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ORIGINAL ARTICLE

Protein profiling of metal-resistant *Bacillus cereus* VITSH1

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Bacillus cereus, chromium stress, metal stress, S-layer protein, stress response.

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Abstract

Aims: The aim of the study was to investigate the proteomic changes and antioxidant enzyme activity in chromium-resistant *Bacillus cereus* VITSH1 in response to heavy metal toxicity.

Methods and Results: The variation in protein expression and antioxidant enzyme activity in the presence and absence of metal was studied in *B. cereus* VITSH1. The differentially expressed proteins in chromium-treated conditions were determined by SDS PAGE. The proteins involved in metal binding, protein biosynthesis, protein folding, energy metabolism and motility were identified by mass spectrometry coupled with bioinformatics search tools. The in gel assays for antioxidant enzymes indicated a change in their activity under metal stress conditions.

Conclusions: The findings of this study suggest that the organism combats metal stress probably by restricting the entry of metal inside the cell. The role of the differentially expressed proteins clearly indicates that the first line of defence is to avoid the entry of metal into the cell either by possessing a modified outer membrane or by moving away from the toxicant. Further work on the identification of other proteins playing a role in resistance would help in integrating the available knowledge on the resistance mechanisms the organisms employ to combat toxicity.

Significance and Impact of the Study: The proteomic changes in the metal-exposed bacteria would give an insight into the proteins involved in metal resistance mechanisms and thereby aid in the development of biosensor-based technology for heavy metal detection.

Introduction

The widespread application of metals and their non-degradable nature has led to their omnipresence in the environment, posing a major concern (Gaur *et al.* 2014; Dixit *et al.* 2015). Chromium contamination is of particular concern due to its widespread use and discharge in the environment (Francisco *et al.* 2002; Hashem *et al.* 2015). The micro-organism isolated from chromium containing places like tanneries involves the use of trivalent chromium in chrome tanning process. Apart from tanning industry, chromium also finds use in steel/electroplating for its corrosion-resistant properties and in textile industries as a fixative for textile dyes.

There are several studies on the hazardous impact of chromium on microflora of soil, humans or other reservoirs. Cr(VI) is actively transported across biological membranes in both prokaryotes and eukaryotes (Wiegand *et al.* 1985; Alexander and Aashet 1995). Once inside the cells, Cr(VI) is reduced to Cr(III) which is responsible for most of the toxic effects. Trivalent chromium has been reported to cause DNA, protein and membrane damage in *E. coli* and *Bacillus subtilis* (Fathima and Rao 2018). A decrease in the viability and proliferation of eukaryotic cells such as murine B 16 melanoma cells and human MCF 10 A neo T transformed human epithelial cells after interaction with trivalent chromium has been reported (Shrivastava *et al.* 2005). Trivalent chromium stimulates nitric oxide formation which alters the metabolism in plants (Ding *et al.* 2009; Kováčik *et al.* 2010). Chromium being one of the redox active metal generates free radicals such as superoxide anion radical (Jomova

and Valko 2011) upon interaction with oxygen resulting in oxidative stress in bacteria.

Micro-organisms adapt to metals on exposure by developing resistance mechanisms (Sabry *et al.* 1997; Bruins *et al.* 2000). The metabolic flexibility of resistant organisms is manifested as alterations in protein expression patterns. Proteins are the versatile macromolecules with diverse functional role in the cell (Amm *et al.* 2014). These biological functions are hampered by environmental perturbations like heavy metal stress, which affects the conformation of the proteins by inhibiting the folding (Sharma *et al.* 2008; Siripornadulsil *et al.* 2014). Interference of heavy metals with protein folding results in the mistranslation of mRNA and misincorporation of amino acids leading to poor protein synthesis (Jacobson *et al.* 2012). Protein synthesis and certain other process like the formation of flagella which is involved in motility is energy demanding (Zarbiv *et al.* 2012). The expression of enzymes involved in energy metabolism was altered in some resistant organisms probably to help the organism to generate more ATP needed to combat additional stress encountered by the organism (Isarankura-Na-Ayudha *et al.* 2009). Certain proteins involved in energy metabolism like ATP synthase, malate dehydrogenase, fructose 1, 6-biphosphatase showed increased levels of expression under hexavalent chromium stress conditions (Kılıç *et al.* 2010). In the present study also the expression of ATP synthase, was found to be increased in metal stress conditions.

In addition to these toxic effects, metal stress also leads to oxidative stress, which generates free radicals (Ramírez-Díaz *et al.* 2008). When cellular macromolecules are exposed to free radicals, their structure and function gets altered disrupting the cellular metabolism (Stadtman 1993). These toxic effects of metals imposed on bacteria are overcome by a cascade of proteins which exert their protective effect and aid in the survival of the organism (Matilda and Shanthi 2017).

The change in protein expression was studied by SDS PAGE and the differentially expressed proteins were identified by LC-MS/MS (liquid chromatography tandem mass spectrometry). The antioxidant profile was studied by in gel assays. Comparative proteomics aid the analysis of differentially expressed proteins under specific environmental conditions and also resolves the functional significance of the fered protein (Matilda and Shanthi 2017; Zorz *et al.* 2018). The present study has been undertaken to investigate the proteomic changes that occur at different concentrations of chromium and to understand how organisms cope with chromium stress. Trivalent chromium released in effluent of certain industries reaches the soil causing changes in bacterial diversity (Altaf *et al.* 2008; Katsaveli *et al.* 2012). The surviving organism develop spectrum of resistance and tolerance mechanisms. In the present study, an attempt has been made to isolate resistant organisms that adapt to stress and determine their proteomic changes. The protein expression in different metals like copper and cadmium was also studied to check if the organism had a common mechanism to resist other metals. This would give an insight into the adaptive strategies involved in chromium resistance mechanism. This differentially expressed protein could also find application in biosensors and bioremediation (Bontidean *et al.* 2000; Xu *et al.* 2010).

