

# Proteomic Analysis of Diversified Extremophilic Strains of *Pseudomonas* in the Presence of Cadmium

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**Abstract** Environmental contamination caused by heavy metals, such as cadmium (Cd), is a serious and growing concern. Cd is a highly toxic and carcinogenic metal for humans, animals and plants and is a widespread pollutant with a long biological life. The presence and persistence of growth promoting metal-resistant microbial community in the soil system facilitates remediation of Cd. This study deals with proteomic characterization of cadmium-resistant diversified isolates of *Pseudomonas* spp., i.e., psychrotolerant *P. putida* (isolate SB32) and alkalophilic *P. monteilli* (isolate SB35). Further, two-dimensional gel electrophoresis (2DE) revealed 24 proteins in SB32 and 21 proteins in SB35, differentially expressed after Cd exposure. On the basis of their increased expression, two protein spots in SB32 and one in SB35 were selected for further analysis. With MS/MS spectra produced by LC–MS analysis, spots were identified through NCBI EST database searching with Mascot MS/MS Ion Search Engines (Matrix Sciences). Spots from cadmium-treated *P. putida* SB32 appear to be ATP-dependent Clp protease subunit and 20S proteasome, A and B subunits, while spot from cadmium-treated *P. monteilli* SB35 appears to be nitroreductase. Protein identification supports that interplay between protein folding and hydrolysis may be a central mechanism for proper cell function survival in the presence of cadmium.

**Keywords** Cadmium · *Pseudomonas* · Proteomics · Psychrotolerant · Alkalophilic

## Introduction

Contamination of soil and groundwater with heavy metals poses a major environmental and human health problem because they are very toxic and have intrinsically persistent nature. In particular, cadmium is very toxic and probably carcinogenic at low concentrations. Cadmium is released into the environment through various industrial discharges of metal plating, Ni–Cd batteries, pigments and plastics, cigarette smoking, smelting operations, incineration of

municipal waste, sewage sludge, chemical fertilizers and polluted groundwater [22, 29]. Cd<sup>2+</sup>, a designated human carcinogen, is one of well-known toxic heavy metal ions, and its toxicity has been extensively studied [1, 8, 28, 32, 37]. It has been proposed that Cd<sup>2+</sup> ions might displace Zn<sup>2+</sup> and Fe<sup>2+</sup> in proteins [34], resulting in their inactivation and in the release of free iron, which might generate highly reactive hydroxyl radicals (OH<sup>•</sup>) [11]. In support of this hypothesis, a major effect of cadmium is oxidative stress [2], particularly lipid peroxidation [33].

Cadmium has no biological function, but hampers the normal functions of life when it accumulates within the living system above the toxicity level. All prokaryotes and eukaryotes have developed mechanisms to prevent excessive accumulation of Cd in the cells. The relatively low intracellular Cd concentration is maintained through the regulation of sequestration or efflux. Microorganisms are well reported to both biotransform [14] and adsorb Cd on

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their cell surface (Watcharamusika et al. 2008). Microbial resistance to Cd is usually based on energy-dependent efflux mechanisms [25, 30]. *Pseudomonas putida* is a ubiquitous saprophytic bacterial species that is known not only for the ability to degrade various biogenic and xenobiotic pollutants [35] but also for the tolerance to heavy metal ions [15, 21, 26, 36]. They have been extensively studied for their well-adapted metal resistance properties [31].

The analysis of plant protein profiles under different treatments is a powerful approach to identify stress-responsive proteins and to elucidate the crucial pathways involved in stress resistance [39]. The combination of two-dimensional differential gel electrophoresis (2D-DIGE) and MALDI-TOF MS/MS analysis allows rapid and accurate separation and identification of hundreds of proteins. The DNA genomic sequence has been considered by some as a basic framework for further investigations into mechanisms of gene action [38]. Because the amount of protein is often not predictable from the amount of mRNA produced in the cell, and post-translational modifications such as phosphorylation and glycosylation can be vital for cellular functions, the study of proteins is crucial to judiciously examine their levels and activities at a given time in a cell, or in response to various environmental conditions [40]. It is inevitable that the study of the cellular responses to different stresses at the proteomic level will inform us what gene products are actually expressed and their changes.

The overall goal of this study was to identify proteins that were expressed in the presence of cadmium by comparing protein expression patterns of diversified extremophilic strains of *Pseudomonas* spp. in the presence and absence of cadmium, respectively.

