Proteomics and bioinformatics analysis reveal potential roles of cadmium-binding proteins in cadmium tolerance and accumulation of *Enterobacter cloacae*

Kitipong Chuanboon [1, 2] , **Piyada Na Nakorn** [3] , **Supitcha Pannengpetch** [2] , **Vishuda Laengsri** [2] , **Pornlada Nuchnoi** [2] , **Chartchalerm Isarankura-Na-Ayudhya** [3] , **Patcharee Isarankura-Na-Ayudhya** [Corresp. 1]

[1] Department of Medical Technology, Faculty of Allied Health Science, Thammasat University, Pathumthani, Thailand

[2] Center for Research and Innovation, Faculty of Medical Technology, Mahidol University, Bangkok, Thailand

[3] Department of Clinical Microbiology and Applied Technology, Faculty of Medical Technology, Mahidol University, Bangkok, Thailand

Corresponding Author: Patcharee Isarankura-Na-Ayudhya
Email address: pujonoy@hotmail.com

Background

Enterobacter cloacae (EC) is a Gram negative bacterium that has extensively been utilized in biotechnological and environmental science applications, possibly resulted from the high capability to adapt itself and survive in hazardous conditions. Searching for the EC from agricultural and industrial areas that possesses high capability to tolerate and/or accumulate cadmium ions has been conducted in this study. Plausible mechanisms of cellular adaptations in the presence of toxic cadmium have also been proposed.

Methods

Nine strains of EC were isolated and subsequently identified by biochemical characterization and MALDI-Biotyper. Minimum inhibitory concentrations (MICs) against cadmium, zinc and copper ions were determined by agar dilution method. Growth tolerance against cadmium ions was spectrophotometrically monitored at 600 nm. Cadmium accumulation at both cellular and protein levels was investigated using atomic absorption spectrophotometer. Proteomics analysis by 2D-DIGE in conjunction with protein identification by QTOF-LC-MS/MS was used to study differentially expressed proteins between the tolerant and intolerant strains as consequences of cadmium exposure. Expression of such proteins was confirmed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Bioinformatics tools were applied to propose the functional roles of cadmium-binding protein and its association in cadmium tolerance mechanisms.

Results

The cadmium-tolerant strain (EC01) and intolerant strain (EC07) with the MICs of 1.6 and 0.4 mM, respectively, were isolated. The whole cell lysate of EC01 exhibited approximately 2 fold higher in cadmium binding capability than those of the EC07 and ATCC 13047, possibly by the expression of Cd-binding proteins. Our proteomics analysis revealed the higher expression of DUF326-like domain (a high cysteine-rich protein) of upto 220 fold in the EC01 than that of the EC07. Confirmation of the transcription level of this gene by qRT-PCR revealed 14 fold induction in the EC01. Regulation of the DUF326-like domain in EC01 was more pronounced to mediate rapid cadmium accumulation (in 6 hours) and tolerance than the other resistance mechanisms found in the ATCC 13047 and the EC07 strains. The only one major responsive protein against toxic cadmium found in these 3 strains belonged to an

antioxidative enzyme, namely catalase. While, the unique proteins found in the ATCC 13047 and EC07 were identified as two groups: i) ATP synthase subunit alpha, putative hydrolase and superoxide dismutase and ii) OmpX, protein YciF, OmpC porin, DNA protection during starvation protein, and TrpR binding protein WrbA, respectively.

Conclusion

All these findings gain insights not only into the molecular mechanisms of cadmium tolerance in EC but also open up a high feasibility to apply the newly discovered DUF326-like domain as cadmium biosorbents for environmental remediation in the future.

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6 Kitipong Chuanboon[1,2], Piyada Na Nakorn[3], Supitcha Pannengpetch[2], Vishuda Laengsri[2],
7 Pornlada Nuchnoi[2], Chartchalerm Isarankura-Na-Ayudhya[3], Patcharee Isarankura-Na-
8 Ayudhya[1,*]
9
10 [1] Department of Medical Technology, Faculty of Allied Health Science, Thammasat University,
11 Pathumthani 12120, Thailand
12 [2] Center for Research and Innovation, Faculty of Medical Technology, Mahidol University,
13 Bangkok 10700, Thailand
14 [3] Department of Clinical Microbiology and Applied Technology, Faculty of Medical
15 Technology, Mahidol University, Bangkok 10700, Thailand
16
17 Corresponding Author:
18 Patcharee Isarankura-Na-Ayudhya[1]
19 99 Moo 18 Paholyothin Road, Klong Nueng, Klong Luang, Pathumthani 12121 Thailand
20 Email address: pujonoy@hotmail.com

22 Abstract

23 **Background**
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25 biotechnological and environmental science applications, possibly resulted from the high
26 capability to adapt itself and survive in hazardous conditions. Searching for the EC from
27 agricultural and industrial areas that possesses high capability to tolerate and/or accumulate
28 cadmium ions has been conducted in this study. Plausible mechanisms of cellular adaptations in
29 the presence of toxic cadmium have also been proposed.
30 **Methods**
31 Nine strains of EC were isolated and subsequently identified by biochemical characterization and
32 MALDI-Biotyper. Minimum inhibitory concentrations (MICs) against cadmium, zinc and copper
33 ions were determined by agar dilution method. Growth tolerance against cadmium ions was
34 spectrophotometrically monitored at 600 nm. Cadmium accumulation at both cellular and protein
35 levels was investigated using atomic absorption spectrophotometer. Proteomics analysis by 2D-
36 DIGE in conjunction with protein identification by QTOF-LC-MS/MS was used to study
37 differentially expressed proteins between the tolerant and intolerant strains as consequences of
38 cadmium exposure. Expression of such proteins was confirmed by quantitative reverse
39 transcription-polymerase chain reaction (qRT-PCR). Bioinformatics tools were applied to

40   propose the functional roles of cadmium-binding protein and its association in cadmium
41   tolerance mechanisms.
42   **Results**
43   The cadmium-tolerant strain (EC01) and intolerant strain (EC07) with the MICs of 1.6 and 0.4
44   mM, respectively, were isolated. The whole cell lysate of EC01 exhibited approximately 2 fold
45   higher in cadmium binding capability than those of the EC07 and ATCC 13047, possibly by the
46   expression of Cd-binding proteins. Our proteomics analysis revealed the higher expression of
47   DUF326-like domain (a high cysteine-rich protein) of upto 220 fold in the EC01 than that of the
48   EC07. Confirmation of the transcription level of this gene by qRT-PCR revealed 14 fold
49   induction in the EC01. Regulation of the DUF326-like domain in EC01 was more pronounced to
50   mediate rapid cadmium accumulation (in 6 hours) and tolerance than the other resistance
51   mechanisms found in the ATCC 13047 and the EC07 strains. The only one major responsive
52   protein against toxic cadmium found in these 3 strains belonged to an antioxidative enzyme,
53   namely catalase. While, the unique proteins found in the ATCC 13047 and EC07 were identified
54   as two groups: i) ATP synthase subunit alpha, putative hydrolase and superoxide dismutase and
55   ii) OmpX, protein YciF, OmpC porin, DNA protection during starvation protein, and TrpR
56   binding protein WrbA, respectively.
57   **Conclusion**
58   All these findings gain insights not only into the molecular mechanisms of cadmium tolerance in
59   EC but also open up a high feasibility to apply the newly discovered DUF326-like domain as
60   cadmium biosorbents for environmental remediation in the future.
61
62   # Introduction
63   Environmental contamination by toxic heavy metals has increased dramatically due to the
64   consequence of global industrialization. Cadmium is one kind of heavy metals that exerts its
65   detrimental toxicity to human, animals, plants and microorganisms (Jaiswal et al. 2018). Cellular
66   adaptation of bacteria to survive under cadmium exposure can be accounted as follows:
67   reduction of cadmium uptake, cadmium sequestration or complexation, cadmium efflux, and
68   secretion of extracellular polysaccharides (Nies 1992; Nies 1999; Xu et al. 2017). Among the
69   other environmental bacteria, *Enterobacter cloacae* (EC), a Gram-negative bacillus belonging to
70   the family of *Enterobacteriaceae*, can localize in gut and in various environmental conditions.
71   The EC has been reported to tolerate against many kinds of heavy metals and antibiotics. For
72   instances, the standard strain of EC (ATCC 13047) has been found to carry many heavy-metal
73   resistance genes belonging to 7 operons (*sil*, *ars*, *mer* and *cop*) (Ren et al. 2010). Moreover,
74   overexpression of extracellular biological components (e.g. exopolysaccharide) has been found
75   to play major roles in chromate resistance by preventing the permeability of chromate into the
76   cells (Iyer et al. 2004; Yang et al. 2007). Some strain of EC that confered lead resistance has
77   been discovered to have increased production of exopolysaccharide (Naik et al. 2012).
78   Accumulation of nickel and vanadium as well as the association between vanadium and
79   multidrug resistance has been reported (Hernandez et al. 1998). Co-existence of heavy metal

80   tolerance (*sil* operon involved in acquired silver resistance) and antibiotic resistance (blaCTX-M
81   and blaKPC acquired extended-spectrum cephalosporin and carbapenem resistance) genes has
82   recently been documented in EC complex (Andrade et al. 2018). With respect to the high
83   tolerance and/or accumulation of metal ions, the EC has been used in many biotechnological and
84   environmental science applications such as biohydrogen production (Khanna et al. 2011), heavy
85   metal bioremediation (Rahman et al. 2015; Xu et al. 2017), nanoparticles-based
86   biotransformation, biocatalyst for decolourization of azo dye (Prasad & Aikat 2014). Redox
87   transformation of toxic selenium oxyanions by the twin arginine translocation system has been
88   discovered (Ma et al. 2007). However, it is noteworthy that the resistance mechanisms against
89   toxic cadmium ions in *Enterobacter cloacae* remain unclear.
90   Domains of unknown/uncharacterized function (DUFs) have been recognized as a series of
91   uncharacterized protein families, which can be accessed through the international database
92   namely Pfam database (available at https://pfam.xfam.org/) (Bateman et al. 2010; Finn et al.
93   2008). Currently, the number of protein families has dramatically been reported of almost upto
94   18,000 entries (El-Gebali et al. 2019). Identification of the DUFs that occur within the protein
95   gains a better understanding on the function of proteins found in nature. The DUFs seem to be
96   essential not only for the normal cellular function (Mashruwala & Boyd 2018) but they also
97   become more important particularly under certain conditions such as stress responses, biofilm
98   formation, pathogenesis, intracellular metal homeostasis, environmental adaptation (Eletsky et
99   al. 2014; Guo et al. 2016; Mashruwala & Boyd 2018; Tong et al. 2016). Many of the DUFs are
100   conserved and shared among prokaryotes and eukaryotes. For instances, the DUF59 has been
101   documented to involve in intracellular Fe homeostasis in most of the organisms (Mashruwala &
102   Boyd 2018). The DUF2233, a protein domain with phosphodiester glycosidase activity, can be
103   found in many bacteria, viruses and mammalian cells (Das et al. 2013). Even though, some of the
104   DUFs (e.g. DUF2525, DUF2526 and DUF2545) have been conserved in many bacteria
105   belonging to the family of *Enterobacteriaceae*, little is known about the regulation of DUFs in
106   *Enterobacter* spp. Moreover, the role of DUFs on metal resistance mechanisms of the EC has
107   never been explored.
108   In this study, isolation of *Enterobacter cloacae* from agricultural and industrial sites has
109   been performed in order to search for the environmental isolates that possessed ability to tolerate
110   and/or accumulate cadmium ions. Minimum inhibitory concentrations (MICs) together with
111   binding capability to cadmium ions have been analyzed. Alterations of protein expression
112   profiles upon exposure to toxic cadmium have subsequently been investigated using two-
113   dimensional-difference in gel electrophoresis (2D-DIGE) in conjunction with protein
114   identification *via* liquid chromatography-mass spectrometry (LC-MS). Differentially-expressed
115   proteins among different strains of EC as consequences of cadmium exposure have been
116   analyzed and confirmed using qRT-PCR. In addition, molecular structure and functions together
117   with their association networks have been determined by bioinformatic tools. Plausible
118   explanations on the underlying mechanisms on how the EC respond and adapt themselves in
119   survival against harmful cadmium have been discussed.

120

Materials & Methods

Isolation and identification of bacteria from water and soil around agricultural and industrial sites

Nine strains of bacteria were isolated from soil and water in various sites around agricultural and industrial areas in the central part of Thailand. Genus and species identification of these isolates was performed by Gram's staining and standard biochemical testings including triple sugar iron agar (TSI), lysine iron agar (LIA), sulfide-indole-motility (SIM), citrate and urease. These bacterial colonies were also subjected to MALDI/TOF mass spectrometry for further confirmation as follows. Isolated colonies were analyzed using a formic acid-based direct on-plate preparation method. One microliter of 70% formic acid (Fluka, Sigma-Aldrich, St. Louis, USA) per well was deposited onto the MALDI-TOF-MS steel anchor plate (BigAnchor 96-well plate, Bruker Daltonics, USA). Colonies were smeared into the formic acid and allowed to dry. The dried mixture was overlaid with 2 μl of matrix solution (α-cyano-4-hydroxycinnamic acid; HCCA) dissolved in 50% acetonitrile, 47.5% water, and 2.5% trifluoroacetic acid (Fluka, Sigma-Aldrich) and allowed to dry prior to analysis using the MALDI Biotyper (Bruker Daltonics). A bacterial test standard (BTS, Bruker Daltonics) was used for instrument calibration. A positive control (*Staphylococcus aureus* ATCC 25923) and a negative control (formic acid and matrix) were included in each run. A MicroFlex LT mass spectrometer (Bruker Daltonics) was used for spectral analysis. Spectra were analyzed using the Bruker Biotyper 3.0 software and library version 3.3.1.0 (4,613 entries), supplemented with mass spectra from an in-house collection of 87 anaerobic isolates encompassing 39 species. Manufacturer-recommended cut-off scores were used for identification, with scores of ≥2.000 indicating identification to the species level, scores between 1.700 and 1.999 indicating identification to the genus level, and scores of <1.700 indicating no identification. Isolates producing scores of <1.700 were retested once with the highest score used for final analysis. These nine isolates of *Enterobacter cloacae* were further deposited at -80°C in the repository room (deposition reference numbers: EC_B-Cd-A series) at the Faculty of Medical Technology, Mahidol University, Salaya campus, Nakornpathom, Thailand.