Materials and methods

The sulphate salts of basic chromium, copper and cadmium from HiMedia (India) were used in this study. Gel purification and PCR cloning kits were procured from Amersham Biosciences (NJ) and Fermentas (Canada) respectively. Formic acid and MS grade acetonitrile was purchased from Sigma Aldrich (St Louis, MO). The chemicals nitroblue tetrazolium (NBT), riboflavin, hydrogen peroxide (H_2O_2), ferric chloride, potassium ferricyanide, 1-chloro-2,4-dinitrobenzene (CDNB) and phenazine methosulphate (PMS) for the antioxidant assay were purchased from Himedia.

Isolation and characterization of chromium-resistant isolate

The metal stock solutions were filter sterilized, and 10% sodium carbonate was used to adjust the pH of the solution to 7. The soil samples were collected in Vellore district, Tamil Nadu, serially diluted and plated onto nutrient agar plate containing 100 mg l^{-1} chromium (pH $7 \pm 0{\cdot}2$) and incubated at 37°C overnight. The bacteria that grew in chromium containing medium was isolated, subcultured and maintained at −80°C in 50% glycerol. The maximum tolerable concentration (MTC) was determined in nutrient broth amended with increasing concentrations of chromium (100–2500 mg l^{-1}) in accordance with previous method (Schmidt and Schlegel 1994). The isolate that tolerated higher concentration of chromium was identified and used for further study. The ability of the isolate to resist copper and cadmium was studied and the MTC was determined.

Identification of the resistant isolate by 16S rRNA gene sequence analysis

The genomic DNA of the isolate was extracted (Babu *et al.* 2009). The PCR amplification of the 16S rRNA gene sequences was carried out (Master cycler personal; Hamburg, Eppendorf, Germany) as per the manufacturer's

instructions using the eubacterial specific primers (24f—5′-AGAGTTTGATCCTGGCTCAG-3′) and (1492r—5′-ACGGCTACCTTGTTACGACTT-3′), purified using GFXTM PCR DNA and gel purification kit and cloned in pTZ57R/T vector according to the manufacturer's instructions. Gene sequencing was done at Macrogen (Seoul, Korea) and the sequence was deposited in NCBI, Genbank.

Growth profile of the isolate

An overnight culture of the organism was inoculated in nutrient broth pH 7 ± 0·2 containing increasing concentrations of chromium and incubated at 100 rev min^{-1} at 37°C. Known volume of culture grown in different concentrations of chromium were plated, the colonies were enumerated after 24 h and expressed as colony-forming units per ml. The experiment was carried out in triplicates. The growth pattern was also studied for copper and cadmium. The growth of organism cultured in the absence of metal was taken as control.

Differentially expressed protein profile by SDS PAGE analysis

The intra and extracellular protein expression changes were monitored in the cells grown in different concentrations of chromium. Protein extracts were prepared from bacterial cells in the late log phase of growth. The centrifuged cells were sonicated (Sonics Vibracell, Sonics & Materials, INC, Newtown, CT) (25 μm, 2 min, 2 pulses) in 50 mmol l^{-1} Tris HCl buffer, pH 8 containing 150 mmol l^{-1} NaCl, 5 mmol l^{-1} EDTA and 0·1% Triton X-100 and precipitated with acetone to get intracellular proteins. The supernatant was subjected to ammonium sulphate precipitation in order to precipitate the extracellular proteins which were centrifuged at 15 294 ***g*** for 10 min at 4°C and suspended in 50 mmol l^{-1} Tris HCl buffer, pH 8. To analyse the alterations in protein profile, same amounts of protein samples were loaded in different lanes on 12% SDS PAGE gels, stained with coomassie brilliant blue R-250 and variations between lanes were compared.

In gel digestion of proteins and MS (ESI-Q-TOF) analysis

The in gel digestion was carried out according to the previously described protocol (Shevchenko *et al.* 2006). The samples were analysed by LC-MS/MS on 1290 infinity LC system coupled with a 6540 UHD accurate-mass Q-TOF mass spectrometer equipped with Agilent jet stream technology ESI source (Agilent Technologies, Santa Clara, CA). The proteins were separated by 1290 HPLC system. The peptide samples were applied and eluted on Agilent Zorbax RRHD SB C-18 column (2·1 × 150 mm, 1·8 μm particle size) by a linear gradient of mobile phase solvents A and B (water and acetonitrile both with 0·1% formic acid (v/v) respectively at a flow rate 0·2 ml min^{-1} as follows 0–5 min 2% B, 5–25 min 45% B, 25–33 min 90% B, followed by 2 min wash at 2% B.

The Q-TOF-MS was operated in MS/MS mode with a detection frequency of 4 GHz. The source conditions were as follows: gas temperature: 200°C; drying gas: 5 l min^{-1}; nebulizer 40 psig; sheath gas temperature: 350°C; sheath gas flow: 11 l min^{-1}; V cap voltage: 3500 V; nozzle voltage: 1000 V; fragmentor voltage: 175 V. The MS was operated in the positive ionization mode in a data-dependent mode, selecting eight most abundant ions per cycle in the m/Z range of 300–1700. The charge state preference was set for selection of peptides with two or more charges for fragmentation with an exclusion time of 0·25 min after one spectrum. An absolute threshold of 1500 counts and relative threshold of 0·1% were maintained for precursor selection. The MS/MS spectra were acquired in the m/Z range of 50–1700. The LC-MS/MS system was operated using Agilent Mass Hunter B.04.00 software (Agilent Technologies) in which data acquisition and analysis were done using mass Hunter workstation B.04.00 and mass Hunter qualitative analysis B.04.00 (Prasanna *et al.* 2014).

