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Protection from metal toxicity by Hsp40-like protein isolated from contaminated soil using functional metagenomic approach

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Abstract

Pollution in the environment due to accumulation of potentially toxic metals results in deterioration of soil and water quality, thus impacting health of all living organisms including microbes. In the present investigation, a functional metagenomics approach was adopted to mine functional genes involved in metal tolerance from potentially toxic metal contaminated site. Eukaryotic cDNA library (1.0–4.0 kb) was screened for the genes providing tolerance to cadmium (Cd) toxicity through a functional complementation assay using Cd-sensitive *Saccharomyces cerevisiae* mutant *ycf1^Δ*. Out of the 98 clones able to recover growth on Cd-supplemented selective medium, one clone designated as PLCc43 showed more tolerance to Cd along with some other clones. Sequence analysis revealed that cDNA PLCc43 encodes a 284 amino acid protein harbouring four characteristic zinc finger motif repeats (CXXCXGXG) and showing partial homology with heat shock protein (Hsp40) of *Acanthamoeba castellanii*. qPCR analysis revealed the induction of PLCc43 in the presence of Cd, which was further supported by accumulation of Cd in *ycf1^Δ/PLCc43* mutant. Cu-sensitive (*cup1^Δ*), Zn-sensitive (*zrc1^Δ*) and Co-sensitive (*cot1^Δ*) yeast mutant strains were rescued from sensitivity when transformed with cDNA PLCc43 indicating its ability to confer tolerance to various potentially toxic metals. Oxidative stress tolerance potential of PLCc43 was also confirmed in the presence of H₂O₂. Present study results suggest that PLCc43 originating from a functional eukaryotic gene of soil community play an important role in detoxification of potentially toxic metals and may be used as biomarker in various contaminated sites.

Introduction

Metal contamination due to increasing anthropogenic activities is a significant problem of the industrial era because of the persistent and irreversible nature of these contaminants (Fernández et al. 2018). Soil contamination by potentially toxic metals not only modify soil physico-chemical properties but also deteriorates its microbial quality, thus leading directly or indirectly to harmful effects on the health of all members of food chain including animals and human beings (Dong et al. 2011; Nabulo et al. 2010). Soil is a rich habitat of highly diverse microorganisms, many of which are still unknown and uncultivable as of yet, but have a high metabolic response potential to survive in polluted environments. Exploring functional microbial communities has come across various cellular mechanisms involved in maintaining homeostasis in potentially toxic metal polluted environment, as chelation, efflux, or cellular compartmentalisation (Clemens 2006; Blindauer and Leszczyszyn 2010;

Gadd [2010](#); Reddy et al. [2014](#), [2016](#)). To harness the hidden functional diversity and in situ mechanisms for revitalizing the polluted soil by attenuation of the detrimental effects of accumulated toxic metals, metatranscriptomics approach has been introduced recently.

Metatranscriptomics is a powerful tool in exploring complex biological system as this approach is designed without any limitation to living organisms and opened to any strata of life (Gonzalez et al. [2018](#); Thavamani et al. [2017](#)). Function-based approach of metagenomics involves conversion of mRNAs into complementary DNA libraries followed by transcription and expression in suitable hosts (Bailey et al. [2007](#); Grant et al. [2006](#)). It has been reported that function-based metagenomic approach may explore the functional diversity by directly using mRNAs, thus providing actively expressed gene repertoire in environmental samples (Bailey et al. [2007](#); Marmeisse et al. [2017](#); Thakur et al. [2018](#)). This approach is a function-driven approach and recognized as an authoritative tool in unearthing various functional genes playing fundamental role in numerous phenomena including biological response mechanisms to environmental conditions (Damon et al. [2011](#); de Menezes et al. [2012](#); Zielinski et al. [2016](#)). Function-based metagenomics has identified numerous ecologically significant genes as those encoding for oligopeptide transporters (Damon et al. [2011](#)), cysteine-rich metallothioneins (Ziller et al. [2017](#)) and candidate proteins involved in metal tolerance (Thakur et al. [2019](#)). This approach provided an unprecedented overview of gene expression profiling in microbial communities, thus providing huge information on functional microbial diversity. The functional microbial diversity of muskoxen rumen (Qi et al. [2011](#)), forest soils (Damon et al. [2011](#)), phenanthrene-contaminated soil (de Menezes et al. [2012](#)), activated sludge from wastewater (Yu and Zhang [2012](#)), acid mine drainage and root microbiome (Chen et al. [2015](#); Gonzalez et al. [2018](#); Teng et al. [2017](#)) are some of the examples.

Metal stress usually alters cell homeostasis by inactivating enzymes and by suppressing protein functions (Hossain et al. [2012](#)). Induction of heat shock proteins (HSPs) may represent a critical protective, eco-physiologically adaptive and genetically conserved response in some living organisms that undergo environmental stress (Hasan et al. [2017](#)). HSPs play a key function by restoring protein conformation and consequently cell homeostasis (Rhee et al. [2009](#)). Among the major categories of HSPs, Hsp40 proteins play the role of molecular chaperones that act either in an independent way or as co-chaperones with Hsp70 (Craig et al. [2006](#)). Hsp40, also known as J- proteins, are categorized into three different classes, types I, II, and III, depending upon the presence or absence of one or more of four structurally defined domains. Type I Hsp40 proteins possess all the four domains similar to those found in *E. coli* DNAJ, i.e. an N-terminal J-domain linked by a glycine/phenylalanine (G/F)-rich region to a cysteine-rich (CXXCXGXG) zinc finger region and followed by a C-terminal substrate-binding domain. Type II Hsp40s are similar to the class I proteins, except the absence of zinc finger domain in their structure, whereas type III Hsp40 proteins only retain the J domain (Tamadaddi and Sahi [2016](#)). In addition, Hsp40 proteins provide tolerance to multiple types of stressors and protect living organisms from oxidative stress (Jiang et al. [2009](#)). These proteins accumulate in a wide range of plant and animal species in response to environmental stressors to maintain cellular homeostasis (Gillis et al. [2013](#); Gupta et al. [2010](#)).