## Materials and Methods

### Strains and Culture Conditions

Psychrotolerant strain SB32 and alkalophilic strain SB35 (pH 9.0), originally isolated from soil samples from Semera mines, Palamau, Jharkhand, were obtained from departmental culture collection. Both the strains were maintained on nutrient agar medium supplemented with 1 mM CdCl<sub>2</sub> concentration with their respective pH and temperature conditions. The plates were incubated at 30 °C.

### Protein Extraction

Proteins were extracted from SB32 and SB35 strains. Single colony of bacterial culture was inoculated in 500 ml

of broth and grown overnight in the presence and/or absence of cadmium, respectively. Culture was harvested at 10,000 rpm for 10 min, and pellet was washed twice with normal saline solution. Pellet was then dissolved in 4 ml of 0.1 M phosphate buffer saline (chilled) and sonicated for 3 min in ice. One mM PMSF (100 µL) was added to the sonicated pellet. Cell suspension was centrifuged for 45 min at 10,000 rpm.

### Two-Dimensional (2D) Gel Electrophoresis

For first dimension electrophoresis (isoelectric focusing, IEF), a 17-cm-long immobilized pH gradient, 3–10 Ready Strip IPG strips (Bio-Rad, Hercules, CA, USA) were passively rehydrated for 18 h at 50 V and 20 °C with 100 µg of protein extract suspended in IEF buffer. IEF was done using a Protean IEF cell (Bio-Rad) at a current limit of 50 µA/strip at 20 °C with the following steps: active rehydration at 250 V for 12 h; 250 V (linear) for 15 min; 10 kV (linear) for 3 h; and 10 kV (rapid) until a total 80 kVh for a combined total of approximately 92 kVh. After IEF separation, each focused IPG strip was equilibrated by soaking, with mild stirring, in 6 ml of equilibration base buffer [8 M urea, 2 % (w/v) sodium dodecyl sulfate (SDS), 50 mM Tris-HCl (pH 8.8), 20 % (v/v) glycerol, 1 % (w/v) DTT] for 20 min. Second dimension electrophoresis (SDS-PAGE) was performed in 12.0 % gradient SDS-polyacrylamide gel. For molecular weight determination, precision protein standard markers (Bio-Rad, Hercules, CA, USA) were used. Proteins were visualized by gel staining with silver nitrate. All 2D experiments were carried out in triplicate.

### Proteomic Profiling and Difference in Gel Electrophoresis

Preparative 2D gels of samples intended for mass spectrometry and loaded with 1.0 mg protein were stained overnight with Bio-Safe Coomassie Brilliant Blue R250 (0.1 % solution) (Bio-Rad Laboratories, Hercules, CA) and destained with water. Spots of interest were excised from the gel; the gel pieces were washed with water and destained with 50 mM NH<sub>4</sub>HCO<sub>3</sub>/50 % acetonitrile (ACN). Proteins were reduced by incubation with 10 mM dithiothreitol in 100 mM NH<sub>4</sub>HCO<sub>3</sub> at 50 °C for 30 min and then alkylated by incubation with 55 mM iodoacetamide in 100 mM NH<sub>4</sub>HCO<sub>3</sub> for 30 min in the dark. After being dehydrated with 100 % ACN and air-dried, the gel pieces were rehydrated for 10 min in a trypsin solution (Promega Corporation, Madison, Wisconsin) in a ratio of approximately 1:10 (w/w) of trypsin/protein. Fifty microliters of 50 mM NH<sub>4</sub>HCO<sub>3</sub> (pH 8.0) was added to each gel piece, and the proteins were digested at 37 °C for 18 h. The

peptides were extracted by vortexing and then concentrated to 10  $\mu$ L in a Speed-Vac. The samples were cleaned using a C-18 ZipTip system (Millipore) and eluted with 5  $\mu$ L of 50 % ACN. One microliter of 1 % formic acid was added to the eluate to protonate peptides. Mass spectrometry was performed using nano-spray injection as the sample delivery method. The 6  $\mu$ L of the sample was injected into nano-LCMS (ThermoFinnigan LCQ Deca). The parental and fragment mass error were 0.2 and 0.1 Da, respectively, and trypsin was set as the digestion enzyme with one missed cleavage allowed. Carbamidomethylation and methionine oxidation were set as the fixed and variable post-translational modifications, respectively.

