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Uptake of Pb^{2+} by a cyanobacterium belonging to the genus *Synechocystis*, isolated from East Kolkata Wetlands

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Abstract East Kolkata Wetlands is a conserved wetland utilizing sewage and garbage, generated by Kolkata Municipal Corporation area for cultivation purpose. Cyanobacteria are the photosynthetic prokaryotes having bioremedial capacity. We have isolated a cyanobacterium from the sewage recycling fish-pond of East Kolkata Wetlands. Partial sequence of 16S rDNA gene of the isolated strain showed 100% similarity with that of genus *Synechocystis*. Isolated strain and *Synechocystis* sp. PCC6803 survived up to 300 μg ml^{-1} Pb^{2+} and growth was completely inhibited at 400 μg ml^{-1} Pb^{2+}. All experiments were carried out with 100 μg ml^{-1} Pb^{2+} in which growth was the maximum. 91.67% of the total Pb^{2+} got adsorbed to the outer surface of the cell and 1% of the total Pb^{2+} entered the cell of the isolated strain as estimated by atomic absorption spectrometry, but in *Synechocystis* sp. PCC6803 72.72% adsorbed and 0.96% penetrated. Intracellular and periplasmic depositions of Pb^{2+} were observed in both the strain. A filamentous structure developed outside the cell wall of the isolated cyanobacterium, but very little change was observed in *Synechocystis* sp. PCC6803. ZiaR—SmtB like regulator gene was expressed in both the strains after Pb^{2+} induction. The cDNA sequence of ZiaR of the isolated cyanobacterium shows 100% homology with that of *Synechocystis* sp. PCC6803. Upon Pb^{2+} induction, expression of SOD gene increased. cDNA sequence of the SOD gene from the isolated strain showed 98% homology with that of *Synechocystis* sp. PCC6803. Enzymatic activity of catalase and SOD was also increased. No DNA damage was monitored upon induction with Pb^{2+}.

Keywords *Synechocystis* sp. PCC6803 · East Kolkata Wetlands · Pb^{2+} uptake · 16S rDNA · Ramsar site

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Introduction

Environmental pollution by heavy metals has become a major issue as mining and industrial activities increased in the late 19th and early 20th century.

Heavy metals such as Cu (II), Fe (II), and Zn are essential to life in trace amount, but are extremely toxic at higher concentrations (Schaefer et al. 2002). Heavy metals like Hg and Pb are not known to perform any useful biological function (Allen 1997). Bioremediation, the use of biological agent to remove or neutralize contaminants is an effective and environmentally friendly way to purify tainted environment (Zhang and Majidi 1994). Cyanobacteria, the oxygen evolving photosynthetic prokaryotes are now reported to possess adaptability and bioremedial capability of heavy metals. For e.g., *Spirulina plantesis* contains detectable amount of Hg and Pb when grown under contaminated condition (Slotton et al. 1989). *Synechococcus cedrorum* 1991 was shown to be tolerant to heavy metals and pesticides (Gothalwal and Bisen 1993). Inactivated biomass of *Synechococcus* sp. was also able to uptake Cd (II), Cr (III), Cr (VI), Cu (II), Ni, Pb from solution (Gardea-Torresdey et al. 1998). The known metal removal pathways in cyanobacteria are: (i) surface adsorption i.e. binding of heavy metals to carboxyl group present on the extracellular surface of cell wall (Gardea-Torresdey et al. 1990), extracellular protein (Gordon et al. 1993), extracellular polysaccharide (Kalpan et al. 1987), (ii) intracellular metal sequestration by metallothionine protein and polyphosphate (Gardea-Torresdey et al. 1998), (iii) efflux through P-Type ATPase (Geisler et al. 1993), (iv) volatilization—a special case by which Hg is removed from cell being vapor (Schaefer et al. 2002).

East Kolkata Wetlands (former name of Kolkata was Calcutta, 22°24′ N–22°36′ N and 88°23′ E–88°32′ E, covering 1,25,000 hectors area), an internationally accredited conserved wetland, was declared Ramsar site on 19th April 2002 by Ramsar Conservation Bureau; the organization that works for world wide wetland conservation (www.ramsar.org and www.ramsar.org/wn/w.n.india_11new.htm). This wetland area is significant for wise use of 600 million liters of sewage and 2,500 metric tons of solid garbage generated by Kolkata Municipal Corporation area everyday. Solid garbage is used for cultivation of vegetable and sewage is being subjected to bioremediation in sewage recycling fish-ponds. In East Kolkata Wetlands bioremediation process is microbe-based. Total 75 of 16S rDNA sequences of cyanobacteria from this site are already reported from our lab (Ray Chaudhuri and Thakur 2006). Cr, Cu, Fe, Mn, Pb, Zn ions are present in waste water in abundance, but strikingly reduction in concentrations of Cu, Fe, Mn, Pb, Zn ions by 25–40% and 95% in case of Cr was found on the course of 40 km long journey of composite waste water from source to river mouth via carrying canals and stabilization ponds (Chattopadhyay et al. 2002). This observation led us to concentrate on metal removal mechanism in sewage water of East Kolkata Wetland and to find out the possible involvement of microorganisms for heavy metals bioremediation. We have focused only on Pb^{2+}, which is known to cause severe health hazards. This is the first report, which describes the capacity of *Synechocystis* to permit Pb^{2+} uptake.