149

Determination of minimum inhibitory concentration (MIC) by agar dilution

Determination of MIC by agar dilution was performed according to a standard guideline from the Clinical and Laboratory Standards Institute, 2012 (CLSI, 2012). Briefly, *E. cloacae* was incubated in the Luria-Bertani (LB) broth (10 g/L tryptone, 5 g/L NaCl and 5 g/L yeast extract, pH 7.2) at 37°C for overnight. Then, cells were collected and adjusted to be equivalent to 0.5 McFarland standard. Then, 2 μl of ten-fold diluted of 0.5 McFarland suspension was spotted onto each plate spreaded with final concentrations of cadmium, copper and zinc at 3.2-0.1 mM, 16-1 mM, and 6.4-0.1 mM, respectively. These plates were further incubated at 37°C for 18 h prior to the MIC analysis.

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Growth curves determination in the presence of sub-lethal dose of cadmium ions

To observe the effect of cadmium ions on bacterial cells growth, overnight cultures of cells in 5 ml LB were adjusted to OD_{600} of 0.001 in a new tube containing 3 ml LB broth. Cells were grown at 37°C with shaking at 200 rpm for 30 min prior to addition of $CdCl_2$ to yield the final concentration of 0.2 mM. Each of bacterial isolates was taken into three replicates. These tubes were incubated in shaking incubator at 37°C at 200 rpm. Growth of cells was monitored by a spectrophotometer at the time interval of 1 h for 24 h. It is noteworthy that the sub-lethal dose of 0.2 mM cadmium ions was equivalent to MIC/2 of intolerant strain of *E. cloacae* (designated as EC07) and was selected as an effective dose for further experiments.

Bacterial cell preparation in the treatment of cadmium ions

Two strains of *E. cloacae* designated as EC01 and EC07 were considered according to their heavy metal tolerant ability as tolerant and intolerant strains, respectively. A standard strain of *E. cloacae* (ATCC 13047) was included for comparison. These isolates from frozen stock were initially grown in LB broth at 37°C for overnight. Then, bacteria were sub-cultured onto LB agar and further incubated at 37°C for overnight. With three biological replicates, three isolated colonies were inoculated into 50 ml of LB broth and incubated at 37°C for 18 h. Cells were collected and adjusted to equal cell density at OD_{600} of approximately 4.0 in 50 ml LB broth. Cells were initially grown at 37°C, 200 rpm for 30 min before treatment with or without 0.2 mM $CdCl_2$. Cells were further incubated for 6 h and harvested by centrifugation at 14,000 rpm for 30 min at 4°C. Pellets were washed five times with Tris-sucrose buffer (1 mM Tris-base, 250 mM sucrose, pH 7.2) at 14,000 rpm for 30 min at 4°C to remove the excess salts and heavy metals. The bacterial cells were divided into two parts: first part was prepared for proteomics analysis and another for cadmium accumulation analysis and every step was done on ice. The pellets were stored at -80°C for proteomics analysis.

Intracellular cadmium accumulation analysis

For cadmium accumulation analysis, cells from the previous step were adjusted to $OD_{600} =$ 1.0 in 3 ml of Tris-sucrose buffer and were centrifuged at 14,000 rpm, 4°C for 30 min. Then, the supernatant was removed and 500 µl of concentrated nitric acid was added to the pellet and kept for overnight. The amounts of cadmium accumulation in bacterial cells were measured by atomic absorption spectroscopy (Varian SpectrAA-640, USA) and SpectrAA 200 software (Varian, USA). Briefly, a cadmium stock standard solution (1,000 µg/ml) was diluted to various concentrations with 0.2% HNO_3 and served as working standard solutions. Then, the calibration curve (0-1,000 µg/L) was prepared using blank and working standard solutions under the preset parameters (wavelength 228.8 nm with 0.5 nm slit width and the characteristic concentration check (~0.2 absorbance unit) at 0.5 mg/L). The sample blank contained all reagents used in the sample preparation. Samples were diluted 20 fold prior to cadmium determination and the amount of cadmium was subsequently recorded.

200 **Proteomics analysis**

201   *Proteins extraction.* The bacterial pellets were resuspened in 2x volumes of sample of lysis
202 buffer (2 M thiourea, 7 M urea, 4% CHAPS, and 1% protease inhibitors cocktail). The bacterial
203 cells were disrupted by sonicator (Amplitude 60, 0.5 cycles) on ice. Then, samples were
204 centrifuged at 14,000 rpm for 30 min at 4°C. Protein concentration was measured by Bradford's
205 method (Bio-Rad protein assay, Bio-Rad Laboratories, USA). The protein samples were
206 collected and stored at -80°C.

207   *Minimal labeling of protein sample.* Protein extracts were initially cleaned-up by using the
208 2-D Clean-up kit (GE Healthcare, USA). Then, the proteins of all samples were labeled with N-
209 hydroxy succinimidyl ester-derivatives of the cyanine dye Cy2, Cy3 and Cy5 (GE Cydye DIGE
210 Fluor (minimal dyes) Labeling Kit (GE Healthcare) following the manufacturer' protocols.
211 Briefly, the internal standard pool was generated by combining equal 25 µg of extracts from all
212 samples and then the pooled sample was labeled with 400 pmol of Cy2 to aid image matching
213 and cross-gel statistical analysis. The 50 µg proteins from control and cadmium treated cell were
214 minimally labeled with 400 pmol of Cy3 or Cy5. The samples were vortexed and kept on ice for
215 30 min in the darkness. Then, the reaction was terminated by addition of 1 µl of 10 mM lysine
216 and subsequently incubated on ice for 10 min in the darkness.

217   *First-dimensional gel electrophoresis.* The total of 50 µg protein of each sample, which
218 randomly labeled with Cy3 or Cy5, and the internal standard Cy2, were mixed together and
219 applied to 120 µl of rehydration buffer (8 M urea, 4% CHAPS, 0.001% (w/v) bromophenol
220 blue), 3 mM of dithiothreitol (DTT) and 1.75 µl 1% IPG buffer 3–10 NL). In parallel, 360 µl of
221 rehydration buffer was applied into rehydration tray and strip was placed. Then, the strips were
222 rehydrated in rehydration solution at 20°C for 16-20 h. The total 150 µg of pooled labeled cy3,
223 cy5 and cy2 samples were focused in a broad range of 18 cm, 3-10 NL of IPG strips (GE
224 healthcare) using cup-loading technique and the isoelectric focusing was carried out using an
225 IPGphor III apparatus (GE Healthcare) according to the following procedures: 500 volts for 500
226 volt-h, 1,000 volts for 800 volt-h, and 10,000 volts to reach 27,000 volt-h.

227   *Second-dimensional gel electrophoresis.* The 12.5% polyacrylamide gel was prepared at
228 room temperature for 16-18 h for gel polymerization. The strips were equilibrated twice (15 min
229 per each) in equilibrium buffer (50 mM Tris pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.03%
230 bromophenol blue) supplemented with 65 mM DTT or 135 mM iodoacetamide. Then, the
231 equilibrated IPG strips were laid onto the surface of a vertical 12.5% polyacrylamide gel and
232 covered with 0.5% agarose gel and 10% bromophenol blue. Separation of protein was carried out
233 at 2.5 watt per gel at 20°C for 30 min following by 10 watt per gel at 20°C until the bromophenol
234 blue dye front reached 0.5 cm from the bottom of the gel.

235   *Image analysis.* 2D-DIGE gel images were acquired using Typhoon TRIO Variable Mode
236 Imager (GE Healthcare). Differential analysis was performed by ImageMaster 2D Platinum
237 version 7.0 (GE Healthcare) software tool. These included spot intensity calibration, spot
238 detection and background subtraction. Quantification of intensity of each spot was performed in
239 terms of spot volume (area × intensity). The total spot volume normalization method was applied

240   in which the percentage of each spot volume on a gel image is calculated relative to the total
241   volume of all spots on that image. Then, determination of differentially expressed proteins was
242   conducted by comparing the ratio of % volume values with control and kept at 4°C until protein
243   identification.
244   *Gel staining.* Gels were stained with Colloidal coomassie brilliant blue staining (10% (w/v)
245   ammonium sulfate, 1% (v/v) phosphoric acid, 0.1% (w/v) Coomassie blue) for overnight.
246   Finally, the gels were destained with milli-Q water until a clear background was observed.
247   *Protein identification.* The QTOF-LC-MS/MS system consisting of a liquid chromatography
248   part (Ultimate 3000, thermo Scientific) in combination with an electrospray ionization (ESI)
249   Quadrupole time of flight mass spectrometer (Model Amazon SL, Bruker, Germany) was used
250   for protein identification. Protein spots of interest were digested by trypsin. First, the protein
251   spots were excised from 2-DE gel and washed twice with 50 µl of 50% acetronitrile (ACN)/25
252   mM ammonium bicarbonate (NH$_4$HCO$_3$) at room temperature for 15 min. Then, the solvent was
253   removed and 50 µl of 100% acetonitrile was added for 10 min or until all gels were in white.
254   After that, the acetonitrile was removed and 5 µl of diluted trypsin (diluted 0.1 mg/ml stock
255   trypsin 1:10 into 25 mM ammonium bicarbonate) was added into each tube at 37°C for 16-24 h.
256   Next, the supernatants were removed to new tubes. Peptides were extracted twice by 15-25 µl of
257   50% acetonitrile, 5% trifluoroacetic acid (TFA) to each tube containing gel slice for 15 min.
258   Then, the extracted peptides were removed and combined with the supernatant in the new tube.
259   Finally, the extracted peptides were dried in a vacuum centrifuge to dryness. For sample analysis
260   by QTOF-LC-MS/MS, peptides were separated on a reversed phase column (Hypersil GOLD
261   50×0.5 mm, 5 µm C18) and eluted at a flow rate of 100 µl/min under gradient condition of 5-
262   80% B over 50 min mobile phase A consists of water/formic acid (99.9:0.1, v/v) and B consists
263   of acetonitrile/water/formic acid (80:20:0.08, v/v). Mass spectral data from 300 to 1,500 m/z
264   were collected in the positive ionization mode. To identify the protein, all MS/MS spectra
265   recorded on tryptic peptides derived from spots were searched against protein sequences from
266   NCBInr databases using the MASCOT search engine program (available at
267   www.matrixscience.com). The functional analysis of protein was performed on UniProtKB
268   database at 'https://www.uniprot.org/help/uniprotkb'.
269
270   Unknown protein searching
271   The amino acid sequences of unknown protein were searched on
272   'https://blast.ncbi.nlm.nih.gov/Blast.cgi' using 'non-redundant protein sequences (nr)' database.
273   Then, the secondary structures of unknown protein were predicted by PSIPRED
274   'server:bioinf.cs.ucl.ac.uk/psipred/'. At last, the tertiary structure was predicted by SWISS-
275   MODEL server 'https://swissmodel.expasy.org/interactive'.
276
277   Protein network analysis
278   Protein association networks were created by subjecting the identified proteins to the
279   STRING software version 9.05 (http://string-db.org/). Direct (physical) and indirect (functional)

280   associations were weighted and integrated by using various data, e.g. genetic context, high-
281   throughput experiments, co-expression and previous knowledge, from the database.
282

283   **Statistical analysis**
284   All data are expressed as mean ± standard deviation (SD). For group comparisons, data were
285   determined by analysis of variance (ANOVA). The strength of each association is presented as
286   the regression coefficient with 95% confidence interval and *P*-value. A *P*-value <0.05 was
287   considered statistically significant.
288

289   **Confirmation of gene expression by qRT-PCR analysis**
290   *RNA isolation and quantification.* Bacterial cell suspension was centrifuged for cells
291   harvesting. A 0.75 ml of TRIzol^{TM} reagent (Thermo Fisher Scientific, USA) was added to 1×10^7
292   cells of EC. Cells were lysed by pipetting-up and -down several times. The homogenized
293   samples were transferred to 1.5 ml microcentrifuge tube and further incubated for 5 min at room
294   temperature for completing the dissociation of the nucleoprotein complex. Then, 0.2 ml
295   chloroform (Sigma-Aldrich Chemical Co., USA) was added. Tubes were further mixed
296   vigorously by hand for 15 sec and incubated for 2 min at room temperature. After that, the
297   samples were centrifuged at 14,000 rpm for 15 min at 4°C. The mixture was separated into a
298   lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. Since
299   the RNA remained in the aqueous phase so the aqueous phase of the sample was removed by
300   angling the tube 45° and pipetting the solution out to new centrifuge tube. For RNA
301   precipitation, 0.5 ml of isopropanol (Sigma-Aldrich Chemical Co., USA) was added to the
302   aqueous phase tubes and incubated at room temperature for 10 min. Tube was centrifuged at
303   14,000 rpm for 10 min at 4°C. Finally, the RNA forms a gel-like pellet on the side and bottom of
304   the tube. After precipitation and centrifugation, the supernatant was removed, leaving only RNA
305   pellet. The RNA pellet was washed with 75% ethanol and vortexed briefly. Then, it was
306   centrifuged at 9,000 rpm for 5 min at 4°C and discarded the wash. The RNA pellet was dried by
307   air for 5-10 min. To quantify the RNA concentration, the RNA pellet was dissolved in 20 μl
308   DEPC-treated water. RNA concentration was quantified with the Nano-Drop Spectrophotometer.
309   RNA quality was evaluated by the absorbance 260/280 nm and 260/230 nm ratios with value
310   >1.8 and >2.0, respectively.
311   *cDNA synthesis.* RNA was reverse transcribed into cDNA using SuperScript$^{®}$ III reverse
312   transcriptase (Thermo Fisher Scientific). The reverse transcription-polymerase chain reaction
313   (RT-PCR) was performed using Mastercycler nexus X2 (Eppendorf, USA). Firstly, each reagent
314   was mixed and briefly centrifuged before use. Starting material of cDNA synthesis was prepared
315   from 5 μg of total RNA, 1 μl of 50 μM oligo(dT)$_{20}$ primer, 1 μl of 10 mM dNTP mix and DEPC-
316   treated water was added until total volume to 10 μl in 0.5 ml tube. Then, the tube was incubated
317   at 65°C for 5 min and placed on ice for at least 1 min. After that, cDNA synthesis was prepared
318   by mixing of 2 μl of 10x RT buffer, 4 μl of 25 mM MgCl$_2$, 2 μl of 0.1 M DTT, 1 μl of
319   RNaseOUT^{TM} (40U/μl) and 1 μl of SuperScript$^{®}$ III RT (200 U/μl). 10 μl of cDNA synthesis mix