MS/MS spectra were used to search against the NCBI non-redundant protein database using MS/MS Ion Search Engine, a computer software program conducting protein identification based on matching the MS/MS spectra of a protein with a protein or DNA sequence database ([http://www.matrixscience.com/search\\_form\\_select.html](http://www.matrixscience.com/search_form_select.html)). The searches were restricted to the bacterial taxonomy. The significance of the protein match with the ion score was based on the Mouse scoring algorithm. The ion score was calculated as  $-10 \times \text{LOG}_{10}(P)$ , where  $P$  is the absolute probability that the observed match is a random event. Thus, a relatively small  $P$  value means that the match of identified protein and the MS/MS spectra is not a random event. A significant specific match increases the ion score, so a high score means highly significant matching (MASCOT Help; [http://www.matrixscience.com/help/scoring\\_help.html#PBM](http://www.matrixscience.com/help/scoring_help.html#PBM)). A single protein having a higher score than the minimum score for the significance level ( $p < 0.05$ ) was judged as a significant match. In each MASCOT search output result, the minimum score for significance level was provided, based on the absolute probability and the size of the sequence database being searched. Identified proteins were aligned to sequences in the Swiss-Prot database using the NCBI BLAST (blastp) tool to confirm the assigned proteins.

## Results and Discussion

### Identification of the cadmium-induced proteins in *P. putida* SB32 and *P. monteylli* SB35

Investigation into the global stress response generated by cadmium on the bacterial strains identified potentially important proteins that involved in cadmium detoxification and metabolism. The protein extract prepared from the cells in the mid-exponential phase were analyzed by 2D gel electrophoresis and visualized by CBB staining. Figures 1 and 2 show the profile of the two-dimensional gel electrophoresis of the extract prepared from non-treated cells

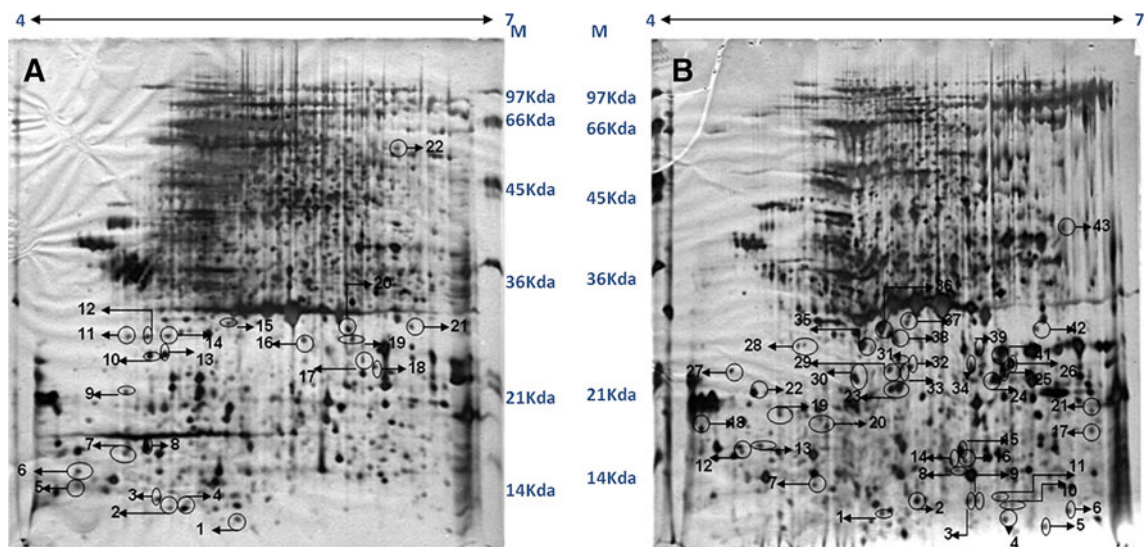
(1a, 2a) and cadmium-treated cells (1b, 2b) of *P. putida* SB32 and *P. monteylli* SB35, respectively. Interestingly, some proteins were highly expressed in the cadmium-treated cells. In order to characterize these cadmium-induced proteins, the overexpressed spots (nos. 15, 24 from *P. putida* SB32 and 5 from *P. monteylli* SB35) in the absence and presence of cadmium were excised from the gel and identified (Fig. 3a–f).

Spot no. 15 appears to be ATP-dependent Clp protease subunit. Energy-dependent proteases have been identified in all organisms analyzed and, although not universally distributed, include the Lon, FtsH (HflB), ClpAP, ClpXP, HslUV (ClpYQ) and proteasome complexes [6, 23]. Several of these proteases are stress or heat shock inducible including ClpXP [10, 18], Lon [9, 12, 27], FtsH [12, 13], HslUV [4], and subunits of the proteasome (17). The Clp protease system was first identified in *Escherichia coli* and consists of two non-homologous components: a proteolytic subunit known as ClpP and an ATPase subunit (ClpA). The ATPase subunit is required for binding and unfolding of the protein substrates and then transferring it to the ClpP protease for degradation [28]. Two distinct ATPase subunits in *E. coli*, ClpA (83 kDa) and ClpX (46 kDa), are known to associate transiently with ClpP (21.5 kDa). Due to their difference in substrate specificity, the subsequent proteases degrade different types of proteins [16].