Materials and methods

Microorganisms

The microorganisms used were cyanobacterium isolated from East Kolkata Wetlands, *Synechocystis* sp. PCC6803, *Escherichia coli* DH5α.

Media and growth conditions

BG11 medium (Rippka et al. 1979) was used for the cultivation of cyanobacteria with minor modification, i.e. EDTA free BG11 was used throughout this study. Isolated cyanobacterium and *Synechocystis* sp. PCC6803 were grown photoautotrophically at 25°C under constant illumination (60 μM photons m^{-2} s^{-1}) by cool white fluorescent light. *Escherichia coli* DH5 α was grown overnight at 37°C in Luria Bertani medium.

Isolation of cyanobacteria

Five hundred milliliters water sample was collected from sewage water recycling fish-pond of East Kolkata Wetlands and centrifuged at 5,000×*g* at 22°C, the resulting pellet was washed twice with BG11 medium, then inoculated in liquid BG11, aerated and kept under the growth condition mentioned above for 7 days. The grown culture was then sub cultured with a series of concentration of Pb^{2+} from 100 μg ml^{-1} to 1 mg ml^{-1} corresponding to 20.7–207 ppm. BG11 plates, supplemented with

100 μg ml^{-1} Pb^{2+}, 50 μg/ml nystatin, were prepared. The liquid culture of cyanobacteria grown in 100 μg ml^{-1} Pb^{2+} was streaked on plates and kept under the same growth condition. Each of the green micro colonies that appeared on the plate was first brought into liquid culture of BG11 with 100 μg ml^{-1} Pb^{2+} for the selection of pure strain and subsequently in Pb^{2+} free BG11 at least for seven generations to get absolutely Pb^{2+} free virgin strain.

Determination of growth curve

Cell density was measured at OD_{730} on a Hitachi spectro-photometer (Keren et al. 2002). A series of culture from both strains were grown in different sets. In one set, Pb^{2+} free medium was used and other sets were supplemented with different concentrations of Pb^{2+} (100 μg ml^{-1} to 1 mg ml^{-1}). Equal number of cells was inoculated from virgin culture. One tube from each set was taken after every 20 h and cell density was measured at OD_{730}.

Isolation of genomic DNA

The genomic DNA was isolated according to Brans (Barns et al. 1994) with few modifications. Four milliliters of virgin culture was harvested at 5,000×*g* for 10 min at 22°C and resuspended in lysis buffer (50 mM Tris–Cl pH 8.3, 5 mM EDTA, 200 mM NaCl, 0.05% TritonX-100), 100 μg/ml lysozyme were added and subjected to three cycles of freeze thaw (−70 and 65°C), then 50 μg/ml Proteinase K followed by 1% SDS was added and incubated at 55°C for 90 min followed by addition of 1% CTAB and 30 min of further incubation at same temperature. Lysed cells were centrifuged at 10,000×*g* at 4°C for 10 min, supernatant was subjected to chloroform extraction and 0.1 volume of 3 M CH_3COONa, 2.5 volume of ethanol were added to the aqueous phase and kept overnight at −70°C. Precipitated DNA was spun down at 15,000×*g* for 30 min at 4°C, then washed with 70% ethanol, air dried, resuspended in 40 μl of sterile single distilled water.

PCR amplification

1,060 bp fragment of SSU rDNA gene 530–1,492 bp was amplified by PCR according to Borneman (Borneman and Triplett 1997).

Isolation of total RNA

Isolated cyanobacterium was cultured in 50 ml BG11 in two different sets. At an $OD_{730} = 0.4$, one set of culture was induced with Pb^{2+} with a final concentration of 100 μg ml^{-1}. Both control and induced culture were kept for 3 h under the same growth condition mentioned earlier. Total cellular RNA was isolated using bacterial total cellular RNA isolation kit (Bangalore GeneI, India) following manufacturer's protocol (Chomezynski and Sacchi 1987).

RT-PCR

RT reaction was carried out with 100 ng of total RNA and random hexamer using RT-PCR kit (Bangalore GeneI, India). Synthesized cDNA was next subjected to PCR amplification for ZiaR gene using ZiarI and ZiarVI primers (Thelwell et al. 1998) and for superoxide dismutase (SOD) gene (Bhattacharya et al. 2004).

Transformation and cloning of PCR product

16S rDNA PCR product and SOD RT-PCR product were purified by PCR product purification kit (Roche, USA). All PCR products were cloned into pGEM T-Easy vector (Promega, USA). Cloned plasmids were transformed in *E. coli* (DH5α) by TSS method (Chung et al. 1989) using 50 μg/ml ampicilin as selection marker.

Agarose gel electrophoresis

Genomic DNA, total cellular RNA and plasmids were run in 1% agarose gel and PCR products were run in 2% agarose gel using TAE buffer. Gels were stained with ethidium bromide, then visualized by Biorad geldoc and subsequently photographs were taken. 100 bp ladder (Promega, USA) was used to determine the size of PCR products.

DNA sequencing and analysis

Recombinant plasmids containing 1,060 bp SSU rDNA gene and SOD gene were isolated by alkali lysis method. Plasmid sequencing was performed by Sanger's dideoxy method using universal forward

M13 primer and fluorescent dye linked ddNTPs (Russell 2002) in ABI Prism automated DNA sequencer. ZiaR RT-PCR products were sequenced directly using ZiaRI primer. Sequence data obtained were analyzed for similarity search using online BLAST (blastn) available on the web at http://ncbi.nlm.nih.gov and for chimera check (http://rdp.cme.msu.edu/html/analyses.html).