Screening of yeast cDNA library constructed from RNA isolated from a metal-contaminated site allowed us to identify several genes involved in Cd tolerance when expressed in yeast (Thakur et al. [2018](#), [2019](#); Mukherjee et al. [2019](#)). In the present study, one of the cDNAs, PLCc43, whose deduced protein sequence showed partial sequence similarity to eukaryotic type I Hsp40 proteins, was characterized and its role in providing tolerance to potentially toxic metals in metal-sensitive yeast mutants was assessed.

Materials and methods

Soil sampling and analysis

Soil samples were collected from the metal contaminated site of Pierrelaye (49° 1' 45" N; 2° 10' 32" E), northwest of Paris, France. Poplar plants are cultivated in this site, which was earlier an agricultural land and became contaminated with potentially toxic metals due to extended use of untreated wastewater for irrigation. Twenty soil cores were collected, mixed in equal volumes to make a composite sample, sieved through 2.0-mm mesh, and preserved in dry ice and then stored at – 70 °C

until further experiments. The physicochemical properties of the soil were determined as described in Thakur et al. (2019) and were as follows: pH 7.15; total phosphorus 291 mg/kg; available phosphorus 14.2 mg/kg; organic carbon 1.6%; and total nitrogen 0.12%. The metal contents (mg/kg) of the soil samples were as follows: Cd 2.5, Zn 385 and Cu 64.

Strains, culture conditions and transformation

The mutant strains of *Saccharomyces cerevisiae* sensitive to cadmium (*ycf1^Δ*), copper (*cup1^Δ*), zinc (*zrc1^Δ*) and cobalt (*cot1^Δ*) were used for transformation (Gietz and Schiestl 2007). The *ycf1^Δ*, *zrc1^Δ* and *cot1^Δ* strains are derived from the wild-type strain BY4741 (*MAT α* , *his3^Δ1*, *met15^Δ0*, *leu2 Δ 0*, *ura3 Δ 0*). The *ycf1^Δ* yeast strain lacks an ABC transporter encoded by gene *YCF1* that mediates the transport of Cd conjugates into vacuoles (Li et al. 1997), while *zrc1^Δ* lacks the transporter protein *ZRC1p* responsible for Zn sequestration into vacuoles (Li and Kaplan 1998). The hypersensitivity of *cot1^Δ* towards Co metal is due to deletion of *COT1* gene that helps in efflux of cobalt ions into the vacuole (Conklin et al. 1992). Similarly, *cup1^Δ* mutant strain is derived from knocking out the copperthionein gene (Longo et al. 1996) from its isogenic wild-type strain *cup1^s* (*MAT α* , *leu2-3*, *112his3^Δ1*, *trp1-1*, *ura3-50*, *gal1*, *CUP1^s*). Yeast strains BY4742 and its derivative superoxide dismutase mutant *sod2^Δ* (BY4742 *sod2::KanMX*) were used for oxidative stress experiments. For yeast complementation assays, minimal medium supplemented with 2% glucose (Hi-Media Laboratories, India) containing dropout supplement without uracil (SD-Ura) (Takara-Clontech, Japan) was used. Yeast strains were maintained on yeast extract peptone dextrose (YEED) (Hi-Media Laboratories, India). Bacterial transformations were carried out by using One Shot[®] TOP10 Electrocomp[™] *E. coli* cells (ThermoFisher, USA). The yeast strains were grown at 30 °C while bacteria at 37 °C.

Construction of sized cDNA library and screening for Cd tolerance

Total RNA was isolated from the soil composite samples using the RNA PowerSoil[®] Total RNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) according to the manufacturer's instructions. Extracted total RNA was checked for quality by agarose gel electrophoresis and Bioanalyzer (Agilent Technologies, USA). The extraction yields ranged from 330 to 980 ng/g of soil and at least 3 μ g of total soil RNA were obtained from each sample. cDNAs were synthesized from total soil RNA with Mint-2 cDNA synthesis kit (Evrogen, Moscow, Russia). Size fractionation of cDNAs was performed as described in Yadav et al. (2014). Size fractions of cDNAs, A (< 0.5 kb), B (0.5–1 kb) and C (> 1 kb) were ligated downstream of PGK1 promoter of *S. cerevisiae* shuttle plasmid vector pFL61 modified with SfiA and SfiB restriction sites (Minet et al. 1992). These recombinant plasmids were introduced into DH10 β electrocompetent *E. coli* cells (One Shot[®] TOP10 Electrocomp[™] *E. coli*, Invitrogen). More than 10⁶ colonies growing on ampicillin-containing Luria agar plates (100 μ g ampicillin per ml of medium) were pooled to represent each of the cDNA size libraries. Plasmid DNA from the pooled cells representing the cDNA library C (> 1.0 kb) was extracted with QIAprep Spin Miniprep Kit (Qiagen, USA) and transformed into Cd-sensitive *ycf1^Δ* yeast mutant by lithium acetate method (Gietz and Schiestl 2007) and were regenerated on SD-Ura medium amended with 40 μ M Cd. The selected clones were treated with 5-fluoroorotic acid (5-FOA) to eliminate false-positive colonies. One of the positive clones, PLCc43, was selected for further characterization based on its ability to tolerate high concentrations of Cd.

Sequence analysis

Plasmid DNA from yeast *ycf1^Δ*/PLCc43 was isolated with Zymoprep yeast plasmid Miniprep II kit (Zymo Research) and sequenced the insert PLCc43 with vector-specific primers NF and NR (Table 1) by using Sanger sequencing technology. Sequence homology search was performed by using BLASTX (<https://blast.ncbi.nlm.nih.gov/Blast.cgi/>). Open reading frame of the sequence was determined by using the webserver <https://www.ncbi.nlm.nih.gov/orffinder/> and molecular weight and theoretical isoelectric point (pI) for the putative encoded protein by http://web.expasy.org/compute_pi/. Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>) was used to align the homologous sequences along with PLCc43. Phosphorylation sites within the deduced polypeptide PLCc43 was predicted with NetPhos 2.0 server (<http://www.cbs.dtu.dk/services/NetPhos/>).

Table 1 Primers used to amplify different genes in this study.