320   was added to RNA-primer mixture, mixed gently, and incubated at 50°C for 50 min. The
321   reaction was terminated at 85°C for 5 min and chilled on ice. Finally, 1 μl of RNaseH was added
322   to each tube and incubated at 37°C for 20 min to hydrolyze specifically the phosphodiester bonds
323   of RNA hybridized to DNA. cDNA was stored at -20°C until use for PCR. cDNA concentrations
324   were measured following the RT reaction with the Nano-Drop Spectrophotometer.
325   *Quantitative reverse transcription-polymerase chain reaction (qRT-PCR).* The resulting
326   cDNA was used in a 20 μl reaction that contained 1 μl cDNA, 10 μl SYBR® Green mix (Bio-Rad
327   Laboratories, USA), 0.4 μl of specific primer sets, the *DUF326* forward: 5′-
328   GTTTGCGCTGATGTGTGTCA-3′ and reverse: 5′-GCCATCCTTCGGCATTGTTC-3′; the
329   *GAPDH* forward: 5′-CAGATGTCATTGGCTCGCAC-3′ and reverse: 5′-
330   AGTGCGGCGTATTTATCAAGCG-3′; Then, DEPC-treated water was added until the total
331   volume of 20 μl. Parameters for PCR amplification were as 95°C for 3 min for enzyme
332   activation, followed by 40 cycles each consisting of denaturation at 95°C for 3 sec, annealing at
333   54°C (*DUF326*) and 59°C (*GAPDH*) for 30 sec and extension at 72°C for 20 sec. The *GAPDH*
334   was used as reference gene to normalize values. Relative quantification calculations were
335   performed by ΔΔCq value.
336

Results

Bacterial identification and minimum inhibitory concentrations (MICs) of cadmium ions in *Enterobacter cloacae* isolated from water and soil

340   All 9 isolates were identified by conventional biochemical identification and further
341   confirmed as *Enterobacter cloacae* with the high scores of 2.013-2.255 by MALDI-Biotyper
342   (Table 1). The MICs of cadmium ions in these isolates were mostly in the range of 0.8-1.6 mM
343   (Table 2). There were only two isolates, namely EC01 and EC08, which exhibited the MICs at
344   1.6 mM. However, it seems that the EC01 could tolerate the other metal ions (e.g. zinc and
345   copper) approximately 2 fold higher than those of the EC08 strain. The same degree of tolerance
346   against these three kinds of metal ions was also found in the standard strain (ATCC 13047). To
347   further elucidate the cellular responses and the underlying mechanism against cadmium ions, the
348   EC07 (isolated from the cleaned area) was selected since it showed 4 fold lower in MIC than that
349   of the EC01 strain while displaying the same degree of tolerance against zinc and copper ions.
350   Therefore, in the present study, 3 strains of *E. cloacae* (EC01, EC07 and ATCC 13047) were
351   selected for further experiments.
352

Growth patterns of *E. cloacae* isolates in the presence of sub-lethal dose of cadmium ions

354   To investigate the growth patterns of these 3 strains in the presence of cadmium ions, the
355   final concentration of 0.2 mM CdCl$_2$ represented as MIC/2 of the most sensitive strain (EC07)
356   was selected. As shown in Fig. 1B, some suppressing effect on the growth arrest of EC01 was
357   found at the lag phase and early-log phase while the rate of cell division at the middle- to late-log
358   phase resembled those in the absence of cadmium ions. Such recovery was not significantly

359 detected in the case of ATCC 13047 (Fig. 1A). Not surprisingly, cadmium ions at 0.2 mM
360 exerted their toxic effects on the growth of EC07 at all phases of cell division (Fig. 1C).
361
362 **Cadmium accumulation at the cellular and protein levels**
363 Next question was addressed on whether the tolerance phenomenon found in EC01
364 associated with the higher cadmium accumulation intracellularly. As illustrated in Fig. 2, the
365 higher cadmium adsorptivity of upto 2.7-3.0 µg per 8×10^8 cells was found in the case of EC01,
366 particularly at 6 h, as compared to the others. Importantly, when quantification of cadmium
367 adsorption was performed in the whole cell lysates, the proteins portion of EC01 provided
368 binding capability of approximately 2 fold higher than those of the ATCC 13047 and EC07
369 strains (Fig. 3). Our findings lend support to the hypothesis that the EC01 expressed some of the
370 cadmium-responsive proteins differently from those of the others.
371
372 **Protein expression profiles of *E. cloacae* in the presence of cadmium ions revealed by 2D-**
373 **DIGE**
374 To further explore the detailed mechanism on how *E. cloacae* initially responded and adopt
375 themselves upon exposure to cadmium toxicity in a short period of time (6 h), two-dimensional-
376 difference in gel electrophoresis (2D-DIGE) in conjuction with protein identification *via* mass
377 spectrometry was utilized. As demonstrated in Fig. 4, approximately 250 protein spots were
378 detected in the master map of pooled proteins (labeled with Cy2) served as an internal standard
379 for these three isolates. Eighty protein spots were then picked up and analyzed by the QTOF-LC-
380 MS/MS system for protein identification (Table 3). Most of the proteins were classified by
381 UniProtKB to be responsive for oxidative stress, protein folding, glycolytic process, translation,
382 metal ion binding, ion transport and homeostasis (Fig. 5). Up- or down-regulations of protein in
383 *E. cloacae* ATCC 13047, EC01 and EC07 strains were summarised in Table 3. In case of ATCC
384 13047, up-regulation of catalase (spots 2 and 4), Hsp20 (spot 28), malate dehydrogenase (spot
385 32), glyceraldehyde-3-phosphate dehydrogenase (spots 36 and 37), superoxide dismutase (spot
386 38), fructose-bisphosphate aldolase (spot 46), GroEL (spots 55, 56 and 59), putative hydrolase
387 (spot 58), ATP synthase (spot 67) was detected in response to cadmium toxicity. For the EC01, a
388 set of protein similar to those of the ATCC strain was up-regulated such as catalase (spots 1, 2
389 and 3), Hsp20 (spot 28), malate dehydrogenase (spot 32), glyceraldehyde-3-phosphate
390 dehydrogenase (spots 36 and 37), fructose-bisphosphate aldolase (spot 46) and GroEL (spots 55,
391 56 and 59). Meanwhile, up-regulation of some proteins including dihydrolipoamide
392 dehydrogenase (spot 10), enolase (spot 52), domain of uncharacterized function (DUF 326) (spot
393 57), aspartate ammonia-lyase (spot 60) and glutamine-binding periplasmic protein (spot 79) was
394 found in the EC01. The protein profile of EC07 showed the up-regulation of catalase (spot 3),
395 hydroperoxidase (spot 11), Dps (spot 19), OmpX (spot 23), YciF protein (spot 27), TrpR binding
396 protein WrbA (spot 29), OmpC porin (spot 45), glucose-specific PTS system (spot 68) and
397 glutamine-binding periplasmic protein (spot 79).

398   To further simplify the groups of protein shown as unique or overlap proteins among these 3
399   strains of *E. cloacae*, Venn diagram was plotted as illustrated in Fig. 6. Catalase was analyzed to
400   be the only one protein found in all 3 strains under cadmium stress. Four proteins namely
401   enolase, DUF326-like domain, dihydrolipoamide dehydrogenase, aspartate ammonia-lyase were
402   detected as unique proteins in the case of tolerant strain (EC01). In the intolerant strain (EC07),
403   the unique proteins were identified as OmpX, protein YciF, OmpC porin, DNA protection during
404   starvation protein, and TrpR binding protein WrbA. However, our finding revealed that
405   glutamine-binding periplasmic protein was the only one protein shared between EC01 and EC07
406   strains.
407
408   **Identification of specific cadmium-induced protein alterations in EC01 (tolerant strain)**
409   **and EC07 (intolerant strain)**
410   Differentially expressed proteins found in EC01 as compared to those of the EC07 strain in
411   the presence of cadmium ions were analyzed as shown in Tables 4 and 5. DUF326-like domain
412   was analyzed to show the highest difference in fold change (+220.92 fold) of protein expression
413   between EC01 and EC07 (Table 4). This indicates that the EC01 normally expresses the
414   DUF326-like domain at high level when grows up in the environmental-contaminated site.
415   Therefore, exposure to cadmium ions at 0.2 mM in a short period of time (6 h) triggered the cells
416   to up-regulate the DUF326-like domain expression of only 1.35 fold higher than that in the
417   absence of cadmium. Similar phenomena were also found in the regulation of antioxidative
418   enzymes such as hydroperoxidase II (+42.3 and +21.3 fold) and superoxide dismutase (+8.14
419   fold), expression of periplasmic protein (+14.3 fold) or outer membrane protein X (+6.98 fold) as
420   well as the metabolic enzyme (MDH; +7.45 fold) as compared to very low/undetectable amount
421   found in the control. Beside these, differences in expression of the other proteins were found in
422   the range of 1.38-6 fold higher than those of the EC07 in response to the toxic cadmium ions.
423   As compared to the results of EC07 shown in Table 5, the EC01 exhibited lower expression
424   of YciF protein of approximately 84.7 fold than that of the EC07. Expression of the OmpC porin
425   and manganese catalase of upto 53.2 and 51.7 fold lower than those of the EC07 was also
426   detected. More than 30 fold difference in the osmotically-inducible protein Y and Dps were
427   recorded. Expression of the other proteins was varied in the range of 1.3-29.2 fold.
428   To further confirm whether cadmium stress affected gene expression of *E. cloacae*, Fig. 7
429   depicts qRT-PCR analysis of DUF326-like domain expression. Data are normalized relative to
430   GAPDH gene (served as a house keeping gene) and expressed as the fold difference ($2^{-\Delta\Delta Cq}$)
431   between control and cadmium-treated group. Expression of DUF326 gene in *E.cloacae* strain 01,
432   07 and ATCC were at 14.13, 1.28, 1.49-fold higher in cadmium-treated than in the control group,
433   respectively.
434

Discussion

436   Cadmium contamination remains a major health concern due to its toxicity to human,
437   animals and microorganisms. Searching for bacteria that possess high capability to bind

438   cadmium ions and/or cadmium tolerance is needed for further applying as bioremediation tool.
439   *E. cloacae* has been isolated and characterized as a potential heavy metal accumulating bacteria
440   (Iyer et al. 2004; Naik et al. 2012; Rahman et al. 2015). However, the global-protein response to
441   cadmium of *E. cloacae* is still unclear. In this study, the cadmium-tolerant *E. cloacae* isolated
442   from agricultural and industrial area were collected. Minimium inhibitory concentrations (MICs)
443   and growth patterns in the presence of cadmium ions have been tested. As expected, the tolerant
444   strain (EC01) with the MIC of 1.6 mM cadmium has been isolated from industrial effluents of
445   heavy metal area whereas the intolerant strain (EC07) (MIC at 0.4 mM) has been isolated from
446   the cleaned area. Even though, the ATCC 13047 exhibited the same MIC as that of the EC01,
447   recovery of growth pattern during exposure to cadmium was in a lesser extent than the EC01.
448   Moreover, the degree of cadmium binding by the whole cell lysate of EC01 was approximately 2
449   fold higher than those of the ATCC 13047 and EC07. Such cadmium binding property opens up
450   a high feasibility of utilizing as biosorbents for cadmium remediation as in case of the
451   exopolysaccharides (Iyer et al. 2005).
452   To explore the underlying mechanisms of *E. cloacae* in response to cadmium toxicity,
453   proteomics profiling of these three strains were carried out by 2D-DIGE technology. DIGE
454   (Difference in Gel Electrophoresis) was conducted in this experiment to overcome the gel-to-gel
455   variation, which might be resulted from gel casting, running and staining. The system is based on
456   the specific properties of CyDye™ DIGE Fluor dyes that enable multiplexing of separate protein
457   mixtures on the same 2-D gel. The mixing sample with internal standard (pooled samples)
458   technique allows for multivariable analyses, as samples can be separated and compared on
459   several DIGE gels that were co-ordinated by the internal standard. The ability to directly
460   compare two samples on the same gel not only avoids the complications of gel-to-gel variation
461   but also enables a more accurate and rapid analysis of differences and reduces the number of gels
462   that need to be run (Westermeier & Scheibe 2008). Up- or down-regulation of protein expression
463   among these three strains is summarized in Tables 3-5 and the presumtive conclusion can be
464   emphasized as followings.
465
466   **Catalase isoforms serve as common adaptive responses of *E. cloacae* upon exposure to toxic**
467   **cadmium**
468   Cadmium ions exert their toxicity on the growth of *E. cloacae* in different degree of
469   inhibition, depending on the protein expression in each strain (Fig. 1). These toxic effects
470   possibly derived from the generation of reactive oxygen and nitrogen species (ROS and RNS)
471   (Brennan & Schiestl 1996; Isarankura-Na-Ayudhya et al. 2018; Stohs & Bagchi 1995). Such free
472   radical was not mainly produced by cadmium itself, however, indirect formation of ROS and
473   RNS e.g. superoxide radical, hydroxyl radical and nitric oxide has been reported (Waisberg et al.
474   2003). Generation of non-radical hydrogen peroxide, which itself may in turn be a significant
475   source of radicals *via* Fenton chemistry, was thought to be involved in this process. Furthermore,
476   cadmium was also proposed to replace copper and iron in various cytoplasmic and membrane
477   proteins such as ferritin, apoferritin. This resulted in the increase amount of free copper and iron,