Study of Pb^{2+} uptake by AAS

Isolated cyanobacteria and *Synechocystis* sp. PCC6803 were grown separately in each 100 ml of BG11 at an OD_{730} = 0.4 in two sets. One set served as control and another set was induced with 100 μg ml^{-1} of Pb^{2+}. Four set of cultures were kept for 48 h at same growth condition as mentioned earlier followed by centrifugation at 5,000×*g* for 10 min at 22°C. The resulting pellet from each of four sets was resuspended in 10 ml of 0.125 M EDTA and kept at room temperature under gentle shaking for 30 min and then centrifuged at 5,000×*g* for 10 min. Supernatants were collected in graduated tubes to measure the volume. EDTA washed pellet from four sets were dried, weighted and digested in nitric acid and perchloric acid and then filtered. Each 10 ml of 0.125 M EDTA used for washing and filtrates of the digested cells were analyzed for Pb^{2+} by atomic absorption spectrometry (Perkin Elmer 5100 model).

Transmission electron microscopy

Virgin cells of the isolated cyanobacterium and *Synechocystis* sp. PCC6803 were streaked on BG11 plates supplemented with 100 μg ml^{-1} of Pb^{2+} and Pb^{2+} free condition. Plates were kept under the described growth condition for 6 days. Then all four samples were fixed in 3% glutaraldedhyde in 100 mM Na-cacodylate buffer (pH 7.4) and post fixed in 1% OsO_4 in same buffer; it was dehydrated first in graded series of alcohol and then in propylene oxide and embedded in Agar100 resin by polymerizing at 60°C overnight. Ultrathin sections were cut in Lica Ultracut UCT ultra microtome, stained with saturated solution of uranylacetate and Reynolds's lead citrate and examined in FE1 Technai Bio Twin TEM.

Study of Pb^{2+} induced DNA damage by CHEF

OD_{730} = 0.4 cells were exposed to Pb^{2+} to a final concentration of 100 μg ml^{-1} for 30 min, 2, 8 and 24 h. Positive controls were also prepared by γ-irradiation of both the strain at 39Gy (130 rads/min, for 30 min). Samples were prepared for CHEF with modification of the protocol described by Churin (Churin et al. 1995). Cell suspension was mixed with equal volume of 1.5% low melting agarose. The cell-agarose suspension was solidified in block former. Cells were lysed in situ by incubating blocks at 55°C over night in lysis solution (125 mM EDTA, 100 μg/ml lysozyme, 50 μg/ml Proteinase K, 1% SDS, 1% N-laurylsarcosine and 1% CTAB). Blocks were then washed in 25 volumes of TE (10 mM Tris–Cl, 100 mM EDTA, pH 8.0). Blocks were loaded into wells of 1% pulse field grade agarose gel and subjected to contour-clamped homogeneous electrophoresis field (CHEF-Biorad) in 0.5X TBE for 25 h with 5 volts/cm, linear ramp, angle 60°.

Preparation of cell lysate

Isolated cyanobacteria, *Synechocystis* sp. PCC 6803 were induced with Pb^{2+} as described earlier. Control and induced cells of both strains were washed with 0.125 M EDTA and resuspended in K-PO_4 buffer (pH 7.0). Pre washed (in sterile distilled water) silica was mixed with cell suspension and vortexed extensively in cold for 5 min. Lysates were centrifuged at 10,000 rpm for 10 min at 4°C and the clear supernatants were collected.

Protein estimation by Bradford's method

All lysates were subjected to protein estimation by Bradford's method (Bradford 1976).

Assay for catalase (CAT) and superoxide dismutase (SOD)

The activity of SOD was determined by the quantification of the pyrogallol auto oxidation inhibition. Auto oxidation of 0.2 mM pyrogallol in Tris–Cl pH 8.2 was measured by increase in absorbance at 420 nm (Marklund and Marklund 1974). One unit of enzyme activity is defined as the amount of enzyme necessary for inhibiting the reaction by 50%. The

activity was expressed as unit/mg protein/min (Luck 1963).

The activity of catalase was determined by directly measuring the decomposition of H_2O_2 at 240 nm for 3 min as described by (Aebi 1984), in 50 mM phosphate buffer (pH 7.0) containing 10 mM H_2O_2 and cell lysate. The activity was expressed as mM H_2O_2 decomposed/mg protein/min (Luck 1963).

Results

Identification of isolated cyanobacterium by 16S rDNA sequence analysis

A 1,060 bp region of SSU rDNA gene was amplified as shown in Fig. 1 and the partial sequence data of 1,060 base pairs product is shown in Table 1A. The sequence was analyzed in BLAST for similarity search. The BLAST search result of the partial sequence of 16S rDNA amplified fragment shows 100% similarity with GenBank sequence accession no.—AY224195.1, AB041938.1, AB041937.1, AB041936.1 which are the partial sequences of 16S rDNA of *Synechocystis* sp. PCC6803, *Synechocystis* sp. PCC6805, *Synechocystis* sp. PCC6714, *Synechocystis* sp. PCC6702, respectively.