Gene	Name	Sequence
pFL61	pFL61 NF	5'-CAGATCATCAAGGAAGTAATTATCTAC-3'
	pFL61 NR	5'-CAGAAAAGCAGGCTGGG AAGC-3'
Actin	qACTF	5'-CGAATTGAGAGTTGCCCCAG-3'
	qACTR	5'-CAAGGACAAAACGGCTTGGA-3'
TAF10	qTAF10F	5'-GCTAACAACAGTCAGGCGAG-3'
	qTAF10R	5'-GAGCCCGTATTTCAGCAACAG-3'
TFC1	qTFC1F	5'-ACGACGCTGCTTTGGAAAAT-3'
	qTFC1R	5'-GCTTTTCATTGTTTCCGCGG-3'
PLCc43	qHspF	5'- GACCGTATGCCCCAAATGTC 3'
	qHspR	5'-GGGAATTACACCAGGTCCT-3'

Relative quantification of PLCc43

Cadmium-sensitive yeast mutant *ycf1^Δ* carrying PLCc43 was tested for its relative quantification in presence of different concentrations of Cd. To accomplish this, flasks containing 50 ml of SD-Ura broth amended with different concentrations of Cd (0, 40, 60 and 80 μM) was inoculated with *ycf1^Δ/PLCc43* cells and incubated for 48 h at 30 °C. The cells were harvested and grounded with liquid nitrogen. Total RNA was isolated by Trizol reagent, and RNase-free DNaseI (Thermo Scientific, USA) was used to remove genomic DNA contamination. cDNA was synthesized from total RNA by using PrimeScript™ first strand cDNA synthesis kit (Takara, Japan) as per manufacturer's instructions. To assess the relative abundance of PLCc43 and their relative basal levels, 25 μl qPCR reaction mixtures were prepared. The reaction contained 12.5 μl SYBR® Premix Ex Taq™ (Tli RNase H Plus) (Takara, Japan) as recommended by the manufacturer, 0.2 μM specific primers (Table 1), 1.0 μL diluted cDNA (20 fold dilution; approximately 50 ng) and H₂O to make the final volume. The qPCR analysis was performed on the Mastercycler® ep realplex system (Eppendorf AG, Hamburg, Germany). The PCR conditions were 95 °C for 2 min (1 cycle), followed by 35 cycles of 95 °C for 15 s, 53 °C for 15 s and 68 °C for 20 s. The E values for PLCc43 were within the range of 0.94 to 1.27. Three reference genes, actin (*ACT*; SGD: S000001855), TATA-binding protein-associated factor (*TAF10*; SGD: S000002574) and transcription factor class C (*TFC1*; SGD: S000000327) were used in this study (Teste et al. 2009). The relative amount of PLCc43 was determined according to 2^{-ΔΔCt} method (Livak and Schmittgen 2001) by using TFC1 as reference gene because of its minimum stability than other two reference genes. The base level expression of PLCc43 in control cells (without Cd augmentation) was considered as one and calculated the fold increase in Cd augmented cells. To confirm that there were no traces of DNA present in RNA extracts, PCR was performed with nonreverse-transcribed RNA samples as control. The reaction mixture without cDNA served as negative control. All qPCR measurements were performed on RNA extracted from three independent biological samples (experimental replicates), and for each RNA extract, three technical replicates were performed.

Tolerance to metal toxicity and metal content

Cd-sensitive (*ycf1^Δ*), Cu-sensitive (*cup1^Δ*), Zn-sensitive (*zrc1^Δ*) and Co-sensitive (*cot1^Δ*) yeast mutants expressing PLCc43 were tested for tolerance towards Cd, Cu, Zn and Co, respectively as follows: serial dilutions (0.1–0.0001) were prepared from calibrated overnight grown transformant yeast cells and empty vector pFL61 carrying control cultures (wild-type BY4741 and mutant strains). Five microliters of each dilution was dropped on SD-Ura plates supplemented with respective metal concentrations, i.e. CdSO₄ 40 μM, CuSO₄ 150 μM, ZnSO₄ 10 mM and CoCl₂ 2 mM. Liquid broth assays were performed in parallel to validate the broad spectrum of metal tolerance. To do this, 20 ml of SD-Ura were inoculated with exponentially cultured cells to achieve an initial OD₆₀₀ = 0.02 and were allowed to grow at 30 °C for 3 h. Then, varying concentrations of CdSO₄ (40 μM–80 μM), CuSO₄ (150 μM–500 μM), ZnSO₄ (10 mM–12 mM) and CoCl₂ (2 mM–4 mM) were added separately to the respective yeast cell

cultures. Growth was monitored via OD measurements at 600 nm at intervals of 3 h for 48 h. Empty vector pFL61 transformed yeast mutants and wild-type BY4741 served as controls.

For metal accumulation studies, all yeast cells were allowed to grow overnight in SD-Ura medium till the $OD_{600} = 1.0$ was attained. Cells were harvested by centrifugation and re-suspended in sterile water. One percent inoculum was used to inoculate 50 ml of SD-Ura broth in 250 ml flasks and was incubated at 30 °C at 200 rpm. After 5 h, various concentrations of $CdSO_4$ (40 μM –80 μM), $CuSO_4$ (150 μM –500 μM), $ZnSO_4$ (10 mM–12 mM) and $CoCl_2$ (2 mM–4 mM) were amended in the media similar to growth kinetics study. After 40 h of growth, cells were harvested and washed 3 times with 10 mM EDTA solution. Final washing was done with sterile deionized water to eliminate residual EDTA and dried for 24 h at 80 °C. A combination of HNO_3 and $HClO_4$ (ratio 2:1 v/v) was used to digest dry yeast biomass (Hseu (2004) and the metal content was determined through ICP-MS (Element XR, Thermo Fisher Scientific, Germany). Experiments were conducted in a 3 × 3 factorial design with yeast strains and concentrations of the metals arranged in a completely randomized block design with three replicates. The experiment consisted of three yeast inoculation treatments (*ycf1^Δ*/pFL61, *ycf1^Δ*/PLCg39 and BY4741) and three concentrations of $CdSO_4$ (40, 60 and 80 μM). Similar design of experiments were conducted for Cu, Zn and Co using the respective yeast mutants.