478 which consequently participated in oxidative stress *via* Fenton reactions (Price & Joshi 1983). In
479 this study, the catalase was found to be the only one protein shared among all three strains in the
480 presence of cadmium stress (Fig. 6). Enhancement of catalase activity was also observed in the
481 EC strain B1 when cultured in the cadmium condition (Banerjee et al. 2015). Importantly, the
482 catalase has been proven to be the major enzyme used to detoxify these harmful effects in the
483 case of ATCC 13047 (Fig. 8C). Even though, the tolerant strain (EC01) did not use the catalase
484 as the primary responses but it showed a specific pattern of catalase expression (spots no. 1, 2, 6
485 and 7). It should also be noted that spot no. 8 was found to be unique in the intolerant strain
486 (EC07) (Table 3). Supportive evidences were documented on the presence of different isoforms
487 of catalase found in *Pseudomonas aeruginosa* and *P. stutzeri* under various growth conditions
488 such as under starvation conditions, induced by exposure to S-nitrosoglutathione (nitric
489 oxide-generating reagent), and induced by exposure to sodium nitroprusside (Kawakami et al.
490 2010; Xie et al. 2014). In addition, *Xanthomonas campestris* responded against cadmium stress
491 by regulation of two monofunctional catalase isozymes (Banjerdkij et al. 2005). Therefore, up-
492 regulation of catalase found herein was proposed not only to associate with an adaptation of *E.*
493 *cloacae* in survival under oxidative stress but also to reduce ROS in the cells due to Cd actions.
494 Moreover, the bacteria selected different isoforms of catalase in manner of Cd responses.
495

496 **DUF326-like domain: a novel molecule participated in detoxification of cadmium stress**
497   It has been suggested that the EC01 could tolerate to toxic cadmium by expressing
498 cadmium-binding proteins (Fig. 3). This coincided with the high level expression of the
499 DUF326-like domain (Table 4 and Fig. 8A). To our knowledge, this is the first report on the
500 involvement of DUF326-like domain in detoxification of cadmium stress in bacteria since the
501 world-wide availability of molecular function of DUF326 is quite limited
502 (www.ebi.ac.uk/interpro/entry/IPR005560). When the DUF326-like domain was subjected to
503 blast on https://blast.ncbi.nlm.nih.gov/Blast.cgi, the results showed 100% identity with four helix
504 bundles copper-binding protein (Fig. 9) of Family 'Enterobecteriaceae'. The copper-binding
505 protein is a cysteine-rich protein that can be found in many copper resistant bacteria. Previous
506 study demonstrated that *Pseudomonas putida* expressed three Cd-binding proteins (CdBP1,
507 CdBP2 and CdBP3), which contain large amounts of cysteine residue. Among these, the CdBP3
508 has the highest cysteine content of 22% (Higham et al. 1983). Compared to our finding, the
509 DUF326 protein comprises of 19 cysteine residues from 108 amino acid (17.6%) (Fig. 9). It can
510 be suggested that the thiol groups derived from these cysteine-rich proteins provide very high
511 binding avidity to Cd. Additionally, protein sequence analysis by searching on UniProtKB
512 database described that the cysteines mostly follow a C-X(2)-C-X(3)-C-X(2)-C-X(3) pattern,
513 though they often appear at other positions in the repeat as well
514 (https://www.ebi.ac.uk/interpro/entry/IPR005560). The result from domain architecture mode
515 analysis had shown two domain types including 4Fe-4S ferredoxin-type, iron-sulphur binding
516 domain (IPR017896) found in the DUF326. Similar observation was also found in the DUF59
517 that involved in FeS protein maturation and/or intracellular Fe homeostasis (Mashruwala & Boyd

518 2018). Supportive evidences on the interconnection between DUF326 and 4Fe-4S ferredoxin
519 protein (YwjF) and other related iron-sulfur containing proteins were identified by the STRING
520 database as shown in Fig. 10.
521   Overexpression of DUF326-like domain was believed to protect the *E. cloacae* from
522 hazardous cadmium ions, possibly by high binding affinity to cadmium. It has extensively been
523 reported the imperative roles of domain of unknown function on stress responses in many
524 organisms as follows. In *P. aeruginosa*, induction of PA1994 (classified as a member of
525 DUF1089) was proposed to be responsive for the bacterial cell-wall stress or host-pathogen
526 interactions (Bakolitsa et al. 2010). The DUF1471 found in many bacteria in the
527 Enterobacteriaceae played roles in stress response, biofilm formation, and pathogenesis (Eletsky
528 et al. 2014). In fungus, the DUF1996 regions involved in the regulation of multiple stress
529 responses and environmental adaptation (Tong et al. 2016). In rice, the DUF1644 and DUF966
530 genes were regulated in response to drought and salinity stresses (Guo et al. 2016; Luo et al.
531 2014).
532
533 **Protein networks involved in the survival of intolerant strain of *E. cloacae* in cadmium**
534 **stress**
535   In case of EC07, the top-ranked of protein expression in survival against cadmium stress
536 included the YciF protein, OmpC porin, manganese catalase, Osmotically-inducible protein Y,
537 DNA protection during starvation protein, and OsmC family protein (Table 5). Expression of the
538 YciF protein, which is a stress-inducible protein, was found to be in the highest level. The
539 structure and function of this protein have not extensively been studied. After searching in
540 UniProtKB/TrEMBL database, the YciF might contain some components closed to the DUF892
541 family that possessed iron sequestration funtion (Liu et al. 2016). It is possible that the YciF is
542 up-regulated when the bacteria encounter stress conditions. From the crystal structure of YciF
543 protein of *Escherichia coli* (Hindupur et al. 2006), a hypothesis can be emphasized that the YciF
544 may possess metal-binding properties since its structure is similar to others metal-binding
545 proteins such as rubrerythrin (a protein that has a di-iron center), ferritin and monooxygenases
546 (Hindupur et al. 2006). The metal-binding sites are proposed to lie in the helix bundle within the
547 two pockets (P1 and P2). The architectural features of YciF are also found to associate with other
548 protein functions such as the manganese catalase domain in the protein bll3758 from
549 *Bradyrhizobium japonicum* (Hindupur et al. 2006).
550   Using the STRING software, the YciF protein has a direct interconnection with the OsmC
551 (encoding peroxiredoxin) (Fig. 11). In addition, it has some indirect connection with a series of
552 outer membrane proteins (OmpC, OmpA and OmpX) linked by the YdeI protein (induced by
553 cadmium, hydrogen peroxide and acid stress) (Lee et al. 2010). The OmpC porin is known to
554 involve in biological process as ion transport system. This protein has been reported mostly to
555 participate in antibiotic resistant mechanism of *E. cloacae*. Disruption of OmpC rendered the
556 cells to be resistant against carbapenem (Uechi et al. 2018). However, little is known about the
557 correlation between cadmium stress and alteration in OmpC porin. In the present study, the fold

558   difference in expression of OmpC porin between the EC07 and EC01 was approximately 53 fold

559   (Table 5). Similar observation was also found in *E. coli* in which deletion of OmpC gene led to

560   decreased cadmium resistance (Egler et al. 2005). Additionally, it is noteworthy that there is a

561   linkage between the OsmC and Dps proteins. More importantly, there is a high possibility that

562   the Dps might be a connecting junction with the other proteins involved in the metabolic

563   processes (glycolysis and tricarboxylic acid cycle), ATP production, chaperone and oxidative

564   scavenging enzyme.

565   The increase of Dps under cadmium-stress condition might be associated with DNA

566   protection of *E. cloacae* to survive. A previous study mentioned that the levels of cellular DNA

567   damage were significantly higher in cadmium adding in cultures than in controls (Shapiro &

568   Keasling 1996). Explanation can be drawn that a mutagenic effect by cadmium is not due to

569   direct interaction with DNA but rather to inhibit DNA repair systems (Serero et al. 2008). The

570   Dps was also reported to be expressed during stationary phase upon starvation or exposure to

571   severe environmental assaults, including oxidative stress and nutritional deprivation (Calhoun &

572   Kwon 2011; Nair & Finkel 2004). It could effectively bind with chromosomal DNA and form

573   the stable Dps-DNA co-crystal. The mutants of Dps also failed to develop starvation-induced

574   resistance to hydrogen peroxide, an agent that can cause oxidative damage to DNA, and show

575   dramatic changes in the pattern of proteins synthesis during starvation in *E. coli* (Almiron et al.

576   1992). Such protection can be mediated by the intrinsic properties such as DNA binding, iron

577   sequestration and ferroxidase activity (Calhoun & Kwon 2011). These properties lend support to

578   the importance of Dps in iron- and hydrogen peroxide-detoxification and acid resistance.

579   Moreover, the prevalence of Dps and Dps-like proteins in bacteria suggests that protection

580   involving DNA and iron sequestration is crucial and widespread in prokaryotes (Calhoun &

581   Kwon 2011). The Dps has also been identified as a ferritin-like protein, partly because of its

582   ferroxidase activity, or more specifically, its ability to oxidize bound ferrous ions to the ferric

583   state and storing it in the form of Fe^{3+} ferrihydrite mineral, which can be released after reduction

584   (Calhoun & Kwon 2011; Nair & Finkel 2004; Velayudhan et al. 2007). In fact, the primary role

585   of Dps may not be iron storage, but to protect macromolecules from the combined lethality of

586   ferrous ions and H_2O_2 (Abdul-Tehrani et al. 1999) and by the fact that Dps strongly prefers H_2O_2

587   over O_2 as an oxidant when preparing iron for storage. The catalase activity of Dps seems to be

588   significant in Dps-mediated protection against hydrogen peroxide stress, as Zhao et al. has

589   identified its ability to decompose hydrogen peroxide as a significant method of detoxification in

590   *E. coli* (Zhao et al. 2002). Altogether, the protective roles of Dps are most likely achieved

591   through various functions associated with the protein-DNA binding and chromosome

592   compaction, metal chelation, ferroxidase activity, regulation of gene expression and a weak

593   catalase activity.

594   Glyceraldehyde-3-phosphate dehydroge nase (GAPDH) is an enzyme of ~37 kDa that

595   catalyzes the sixth step of glycolysis and thus serves to break down glucose for energy and

596   carbon molecules. In addition to this long established metabolic function, GAPDH has

597   activation, initiation of apoptosis (Tarze et al. 2007). Metabolic adaptation and energy

598 production are crucial for survival under heavy metal load/stress. This enzyme facilitates the
599 enhanced metabolic needs for survival followed by an enhanced expression of proteins (Vranakis
600 et al. 2014). Additionally, the alterations in the flux of metabolites can create metabolic
601 networks, allowing an organism to go along in a changing environment (Mailloux et al. 2011).
602 The GAPDH and related enzymes were also shown to be involved in the adaptive response to
603 oxidative stress (Oh et al. 2002; Ralser et al. 2007). It can also act as a reversible metabolic
604 switch under oxidative stress. The temporary inactivation of GAPDH by oxidant treatment may
605 re-route the metabolic flux from glycolysis to the pentose-phosphate pathway, allowing the cell
606 to generate more NADPH as an antioxidant cofactor (Ralser et al. 2007).
607

Conclusions

609   With respect to the performances of proteomics and bioinformatics tools, this work
610 successfully explores that the DUF326-like domain of *Enterobacter cloacae* strain EC01
611 (cadmium-tolerant strain) has been found to play imperative roles in detoxification of cadmium
612 stress, possibly by binding to cadmium ions. Such protective effect helps to recover the rate of
613 cell division particularly at the middle- to late-log phase of growth curve in the presence of
614 cadmium ions. This also coincides with the capability to accumulate cadmium ions
615 intracellularly at 6 h. The remarkable binding capability due to the presence of cysteine-rich
616 domains of DUF326-like domain has been observed in the case of EC01 of approximately 2 fold
617 higher than those of the EC07 (cadmium-intolerant strain) and ATCC 13047. Expression of the
618 DUF326-like domain is more pronounced of upto 220 fold higher than the others. Results from
619 the qRT-PCR confirm that the transcription level of *DUF326* gene in the EC01 is approximately
620 14 fold higher than those of the others. This indicates that the cadmium-tolerant strain of EC
621 adapts itself by complexation of cadmium ions by metal-binding proteins rather than using the
622 proteins/enzymes involved in oxidative deterioration, stress responses or outer membrane portion
623 as observed in the case of intolerant or standard strains. Taken altogether, our findings explore
624 the molecular mechanisms of cadmium tolerance in EC and also open up a high possibility of
625 applying either the DUF326-like domain or the EC01 cells as potential tools for cadmium
626 bioremediation in the future.
627

Acknowledgements
629

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Genus and species identification of 9 environmental isolates and ATCC strain of *E. cloacae* by MALDI-TOF mass spectrometry

1 **Table 1** Genus and species identification of 9 environmental isolates and ATCC strain of *E.*
2 *cloacae* by MALDI-TOF mass spectrometry

No.	Name	MALDI-TOF/MS			
		Organism (best match)	Score Value	Organism (second best match)	Score Value
1	*E. cloacae* 01	*Enterobacter cloacae*	2.013	*Enterobacter cloacae*	2.002
2	*E. cloacae* 02	*Enterobacter cloacae*	2.122	*Enterobacter kobei*	2.099
3	*E. cloacae* 05	*Enterobacter cloacae*	2.072	*Enterobacter cloacae*	2.011
4	*E. cloacae* 06	*Enterobacter cloacae*	2.177	*Enterobacter cloacae*	2.004
5	*E. cloacae* 07	*Enterobacter cloacae*	2.255	*Enterobacter cloacae*	2.172
6	*E. cloacae* 08	*Enterobacter cloacae*	2.142	*Enterobacter cloacae*	2.068
7	*E. cloacae* 09	*Enterobacter cloacae*	2.173	*Enterobacter cloacae*	2.046
8	*E. cloacae* 10	*Enterobacter cloacae*	2.099	*Enterobacter cloacae*	1.997
9	*E. cloacae* 11	*Enterobacter cloacae*	2.08	*Enterobacter kobei*	2.066
10	ATCC 13047	*Enterobacter cloacae*	2.001	*Enterobacter cloacae*	1.965

5 Note Meaning of score values derived from MALDI/TOF mass spectrometry are as follows:
6   -   Values in the range of 2.300-3.000 denote the highly probable species identification (represented in
7   green)
8   -   Values in the range of 2.000-2.299 denote the secure genus identification, probable species
9   identification (represented in green)
10   -   Values in the range of 1.700-1.999 denote the secure genus identification (represented in yellow)
11   -   Values in the range of 0.000-1.699 denote the unreliable identification (represented in red)

The minimum inhibitory concentrations (MICs) of cadmium, zinc and copper ions of 9 environmental strains and ATCC 13047 strain of *E. cloacae*

1 **Table 2** The minimum inhibitory concentrations (MICs) of cadmium, zinc and copper ions of 9
2 environmental strains and ATCC 13047 strain of *E. cloacae*

No.	Name	Minimal Inhibitory Concentration (MIC) test		
		Cd^{2+} (mM)	Zn^{2+} (mM)	Cu^{2+} (mM)
1	ATCC 13047	1.6	3.2	8
2	*E. cloacae* 01	1.6	3.2	8
3	*E. cloacae* 02	0.8	1.6	8
4	*E. cloacae* 05	0.8	1.6	8
5	*E. cloacae* 06	0.8	1.6	4
6	*E. cloacae* 07	0.4	3.2	8
7	*E. cloacae* 08	1.6	1.6	4
8	*E. cloacae* 09	0.8	1.6	8
9	*E. cloacae* 10	0.8	1.6	8
10	*E. cloacae* 11	0.8	1.6	4

Differentially expressed proteins of *E. cloacae* represented as fold changes in response to cadmium treatment. Proteins were identified by QTOF mass spectrometry.