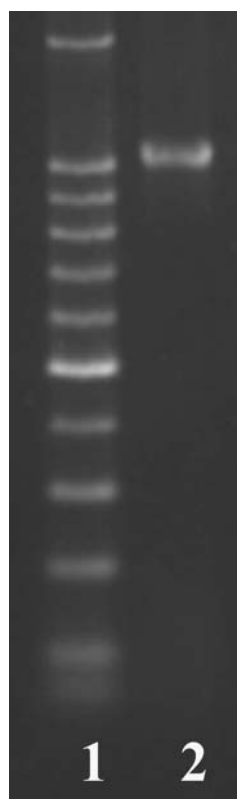

Fig. 1 Amplified SSU rDNA fragments; Lane 1 is 100 bp Ladder, the brightest band is 500 bp; Lane 2 is the amplified fragment

Table 1 Partial sequence of 16S rDNA gene [A]; ZiaR RT-PCR product [B] of isolated cyanobacteria and SOD RT-PCR product [C]

[A]

5′ACTCACTATAGGGCGAATTGGGCCCGACGTCGCATG
CTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTT
GACTGACTGAGTGCCAGCAGCCGCGGTAATACGGA
GGATGCAAGCGTTATCCGGAATTATTGGGCGTAAA
GCGTCCGTAGGTGGTTATGCAAGTCTGCCGTTAAAG
AATGGAGCTTAACTCSACTCACTATAGGGCGAATTG
GGCCCGACGTCGCATGCTCCCGGCCGCCATGGCGGC
CGCGGGAATTCGATTTGACTGACTGAGTGCCAGCA
GC ACTCACTATAGGGCGAATTGGGCCCGACGTCGC
ATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCG
ATTTGACTGACTGAGTGCCAGCAGCCGCGGTAATAC
GGAGGATGCAAGCGTTATCCGGAATTATTGGGCGTA
AAGCGTCCGTAGGTGGTTATGCAAGTCTGCCGTTAA
AGAATGGAGCTTAACTCSACTCACTATAGGGCGAAT
TGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCG
GCCGCGGGAATTCGATTTGACTGACTGAGTGCCAGC
AGC ACTCACTATAGGGCG3′

[B]

5′TCACATGATTATCCGCCAAGCTGTAGTAAACATTAC
GGCCGACCCGGCGATACTTTACCAGGCGCTGCGAT
CGTAAAATTCGTAATTGATGGGAAACTGCCGATTCA
CTCACTTTCATCGCCGCTGCTAAATCACAGACACAG
AGTTCTTGGCGGGCCAATGCCGACATTAAACGCAA
CCGACTCGGATCAGCtTCACATGATTATCCGCCAAG
CTGTAGTAAACATTACGGCCGACCCGGCGATACTTT
ACCAGGCGCTGCGATCGTAAAATTCGTAATTGATG
GG TCACATGATTATCCGCCAAGCTGTAGTAAACAT
TACGGCCGACCCGGCGATACTTTACCAGGCGCTGC
GATCGT3′

[C]

5′GGCCGCGGGAATTCGATTCATATGGCTTACGCACTA
CCTAACTTACCTTACGACTACACCGCTCTGGAACCC
TGCATTTCCAAAAGCACCCTGGAGTTCCATCACGAC
AAACACCATGCCGCCTACGTTAACAATTTCAATAAT
GCAGTGGCGGGTACTGATTTAGATAATCAATCCATT
GAAGATGTAATTA_GGCCGCGGGAATTCGATTCATA
TGGCTTACGCACTACCTAACTTACCTTACGACTACA
CCGCTCTGGAACCCTGCATTTCCAAAAGCACCCTG
GGCCGCGGGAATTCGATTCATATGGCTTACGCACT
ACCTAACTTACCTTACGACTACACCGCTCTGGAACC
CTGCATTTCCAAAAGCACCCTGGAGTTCCATCACGA
CAAACACCATGCCGCCTACGTTAACAATTTCAATAA
TGCAGTGGCGGGTACTGATTTAGATAATCAATCCAT
TGAAGATGTAATTA_GGCCGCGGGAATTCGATTCAT
ATGGCTTACGCACTACCTAACTTACCTTACGACTAC
ACCGCTCTGGAACCCTGCATTTCCAAAAGCACCCTG
GGCCGCGGGAATTCGATTCATATGGCT3′

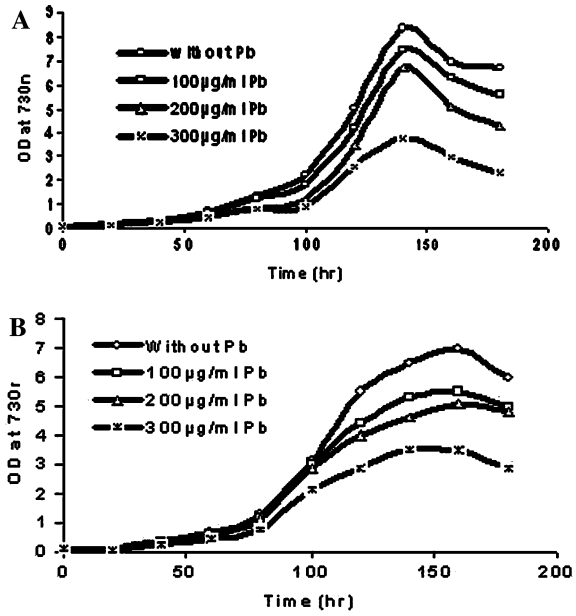

Fig. 2 Growth curve; (**A**) isolated cyanobacteria and (**B**) *Synechocystis* sp. PCC6803. Mean of three experiments were plotted

Growth curve

Growth curves of isolated cyanobacterium and *Synechocystis* sp. PCC6803 at different concentration of Pb^{2+}, which was determined by measuring OD_{730}, are shown in Fig. 2A, B, respectively. Growth of the isolated strain as well as *Synechocystis* sp. PCC6803 gets retarded gradually with the increasing concentrations of Pb^{2+}. Growth of both the strains was completely inhibited with 400 µg ml^{-1} Pb^{2+} (not shown in graph). Since at 100 µg ml^{-1} Pb^{2+} growth was found to be the highest, 100 µg ml^{-1} concentration was chosen for the rest of the experiments.