Protein identification

The multiple runs of the same sample were combined in a single file and the peak list was created with the spectrum mill data extractor program with the following parameters: the minimum-maximum mass was set between 600 and 4000; sequence tag length > 1; scans with the same precursor ±1·4 m/Z with a retention time window of ±30 s were merged. The S/N ratio of the precursor ion was set at 25. Protein identification was attempted using the SWISSPROT database by querying the MS data against the bacterial proteins. The search parameters were set as follows: cysteine carbamidomethylation modification (fixed) and methionine oxidation (variable), precursor mass tolerance of ±50 mg l^{-1}, missed cleavage-two, the minimum matched peak intensity at 50%. A spectrum mill auto validation was carried out (Prasanna *et al.* 2014). Only the validated peptides and the proteins with a threshold score of 4 and above were considered.

Detection of antioxidant enzymes on native PAGE

Superoxide dismutase

The intracellular protein was loaded on 8% native PAGE gel and run. The gel was incubated in solution containing 0·1% NBT and 28 μmol l^{-1} riboflavin with shaking for

45 min in dark (Branco and Morais 2016). Gels were incubated in 1% TEMED in the dark for 15 min and immersed in distilled water. The gels were illuminated with white light until superoxide dismutase (SOD) bands appeared as a clear achromatic zone in blue-violet background. The SOD activity was measured as per the protocol previously reported with slight modifications (Beauchamp and Fridovich 1973; Rukmini *et al.* 2004). Blank for SOD assay consists of the reaction mixture (phosphate buffer, NBT, EDTA, methionine and riboflavin) without enzyme. Control consists of the reaction mixture (phosphate buffer, NBT, EDTA, methionine and riboflavin) with enzyme from chromium untreated culture. The incubation time for SOD assay was 20 min.

Catalase
Staining of the 8% native PAGE gel was carried out by washing the gel twice with distilled water, followed by incubation with 0·01% (v/v) H_2O_2 for 5 min. Gel was washed again with distilled water, followed by incubation in freshly prepared mixture of 0·5% ferric chloride and 0·5% potassium ferricyanide. The catalase bands appeared as achromatic bands in the Prussian blue background. Catalase activity was measured spectrophotometrically as previously reported with slight modifications (Beers and Sizer 1952; Sinha 1972).

Glutathione peroxidase
The 8% native PAGE gel was washed three times for 10 min in 1 mmol l^{-1} glutathione. This is followed by incubation in 0·12 μmol l^{-1} H_2O_2 for 10 min. The gel was washed twice with water, followed by addition of staining solution (0·5% ferric chloride and 0·5% potassium ferricyanide in glutathione solution). The glutathione peroxidase activity (GPX) was measured spectrophotometrically as previously reported with slight modifications (Rotruck *et al.* 1973).

Glutathione S transferase
The protein was run on a 10% native PAGE gel. Gel was incubated in a solution mixture containing 0·1 mol l^{-1} phosphate buffer pH 6·5, 4·5 mmol l^{-1} reduced glutathione, 1 mmol l^{-1} NBT and 1 mmol l^{-1} CDNB at 37°C for 10 min under gentle agitation. The gel was washed with water and incubated with a mixture of 0·1 mol l^{-1} Tris HCl buffer pH 9·6 and 3 mmol l^{-1} PMS at room temperature for 3–5 min. The glutathione S transferase (GST) activity was measured spectrophotometrically as previously reported (Moatamedi *et al.* 2014).

All the above mentioned antioxidant enzyme assays were carried out in triplicates and the activity of antioxidant enzymes was measured as average values ± standard deviation.

Results

Isolation, screening, identification and maximum tolerable concentration of chromium-resistant isolate

Resistant bacteria were isolated by the spread plate method in nutrient agar amended with basic chromium sulphate. To prevent precipitation of chromium at neutral pH, chromium solution was chelated with citrate, pH adjusted to 7 and added to the media. Metal to citrate at a ratio of 1 : 1 prevented precipitation of metal ion at pH 7. The isolate that showed tolerance to higher concentration chromium was identified by Gram staining and 16S rRNA gene sequencing. The isolate was Gram positive showing 99% similarity to the sequences of *Bacillus cereus* and designated as *B. cereus* VITSH1 assigned with the NCBI accession number KC731425. The phylogenetic tree is depicted in Fig. 1. The MTC of the metal was assessed in nutrient broth containing trivalent chromium. At a concentration of 2000 mg l^{-1} of chromium, *B. cereus* VITSH1 remained in lag phase up to 12 h above which no growth was observed up to 48 h and 2000 mg l^{-1} was recorded as the MTC of *B. cereus* VITSH1. The chromium-resistant isolate exhibited co-resistance to cadmium and copper showing a MTC of 60 and 300 mg l^{-1} respectively.

Growth profile of chromium resistant bacteria

The growth response of metal-resistant *B. cereus* VITSH1 in the presence and absence of metals is illustrated in Fig. 2. The growth profile of the organisms was studied at varying concentrations of chromium based on MTC. The growth of the isolates in chromium supplemented broth indicated that the growth in 300 mg l^{-1} chromium was comparable to that of the control containing organisms grown in the absence of metal. At higher concentrations of chromium the organism was able to resist after an initial lag in growth. *Bacillus cereus* VITSH1 exhibited a lag phase of 4, 7 and 10 h at 800, 1500 and 2000 mg l^{-1} of chromium respectively (Fig. 2a). The growth of the organism in 50 mg l^{-1} of copper was comparable to that of the control. The organism exhibited a lag phase of 4 h at 300 mg l^{-1} of copper and 6 and 9 h at 40 and 60 mg l^{-1} of cadmium (Fig. 2b). There was a delay in the onset of log phase as the metal concentration increased.