Furthermore, the involvement of PLCc43 in providing the tolerance to metal toxicity was tested by transforming PLCc43 into the wild-type strain BY4741, which already consists of gene for yeast Hsp40 protein. The transformed cells were grown in presence of 60 μM of $CdSO_4$. The wild-type strain BY4741 transformed with empty vector pFL61 was used as positive control. Calibrated inoculums consisting of 2×10^6 cells from actively growing cultures were used as starting materials and the growth of both the strains was recorded for 36 h with an interval of 3 h.

Oxidative stress

The superoxide dismutase yeast mutant *sod2^Δ* was used to examine the role of PLCc43 in oxidative stress. To do this, functional complementation of *sod2^Δ* using PLCc43 was evaluated in SD-Ura medium supplemented with H_2O_2 (0–0.5 mM) following drop assays and growth kinetics study. For controls, the parent-type BY4742 and *sod2^Δ* both transformed with empty vector pFL61 were used. Growth response was determined by recording the OD_{600} until 30 h at an interval of 5 h.

Statistical analysis

The statistical tests were carried out by using GraphPad Prism (version 5.04) (GraphPad Prism, San Diego California, USA) software. Two-way analysis of variance was performed to examine the effect of various concentrations of metals on the cell growth and metal uptake of different yeast strains and their interactions. The significant differences among the means were compared with Tukey's test at $P < 0.05$ when one way-ANOVA was used

Results and discussion

Screening of cadmium tolerant cDNAs

Yeast mutant sensitive to Cd (*ycf1^Δ*) was used for screening of the environmental cDNAs (library C, > 1.0 kb insert size) synthesized from total soil RNA and the quality of total RNA was determined by agarose gel. Among various clones screened through functional complementation by drop assay, 98 clones were retrieved, which are capable of growing in Cd-amended medium (Supplementary figure S1). These results indicated that some of the transformed clones were able to rescue *ycf1^Δ* cells from Cd sensitivity. The clone *ycf1^Δ*/PLCc43 was selected for further studies based on its tolerance to high Cd concentrations. Thakur et al. (2018) reported a 695-bp-long eukaryotic transcript PLBe1 providing tolerance towards potentially toxic metals such as Co, Cu, Zn and Cd. Numerous ecologically important genes playing a significant role in many biological processes have been identified using function-based metagenomics approach (Damon et al. 2012; Lehembre et al. 2013; Ren et al. 2018; Ziller et al. 2017).

Sequence analysis of PLCc43

Sequence analysis of PLCc43 revealed that it consisted of 1269 bp with 855 bp open reading frame encoding a putative protein of 284 amino acids with theoretical molecular mass of 31.2 kDa and calculated isoelectric point of 8.99. The sequence showed 51% sequence similarity (65% positives) with Hsp40 protein of *Acanthamoeba castellanii*. Generally, Hsp40 proteins are categorised based on

presence of different combinations of three domains, namely, J domain that helps in stimulating the ATPase activity of Hsp70, long stretch of G/F amino acid-rich region and cysteine-rich region that contains zinc-binding CXXCXGXG motif repeats and a carboxy-terminal substrate-binding domain (Caplan and Douglas 1991; Qiu et al. 2006). Putative PLc43p may display the fundamental structural similarities with type I Hsp40 proteins, i.e. a partial J domain with the conserved tri-peptide HPD (histidine, proline and aspartic acid) motif, a stretch of glycine (G)/phenylalanine (F)-rich region and four zinc finger motif repeats although the final G amino acid in the fourth stretch CXXCXGXG was missing (Fig. 1a). Sequence alignment with other homologous type I Hsp40 proteins showed highly conserved pattern of PLc43 (Fig. 1b).



Fig. 1
a Deduced amino acid sequence of type I Hsp40-like protein encoded by cDNA PLc43: highly conserved tri-peptide HPD (green highlighted) and G/F-rich region (boxes in bold) are shown. Four cysteine-rich domains CXXCXGXG (yellow-highlighted bold letters) are underlined in the polypeptide sequence. Putative phosphorylation sites are highlighted in red. **b** Alignment of sequences of type I Hsp40 proteins. The fully conserved tripeptide HPD is highlighted in yellow; the cysteine residues from cysteine-rich domains CXXCXGXG are coloured in red, respectively. Hsp40 from *Acanthamoeba castellanii* (XP_004349556) and DnaJ homolog members from *Danioreerio* (NP_998658) and *Xenopus tropicalis* (NP_001004807) are shown

Hsp40 proteins are highly conserved proteins that play an important role in protein degradation, translocation, folding and unfolding of protein by stimulating the ATPase activity of another 70-kDa chaperone, Hsp70, which is induced by stress (Caplan and Douglas 1991; Cyr et al. 1994; Qiu et al. 2006). Biochemical and genetic studies have reported the functional redundancy between *E. coli* DnaJ proteins and eukaryotic Hsp40 proteins; hence, the concept of regulation of Hsp70 by Hsp40 via stimulating ATPase activity with the help of J domain as reported in *E. coli* has also been conserved in higher organisms as well (Cyr et al. 1994). Similarly, the tri-peptide motif, HPD, has also been evidenced to play crucial role in functional regulation of Hsp70 as mutations in this conserved motif block the activity of the Hsp70 ATPase (Tsai and Douglas 1996; Wall et al. 1994). Although J domain is considered as an essential feature for functioning of Hsp40 proteins, studies have identified Hsp40 proteins that do not need J domain to perform all their functions (Hageman et al. 2010; Sahi et al. 2010). This further suggests that Hsp40 proteins can perform J domain-independent functions and not all the functions of Hsp40 proteins are Hsp70 dependent. For instance, DnaJA1, a class I Hsp40 protein in mammals, which acts as host factor for influenza A virus replication, could associate itself with the PA and PB2 subunits of RNA polymerase by C-terminal substrate-binding domain, which lacks J domain (Cao et al. 2014). Further, cysteine-rich zinc finger domains of polypeptide encoded by cDNA PLc43 possess same sequence pattern as in type I Hsp40 protein of *S. cerevisiae* Ydj1 (Caplan and Douglas 1991). When analysing the structure of Hsp40 proteins, Banecki et al. (1996) reported that the four cysteine-rich zinc finger domains combine by pairs, each of the pairs binding to one unit of Zn²⁺ ion, hence allowing the Hsp40 proteins to complex two Zn²⁺ ions. Zinc finger domains known to prevent the aggregation of denatured proteins, is also a part of peptide-binding domain, which is responsible for chaperone function of Hsp40 proteins (Banecki et al. 1996; Szabo et al. 1996). The fourth domain found in the carboxyl-terminal part of the protein was less conserved and plays a significant role in substrate binding (Banecki et al. 1996). Lu and Cyr (1998) reported that the mutant Ydj1¹⁷⁹⁻³⁸⁴ protein with completely lacking J domain, G/F region and a large part of cysteine rich zinc finger region, was able to

suppress the aggregation of rhodanese in vitro thus arguing that some functions rely on the C-terminal substrate-binding fragment only. Similarly, Sahi et al. (2013) advocates that C-terminal region of Hsp40 Class I proteins is the most evolved region of Hsp40 proteins, which results into a more diverse and specific Hsp40 class I protein family with binding capability towards different polypeptides.