Pb^{2+} uptake study

Atomic absorption spectrometry was employed to determine the uptake of Pb^{2+} by the isolated strain. To check whether the wild type of *Synechocystis* sp. PCC6803 could be able to uptake lead; same experiment was also carried out with it. Data of total Pb^{2+} uptake by both cell masses are tabulated in Table 2. Atomic absorption spectrometry data revealed that in case of isolated strain 91.67% of the total Pb^{2+} got adsorbed to the outer surface of the cell and 1% of the total Pb^{2+} entered the cell. The values are 72.72 and 0.96%, respectively in case of *Synechocystis* sp. PCC6803.

Transmission electron microscopy

Thin sections of isolated cyanobacterium and *Synechocystis* sp. PCC6803 cells grown in the absence and presence of Pb^{2+} are shown in Fig. 3A–D, respectively. Stained micrograph of the two strains clearly showed intracellular and periplasmic deposition of Pb^{2+}. A thick filamentous structure outside the cell wall of the isolated cyanobacterium is observed, but *Synechocystis* sp. PCC6803 exhibits minor change in cell wall structure.

Study of heavy metal resistance mechanism

RT-PCR product of about 429 bp is amplified only in Pb^{2+} induced cells of both the strain (Fig. 4) and sequence of that product from the isolated strain is shown in Table 1B. BLAST search result shows 100% similarity of the RT-PCR product with ZiaR—a SmtB like regulator of P-Type ATPase of *Synechocystis* sp. PCC6803.

Table 2 Pb^{2+} uptake data

Samples	Dry weight or volume	AAS data of Pb^{2+} content	Amount of Pb^{2+} present in given weight/volume (µg)	Total Pb^{2+} used (mg)	% Uptake
Isolated Cyanobacterium (Cell mass)	55. 33 mg	373.66 µg/g	20.7	2.07	1
Isolated Cyanobacterium (Washed fluid)	10 ml	189.76 µg/ml	1897.6	2.07	91.67
Synechocystis sp. PCC6803 (Cell mass)	49.54 mg	400.18 µg/ml	19.8	2.07	0.96
Synechocystis sp. PCC6803 (Washed fluid)	10 ml	150.52 µg/ml	1505.2	2.07	72.72

Note: $n = 3$

Pb^{2+} content of fish-pond water sample = 0.046 ppm (0.046 µg L^{-1})

Pb^{2+} content of control cell (grown without Pb^{2+}) = nil

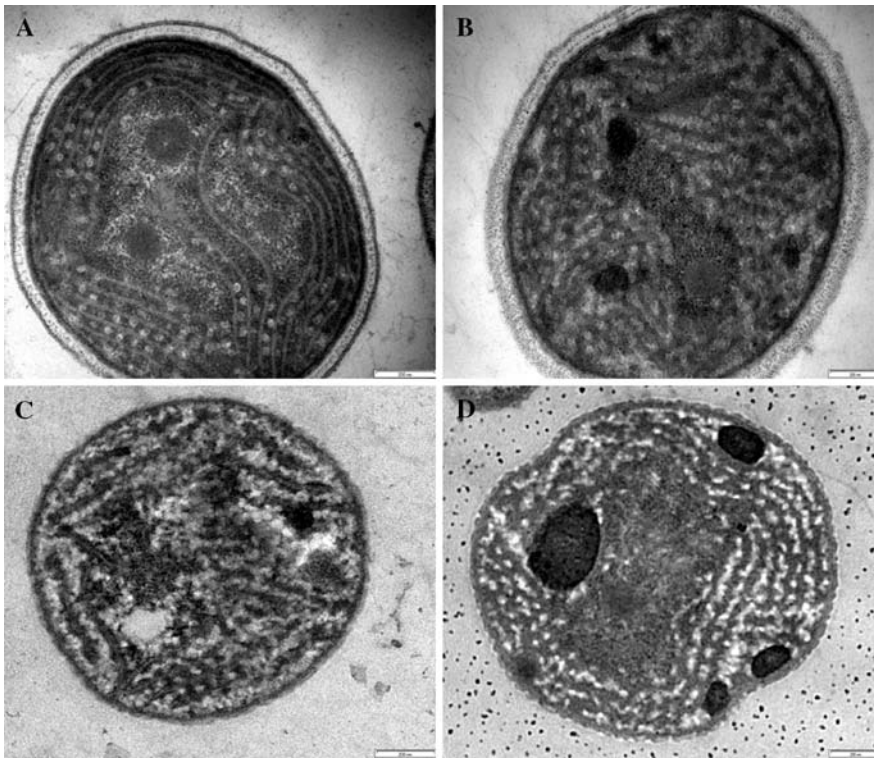

Fig. 3 Transmission electron micrograph of thin sections; (**A**) isolated cyanobacteria without Pb^{2+}; (**B**) isolated cyanobacteria with Pb^{2+}; (**C**) *Synechocystis* sp. PCC6803 without Pb^{2+}; (**D**) *Synechocystis* sp. PCC6803 with Pb^{2+}

Expression of SOD gene

RT-PCR product of about 600 bp was derived from the isolated strain and *Synechocystis* sp. PCC6803 (Fig. 5). The expression of SOD gene increased after induction with Pb^{2+}. Sequence of the RT-PCR product of SOD gene showed 98% homology with that of *Synechocystis* sp. PCC6803.