Protein expression and identification of differentially expressed protein

On comparison of intracellular protein on SDS gels, three newly expressed proteins around a molecular weight of

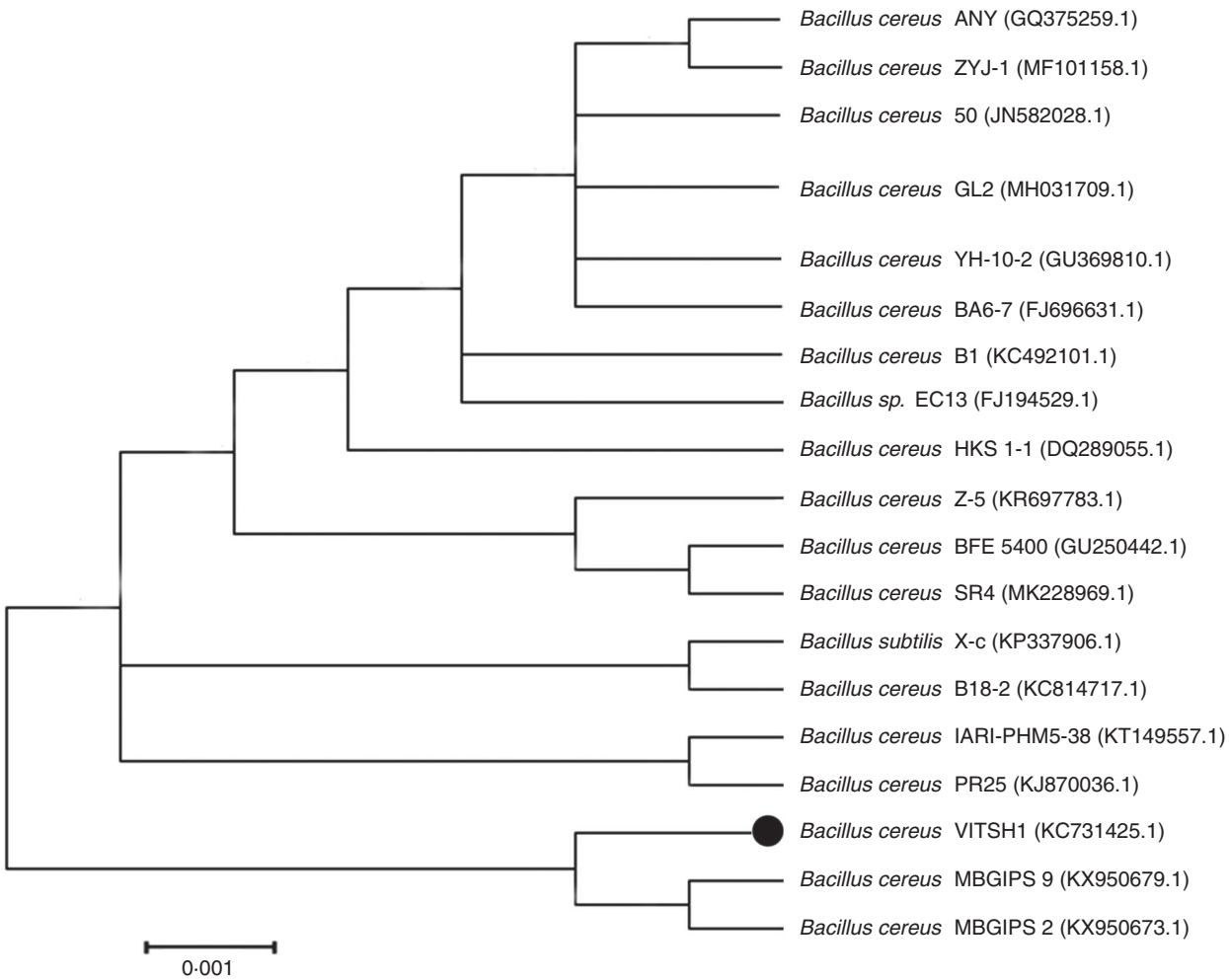

Figure 1 Neighbour-joining phylogenetic tree from 16S rRNA gene sequence of *Bacillus cereus* VITSH1 and reference strains. The dot mark indicates the isolate *B. cereus* VITSH1 (KC731425.1) employed in the present study.

87, 50 and 22 kDa was seen in response to chromium (Fig. 3a). These altered proteins studied by SDS PAGE and identified by mass spectrometry were marked as IP1, IP2 and IP3 in the Fig. 3a. These proteins were also expressed in copper and cadmium-treated conditions and probably a common mechanism of resistance occurs between different classes of metals. The extracellular protein pattern on SDS PAGE displayed alteration in expression of proteins marked as EP1 and EP2 in Fig. 3b. EP1 with a molecular weight above 55 kDa was overexpressed in chromium and copper-treated condition, but repressed in control and cadmium-treated condition. EP2 with a molecular weight around 42 kDa was expressed in control and copper-treated condition and repressed in chromium and cadmium-treated condition. The characteristics of differentially expressed proteins are given in Table 1.

