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WHAT MAKES *A. guillouiae* SFC 500-1A ABLE TO CO-METABOLIZE PHENOL AND Cr(VI)? A PROTEOMIC APPROACH

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HIGHLIGHTS

Phenol induced β-ketoadipate pathway in presence and absence of Cr(VI)

Products of phenol degradation were metabolized through TCA and glyoxylate cycles

Two flavoproteins may be involved in Cr(VI) reduction to Cr(III)

Both phenol and Cr(VI) caused cellular stress and induced antioxidant response

Stress mitigation was based on redox reactions, cellular repairing and remodeling

ABSTRACT

Acinetobacter guillouiae SFC 500-1A is an environmental bacterium able to efficiently co-remediate phenol and Cr(VI). To further understand the molecular mechanisms triggered in this strain during the bioremediation process, variations in the proteomic profile after treatment with phenol and phenol plus Cr(VI) were evaluated.

The proteomic analysis revealed the induction of the β-ketoadipate pathway for phenol oxidation and the assimilation of degradation products through TCA cycle and glyoxylate shunt. Phenol exposure increased the abundance of proteins associated to energetic processes and ATP synthesis, but it also triggered cellular stress. The lipid bilayer was suggested as a target of phenol toxicity, and changing fatty acids composition seemed to be the bacterial

response to protect the membrane integrity. The involvement of two flavoproteins in Cr(VI) reduction to Cr(III) was also proposed. The results suggested the important role of chaperones, antioxidant response and SOS-induced proteins in the ability of the strain to mitigate the damage generated by phenol and Cr(VI).

This research contributes to elucidate the mechanisms involved in *A. guillouiae* SFC 500-1A tolerance and co-remediation of phenol and Cr(VI). Such information may result useful not only to improve its bioremediation efficiency but also to identify putative markers of resistance in environmental bacteria.

KEYWORDS

Simultaneous Bioremediation; Phenol; Chromium(VI); Proteomics; *Acinetobacter*

1. INTRODUCTION

Phenol and chromium are listed among the most dangerous environmental pollutants due to their toxicity and persistence [1, 2]. They are usually discharged together in diverse industrial effluents insufficiently treated, such as those from leather tannery, wood treatment plants, car manufacturing and petroleum refineries, which causes the high concentrations detected in natural waters worldwide [3]. Therefore, the development of cost-effective and non-disruptive techniques for their remediation has become a global priority in the last years, and bioremediation could be an interesting option for such purpose [4].

In this sense, many bacterial strains have demonstrated to be useful for biodegradation of phenols and other monoaromatic compounds through different oxidation pathways. However, heavy metals are known to be powerful inhibitors of such activity [5]. Similarly, several microorganisms display good performance for enzymatic reduction of Cr(VI) to the less toxic species Cr(III) employing aliphatic compounds as electron donors, but there is a limited number of strains capable of coupling aromatics degradation to Cr(VI) reduction [6-8]. Moreover, there is scarce information about physiological and molecular responses in bacteria during the simultaneous removal of these pollutants.

Acinetobacter guillouiae SFC 500-1A is an environmental isolate that efficiently co-remediates phenol and Cr(VI) in short time periods. Its ability to metabolize phenol through *ortho*-oxidation reactions was previously demonstrated. In addition, its enzymatic potential to reduce Cr(VI) to Cr(III) and immobilize it into the biomass was also studied [9]. Nevertheless, there are still many questions to be answered, such as the routes that drive the assimilation of phenol degradation intermediates, the main enzymes involved in Cr(VI) reduction, destination of contaminants and their derivatives, cellular damage caused by stress and antioxidant

response, among others. In this sense, having an overview of protein profiles during exposure to these contaminants might help to elucidate the molecular networks involved in the bioremediation potential of this strain.