Study of DNA damage

Cells were exposed to Pb^{2+} for different time intervals to find out the occurrence of Pb^{2+} induced DNA damage in first 24 h of growth phase but no DNA damage was monitored. All DNA remained in the groove; only in case of γ-irradiated cells DNA came out in the gel (data not shown).

Activity of CAT and SOD

Changes of the activity of CAT and SOD were determined in cell lysates of both the strain. As evident from the mean ± S.E.M values of three independent experiments the activity of both enzymes increased 3 h after induction with 100 μg ml^{-1} Pb^{2+} (data not shown).

Discussion

We have isolated a cyanobacterium from the sewage recycling fish-pond of East Kolkata Wetland. Partial sequence of 16S rDNA gene of isolated strain showed 100% similarity with that of genus *Synechocystis*. The cells were grown in EDTA free BG11 throughout the experiment to avoid any chelation of Pb^{2+} by EDTA, because chelation could decrease the desired concentration of Pb^{2+} required for the experiments. During the last few years there have been reports about the uptake of Co^{2+}, Ni^{2+}, Zn^{2+} by *Synechocystis* sp. PCC6803 (Garcia-Dominguez et al. 2000), but no report was available on Pb^{2+} uptake by this organism. Hence, we have focused on Pb^{2+}. Determination of sub-toxic concentration of a pollutant for an organism is essential for effective bioremedial purpose (Gadd and White 1993). The effect of Pb^{2+} on the growth of the isolated strain was monitored by expressing OD values at 730 nm. Cells were observed to survive up

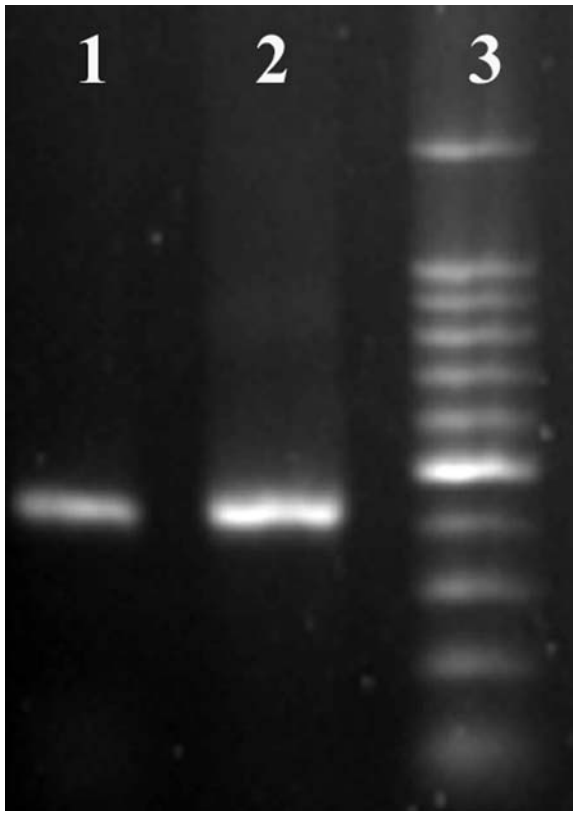

Fig. 4 Pb^{2+} induced expression of ZiaR gene; Lane 1 *Synechocystis* sp. PCC6803; Lane 2 isolated cyanobacteria; Lane 3 100 bp ladder

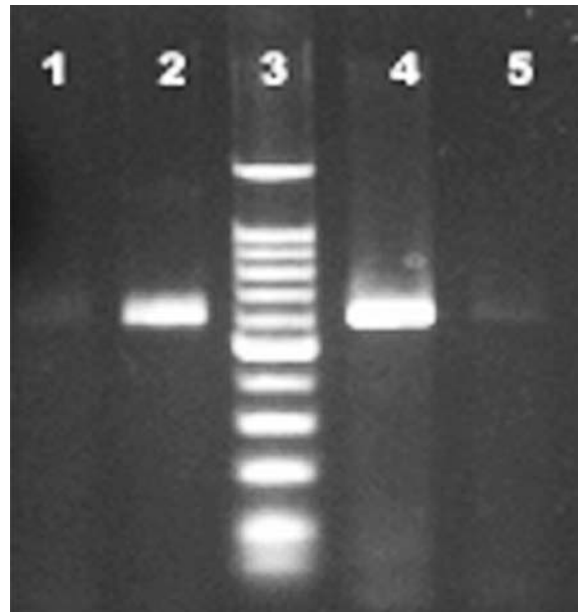

Fig. 5 Expression of SOD gene; Lane 1, 5 are control cells of the isolated cyanobacteria and *Synechocystis* sp. PCC6803, respectively; Lane 2, 4 are Pb^{2+} induced cells of the isolated cyanobacteria and *Synechocystis* sp. PCC6803, respectively; Lane 3 is 100 bp ladder

to 300 μg ml^{-1} Pb^{2+} and complete inhibition of growth was observed at 400 μg ml^{-1} Pb^{2+}. Maximum growth was observed with 100 μg ml^{-1} Pb^{2+}; so this concentration was considered as the optimal one to carry out all the experiments.