Detection of antioxidant enzymes on Native PAGE

The native PAGE revealed three isoenzyme bands for SOD of molecular weight around 30 kDa in chromium-treated condition (Fig. 4a,b). In spite of the protective role exerted by the enzyme in other organisms under stress condition, the results of this study indicate a reduction in SOD activity on metal treatment (Table 2). Catalase enzyme activity analysed in 8% native polyacrylamide gel (Fig. 4c,d) indicated the presence of two major bands around 200 and 180 kDa in the presence of metal. The activity of catalase was found to increase when chromium concentration increased from 300 to 2000 mg l^{-1} (Table 2) which correlates with the previous findings on copper-resistant *B. cereus* (Behera *et al.* 2014). Two white bands in purple background around 60–90 kDa in gel assay indicated GPX activity

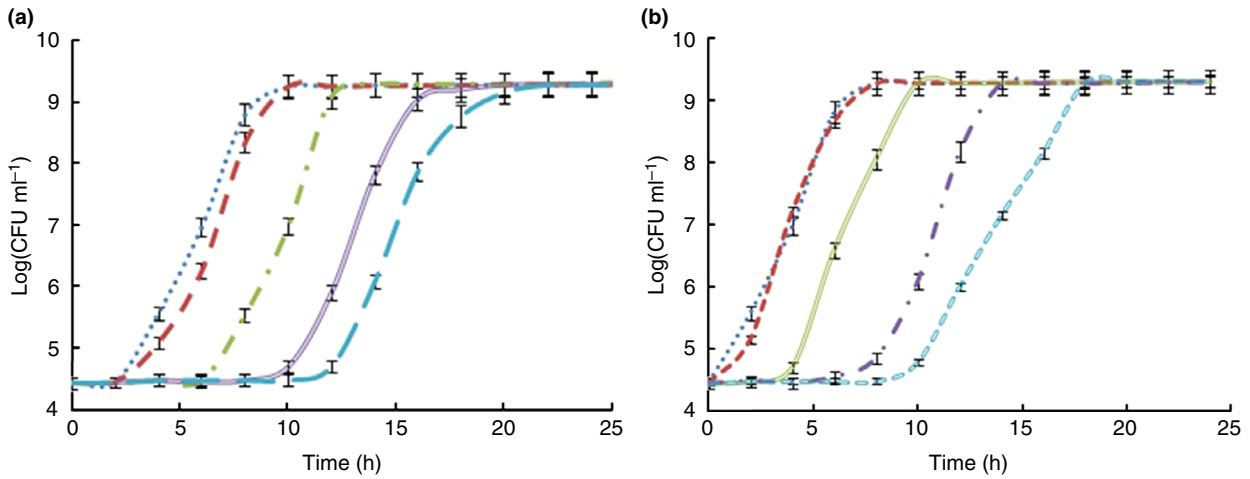

Figure 2 Growth curve of *Bacillus cereus* VITSH1 in (a) control, chromium, (b) copper and cadmium stress conditions. In (a), control without metal; chromium 300 mg l^{-1}; chromium 800 mg l^{-1}; chromium 1500 mg l^{-1}; chromium 2000 mg l^{-1}. In (b), control without metal; copper 50 mg l^{-1}; copper 300 mg l^{-1}; cadmium 40 mg l^{-1}; cadmium 60 mg l^{-1}. [Colour figure can be viewed at wileyonlinelibrary.com]

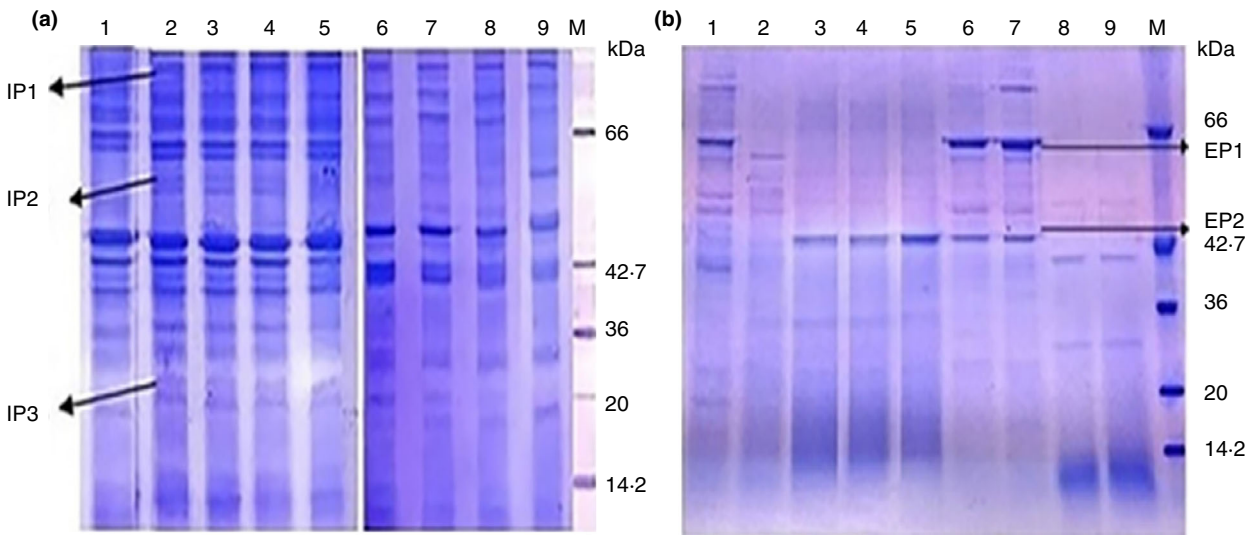

Figure 3 Proteins variations of *Bacillus cereus* VITSH1 on SDS PAGE (a) intracellular proteins (b) extracellular proteins. Lane 1—control; lane 2—chromium 300 mg l^{-1}; lane 3—chromium 800 mg l^{-1}; lane 4—chromium 1500 mg l^{-1}; lane 5—chromium 2000 mg l^{-1}; lane 6—copper 50 mg l^{-1}; lane 7—copper 300 mg l^{-1}; lane 8—cadmium 40 mg l^{-1}; lane 9—cadmium 60 mg l^{-1}; lane M—protein marker. [Colour figure can be viewed at wileyonlinelibrary.com]

(Fig. 4e,f) in the intracellular extract of metal-treated organism. The native PAGE in gel assay showed the presence of GPX activity in control as well as in metal-treated conditions, and the spectrophotometric enzymatic assay showed an increase in GPX activity with an increase in chromium concentration (Table 2). The native PAGE analysis of GST had shown the presence of two isoenzymes having molecular weight around 35–40 kDa in the presence of metal (Fig. 4g,h). The activity of the enzyme was high in metal-treated condition compared to control (Table 2).