In the present work, the proteomic variations of *A. guillouiae* SFC 500-1A in response to phenol and phenol plus Cr(VI) were evaluated by two dimensional electrophoresis coupled to mass spectrometry. The obtained results may help to understand the molecular mechanisms triggered by this strain and provide potential biomarkers for selecting new bacteria able to cope with these contaminants.

2. MATERIALS AND METHODS

2.1. Bacterial strain and culture conditions

A. guillouiae SFC 500-1A previously isolated from contaminated tannery sediments was used in this study [9, 10].

For proteomic experiments, the strain was pre-grown during 20 h in TY broth [11] supplemented with phenol and Cr(VI), centrifuged (10,000 g, 4°C, 15 min) and re-suspended in mineral medium plus 0.3% yeast extract (MMYE) [9] up to a cellular concentration of 2 x 10^9 CFU/ml. This bacterial suspension was employed to inoculate Erlenmeyer flasks (20% v/v) containing MMYE medium with and without pollutants and incubated at 28±2°C. Three conditions were tested by triplicate: medium MMYE (**control** condition), medium MMYE supplemented with phenol 300 mg/l (**phenol** condition) and with 300 mg/l phenol plus 10 mg/l Cr(VI) [**phenol+Cr(VI)** condition].

Residual phenol was measured hourly until the removal was around 50-75%. At this point growth and residual Cr(VI) concentration were also determined and cells were harvested and centrifuged (10,000 g, 4°C, 15 min). Pellets were washed three times with 0.85% NaCl and kept at -20°C.

Cr(VI) and phenol removal were evaluated by spectrophotometric methods, according to APHA-AWWA [12] and Wagner and Nicell [13], respectively.

2.2. Samples preparation for two dimensional electrophoresis (2DE)

Proteins extraction was carried out according Kim et al. [14] with modifications. For that, pellets were resuspended in Tris-HCl (20 mM; pH 8.0) and disrupted by ultrasonication. The obtained suspensions were treated with nucleases (final concentration: 50 µg/ml) and cell debris was removed by centrifugation (15,000 g, 30 min). Resulting supernatants were lyophilized and further rehydrated with buffer I [50 mM Tris-HCl; 0,3% w/v Sodium Dodecyl Sulphate (SDS); 0.2M dithioerythritol (DTE)], heated (95°C, 5 min) and then resuspended in IEF

buffer composed of 7M urea; 2 M thiourea; 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate (CHAPS); 1% DTE; 0.5% IPG buffer.
Protein concentration was determined using Bradford protein assay (BioRAD) and bovine serum albumin as standard.

2.3. 2DE

2DE was performed using the Immobiline polyacrylamide system. Isoelectric focusing (IEF) was carried out with pH 4-7 nonlinear, immobilized pH gradient strips (18 cm) employing Ettan™ IPGphor™ system (GE Healthcare, Uppsala, Sweden). Dry strips were rehydrated with 350 μL of IEF buffer containing 60 μg of proteins for 2D gel map construction, and 600 μg for protein identification through preparative gels. Electrical conditions were: 200 V for 7 h, from 200 V to 3500 V for 2 h, 3500 V for 2 h, from 3500 to 5000 V for 2 h, 5000 V for 3 h, from 5000 to 8000 V for 1 h, 8000 V for 3 h, 8000 V for a total of 80,000 Vh.
After IEF, strips were equilibrated using two buffers, the first composed of 6 M urea, 2% w/v SDS, 2% w/v DTE, 30% v/v glycerol and 0.05 M Tris-HCl pH 6.8 for 12 min and the second of 6 M urea, 2% w/v SDS, 2.5% w/v iodoacetamide, 30% v/v glycerol, 0.05 M Tris-HCl pH 6.8 and a trace of bromophenol blue for a further 5 min. SDS-PAGE was carried out at 40 mA/gel constant current on 9-16% SDS polyacrylamide linear gradient gels at 9°C. Analytical gels were stained with ammoniacal silver nitrate [15] and digitized with a Molecular Dynamics 300S laser densitometer (4000 × 5000 pixels, 12 bits/pixel; Sunnyvale, CA, USA) for spot detection and protein map construction. For protein identification, preparative gels were attached to a glass surface using Bind-Silane (γ-methacryloxypropyltrimethoxysilane) (LKB-Produkter AB, Bromma, Sweden), stained with SYPRO Ruby (Bio-Rad Laboratories, Hercules, CA) and digitalized with a Typhoon 9400 laser densitometer (GE Healthcare) [16, 17].