Atomic absorption spectrometry data revealed that 91.67 and 72.72% of the total Pb^{2+}, to which the cells were exposed, gets adsorbed to the outer surface of the isolated strain and *Synechocystis* sp. PCC6803, respectively. The adsorbed Pb^{2+} came out when washed with 0.125 M EDTA. High concentration of EDTA was used to nullify the masking effect of other divalent cations present. EDTA cannot cross the membrane, so the entire amount of Pb^{2+} entered the cells can be quantified only upon digestion of the cells with strong acid.

TEM studies were employed to investigate any ultra structural change during exposure to high Pb^{2+} concentration. No change in the cellular shape or any distortion in cellular morphology was observed, but the development of filamentous structure outside the cell wall was observed. This structure could be fibriae that generated from cell wall and extended through it. These projections have been reported to serve as a defense mechanism against heavy metal ions (Gardea-Torresdey et al. 1996). Metal ions binds to the different functional groups (mainly –COOH) present in this sheath, thus decreasing the availability of metal ions to enter the cell (Gardea-Torresdey et al. 1990). Our atomic absorption spectrometry data correlates with the TEM data. This observation is also consistent with the previous report of the development of filamentous structure in *Synechocystis* sp. PCC6803 in response to exposure of high concentration of Cu^{2+} (Gardea-Torresdey et al. 1996). It is reported that in *Synechocystis* sp. PCC6803 P-Type ATPase is involved in heavy metal resistance mechanism. ZiaR—a SmtB like transcriptional regulator of P-Type ATPase is situated upstream to the ORF of P-Type ATPase (Thelwell et al. 1998). Expression of the gene homologues to ZiaR upon Pb^{2+} induction in both the strains indicates the involvement of P-Type ATPase mediated defense mechanism against Pb^{2+}.

The circular genome of *Synechocystis* sp. PCC6803 is 3.57 Mbp (Kaneko et al. 1996). Upon γ-ray irradiation cellular DNA of *Synechocystis* sp. PCC6803 damaged, a fraction came out as smear in the gel, but DNA of the control and Pb^{2+} exposed cells remain within the groove. This observation nullifies the possibility of the Pb^{2+} induced DNA damage. To find out the possible resistant mechanism against Pb^{2+} induced oxidative stress; profile of CAT and SOD were studied. We have looked at SOD gene expression as well as the biochemical assay for SOD and CAT enzymes. The primers used in this study, was previously used to amplify SOD gene from *Synechocystis* sp. PCC6803 (Bhattacharya et al. 2004). The same primers were used in RT-PCR. 607 bp RT-PCR product was amplified from the isolated strain and the sequence of that product showed 98% homology with SOD of wild type *Synechocystis* sp. PCC6803. This finding not only supports that the isolated strain belongs to the genus *Synechocystis*, but also confirms the involvement of SOD in the prevention of Pb^{2+} induced oxidative stress.

Expression of SOD increased in Pb^{2+} induced cells. Generation of heavy metal induced $O_2^{\cdot -}$ occurs in the light dependent phase of photosynthesis (PSI). Elevated level of $O_2^{\cdot -}$ increased the expression of SOD. A small amount of the $O_2^{\cdot -}$ is generated during the electron transfer through respiratory chain complex which accounts for the expression of SOD in control cell. CAT activity was also increased. Based on these results, a conclusion may be drawn that Pb^{2+} induced ROS generated is scavenged by SOD-CAT pathway ultimately preventing DNA damage. The retarded growth with increasing concentrations of Pb^{2+} could be due to the immediate expression of intracellular proteins involved in cellular defense against Pb^{2+}, like metallothionine or P-type ATPase, rather than the proteins necessary for cell division. The other reason for this delay might be the time required to repair any heavy metal induced DNA damage. The second possibility was ruled out by CHEF data and the expression of ZiaR upon Pb^{2+} supports the first possibility. Heavy metals are potent inhibitor of metabolic processes like respiration and photosynthesis (Kleiner 1978; Mallick and Rai 1992). When cells are exposed to very high concentration of heavy metal ions, the cellular defense machinery gets saturated; as a result excess ions inhibit metabolic processes leading to cell death. Our findings to some extent correlate the previous observation of reduction in Pb^{2+} content by 25–40% of sewage water of East Kolkata Wetland (Chattopadhyay et al. 2002). It may be the cyanobacteria belonging to genus *Synechocystis* present in sewage water, which permit Pb^{2+} uptake and thereby decrease Pb^{2+} concentration in sewage.

