0.003	0.52± 0.005	0.42± 0.004	0.20± 0.002
160	↓25± 1.5	↓13± 1.43	↓13± 0.8	↓13.5± 1.25	↓37.5± 1.66	↔	↔	↔	↓7± 0.65	↓10± 0.9
180	↓43± 1.2	↓35± 1.4	↓45± 2.65	↓67.5± 2.35	↓75± 2.97	↓5± 0.5	↓12± 0.9	↓30± 1.2	↓43± 2.6	↓45± 2.3

↓ indicates % decrease of respective controls.
↔ indicates no change.

Cr^{6+} (zero to 220 μM). After 12 days, cell population of both strains in media devoid of Cr^{6+} increased to $84\pm6\times10^6$ cells ml^{-1} indicating that their growth rates were the same. However, a concentration dependent decline in cell population of both *A. nidulans* and strain Cr^rI8 was observed in Cr^{6+} supplemented media, where decrease was more pronounced in *A. nidulans* as compared to resistant strain Cr^rI8. Cell density of *A. nidulans*, after 12 days, was almost half ($46\pm2\times10^6$ cells ml^{-1}) and one tenth ($8.2\pm1.6\times10^6$ cells ml^{-1}) of parent cultures in 100 μM and 160 μM Cr^{6+}, respectively. The cells did not divide and lysed in 180 μM Cr^{6+}. Chromium concentrations in which there was 50% and 10% growth of control cultures were taken as LD_{50} and sublethal concentrations, while the concentrations in which no growth but cell lysis occurred were considered lethal concentrations. For metal resistant strain Cr^rI8, these concentrations were substantially high. On the basis of increase in cell number, the LD_{50}, sublethal, and lethal concentrations of Cr^{6+} for resistant strain Cr^rI8 were 180 μM ($52\pm4\times10^6$ cells ml^{-1}), 210 μM ($7.4\pm1.8\times10^6$ cells ml^{-1}), and 220 μM Cr^{6+}, respectively. Thus, Cr^{6+} concentration which was otherwise lethal for *A. nidulans* supported 50% growth of control cultures of the strain Cr^rI8. Toxic effects of heavy metals may be "short term" or "long term". The former include vital physiological processes such as CO_2 fixation, depletion of ATP and reductant, inhibition of photosynthesis etc., while the latter result in overall decrease in general growth [19, 24]. Thus, long-term effects are as a consequence of short-term effects.

Experiments were performed to study toxic effects of Cr^{6+} (160 and 180 μM Cr^{6+} which are sublethal and lethal, respectively, to *A. nidulans*) on photosynthetic pigments, nitrogen assimilation, and phosphate uptake to see which of these processes are more affected by Cr^{6+} and to ascertain the level of resistance exhibited by the strain Cr^rI8.

Strain Cr^rI8 exhibited lower amounts of Chl *a* and carotenoids and high amounts of PC and APC in Cr^{6+}-free medium as compared to *A. nidulans* (Table 1). Sublethal concentration of Cr^{6+} affected all the photosynthetic pigments of *A. nidulans* (13 to 37.5% decrease) with maximum reduction in PE followed by Chl *a*. A slight decrease (7–10%) in APC and PE of the strain Cr^r18 was observed in sublethal concentration of Cr^{6+}, while Chl *a*, carotenoids, and PC were not affected. Lethal concentration of Cr^{6+}, on the other hand, drastically decreased all the pigments of *A. nidulans* with maximum reduction in PE (75%) and APC (67.5%), while in the same concentration Chl *a* and carotenoids of resistant strain Cr^rI8 were least affected (5–12% decrease) (Table 1). Thus, for the survival of the organism, Chl *a* and carotenoids are more important. The toxic effects of lethal concentration of Cr^{6+} on photosynthetic pigments were more pronounced in *A. nidulans* as compared to strain Cr^rI8. Pigment content has been identified as a valuable parameter for defining toxicity of heavy metals [2, 26, 27, 28, 41]. It has been demonstrated that in the presence of heavy metal ions like Zn^{2+}, Cu^{2+}, Hg^{2+}, and Pb^{2+}, photosynthetic electron transport chain of cyanobacteria is inhibited [4, 17, 18]. Decrease in photosynthetic pigments of *A. nidulans* in presence of Cr^{6+}, thus, this indicates that biosynthesis of these pigments is affected by metal ions. On the other hand, biosynthesis of photosynthetic pigments of the resistant strain Cr^rI8 is not much affected in sublethal concentration of Cr^{6+}, leading to normal levels of photosynthetic pigments and growth in presence of Cr^{6+} ions.