Polypeptide analysis of PLCc43p coding for Hsp40-like protein also marked the presence of 22 metabolically important phosphorylation sites (Fig. 1). The sequence characterization of cDNA PLCc43 thus evidenced features supporting that the encoded polypeptide may belong to the class I of Hsp40 proteins. The sequence of PLCc43 obtained in this study was deposited at NCBI database under the accession number MK079355.

Relative quantification of PLCc43

Expression levels of PLCc43 increased with increase in Cd concentration and the maximum expression of 68-fold increase was observed at 80 μ M of CdSO₄. Eighteen-fold increase at 40 μ M and 55-fold increases at 60 μ M CdSO₄ was recorded, respectively, in *ycf1^Δ*/PLCc43 cells compared to control cells (Fig. 2). Several studies have reported the over-expression of Hsp40 proteins along with other heat shock proteins and their role in degradation and refolding of proteins in various organisms in response to various accumulated metals (Barque et al. 1996; Cai and Zhang 2000; Fusco et al. 2005).

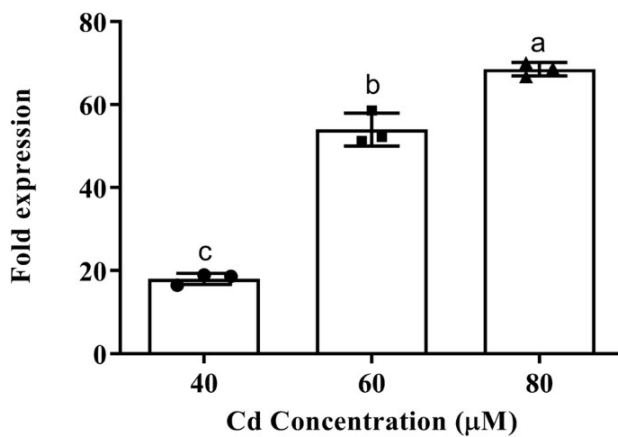


Fig. 2

Fold increase in expression levels of cDNA *ycf1^Δ*/PLCc43 after 48-h incubation in medium supplemented with different concentrations of Cd. Values plotted are referred to the control condition (expression level in transformant *ycf1^Δ*/PLCc43 without metal treatment). Individual data points are shown in each bar. Error bars are \pm SD. Bars sharing a common letter are not significant at $P < 0.05$

Tolerance to metal toxicity and metal content

The role of PLCc43 in conferring metal tolerance when over-expressed within different hypersensitive mutants of *S. cerevisiae* was studied. The heterologous expression of PLCc43 allowed all hypersensitive yeast mutant strains used (*ycf1^Δ*, *cup1^Δ*, *zrc1^Δ* and *cot1^Δ*) to tolerate towards Cd, Cu, Zn and Co toxicity compared to yeast strains carrying empty vector (Fig. 3A, B, C & D). The tolerance potential of PLCc43 towards the four metals used in this study was further authenticated by growing the transformed yeast mutants into metal-amended liquid media (Fig. 4). The transformant *ycf1^Δ*/PLCc43 showed high tolerance towards Cd in the medium compared to wild-type strain BY4741 and *ycf1^Δ* transformed with empty vector. However, with increase in external metal concentration, the growth was significantly reduced. Maximum growth was observed when *ycf1^Δ*/PLCc43 cells were grown in presence of 40 μ M of Cd compared to other concentrations (Fig. 4a). The growth of yeast cells decreased with increase in concentrations of external CuSO₄ in the medium. The *cup1^Δ*/PLCc43 cells showed more growth in presence of CuSO₄ compared to wild-type strain BY4741, the maximum growth being at 150 μ M of CuSO₄ (Fig. 4B). The growth of *zrc1^Δ* cells carrying PLCc43 showed more tolerance to Zn compared to wild-type strain as well as *zrc1^Δ* cells carrying empty vector. The growth of yeast cells decreased significantly with increase in concentrations and the maximum growth was observed in *zrc1^Δ*/PLCc43 cells at 10 mM ZnSO₄ (Fig. 4c). In the case of Co, over-expression of PLCc43 in hypersensitive yeast mutant *cot1^Δ* provided more tolerance against Co toxicity compared to the wild-type strain BY4741 and the *cot1^Δ* cells transformed with empty vector. *cot1^Δ* cells transformed with PLCc43 showed maximum growth at 2 mM of CoCl₂ compared to other concentrations (Fig. 4d). Significant variation among the treatments of yeast strains and concentrations of metals along with their interaction effect was observed when analysed by ANOVA (Table 2).