1   **Table 3** Differentially expressed proteins of *E. cloacae* represented as fold changes in response to cadmium treatment. Proteins were identified by QTOF mass spectrometry.

Spot no.	Accession no.	Protein names	Fold change ATCC 13047 + Cd	Fold change EC01 + Cd	Fold change EC07 + Cd	Anova	M.W.	pI	Score	% protein seq. coverage
1	KGB06270.1|	catalase HPII	-1.07	1.76	-1.11	3.42E-03	83514	5.63	331	14
2	KGB06270.1|	catalase HPII	2.23	2.33	-1.04	2.89E-04	83514	5.63	314	7
3	KGB06270.1|	catalase HPII	-1.15	1.34	1.31	5.33E-01	83514	5.63	1587	16
4	KGB06270.1|	catalase HPII	1.57	1.14	1.21	1.02E-05	83514	5.63	1396	22
5	KJC00120.1|	hydroperoxidase	-1.56	-1.59	-2.04	1.70E-07	83488	5.66	298	13
6	CZY07068.1|	hydroperoxidase II	Undetectable	-1.85	Undetectable	4.33E-02	83493	5.76	151	7
7	SAG51425.1|	hydroperoxidase II	Undetectable	-1.06	Undetectable	1.52E-02	83473	5.61	1471	24
8	KJC00120.1|	hydroperoxidase II	Undetectable	Undetectable	-1.87	2.82E-02	83488	5.66	791	18
9	KJX08087.1|	succinate-semialdehyde dehydrogenase	1.22	-1.06	-1.13	7.38E-10	49930	5.71	516	25
10	KTI00918.1|	dihydrolipoamide dehydrogenase	-1.03	1.46	-1.05	1.66E-06	50867	5.79	302	16
11	KJC00120.1|	hydroperoxidase	Undetectable	Undetectable	3.88	3.11E-04	83488	5.66	580	20
12	ADF60390.1|	chaperone protein DnaK	-1.38	-1.06	-1.98	1.39E-01	69092	4.82	120	13
13	SAB00249.1|	30S ribosomal protein S1	-2.14	-1.28	-2.04	1.40E-01	61289	4.89	134	14
14	KLG03309.1|	peroxiredoxin OsmC	-7.07	-2.44	-1.15	6.58E-03	15005	6.04	217	52
15	KGB03490.1|	DNA protection during starvation protein	-7.51	-3.74	-1.01	1.39E-02	18720	5.94	1022	76
16	KPU05892.1|	superoxide dismutase	1.26	-1.16	-1.23	2.94E-02	17768	6.43	766	33
17	ADF61100.1|	type VI secretion system secreted protein Hcp	Not analysis	Not analysis	Not analysis	Not analysis	16996	5.76	432	40
18	SAH00153.1|	OsmC family protein	Undetectable	Undetectable	-1.91	5.04E-08	15149	5.74	156	52
19	SAG00330.1|	DNA protection during starvation protein	Undetectable	Undetectable	2.01	4.55E-05	18677	5.72	819	36
20	SAG00330.1|	DNA protection during starvation protein	Undetectable	Undetectable	-5.1	4.68E-07	18677	5.72	506	38
21	KJQ00352.1	50S ribosomal protein L21	-1.33	-1.09	1.1	6.97E-06	11543	9.85	58	7
22	EUM00092.1|	Universal stress protein A	-1.84	-1.15	-1.38	7.28E-05	16200	4.98	53	23
23	AQT90079.1|	outer membrane protein OmpX	-1.28	-1.22	1.3	1.35E-07	18654	6.23	1064	22
24	SAF00184.1|	protein YciE	Undetectable	Undetectable	-1.74	2.68E-04	19036	4.85	173	28
25	SAF00211.1|	protein YciF	Undetectable	Undetectable	-1.06	4.32E-09	18313	5.04	1838	29
26	OUF00075.1|	osmotically-inducible protein Y	Not analysis	Not analysis	1.1	3.72E-03	21426	6.17	1181	35
27	CZX11689.1|	protein YciF	Undetectable	Undetectable	2.45	3.65E-05	18325	4.95	535	60
28	KPU03204.1|	heat-shock protein Hsp20	1.3	2.39	1.29	6.21E-05	21493	5.32	687	48
29	SAD00058.1|	TrpR binding protein WrbA	-1.31	-1.01	1.41	8.72E-10	20847	5.78	255	25
30	KJX08128.1|	periplasmic protein	-1.04	-1.78	-1.75	1.69E-05	21374	8.63	1272	54
31	CZW02624.1|	periplasmic binding protein/LacI transcriptional regulator	-2.05	1.011	-1.34	6.04E-09	35617	5.88	1576	53
32	OUF11927.1|	malate dehydrogenase	1.32	1.43	1.05	1.17E-09	32673	5.9	1086	42
33	CZX00043.1|	2,5-diketo-D-gluconate reductase A	-1.09	1.21	-1.07	1.46E-02	30934	5.49	219	54
34	SAD00983.1|	cationic amino acid ABC transporter substrate-binding protein	-1.37	-1.27	-1.45	4.27E-01	28179	7.6	333	23
35	KGB04009.1|	bacterial extracellular solute-bindings, 3 family protein	-1.49	-1.91	1.14	6.37E-04	33362	8.8	565	42
36	KJC00085.1|	glyceraldehyde-3-phosphate dehydrogenase	1.37	1.45	1.06	1.32E-02	35648	6.61	585	42
37	SAJ08363.1|	glyceraldehyde-3-phosphate dehydrogenase	1.62	1.72	1.07	5.94E-06	35650	6.61	189	17
38	ADF61881.1|	superoxide dismutase	1.59	-1.05	1.17	1.01E-02	21457	5.54	46	17

Spot no.	Accession no.	Protein names	Fold change ATCC 13047 + Cd	Fold change EC01 + Cd	Fold change EC07 + Cd	Anova	M.W.	pI	Score	% protein seq. coverage
39	SAD00058.1|	TrpR binding protein WrbA	-1.35	-1.7	-1.06	5.17E-02	20847	5.78	1160	35
40	KTI00016.1|	superoxide dismutase	-1.08	-1.05	1.19	4.00E-01	22995	6.23	204	13
41	KTI01411.1|	hypothetical protein ASV14_00320	-1.21	-1.17	1.17	2.53E-07	37677	5.33	1254	47
42	CZW04458.1|	outer membrane protein A	-1.14	-1.43	1.24	4.62E-08	37577	5.2	1791	48
43	KJX05875.1|	porin [Enterobacter cloacae subsp. cloacae]	-1.36	-1.26	1.27	5.81E-09	39231	4.58	493	32
44	5FVN|A	Chain A, X-ray Crystal Structure of *Enterobacter cloacae* Ompe36 Porin	-1.28	1.06	1.09	2.01E-07	37857	4.39	3805	35
45	SAF58867.1|	OmpC porin	-1.24	-2.06	1.38	8.59E-07	39850	4.44	573	35
46	KJQ00573.1|	fructose-bisphosphate aldolase	1.5	1.6	-1.18	3.50E-03	39384	5.52	124	23
47	SAH00347.1|	alcohol dehydrogenase	-1.25	1	-1.15	2.21E-05	35832	5.7	1725	60
48	KPU01695.1|	elongation factor Tu	-1.57	-1.55	1.12	2.09E-03	43460	5.25	494	27
49	KPU01695.1|	elongation factor Tu	-1.59	-1.17	-1.46	4.33E-02	43460	5.25	481	29
50	CZW60501.1|	isocitrate dehydrogenase	-1.36	1.17	-1.25	9.02E-03	46145	5.14	127	23
51	CZW60501.1|	isocitrate dehydrogenase	-1.18	1.18	-1.51	1.49E-08	46145	5.14	79	9
52	SAH00247.1|	enolase	1.19	1.56	-1.35	1.65E-03	45632	5.19	85	10
53	SAI00778.1|	elongation factor Tu	-1.39	1.25	-1.8	3.38E-02	43444	5.25	673	33
54	WP_075208257.1|	translation elongation factor Tu	-1.37	1.04	-1.84	2.35E-05	40036	5.01	495	40
55	KJJ00122.1|	molecular chaperone GroEL	2.29	2.51	-1.29	7.48E-06	57205	4.88	800	38
56	KPU06091.1	molecular chaperone GroEL	1.32	1.33	-1.09	3.85E-06	57209	4.85	484	36
57	SAB11354.1|	Domain of Uncharacterised Function (DUF326)	Undetectable	1.35	Undetectable	9.38E-11	13306	5.27	3402	66
58	CZV11460.1|	Putative hydrolase	1.59	-1.1	-1.24	1.98E-02	72227	5.28	213	10
59	KPU06091.1|	molecular chaperone GroEL	1.85	1.36	1.09	1.56E-02	57209	4.85	800	27
60	KJJ00126.1|	aspartate ammonia-lyase	-1.59	1.55	-1.37	9.13E-06	52776	5.05	140	23
61	SAB00249.1|	30S ribosomal protein S1	-4.92	-2.24	1.03	2.31E-03	61289	4.89	139	10
62	SAJ00217.1|	F0F1 ATP synthase subunit beta	1.29	1.29	-1.14	2.46E-04	50268	4.89	305	19
63	CZY00919.1|	maltoporin	-1.25	1.08	1.21	1.38E-04	48457	4.79	471	27
64	SAB00172.1|	Tellurium resistance protein TerE	-1.75	-1.66	-1.27	1.60E-02	20437	4.69	688	30
65	SAB00191.1|	Tellurium resistance protein TerD	-1.1	-1.61	-1.28	3.66E-05	20573	4.64	1803	36
66	KTI00836.1|	molecular chaperone DnaK	-1.73	-1.61	1.22	4.85E-04	69092	4.82	473	9
67	WP_004144995.1|	MULTISPECIES: ATP synthase subunit alpha	2.98	1.24	-1.19	4.42E-03	55363	5.73	99	19
68	CZZ00122.1|	glucose-specific PTS system component	Undetectable	Undetectable	2.16	1.38E-01	18313	5.04	104	7
69	CZZ00122.1|	glucose-specific PTS system component	-3.03	-1.71	-1.61	6.63E-05	18222	4.73	660	27
70	KGZ01983.1|	chemical-damaging agent resistance protein C note like to terD domain protein and Tellurium resistance protein TerE	-3.64	-1.5	1.02	1.71E-01	20463	4.69	392	39
71	SAF00152.1|	manganese catalase	Undetectable	Undetectable	1.13	3.21E-05	31431	4.8	338	20
72	KJJ00123.1|	molecular chaperone GroES	-1.05	1.11	1.27	1.02E-05	10354	5.38	83	27
73	KJJ00123.1|	molecular chaperone GroES	Undetectable	-1.13	1.29	4.92E-03	10354	5.38	70	14
74	KJJ00123.1|	molecular chaperone GroES	-1.21	-1.32	-1.25	1.44E-01	10354	5.38	555	58
75	Q84FI1.1|DPS_ENTCL	RecName: Full=DNA protection during starvation protein	Undetectable	Undetectable	-1.21	8.29E-07	18691	5.95	133	23
76	SAC00082.1|	LysM domain/BON superfamily protein	-3.09	-1.52	Undetectable	5.26E-06	15895	5.4	155	24
77	KJC00085.1|	glyceraldehyde-3-phosphate dehydrogenase	-3.26	-2.55	Undetectable	3.38E-07	35648	6.61	227	16

Spot no.	Accession no.	Protein names	Fold change ATCC 13047 + Cd	Fold change EC01 + Cd	Fold change EC07 + Cd	Anova	M.W.	pI	Score	% protein seq. coverage
78	KPU04452.1|	ecotin	-1.08	-1.56	Undetectable	1.79E-08	19034	8.35	208	28
79	ESM17978.1|	glutamine-binding periplasmic protein	-1.03	2.38	1.8	1.04E-02	26950	8.91	130	15
80	KGB04009.1|	bacterial extracellular solute-bindings, 3 family protein	Not analysis	Not analysis	Not analysis	Not analysis	33362	8.8	884	19

Regulation of major responsive proteins found in EC01 (cut-off at 1.3 fold)

1   **Table 4** Regulation of major responsive proteins found in EC01 (cut-off at 1.3 fold)