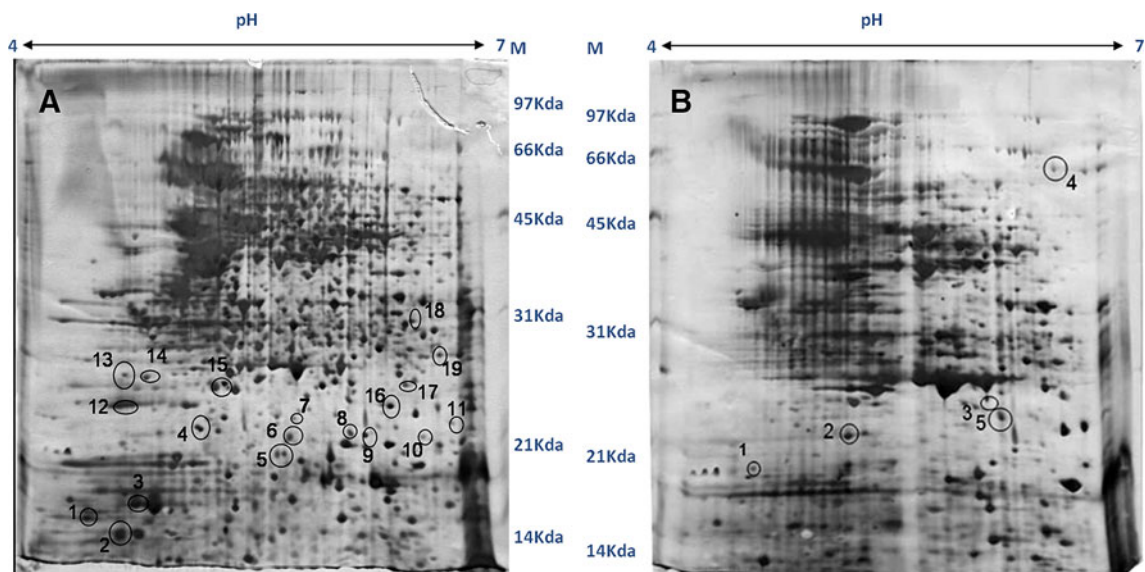
The up-regulation of ATP-dependent C1 protease subunit reveals that this protein is an induced stress protein that may be involved in bacterial acclimation to cadmium. Penaud et al. [27] reported the induction of ATP-dependent C1 protease proteolytic subunit during acid adaptation in *Lactobacillus bulgaricus*.

Spot no. 24 appears to be proteasome, A and B subunits. The 20S proteasome, the proteolytic core in the 26 proteasome, is known to be involved in the degradation of proteins modified by oxidation. The 20S proteasome homologues were discovered within the genome of Actinomycetes [24], a lineage of high-GC Gram-positive bacteria, including *Streptomyces coelicolor* A3(2) [6, 24], *Mycobacterium leprae* and *Mycobacterium smegmatis* [17], *Mycobacterium tuberculosis* [5] and *Rhodococcus erythropolis* [41]. No known function could be attributed to the 20S proteasome in bacteria so far, except for the *M. tuberculosis* proteasome that has been found to protect the organism against oxidative or nitrosative stress [5].

Amino acids of proteins can be modified by the oxygen radicals or other activated oxygen produced as by-products of cellular metabolism or from the response to abiotic or biotic stress. Subsequently, oxidative modified proteins can undergo chemical fragmentation or form aggregates due to covalent cross-linking reactions and increased surface hydrophobic. Abnormal proteins that accumulate after exposure to stresses such as heavy metals stress, heat shock