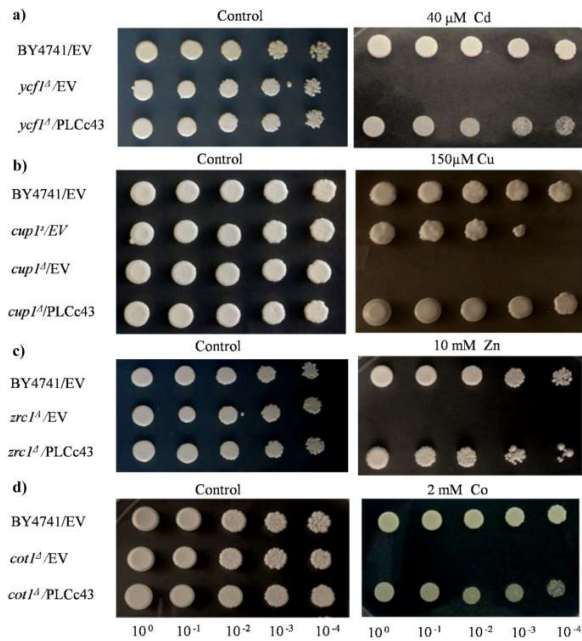


Fig. 3

Drop assay of metal-sensitive mutant strains *ycf1 Δ* , *cup1 Δ* , *zrc1 Δ* and *cot1 Δ* expressing PLcC43 at various concentrations **a** Cd 40 μ M, **b** Cu 150 μ M, **c** Zn 10 mM and **d** Co 2 mM. Wild strain BY4741/EV, *cup1 Δ* /EV serves as positive controls and *ycf1 Δ* /EV, *cup1 Δ* /EV, *zrc1 Δ* /EV and *cot1 Δ* /EV as negative controls, respectively. EV stands for empty vector

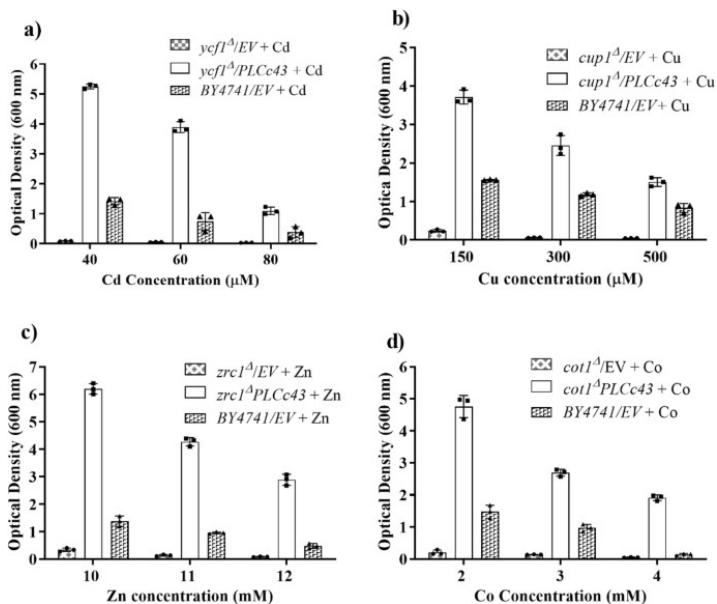


Fig. 4

Growth assay of metal-sensitive mutant strains *ycf1 Δ* , *cup1 Δ* , *zrc1 Δ* and *cot1 Δ* expressing cDNA PLcC43 in SD-Ura medium supplemented with **a** Cd 40–80 μ M, **b** Cu 150–500 μ M, **c** Zn 10–12 mM, **d** Co 2–4 mM. Wild-type strain BY4741 and the corresponding mutants transformed with empty vector (EV) are shown as controls. Individual data points are shown in each bar. Error bars are \pm SD

Table 2 The factorial ANOVA results indicating the effects of different concentrations of metals on cell growth and metal accumulation by different yeast strains. The *P* values < 0.001 are highly significant

Variables	Yeast strains (YS)	Metal concentrations (MC)	YS x MC
Cell growth (O.D)	df 2, 18	2, 18	4, 18
In presence of Cd	< 0.001	< 0.001	< 0.001
In presence of Cu	< 0.001	< 0.001	< 0.001
In presence of Zn	< 0.001	< 0.001	< 0.001
In presence of Co	< 0.001	< 0.001	< 0.001
Metal accumulation			

Variables	Yeast strains (YS)	Metal concentrations (MC) YS x MC	
Cd accumulation	< 0.001	< 0.001	< 0.001
Cu accumulation	< 0.001	< 0.001	< 0.001
Zn accumulation	< 0.001	< 0.001	< 0.001
Co accumulation	< 0.001	< 0.001	< 0.001
Oxidative stress			
	df 2, 42	6, 42	12, 42
	Yeast strains (YS) Days (D)		YS x D
Control	0.141	< 0.001	0.349
0.25 mM H ₂ O ₂	< 0.001	< 0.001	< 0.001
0.5 mM H ₂ O ₂	< 0.001	< 0.001	< 0.001

1. *df* degrees of freedom

Accumulation of metal by respective yeast mutant strain carrying cDNA PLCc43 was further estimated by ICP-MS. The *ycf1^Δ* cells transformed with PLCc43 showed significantly higher accumulation of Cd at different concentrations compared to wild-type BY4741 as well as *ycf1^Δ* cells transformed with empty vector in all concentrations of Cd. The accumulation of Cd levels increased up to 60 μM of CdSO₄ and decreased at 80 μM of CdSO₄, which might be due to decrease in the growth of yeast cells (Fig. 5a). Similarly, the level of Cu accumulation increased with concentrations up to 300 μM of CuSO₄ after which a slight but not significant decrease was observed at 500 μM of Cu concentration (Fig. 5b). In case of Zn and Co metals, maximum metal accumulation was observed for 10 mM of ZnSO₄ and 2 mM of CoCl₂, respectively, after which a sharp decrease was observed for either concentration of metal used (Fig. 5c and d). Overall, metal accumulation levels were significantly higher in yeast mutants expressing PLCc43 than in mutants or wild-type strain transformed with empty vector. A significant variation was observed among the yeast strains, metal concentration, and their interaction in relation to the metal accumulation (Table 2).