Discussion

Metals used in industries like chrome plating, mining, tanning leaches into the surrounding soils from the effluents and solid wastes disposed around the industry. The biodiversity around the contaminated area is markedly reduced (Xie *et al.* 2016) and only some organism adapt to the changes in the stressed environment. Studying the changes in the organisms could help in establishing the probable resistant mechanism based on which the bacteria could be exploited in bioremediation

Table 1 Characteristics of the differentially expressed proteins of *Bacillus cereus* VITSH1 identified in MS (ESI-Q-TOF) analysis

Protein name	Accession	Peptides	Mol wt (kDa)	PI	Sequence
S layer protein	315308170	18	87·2	5·44	(K)AIEGKPDGTFAPTEAIDR(A) (K)ALEVIFFNDEVAPTVSTVSTPDGNVK(V) (K)DAQDSWAAK(Y) (K)DAVTVVIDGK(L) (K)DLVGNTMEMYEGK(A) (K)DVTAANFAVVEGSK(E) (K)DVTAPEVK(D) (K)DVVAPNLVK(V) (K)FTVANVANK(D) (K)LPEGTLIVVNADGK(S) (K)MGTTNLLVNVVDTK(A) (K)NGEAFNVIVTGAK(D) (K)SATITLK(N) (K)VEFVGYK(D) (R)VINLDTSK(D) (K)YIAAVEK(A)
F0F1ATP synthase subunit β	30265328	2	51·1	4·93	(K)AISVPVGDATLGR(V) (K)IGLFGGAGVGK(T)
50S ribosomal protein L4	30018381	4	22·5	9·95	(K)DMLAVLK(G) (K)LIMTK(A) (R)NIPGVTVITADGVNVLDVLHHDK(L) (K)VVNNIVVLEDLVLNAPK(T)
Molecular chaperone	446952738	6	57·1	4·84	ENTTVVEGVGSTEQIEAR AQLEETTSEFDR VGAATETELKER VGAATETELK IEDALNSTR SATVESLGR
Flagellin	447145068	6	47·3	5·64	SIADTAATGTGAAK INNASDDAAGLAIATR GNLYTGLTADQK TADSALGSVSNILLR QITYIADNTQFNDK

of metals, in improving the plant growth in metal contaminated sites, in the construction of metal-based biosensors, as indicators of pollution and in the synthesis of nanoparticles (Ayangbenro and Babalola 2017; Igiri *et al.* 2018).

Metals can be classified as hard, borderline and soft (Pearson 1963). The different categories of metals, namely copper from borderline and cadmium from soft category were chosen to study their effect on chromium-resistant organisms. The MTC to cadmium was the least among the metals tested. Hard metals are usually nontoxic and are required as micronutrients for the growth of microbes whereas the soft metals possess high toxicity (Pearson 1963) emphasizing that cadmium possesses more toxicity and is also confirmed in this study by the increase in the duration of lag phase even at lower concentrations of cadmium compared to copper and chromium (Fig. 2). Previous studies have reported that the long lag phase observed is due to the acclimatization of the bacteria to the toxic environment (Kader *et al.* 2007). The protein expression changes under chromium stress (Fig. 3a) were compared with that of other metal stress conditions ,that is, copper and cadmium to compare the effect of different categories of metals (Fig. 3b) and to analyse whether the same type of differentially expressed proteins is present in all three metal stress conditions. Protein was extracted from the bacterial cells in the late log phase to avoid heterogeneous cell population that might arise in the early log phase after immediate adaptation in the lag phase (Koch 1993). The protein pattern of the isolate on the SDS PAGE (Fig. 3) revealed pronounced alterations. Change in protein expression was observed, even though there was no difference in growth at a concentration of 300 mg l^{-1} chromium and 50 mg l^{-1} copper compared to their control. The organism probably coped with growth at lower concentration of metal where as at higher concentrations there was a delay in the lag phase of growth. The differentially expressed proteins. IP1, IP2