Nitrogen assimilation can be studied in two independent assimilatory pathways, i.e., nitrate and ammonium assimilation. Nitrate assimilation involves uptake of nitrate by the cells from the medium and its reduction to ammonia by nitrate reductase and nitrite reductase. Incorporation of ammonia into the carbon skeleton is catalyzed by the enzyme glutamine synthetase (GS). Strain Cr^rI8 took up higher amounts of nitrate as compared to *A. nidulans* (Fig. 1). Addition of sublethal concentration of Cr^{6+} drastically reduced (71.5%)

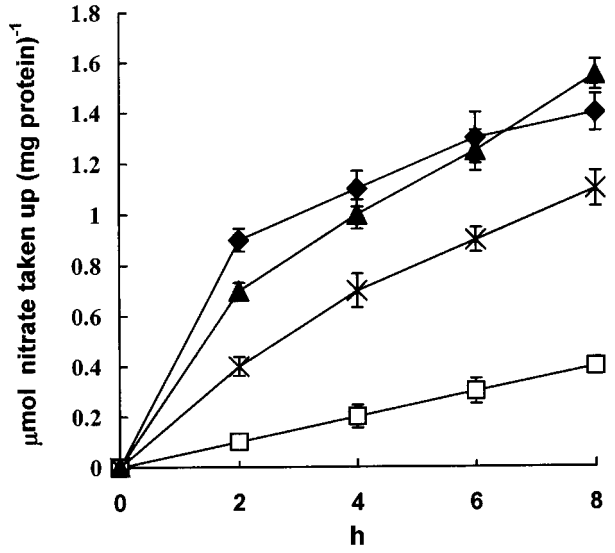

Fig. 1. Nitrate taken up from control medium (–◆– *A. nidulans*, –▲– Cr^{r}I8) and metal supplemented (160 µM Cr^{6+}) medium (–□– *A. nidulans*, –✕– Cr^{r}I8).
6-day-old cells equivalent to 50 µg protein ml^{-1} were incubated in 50 ml medium containing 100 µM NO_3^- and nitrate taken up by the cells was monitored as described in the text. Values are mean±SD.

nitrate uptake by *A. nidulans*, whereas the same concentration caused 29% decrease in nitrate uptake by resistant strain Cr^{r}I8. Short-term (2-day) exposure of *A. nidulans* and the strain Cr^{r}I8 to Cr^{6+} did not affect enzymes NR and GS significantly (Figs. 2 and 3). Longer exposure (10 days) to Cr^{6+}, however, reduced NR (61%) and GS (62%) of *A. nidulans* but to a lesser extent of strain Cr^{r}I8. Thus, the immediate target of Cr^{6+} toxicity is the nitrate uptake system rather than enzymes involved in nitrate reduction. Decrease in NR and GS of *A. nidulans* with longer exposure to Cr^{6+} may be due to the fact that lower amounts of NR and GS are required since very low nitrate is entering the cells. These results are similar to the observations of Kashyap and Gupta [9], where Cu^{2+} was shown to decrease nitrate uptake by *A. nidulans.* Rai *et al.* [28] reported inhibition of nitrate uptake due to Cr^{6+}, Pb^{2+}, Ni^{2+}, and Ag^{2+} toxicity. Vanadium has been reported to inhibit NR activity of *Nostoc linckia* [12, 16].