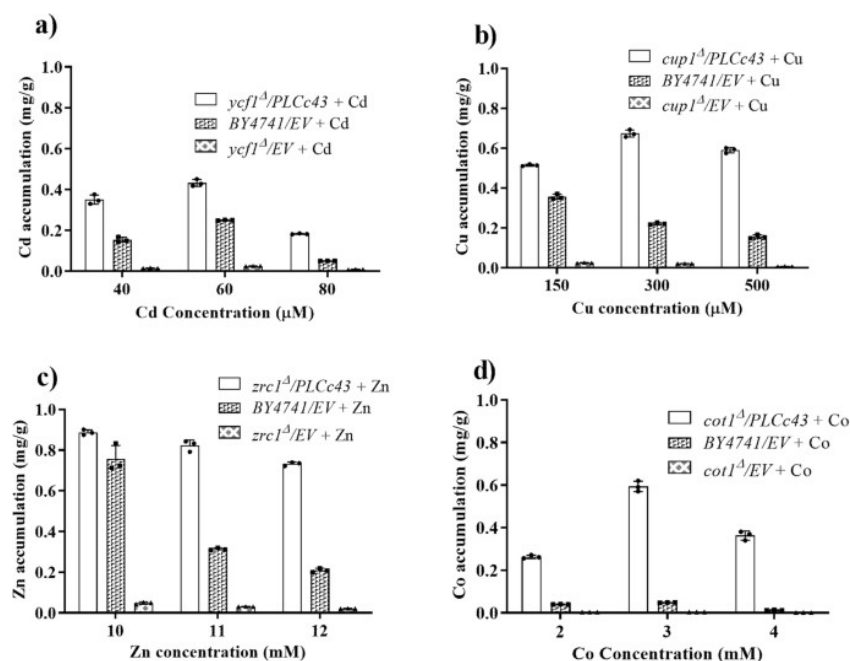


Fig. 5

Accumulation of **a** Cd, **b** Cu, **c** Zn and **d** Co in mutant yeast cells *ycf1^Δ*, *cup1^Δ*, *zrc1^Δ* and *cot1^Δ* carrying cDNA PLCc43 and BY4741/EV. Wild-type BY4741 transformed with only empty vector pFL61 (EV) and mutant strains with EV were used as positive and negative controls, respectively. Individual data points are shown in each bar. Error bars are ± SD

To investigate the combined effect of the yeast Hsp40 of BY4741 and PLCc43, the BY4741 transformed cells were grown in SD-Ura amended with 60 μM of CdSO₄. When compared to BY4741 carrying empty

vector, growth of BY4741 transformed with PLCc43 showed significantly higher growth. This result clearly indicated that PLCc43 is providing additional tolerance to yeast cells compared to control in presence of 60 μM of CdSO_4 (Fig. 6).

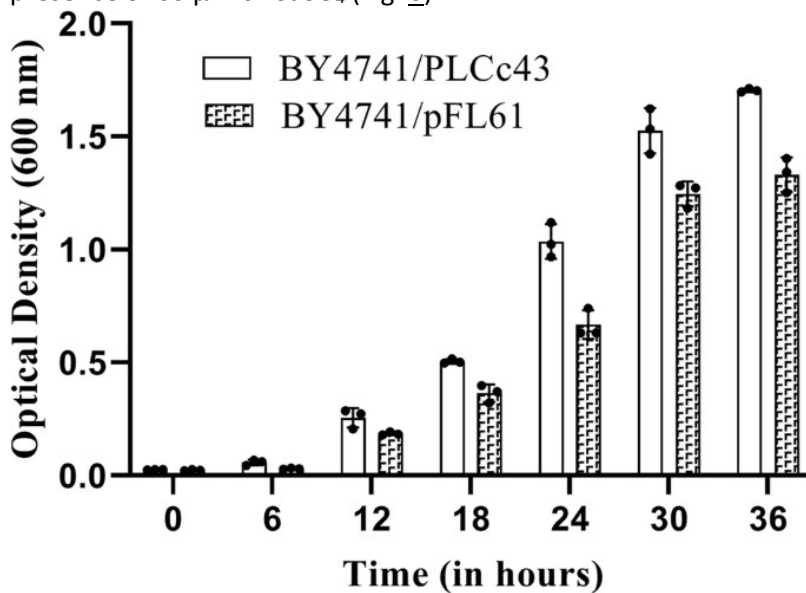


Fig. 6

Growth response of BY4741 transformed with PLCc43 and empty vector pFL61 in SD-Ura medium supplemented with 60 μM of Cd. Individual data points are shown in each bar. Error bars are $\pm\text{SD}$

Nover (1991) reported that the divalent cations of transition metal series such as Cd, Cu, Zn and Hg act as heat stress-like chemical stressors, which induce the production of various stress combating proteins such as Hsp40s. In many plant species, high expression of HSPs especially Hsp40s was observed under a variety of metal stress. Degradation of non-native proteins or proteolytic inactivation of regulatory proteins is a major cellular response of living organisms in metal-contaminated environments. It has been reported that Hsp40 proteins are involved in maintaining the cell homeostasis through translocation, refolding or degradation of aberrant proteins during cellular stress such as metal toxicity (Cyr et al. 1994; Suzuki et al. 2001). It was observed that Hsp40 in association with Hsp70 prevents aggregation of proteins and assist in refolding of non-native proteins under both normal and stressed environments (Frydman 2001; Hartl 1996). In a study, a total of twenty-one cDNAs including Hsp40 were reported in birch *Betula pendula* playing significant role in maintaining the homeostasis and in alleviating the toxic effects of accumulated Cu metal (Keinänen et al. 2007). Similarly, higher expression of Hsp40-like protein encoding gene conferred tolerance to beans when exposed to mercuric chloride (Chai et al. 2000). The mechanism behind this might be direct binding and trafficking, which has also been observed experimentally with copper (Field et al. 2002), or by refolding of denatured proteins due to the stress conditions (Meacham et al. 1999; Shen and Hendershot 2005). The expression of Hsp40 and Hsp70 limits the proteotoxic symptoms of metal ions and helps the detoxification and sequestration of these metal ions by metallothioneins (Haap et al. 2016).

Furthermore, in a study, deletion of Hsp40 protein Mdj1 in the filamentous entomopathogenic fungus *Beauveria bassiana* resulted into sensitivity towards various metals such as Zn, Cu and Fe along with some growth defects such as impaired cell wall integrity, reduced osmo-tolerance and antioxidant activities (Wang et al. 2017). Similarly, DnJ3 protein, an Hsp40 protein in honeybee, *Apis cerana cerana* has regulated the ill effects of various stress factors including metal ions (Zhang et al. 2019). Hence, overall, significant tolerance towards potentially toxic metals used in this study, i.e. Cd, Cu, Zn and Co was shown in model eukaryotic living organism that expressed soil originating cDNA, PLCc43.