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References

Aebi H (1984) Catalase in vitro. Methods Enzymol 105: 121–126

Allen R (1997) Introduction: mining and metals in the environment. J Geochem Explor 58:95–100

Barns SM, Fundyga RE, Jeffries MW, Pace NR (1994) Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. Proc Natl Acad Sci USA 91:1609–1613

Bhattacharya J, GhoshDastidar K, Chatterjee A, Majee M, Lahiri Majumder A (2004) Synechocystis Fe superoxide dismutase gene confers oxidative stress tolerance to *Escherichia coli*. Biochem Biophys Res Commun 316:540–544

Borneman J, Triplett EW (1997) Molecular microbial diversity in soil from Eastern Amazonia: evidence from unusual microorganism and microbial population shift associated with deforestation. Acad Emerg Med 63:2647–2657

Bradford MM (1976) A rapid and sensitive for the quantitation of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Chattopadhyay B, Chatterjee A., Mukhopadhyay SK (2002) Bioaccumulation of metal in the East Calcutta Wetland ecosystem. Aquat Ecosyst Health Manage 5:191–203

Chomezynski P, Sacchi N (1987) Single step method of RNA isolation by acid guanidiumthiocyanat-phenol chloroform extraction. Anal Biochem 162:156–159

Chung CT, Suzzane LN, Miller RH (1989) One step preparation of competent *E.coli*: transformation and storage of bacterial cells in the same solution. Proc Natl Acad Sci USA 86:2172–2175

Churin YN, Shalak IN, Thomas B, Shestakov SV (1995) Physical and genetic map of the chromosome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803. J Bacteriol 177(11):3337–3343

Experimental Biosciences Introductory Laboratory—Bios 211 available at http://www.ruf.rice.edu/~bioslabs/methods/microscopy/cellcounting.html

Gadd GM, C White (1993) Microbial treatment of metal pollution—a working biotechnology. Trends Biotechnol 11:353–359

Garcia-Dominguez M, Lopez-maury L, Florencio FJ, Reyes JC (2000) A gene cluster involved in metal homeostasis in the cyanobacterium *Synechocystis* sp. Strain PCC6803. J Bacteriol 182:1507–1514

Gardea-Torresdey JL, Becker-Hapak MK, Hosea JM, Darnell DW (1990) Effect of chemical modification of algal carboxyl group on metal ion binding. Environ Sci Technol 19:1372–1379

Gardea-Torresdey JL, Arenas JL, Webb R, Tiemann K, Gonzalez J (1996) Uptake of metal ions from solution by inactivated cells of cyanobacteria. Proceedings for 1996 HSRC-WERC Joint Conference of the Environment, pp 48–58

Gardea-Torresdey JL, Arenas JL, Francisco NMC, Tiemann K, Webb R (1998) Ability of immobilized cyanobacteria to remove metal ions from solution and demonstration of the presence of metallothionine genes in various strain. J Hazard Subst Res 1:2–18

Geisler M, Rickter J, Schumann J (1993) Molecular cloning of a P-type ATPase gene from the cyanobacterium *Synechocystis* sp. PCC 6803. Homology to eukaryotic Ca(2+)-ATPases. JMB 234:1284–1289

Gothalwal R, Bisen PS (1993) Isolation and physiological characterization of *Synechococcus cedrorum*1191 strain tolerant to heavy metals and pesticides. Biol Environ Sci 6:187–194

Gordon AS, Harwood VJ, Sayyar S (1993) Growth, copper-tolerant cells, and extracellular protein production in copper-stressed chemostat cultures of vibrio alginolyticus. Acad Emerg Med 59:60–66

Kalpan D, Christiaen D, Arad SM (1987) Chelating properties of extracellular polysaccharides from *Chlorella* sp. Acad Emerg Med 53:2953–2956

Kaneko TS, Sato H, Kotani A (1996) Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. DNA Res 3:109–136

Keren N, Kidd MJ, Penner-Hahn JE, Pakrasi HB (2002) A light dependent mechanism for massive accumulation of manganese in the photosynthetic bacterium *Synechocystis* sp. PCC6803. Biochemistry 41:5085–15092

Kleiner D (1978) Inhibition of the respiratory system in *Azotobacter vinelandii* by divalent transition metal ions. FEBS Lett 96:364–366

Luck H (1963) A spectrophotometric method for the estimation of catalase. In: Bergmeyer HV (ed) Methods of enzymatic analysis. Academic press, New York, pp 886–888

Mallick N, Rai LC (1992) Metal induced inhibition of photosynthesis, photosynthetic electron transport chain and ATP content of *Anabaena doliolum* and *Chlorella Vulagaris*: interaction with exogenous ATP. Biomed Environ Sci 5:241–250

Marklund S, Marklund G (1974) Involvement of the superoxide anions radicals in the inhibition of autooxidation of pyrogallol and convenient assay for superoxide dismutase. Eur J Biochem 47:469–474

Ray Chaudhuri S, Thakur AR (2006) Microbial genetic resource mapping of East Calcutta wetlands. Curr Sci 91:212–217

Rippka R, Deruelles J, Waterbury JB, Herdman M, Stanler RY (1979) Genetics assignments, strain histories and properties of pure culture of cyanobacteria. J Gen Microbiol 111:1–61

Russell P (2002) iGenetics. Pearson Education, Inc., San Francisco, pp 187–189

Schaefer J, Letowski KJ, Barkay T (2002) *mer*-Mediated Resistance and Volatilization of Hg(II) Under Anaerobic Conditions. Geomicrobiol J 19:87–102

Slotton DG, Goldman CR, Frank A (1989) Commertially grown *Spirulina* found to contain low level of mercury and lead. Nutr Rep Int 40:1165–1172

Thelwell C, Robinson NJ, Turner-Cavet JS (1998) An SmtB-like repressor from *Synechocystis* sp. PCC6803 regulates a zinc exporter. Proc Natl Acad Sci USA 95:10728–10733

Zhang W, Majidi V (1994) Monitoring the cellular response of *Stichococcus bacillaris* to exposure of several different metals using *in vitro* NMR and other spectroscopic technique. Environ Sci Technol 28:1372–1379