Another metabolic process reported to be sensitive to chromium toxicity is NH_4^+ uptake. Short-term (8 h) experiments were performed to study ammonium uptake by *A. nidulans* and by strain Cr^{r}I8 in absence and presence of Cr^{6+}. Rate of ammonium uptake (3.9 µmol NH_4^+ mg^{-1} protein in 8 h) by the resistant strain Cr^{r}I8 from medium devoid of Cr^{6+} was comparable to *A. nidulans* and was not affected by Cr^{6+}. This suggests that both strains can do better in the presence of Cr^{6+} with ammonium ions rather than with nitrate ions. Bolanos *et al.* [2] demonstrated that NH_4^+ uptake was considerably reduced in *Anabaena* strain PCC 7119. Inhibition of ammonium uptake in *A. nidulans* due to Cd^{2+} has also been reported [39].

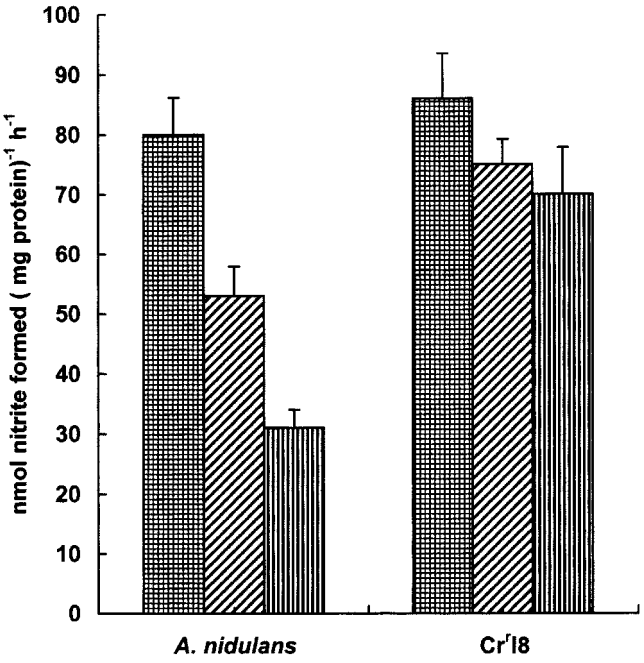

Fig. 2. NR activity in control (▦) and metal-supplemented (160 µM Cr^{6+}) medium on 2nd (▨) and 10th (▥) d.
6-day-old cells were incubated in respective medium with 5 mM KNO_3^-. Values are mean±SD.

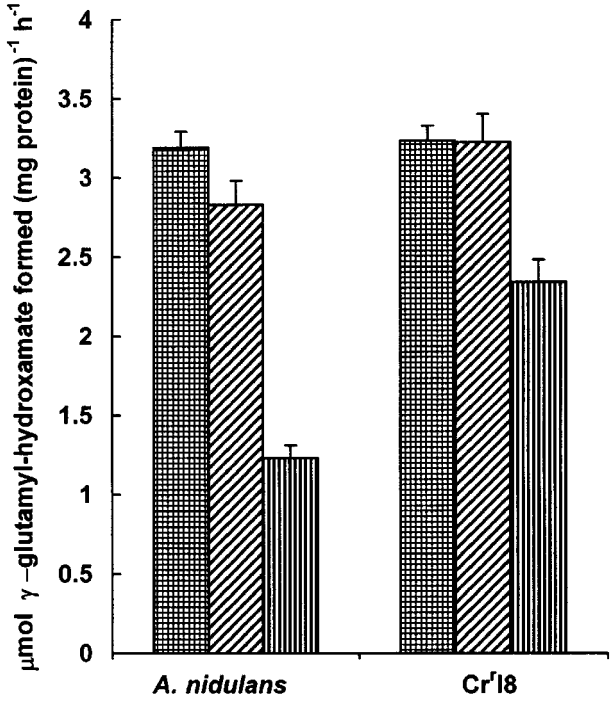

Fig. 3. GS activity in control (▦) and metal-supplemented (160 µM Cr^{6+}) medium on 2nd (▨) and 10th (▥) d.
Conditions were same as in Fig. 2. Values are mean±SD.