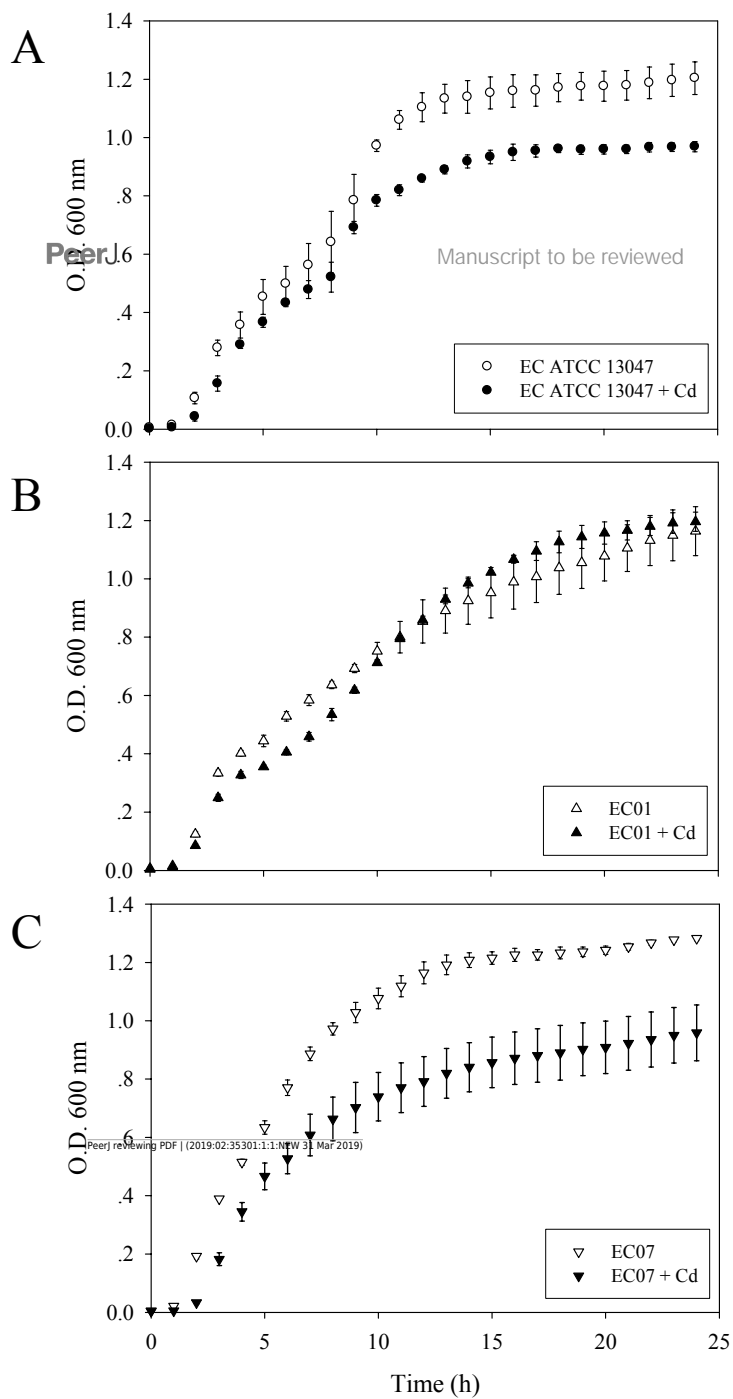
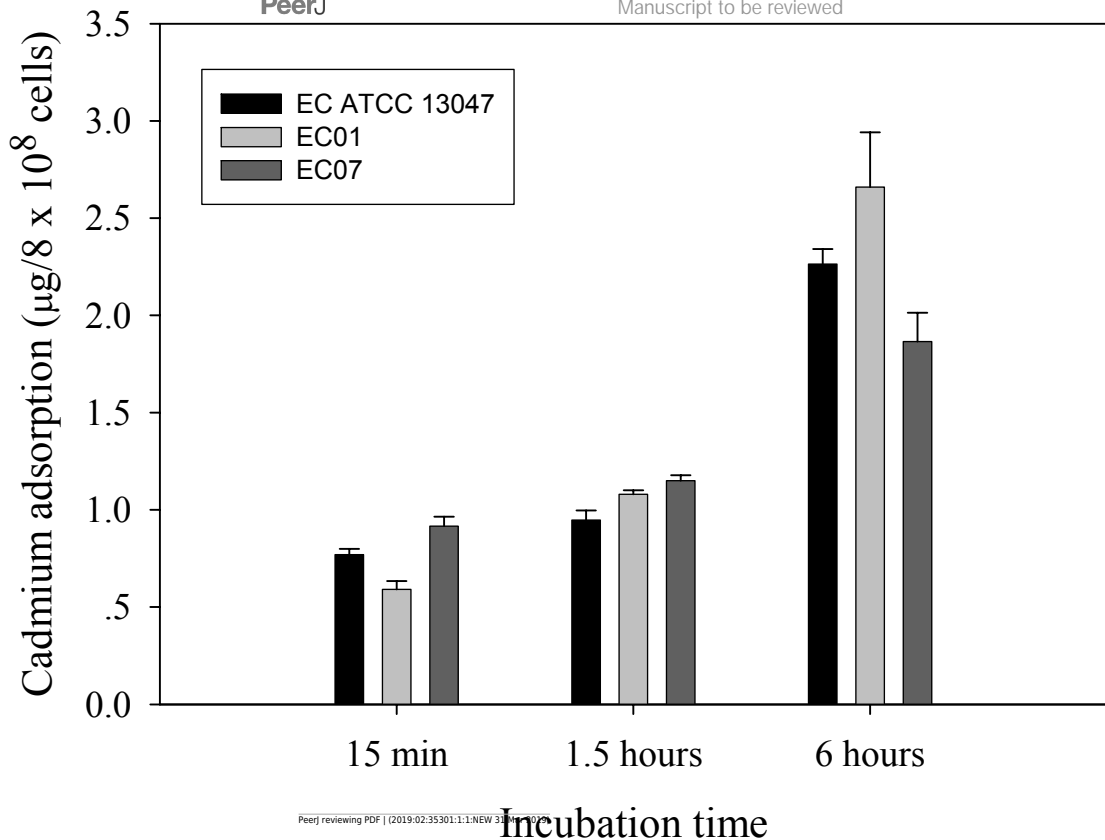
Spot no.	Accession no.	Protein names	Ratio of 01-Cd/07-Cd	Ratio of 01-Cd/01-control	Anova
57	SAB11354.1|	Domain of Uncharacterized Function (DUF326)	+220.92	+1.35	9.38E-11
6	CZY07068.1|	hydroperoxidase II	+42.29	U.D.	4.33E-02
7	SAG51425.1|	hydroperoxidase II	+21.28	U.D.	1.52E-02
30	KJX08128.1|	periplasmic protein	+14.29	-1.78	1.69E-05
16	KPU05892.1|	superoxide dismutase	+8.14	-1.16	2.94E-02
32	OUF11927.1|	malate dehydrogenase	+7.45	+1.43	1.17E-09
23	AQT90079.1|	outer membrane protein OmpX	+6.98	-1.22	1.35E-07
41	KTI01411.1|	hypothetical protein ASV14_00320	+6.63	-1.17	2.53E-07
38	ADF61881.1|	superoxide dismutase	+5.94	-1.05	1.01E-02
2	KGB06270.1|	catalase HPII	+5.02	+2.33	2.89E-04
56	KPU06091.1	molecular chaperone GroEL	+4.59	+1.33	3.85E-06
1	KGB06270.1|	catalase HPII	+3.41	+1.76	3.42E-03
15	KGB03490.1|	DNA protection during starvation protein	+3.00	-3.74	1.39E-02
14	KLG03309.1|	peroxiredoxin OsmC	+2.74	-2.44	6.58E-03
59	KPU06091.1|	molecular chaperone GroEL	+2.21	+1.36	1.56E-02
76	SAC00082.1|	LysM domain/BON superfamily protein	+2.07	-1.52	5.26E-06
65	SAB00191.1|	Tellurium resistance protein TerD	+1.90	-1.61	3.66E-05
78	KPU04452.1|	ecotin	+1.87	-1.56	1.79E-08
77	KJC00085.1|	glyceraldehyde-3-phosphate dehydrogenase	+1.71	-2.55	3.38E-07
70	KGZ01983.1|	chemical-damaging agent resistance protein C note like to terD domain protein and Tellurium resistance protein TerE	+1.62	-1.50	1.71E-01
48	KPU01695.1|	elongation factor Tu	+1.47	-1.55	2.09E-03
35	KGB04009.1|	bacterial extracellular solute-bindings, 3 family protein	+1.38	-1.91	6.37E-04

Regulation of major responsive proteins found in EC07 (cut-off at 1.3 fold)

1 **Table 5** Regulation of major responsive proteins found in EC07 (cut-off at 1.3 fold)

Spot no.	Accession no.	Protein names	Ratio of 07-Cd/01-Cd	Ratio of 07-Cd/07-control	Anova
25	SAF00211.1|	Protein YciF	84.7	-1.06	4.32E-09
45	SAF58867.1|	OmpC porin	53.2	1.38	8.59E-07
71	SAF00152.1|	Manganese catalase	51.7	1.13	3.21E-05
26	OUF00075.1|	Osmotically-inducible protein Y	42.8	1.10	3.72E-03
75	Q84FI1.1|DPS_ENTCL	DNA protection during starvation protein	32.7	-1.21	8.29E-07
18	SAH00153.1|	OsmC family protein	29.2	-1.91	5.04E-08
8	KJC00120.1|	hydroperoxidase II	24.2	-1.87	2.82E-02
27	CZX11689.1|	Protein YciF	19.8	2.45	3.65E-05
4	KGB06270.1|	Catalase HPII	18.5	1.21	1.02E-05
11	KJC00120.1|	Hydroperoxidase	13.9	3.88	3.11E-04
19	SAG00330.1|	DNA protection during starvation protein	12.3	2.01	4.55E-05
47	SAH00347.1|	Alcohol dehydrogenase	11.7	-1.15	2.21E-05
43	KJX05875.1|	Porin	10.1	1.27	5.81E-09
20	SAG00330.1|	DNA protection during starvation protein	9.9	-5.1	4.68E-07
42	CZW04458.1|	Outer membrane protein A	6.6	1.24	4.62E-08
51	CZW60501.1|	Isocitrate dehydrogenase	5.4	-1.51	1.49E-08
5	KJC00120.1|	Hydroperoxidase	3.7	-2.04	1.70E-07
54	WP_075208257.1|	Translation elongation factor Tu	3.6	-1.84	2.35E-05
9	KJX08087.1|	Succinate-semialdehyde dehydrogenase	3.4	-1.13	7.38E-10
60	KJJ00126.1|	Aspartate ammonia-lyase	3.4	-1.37	9.13E-06
55	KJJ00122.1|	Molecular chaperone GroEL	3.1	-1.29	7.48E-06
72	KJJ00123.1|	Molecular chaperone GroES	2.8	1.27	1.02E-05
44	5FVN|A	Chain A, X-ray Crystal Structure Of Enterobacter Cloacae Ompe36 Porin	2.6	1.09	2.01E-07
24	SAF00184.1|	Protein YciE	2.4	-1.74	2.68E-04
29	SAD00058.1|	TrpR binding protein WrbA	2.0	1.41	8.72E-10
46	KJQ00573.1|	Fructose-bisphosphate aldolase	2.0	-1.18	3.50E-03
10	KTI00918.1|	Dihydrolipoamide dehydrogenase	2.0	-1.05	1.66E-06
61	SAB00249.1|	30S ribosomal protein S1	1.9	1.03	2.31E-03
73	KJJ00123.1|	Molecular chaperone GroES	1.8	1.29	4.92E-03
31	CZW02624.1|	Periplasmic binding protein/LacI transcriptional regulator	1.7	-1.34	6.04E-09
67	WP_004144995.1|	MULTISPECIES: ATP synthase subunit alpha	1.6	-1.19	4.42E-03
62	SAJ00217.1|	F0F1 ATP synthase subunit beta	1.6	-1.14	2.46E-04
52	SAH00247.1|	Enolase	1.5	-1.35	1.65E-03
33	CZX00043.1|	2,5-diketo-D-gluconate reductase A	1.4	-1.07	1.46E-02
49	KPU01695.1|	Elongation factor Tu	1.4	-1.46	4.33E-02

Spot no.	Accession no.	Protein names	Ratio of 07-Cd/01-Cd	Ratio of 07-Cd/07-control	Anova
53	SAI00778.1|	Elongation factor Tu	1.3	-1.8	3.38E-02

Growth curves of *E. cloacae* strains ATCC 13047 (A), EC01 (B) and EC07 (C) in the absence (opened symbol) and presence (closed symbol) of 0.2 mM CdCl$_2$. Optical density at 600 nm was monitored at 1-h intervals for 24 h.

Time-course cadmium adsorption by *E. cloacae* tolerant strain (EC01), intolerant strain (EC07) and ATCC strain grown in LB broth containing 0.2 mM CdCl$_2$ at 37°C. Error bars represent as mean ± SD.

Cadmium adsorptivity of whole cell proteins from *E. cloacae* tolerant strain (EC01), intolerant strain (EC07) and ATCC strain. Error bars represent as mean ± SD.