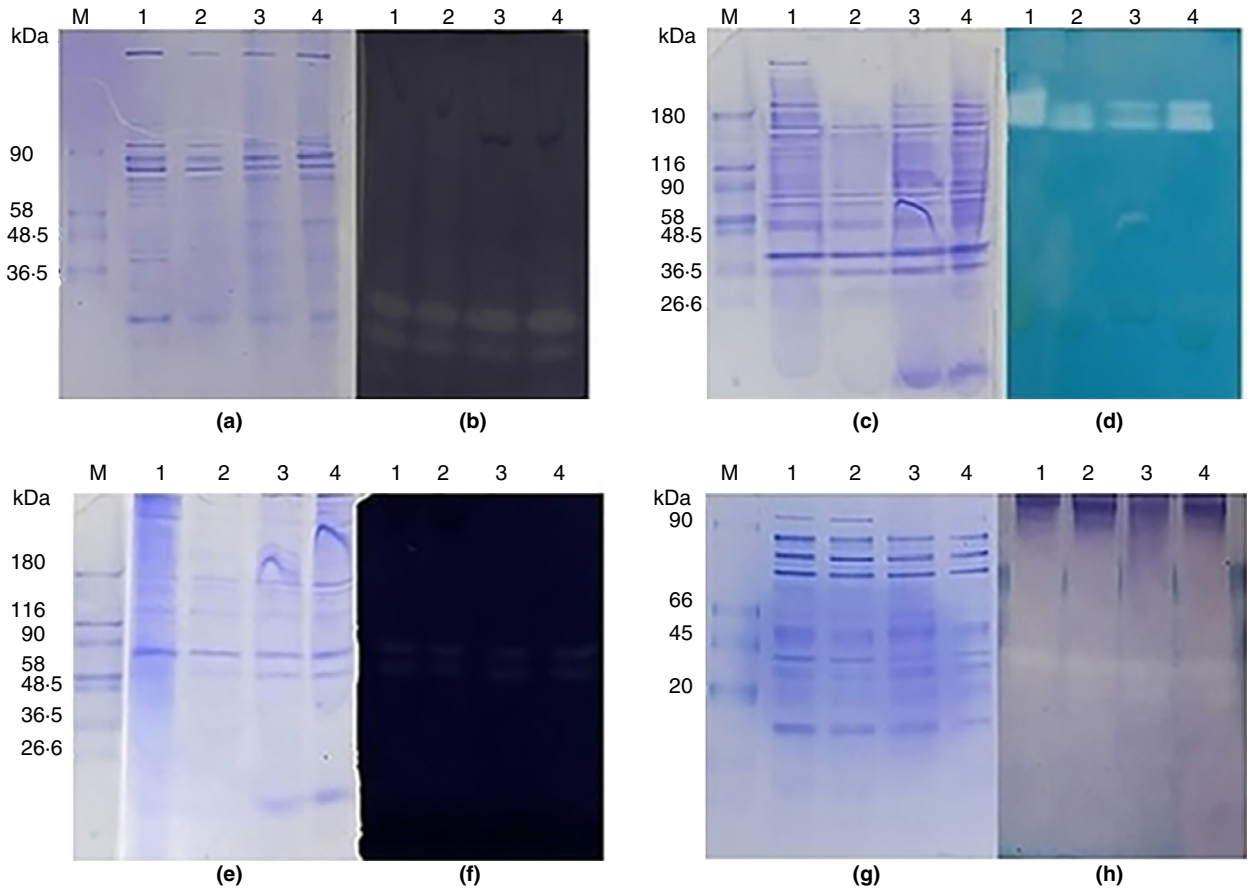

Figure 4 Native PAGE of *Bacillus cereus* VITSH1 for antioxidant enzyme profile. a, c, e, g represents coomassie brilliant blue R-250 staining of SOD, catalase, GPX and GST respectively. b, d, f, g represents gel activity staining of SOD, catalase, GPX and GST respectively. Lane M-protein marker; lane 1—control; lane 2—chromium 300 mg l^{-1}; lane 3—chromium 800 mg l^{-1}; lane 4—chromium 2000 mg l^{-1}. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 2 Activity of antioxidant enzymes in chromium-resistant *Bacillus cereus* VITSH1 at different chromium concentration

Enzymes (U mg^{-1})	Control	Chromium (300 mg $l^{-1)}$	Chromium (800 mg l^{-1})	Chromium (2000 mg l^{-1})
SOD	2·39 ± 0·292	2·02 ± 0·029	1·93 ± 0·024	1·92 ± 0·042
Catalase	3·08 ± 0·775	2·19 ± 0·242	3·98 ± 1·391	6·86 ± 2·184
GPX	2·0 ± 0·171	2·60 ± 0·019	4·30 ± 0·031	4·28 ± 0·046
GST	1·40 ± 0·398	2·48 ± 0·142	4·56 ± 1·211	6·47 ± 0·806

and IP3 in Fig. 3a and EP1 and EP2 in Fig. 3b) were identified by MS/MS analysis and searched against the NCBI database for micro-organisms, that matched single proteins. The IP1 protein of 87·2 kDa was identified to be S-layer protein. The other intracellular proteins were ATP synthase FoF1 β subunit (IP2) and 50S ribosomal L4 protein (IP3) with molecular weights of 51·1 and 22·5 kDa respectively. The extracellular proteins were identified to be molecular chaperone (EP1) and flagellin (EP2) with molecular weights 57·1 and 47·3 kDa respectively.

Intracellular proteins

S-layer protein

The S-layer proteins acts as an ion trap enabling the cells to resist changes in a dynamic environmental conditions (Engelhardt and Peters 1998; Claus *et al.* 2005). The S-layer proteins was found to possess uranium binding properties in *Lysinibacillus sphaericus* JG A12 and thus protecting the organism from toxic effects of the metal (Merroun *et al.* 2005). *Bacillus sphaericus* JG-A12 was also capable of binding lead and transmission electron

microscopy analysis revealed the presence of metal particles on cells. The S-layer proteins are known to be rich in amino acids with carboxylic side chains which probably aid metal binding (Pollmann *et al.* 2005). In *Bacillus thuringenesis*, the property of the organism to biosorb copper reduced upon the removal of the S-layer proteins (Allievi *et al.* 2011). The S-layer proteins have been reported to act as a protective layer in *Lactobacillus kefiri* in the presence of lead (Gerbino *et al.* 2015). The increase in the expression of S-layer proteins in the isolated organism on metal exposure probably helps the organism in sequestering metals and preventing their entry.