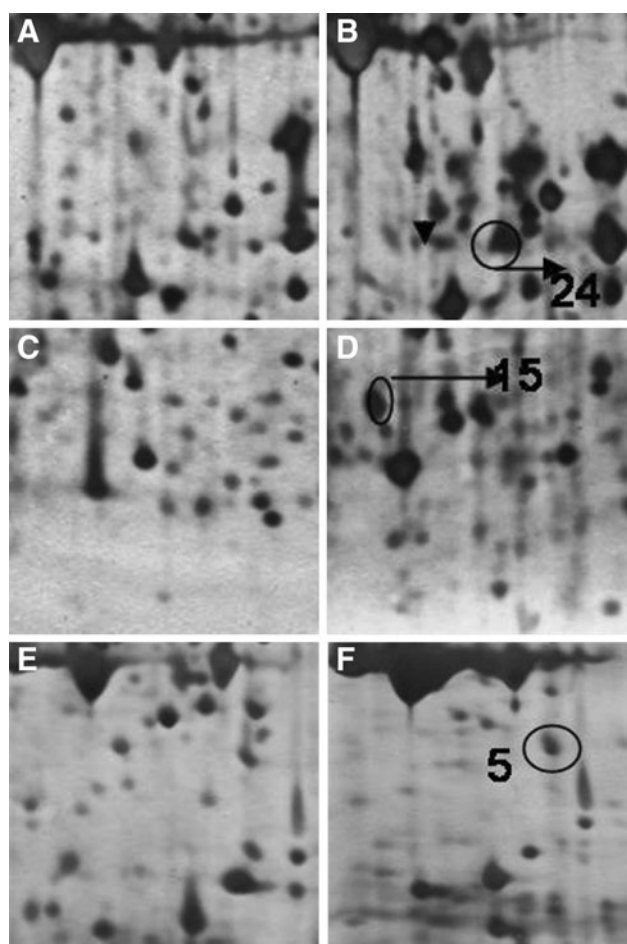
**Fig. 1** Two-dimensional gel depicting total bacterial (*P. putida* SB32) protein expressed in the absence (a) and presence of cadmium (b), wherein pH range was 4–7, and *M* represent molecular weight markers in kDa



**Fig. 2** Two-dimensional gel depicting total bacterial (*P. montelli* SB35) protein expressed in the absence (a) and presence of cadmium (b), respectively, wherein pH range was 4–7, and *M* represent molecular weight markers in kDa

or oxidative damage are specifically targeted for hydrolysis. The up-regulation of 20S proteasome in this study may be involved in the defence against oxidative stress, which is consistent with the up-regulation of several antioxidant proteins in cadmium stress. The effects of cadmium (Cd) on cellular proteolytic responses were investigated in the roots and leaves of tomato (*Solanum lycopersicum* L., var Ibiza) plants. The up-regulation of the 20S proteasome gene expression and proteolytic activity argues in favor of the involvement of the 20S proteasome in the degradation of oxidized proteins in plants [7].

Spot no. 5 appears to be nitroreductase. Nitroreductase reduces nitro groups on explosives and other nitroaromatic compounds and removes nitrogen from rings structure. The nitroreductase can utilize either NADH or NADPH as a source of reducing equivalents and can reduce a variety of nitroaromatic compounds including nitrofurans and nitrobenzenes as well as quinones. Nitroreductase have been reported in *Clostridium acetobutylicum* ATCC 824 [19], *E. coli*, *Enterobacter cloacae* [3] and in some plants [34]. In a study performed by Kwak et al., [20], chromate-reducing activities of *E. coli* DH5 and *Vibrio harveyi*



**Fig. 3** Depiction of relative abundance of the protein spot (circled) in the presence of cadmium, wherein **a** untreated *P. putida* SB32, **b** Cd-treated *P. putida* SB32, **c** untreated *P. putida* SB32, **d** Cd-treated *P. putida* SB32, **e** untreated *P. monteilli* SB35, **f** Cd-treated *P. monteilli* SB35

KCTC 2720 nitroreductases were determined. Similarly, the chromate reductase purified from *Pseudomonas ambigua* was found to be homologous with several nitroreductases [20].

## Conclusion

Heavy metal-resistant bacteria containing different detoxification mechanisms have been previously shown to alleviate the inhibitory effects of various heavy metals on plant growth. However, how the bacteria react to the heavy metals stresses has not been previously characterized. In this work, an examination of the proteome of both the isolates *P. putida* SB32 and *P. monteilli* SB35 revealed systematic cadmium resistance responses of these bacteria including general stress adaptation and anti-oxidative stress, which may be useful in the development of bioremediation protocols.

Based on the elevated production of ATP-dependent Clp protease and 20S proteasome, A and B subunits, it appears that cells growing in the presence of cadmium have an increased demand for the recognition and elimination of abnormal proteins accumulate after exposure to oxidative damage. Nitroreductase reduces nitro groups on explosives and nitroaromatic compounds and removes nitrogen from ring structures, but the role of nitroreductase in the presence of heavy metal has not been deciphered till now. To the best of our knowledge, the occurrence of ATP-dependent Clp protease, 20S proteasome, A and B subunits, and nitroreductase has never been reported in bacteria in the presence of cadmium so far and might constitute a link between these proteins and cadmium resistance.

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**Conflict of interest** None.

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