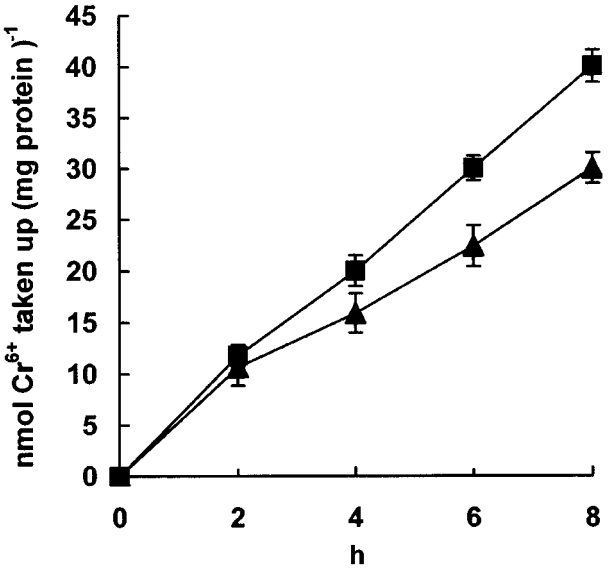

Fig. 4. Cr^{6+} uptake by (150 μg protein ml^{-1}) of *A. nidulans* (–▲–) and $Cr^{r}I8$ (–■–) from Chu-10 medium (30 μM Cr^{6+}).
Cells grown in medium devoid of Cr^{6+} for 6 days were used for Cr^{6+} uptake. Values are mean±SD.

Phosphate uptake is another target of metal toxicity [5]. Phosphate uptake was found to be inhibited in a competitive manner in *A. doliolum* under Cd^{2+}, Cu^{2+}, Ni^{2+}, Fe^{3+}, and Al^{3+} stress [15]. Rai *et al.* [29] reported that growth along with nitrogen and phosphorus metabolism was affected due to $AlCl_3$ and AlF_3 in *N. linckia.* During the present study, both *A. nidulans* and resistant strain $Cr^{r}I8$ showed steady rate of phosphate uptake, and removed 1.48 and 1.68 μmol of phosphate from the basal medium, respectively in 8 h. Supplementation of 160 μM Cr^{6+} decreased phosphate uptake by 14% and 12% by *A, nidulans* and by strain $Cr^{r}I8$, respectively. Results obtained thus revealed that sublethal and lethal concentrations of Cr^{6+} affected photosynthetic pigments, nitrogen assimilation, and phosphate uptake of *A. nidulans* leading to less/no growth, while ammonium uptake was not affected. On the other hand, strain $Cr^{r}I8$ was able to resist toxic concentrations of Cr^{6+} and could grow in the presence of Cr^{6+}. The resistance exhibited by a metal resistant strain may be at extracellular or intracellular level [13, 33, 38]. At extracellular level, an organism may have a defective uptake system so that metal ions are not taken up [31] or it may bind metal ions extracellulary on cell walls [33, 38]. Cell wall surfaces of microorganisms contain negatively charged groups as carboxyl-, hydroxyl-, phosphoryl-, amino-, and mercapto- groups to which metal ions bind through electrostatic interactions [33, 44]. Intracellularly, metal ions may bind with phytochelatin complexes [6], polyphosphate bodies [8], or may be precipitated within cytoplasm/vacuole [30]. In prokaryotes metal resistance has been shown to be plasmid-borne [36, 37]. Another important resistance mechanism employed by prokaryotes is efflux of metal ions from cells, and efflux-mediated heavy metal resistance in prokaryotes has been reviewed [22].

To test whether strain $Cr^{r}I8$ resisted metal toxicity extracellularly or intracellularly, metal uptake experiments in batch cultures were performed. It was found that both strains were able to take up Cr^{6+} ions intracellularly, and strain $Cr^{r}I8$ removed 33% more Cr^{6+} 8 h than *A. nidulans.* Washing of cells with 1 mM EDTA revealed that metal ions did not bind extracellularly with cell walls. Since resistant strain $Cr^{r}I8$ took more Cr^{6+} intracellularly, it can prove to be a good candidate for bioremediation of Cr^{6+} ions from polluted water. A number of cyanobacteria have been employed to study their potential in metal bioremediations [3, 10, 11]. It will also be interesting to find out how this strain protects itself from the toxic effects of metal ions while taking these up intracellularly.

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