Proteins involved in energy metabolism: ATP synthase FoF1β subunit

The F1 ATPase contains five different kinds of subunits and β subunit being one of them present in three copies. The β subunit of the F1 ATPase contains three catalytic sites and plays a vital role in ATP synthesis (Ono *et al.* 2003). Increase in expression of gene coding for β subunit was also observed in four organisms that tolerated radiation stress (Gao *et al.* 2009). The increased expression of the β subunit in stress condition probably implicates the need for increased energy metabolism that might result in the production of additional proteins to counteract stress.

Proteins involved in protein biosynthesis: 50S ribosomal L4 protein

In this study, the 50S ribosomal L4 protein was overexpressed upon exposure to metals compared to the control. The 50S ribosomal L4 protein is a rRNA binding protein belonging to the L4 family having multiple interactions with 23S rRNA of the 50S subunit of the ribosome. These proteins are probably involved in the process of transcription and translation (Lindahl and Zengel 1979; Yates and Nomura 1980). Bacterial growth rate might decrease during stress conditions. Starved micro-organisms decrease its growth rate (Reeve *et al.* 1984) accompanied by poor synthesis of proteins, rRNA and tRNA. The organism expresses the ribosomal protein probably to increase the growth rate and protein synthesis providing an adaptive environment for bacteria upon exposure to stress. Such protective feature of L4 protein in stress conditions might have played special functional roles in the isolated organism to combat metal stress.

Extracellular proteins

Stress proteins: molecular chaperones

Molecular chaperones (GroEL) are not expressed in chromium and cadmium stress, but expressed in copper stress conditions. Some proteins are specifically induced by specific metals (Mehta *et al.* 2014). Molecular chaperones are known to help in the folding of the newly synthesized proteins and refolding of proteins altered under stress, providing protection to cells under stress (Vlamis-Gardikas 2008; Saibil 2013). In environmental stress conditions, the chaperonins encapsulates the polypeptide chains and facilitates folding until the hydrophobic regions are buried (Ellis and Hartl 1996). The expression of the stress protein GroEL in the isolated organism under copper stress was probably to aid folding of protein. In certain bacterial secretion systems, the molecular chaperones are also secreted extracellularly to assist in protein folding of the unfolded proteins translocated across the membrane (Lund 2001; Zhou and Xu 2005).

Structural protein: flagellin

The expression of flagellin appears to have a role in chromium and copper resistance. Secretion of flagellin helps the organism move to optimal colonization sites, thereby evading toxicants and also improving nutrient acquisition (Ottemann and Miller 1997). The overexpression of flagellin in *B. cereus* CMG2K4 has contributed to nickel tolerance and to cadmium tolerance in *Pseudomonas* sp. (Shoeb *et al.* 2010; Izrael-Živković *et al.* 2018). The present study indicates that the probable impact of metals on the expression of flagellin is to assist in motility away from the toxic conditions. Flagellin proteins are translocated by the flhA protein a component of the flagellar type 3 secretion system indicating that flagellin might also have a role outside the bacterial cell (Ibuki *et al.* 2013).

Detection of antioxidant enzymes on native PAGE

Superoxide dismutase causes elimination of superoxide radical, a reduction product of oxygen. The activity of the enzyme was determined by its ability to inhibit the reduction of NBT to formazan by superoxide radicals. In control, photoreduction of NBT when exposed to light by superoxide radicals gives a blue coloured formazan whereas in presence of SOD there is a corresponding decrease in NBT reduction giving a less coloured complex compared to control. chrC, a SOD gene has been identified as one of the chromate resistant genes in resistant bacteria (Liu *et al.* 1997) that helps in repairing the chromate induced damage by the formation of superoxide radicals. Previous studies have reported the role of SOD in sequestration of metals (So *et al.* 2001). In another study, a thermostable Fe/Mn SOD with antioxidative potential from *Bacillus licheniformis* isolated from hot-springs of Himalayan region enabled survival of the organism (Thakur *et al.* 2018). SOD was also found to possess lead binding capability in *Streptomyces subrutilis* which precipitated upon complexing with lead ions (So

et al. 2001). Similarly, Mn SOD from *Aeromonas hydrophila* was found to play an important role in protecting bacterial cells from oxidative stress (Leclère *et al.* 2004). Ose and Fridovich (1979) observed that exposure to metal increases the incorporation of the toxic metal in the active site of SOD and thereby causing reduction in the enzyme activity. Furthermore, strict cofactor specificity of SOD may lead to activity loss if the exposed metal is incorporated in the active site. (Kirby *et al.* 1980). There may be a high persisting oxidative stress due to heavy metal toxicity and hence reduction in SOD activity as the organism was not able to cope up with free radicals generated on metal exposure (Scandalios *et al.* 1997). The reduction could probably be due to the toxic effect of the metal (Behera *et al.* 2014) or as a result of exposed metal chelation to the sulfhydryl group of SOD (Imai and Nakagawa 2003).

Catalases cause the dismutation of H_2O_2 to oxygen and water generated from the reaction catalysed by SOD. Catalases have been reported to play a protective role under stress condition (Jia *et al.* 2017).

GPX utilizes reduced glutathione to scavenge reactive oxygen species and catalyze the decomposition of hydroperoxides and H_2O_2 (Imai and Nakagawa 2003). GPX was found to be one of the proteins upregulated in the presence of cobalt in *Synechocystis* and helped the organism to combat oxidative stress upon exposure to cobalt (Mehta *et al.* 2014).

Glutathione S transferase catalyses the conjugation of glutathione to electrophilic substrates resulting in the formation of less toxic and hydrophilic compounds which can be removed from the cell. In *Caulobacter crescentus* the GST was upregulated in cadmium and chromate stress (Hu *et al.* 2005) to combat the oxidative attack. On the whole, an alteration in the activity of the antioxidant enzymes was observed confirming the role of these enzymes in helping the organism overcome the stress.

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Conflict of Interest

The authors declared no conflict of interest.

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