Oxidative stress

The potential role of PLCc43 in response to oxidative stress was examined using the superoxide dismutase yeast mutant *sod2^Δ* expressing the cDNA PLCc43. The growth of wild-type BY472, *sod2^Δ* transformed with empty vector and *sod2^Δ*/PLCc43 showed similar growth pattern when grown in absence of H_2O_2 . The *sod2^Δ* cells expressing PLCc43 were able to confer tolerance to 0.25 mM and 0.5 mM of H_2O_2 . The growth of BY4742 and *sod2^Δ*/PLCc43 were comparable at these concentrations and

were significantly higher than the *sod2^Δ* transformed with empty vector (Fig. 7). Significant variation among the yeast strains and the days along with their interaction was observed (Table 2). Oxidative stress is a result of uncontrolled oxidation process, which results in the accumulation of reactive oxygen species (ROS). The generation of ROS because of metal toxicity is very common. Mostly, metals cause inhibition of metabolic processes and are involved in direct electron transfer involving metal cations (Grimsrud et al. 2008). The abiotic stress due to formation of ROS is confronted by various stress-responsive proteins, out of which heat shock proteins play an important role. In a study by Qiu et al. (2006), over-expression of *LeCDJ1* DnaJ gene in tomato not only resulted in improving heat tolerance but also in increasing superoxide dismutase activity, which reduced accumulation of O₂⁻. Similar studies on the effects of HSPs on ROS scavenging proteins and related to heat stress have been investigated. In *Arabidopsis*, over-expression of *RcHSP17.8* improved SOD activity (Jiang et al. 2009). Also, in tobacco, when the HSP *ZmHSP16.9* was over-expressed, it confirmed increase in the levels of peroxidase, catalase and superoxide dismutase activity (Sun et al. 2012). A study by Kim et al. (2008) indicated a possible mechanism of H₂O₂ resistance by HSPs. It was concluded that the increase in resistance against oxidative stress conferred by HSPs was due to the prevention of proteasome activity, which ultimately provided resistance against cell toxicity. Furthermore, these findings illustrate the role of proteins like type I Hsp40 in combating the metal toxicity and advocate the significance of functional approach of functional metagenomics in mining, the hidden treasures of soil for remediation of various contaminated sites.

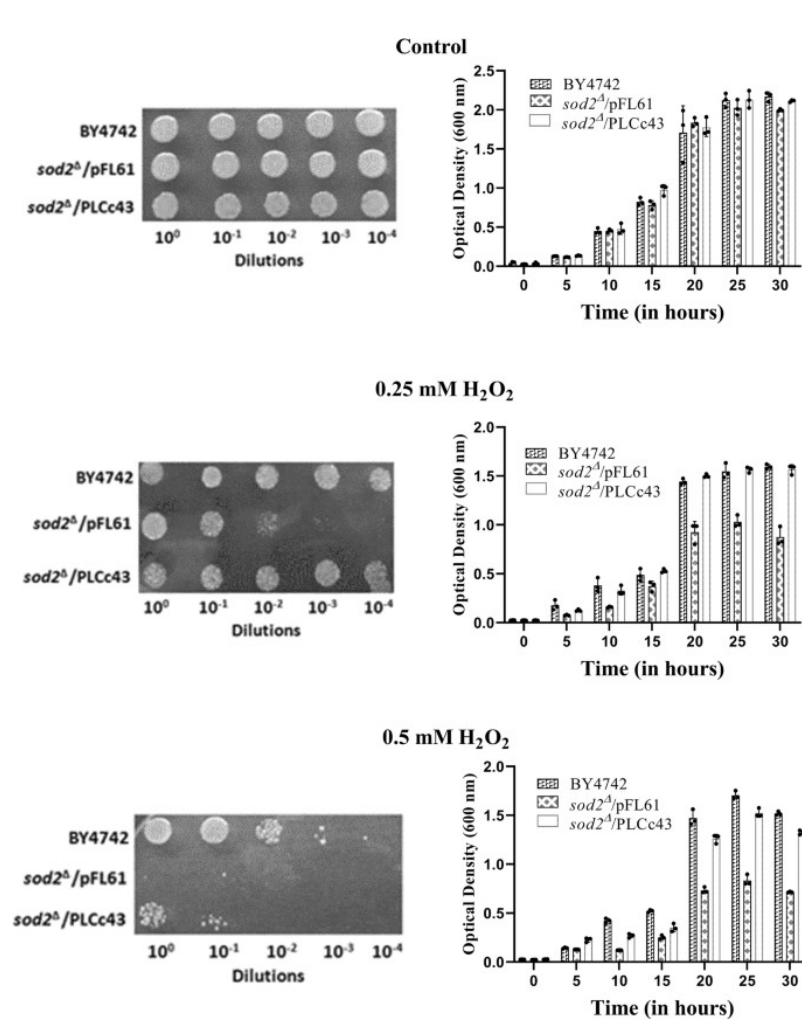


Fig. 7

Drop assay and growth response of yeast mutant *sod2^Δ* strain expressing cDNA PLCc43 in presence of different concentrations of H₂O₂. Wild-type strain BY4742 and *sod2^Δ* transformed with empty were used as controls. Individual data points are shown in each bar. Error bars are ±SD

Conclusions

The potential of cDNA PLCc43 to tolerate toxic concentrations of toxic metals was quite evident from the experimental observations. The translated protein had the characteristics of a type I Hsp40-like protein. Functional complementation of *S. cerevisiae* using PLCc43 confers metal tolerance to the

mutant strains of yeast, which categorize PLCc43 as potential member of functional repertoire participating in metal tolerance. Hsp40-like proteins might play an important role not only in targeted specific hosts as previously evidenced in numerous studies, but also in still uncharacterized living organisms that prevail in environments such as metal contaminated soils. This study described Hsp40-like protein as a protein originating from metal-polluted soil that may help living organisms to maintain homeostasis between flora and fauna of the soil environment through tolerating high concentrations of metals. Thus, function-based metagenomics approach can reveal diversity of functions related to stress tolerance, when applied to complex environments like polluted soil.

Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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RM, LFT, DM and MSR conceived the idea of the experiments and provided technical guidance. RY, BT and AM had a major contribution to overall preparation and carrying out of the research. MSR, DM, BT and AM revised and edited the manuscript.