Proteins from different strains of *Enterobacter cloacae*

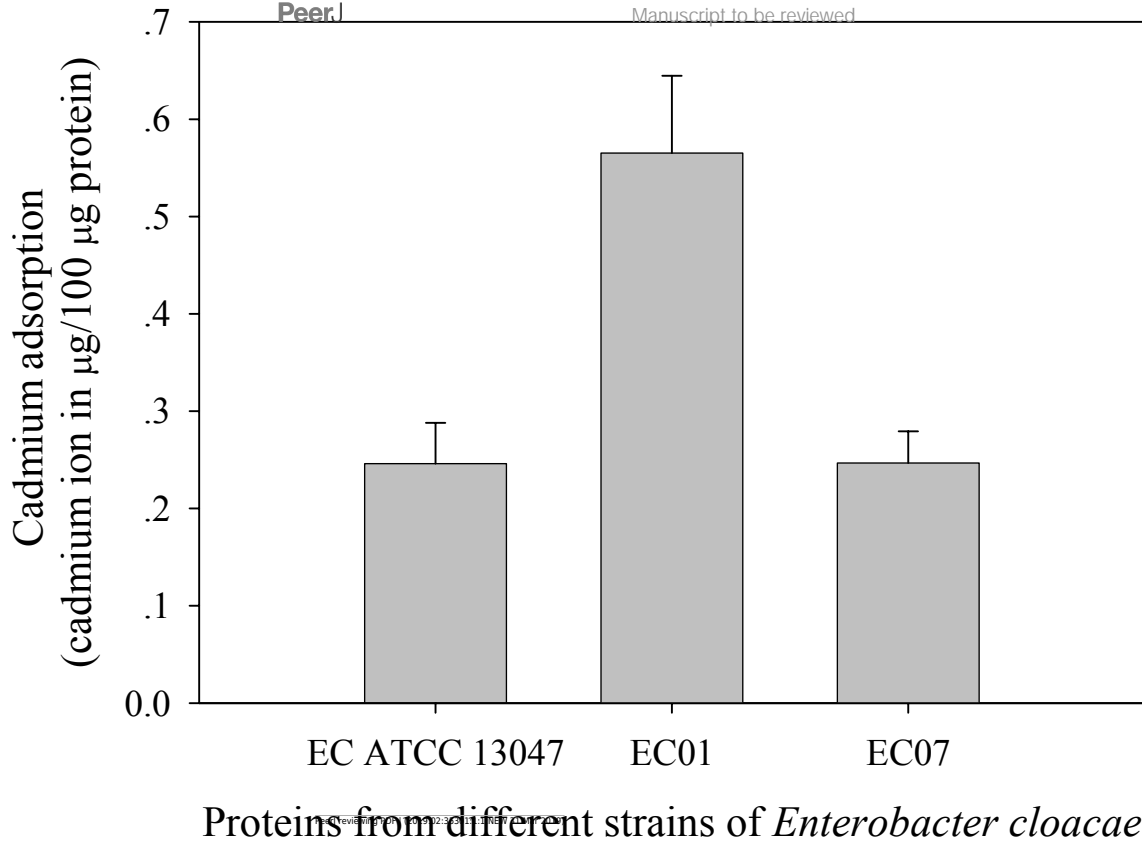
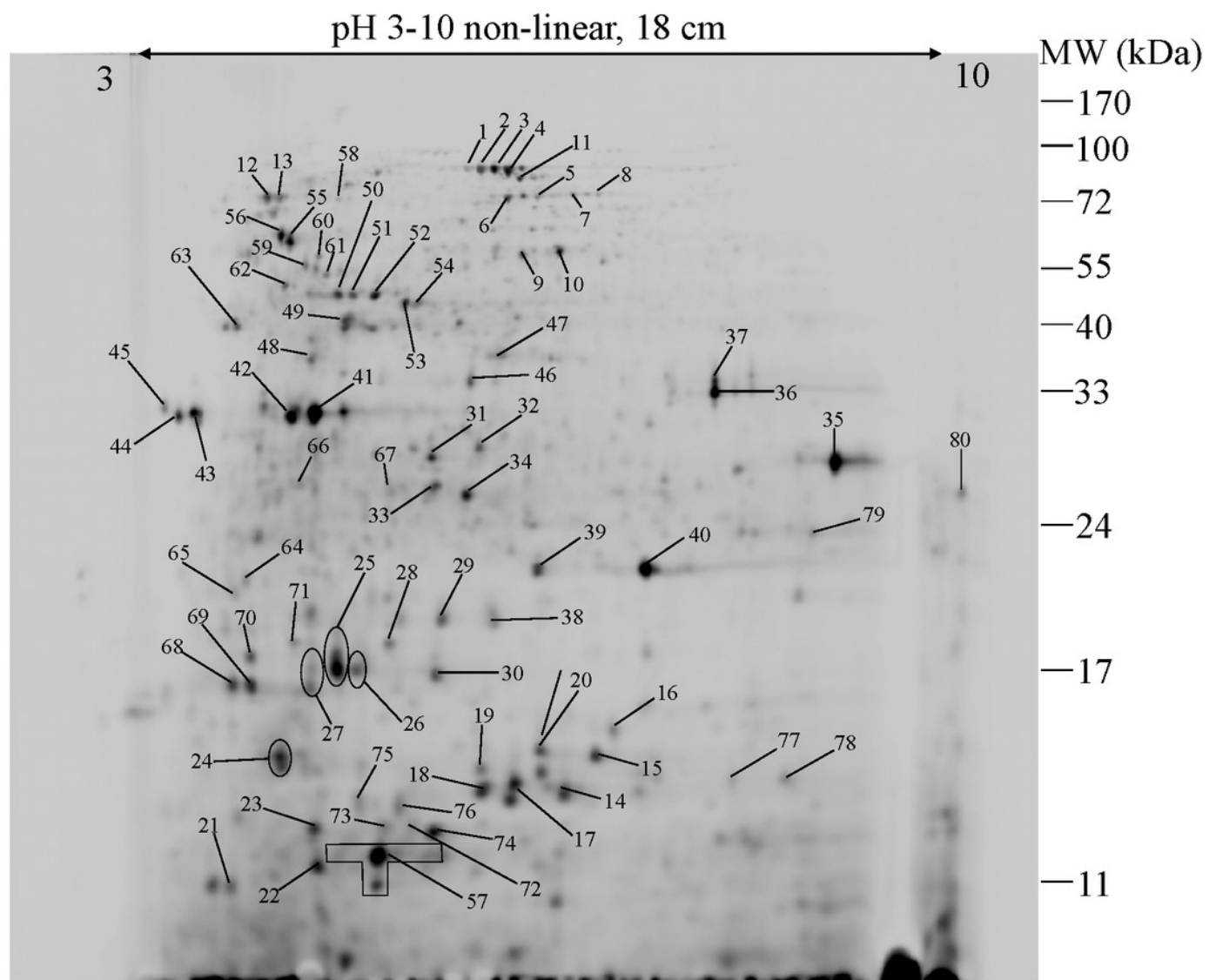



Figure 4

2-DE image of 350 μg of total soluble proteins extracted from *E. cloacae* ATCC 13047.

The proteins were immobilized on Immobiline Dry strips with nonlinear pH gradient from 3 to 10 followed by SDS-PAGE on 12.5% polyacrylamide gels. Protein spots were visualized by coomassie brilliant blue R-250 (CBB) staining. Eighty protein spots were identified as marked with arrows.

Numbers of proteins involved in various biological processes of *E. cloacae* ATCC 13047.

Functional analysis of proteins was identified by UniProtKB database.

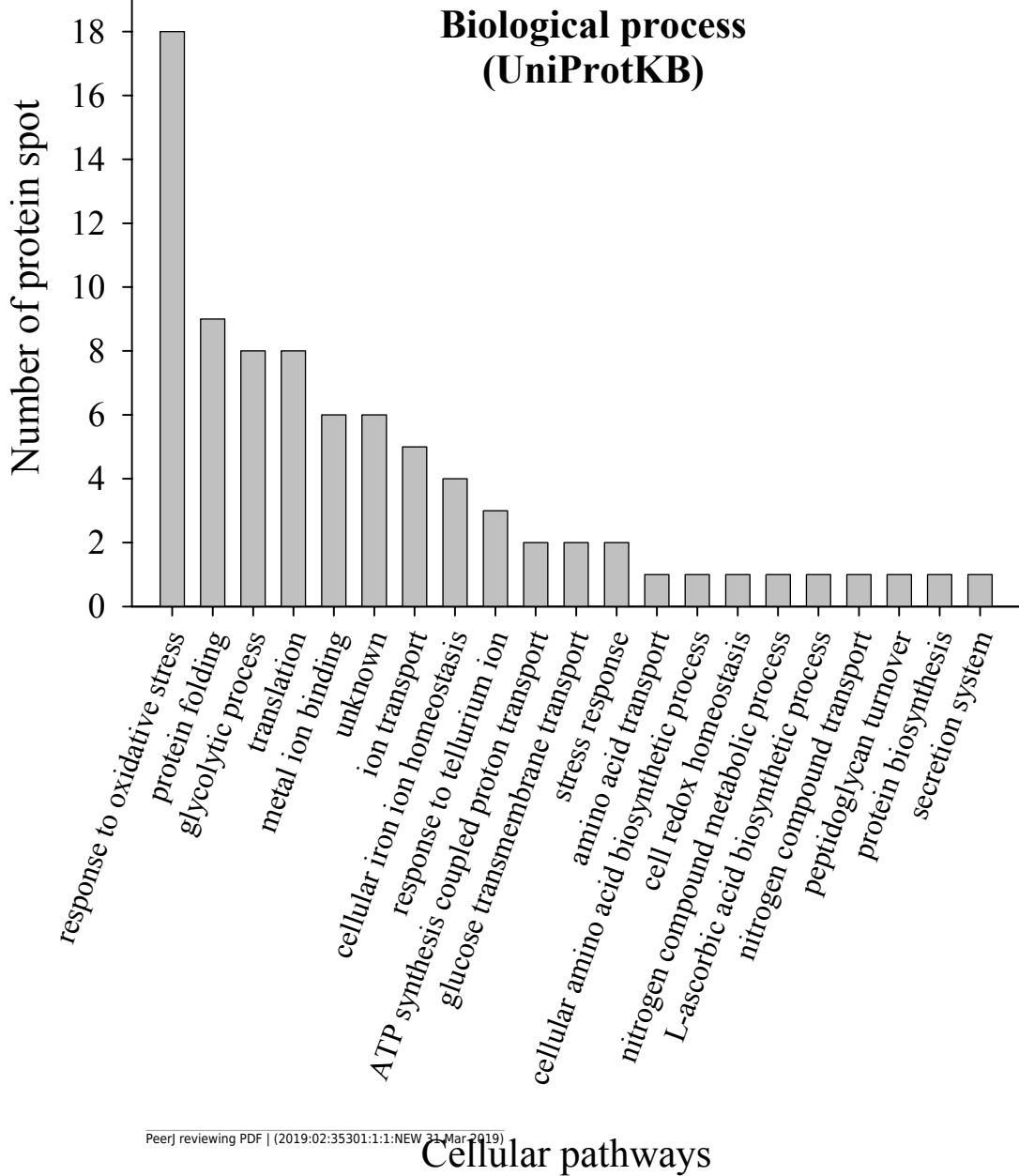
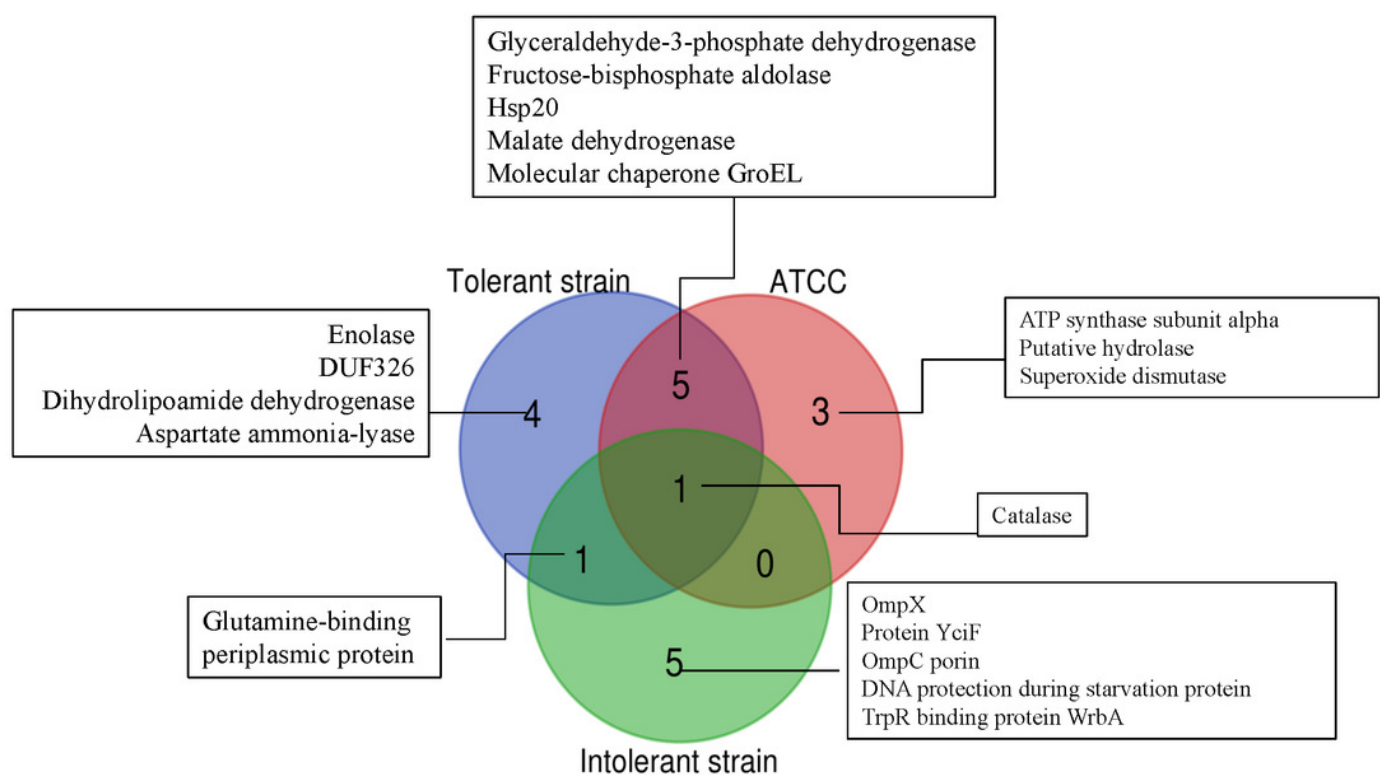



Figure 6

Venn diagram showing the number of significantly up-regulated proteins from 3 strains of *E. cloacae* with *P*-value ≤0.05 and fold change ≥1.3 under cadmium stress.

The diagram indicates the overlap between the tolerant (EC01), intolerant (EC07) and ATCC 13047 strains.

Fold differences in transcription level between *DUF326* gene (target gene) and *GAPDH* gene (hoursekeeping gene) in *E. cloacae* strains EC01, EC07 and ATCC 13047 in the absence and presence of cadmium ions.

Condition	DUF326 gene		GAPDH gene		ΔCq control	ΔCq cadmium	ΔΔCq	Fold difference
	Mean	S.D.	Mean	S.D.				
EC01 control	23.28	4.24	31.61	4.30	-8.33	-	-3.82	14.13
EC01 cadmium	21.49	1.49	26.00	2.72	-	-4.51		
EC07 control	32.57	1.87	34.88	2.36	-2.31	-	-0.35	1.28
EC07 cadmium	31.16	1.53	33.11	1.71	-	-1.96		
ATCC control	31.56	0.99	25.24	2.86	6.32	-	-0.57	1.49
ATCC cadmium	33.59	2.39	26.70	1.49	-	6.89		

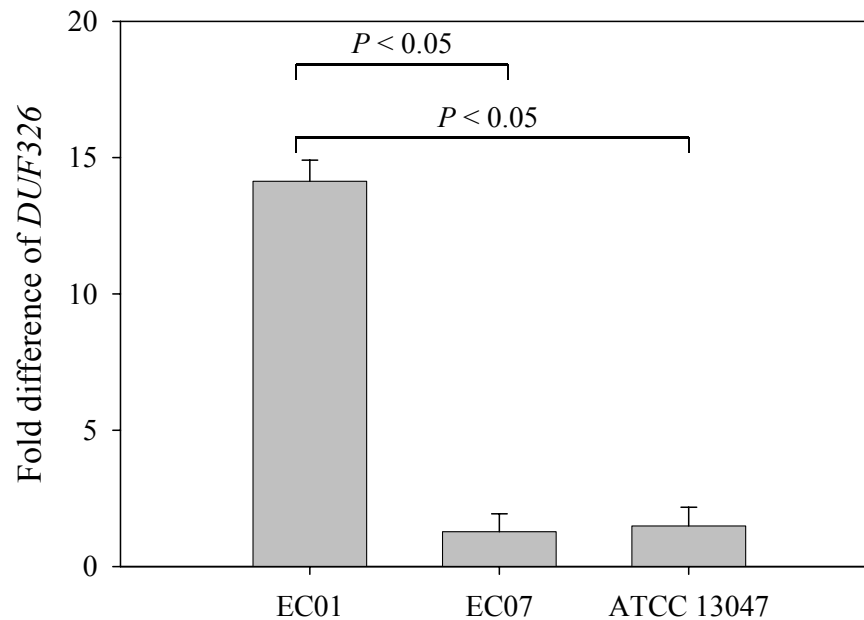



Figure 8

The superimposed of 2D-DIGE gels representing differentially expressed proteins of *E. cloacae* strains EC01 (A), EC07 (B) and ATCC 13047 (C) in the presence of cadmium ions at different locations (zoomed gels)

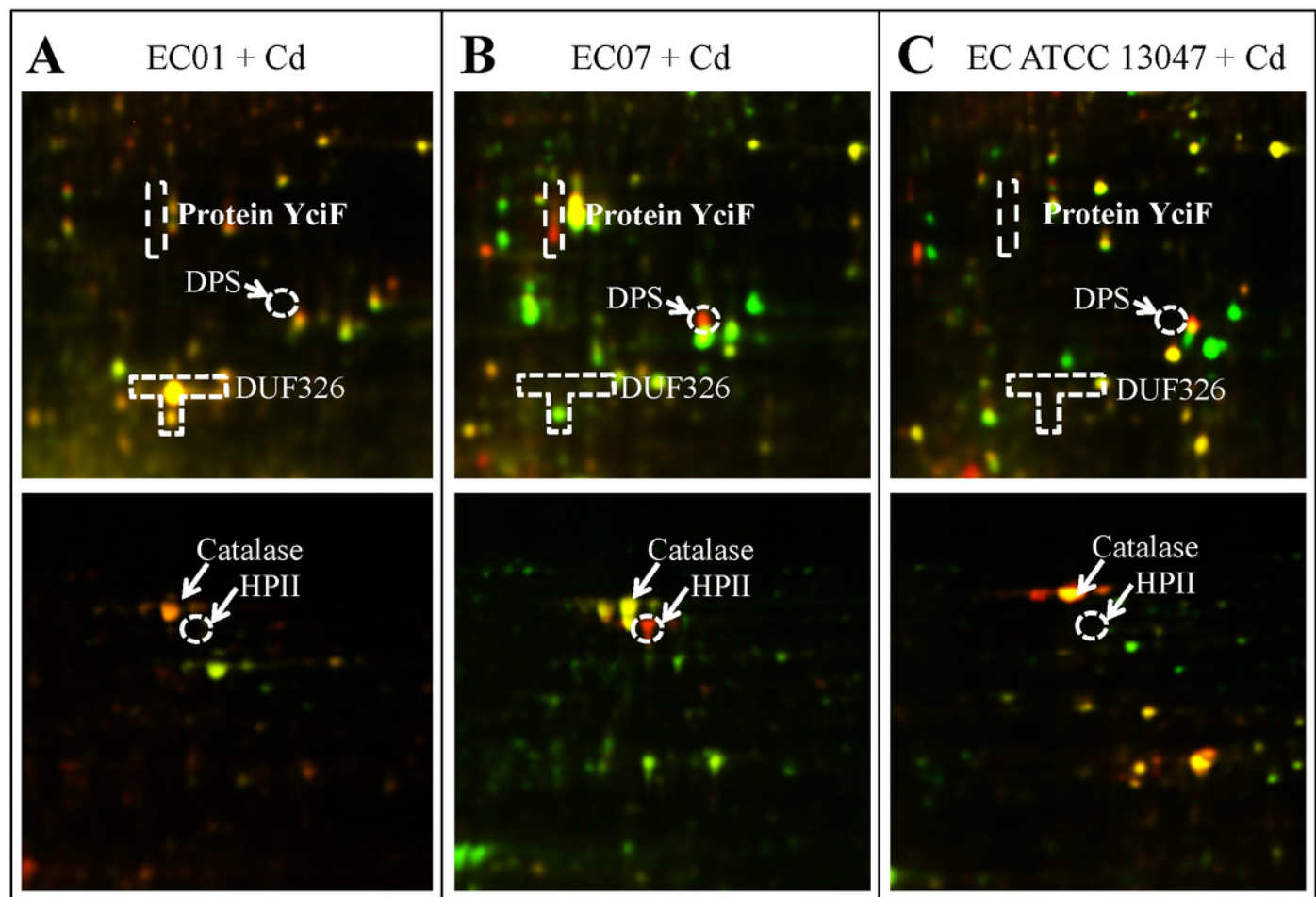



Figure 9

Amino acid sequences and the predicted tertiary structure of Domain of Uncharacterized Function (DUF326)

MLDEFKKCIESCYLCAVACDHCAASCLEEENLEMMRECIKLDMQCANICRLAAQ
FMALNSESARELCRVCADVCQQCGDECAKHEHEHCQNCSRACHHCAEQCRRMAA

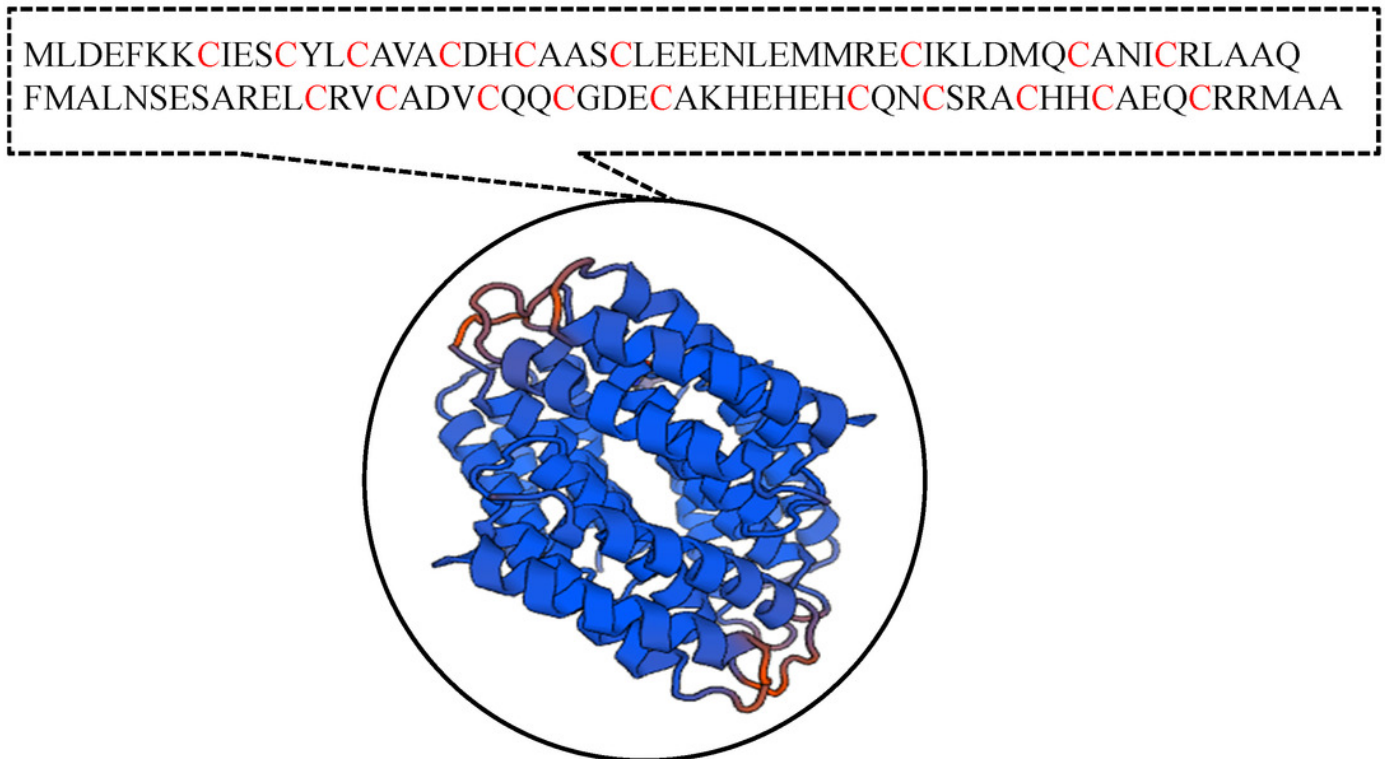



Figure 10

Relationship between genes encoding protein of hypothetical function DUF326 (from *Sporosarcina newyorkensis*) and other proteins as identified by the STRING software

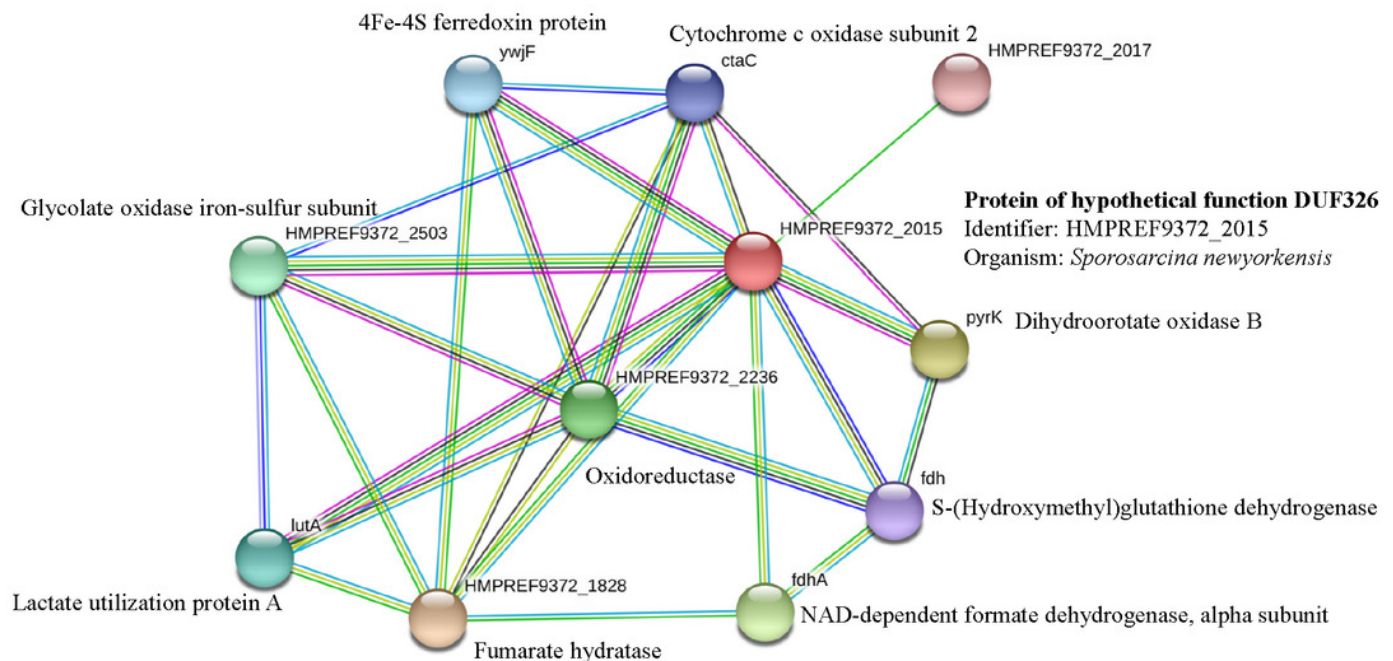



Figure 11

Relationship between genes encoding proteins of *E. cloacae* strains EC01, EC07 and ATCC 13047 involved in responses to cadmium stress as identified by the STRING software