2.4. Image analysis and statistics

Two-dimensional image analysis was performed with Image Master Platinum 7.0 software (GE Healthcare). Spots were first detected and clarified by setting parameters such as filtering and smoothing. After that, gels belonging to each group were matched with intra-class reference gels known as *master gels* and then the three master reference gels were matched with each other. The algorithm of the software emphasized quantitative differences between gels, considering a spot to be differentially regulated when the mean relative percentage volume ratio (*%V=V single spot/V total spots)* was ± 2 and satisfied the T-test ($p \leq 0.05$).
Statistical analysis was performed by ANOVA test followed by *post hoc* Tukey test.

2.5. Protein identification by peptide mass fingerprinting

Differentially regulated spots were identified by peptide mass fingerprinting (PMF) using mass spectrometry (MS) as previously described [17]. Spots stained with Sypro ruby were mechanically excised with Ettan Spot Picker (GE Healthcare) and destained in 2.5 mM ammonium bicarbonate and 50% acetonitrile (ACN). After dehydration in ACN, the spots were rehydrated in trypsin solution and digested overnight at 37°C. Each digested protein was spotted onto the MALDI target, dried, covered with a matrix solution of α-cyano-4-hydroxycinnamic acid in 50% v/v acetonitrile and 0.5% v/v trifluoroacetic acid, and allowed to dry again. Peptide masses were acquired by ultrafleXtreme™ MALDI-ToF/ToF (Bruker Corporation, Billerica, MA, United States). PMF search was performed using MASCOT software (Matrix Science Ltd., London, UK, http://www.matrixscience.com). The search parameters were set as follows: Swiss-Prot/TrEMBL and NCBInr as databases, *Proteobacteria* as taxonomy, 100 ppm as mass tolerance, one missed cleavage site as acceptable, carbamidomethylation (iodacetamide alkylation of cysteine) as fixed modification, and oxidation of methionine as a possible modification. The biological functions of the identified proteins were searched in the UniProt database (www.ebi.uniprot.org) and NCBI database (www.ncbi.nlm.nih.gov).

3. RESULTS AND DISCUSSION

3.1. Growth and removal of phenol and Cr(VI) by *A. guillouiae* SFC 500-1A

A. guillouiae SFC 500-1A was able to grow in the presence of phenol and phenol plus Cr(VI), and to remove them in a few cultivation hours. Table 1 shows how bacterial growth was stimulated by phenol but negatively affected by Cr(VI).

Phenol degradation above 50% was detected in both conditions after 7 h, while Cr(VI) removal was around 38% in the same time period. At this time, samples were collected and proteomic assays were carried out.

These results were in agreement with previous reports that indicated the ability of *A. guillouiae* SFC 500-1A to simultaneously degrade phenol and reduce Cr(VI) to Cr(III) through enzymatic mechanisms [9].

3.2. Proteome variations associated with “phenol” and “phenol plus Cr(VI)” treatments

Phenol and phenol plus Cr(VI) caused statistically significant changes in the relative abundance of 87 identified protein spots, which were classified into 10 functional groups (Fig. 1). Not surprisingly, most of them are involved in cellular metabolism of carbon, nitrogen and fatty acids (CM, NM, FA/LPS, respectively), phenol degradation (PD), and energetic processes (EP).

The concentration of membrane proteins (MP) and proteins involved in signaling and chemotaxis also varied with the addition of pollutants.

Some enzymes related to the metabolism of aromatic compounds other than phenol (AM) were identified when the strain was exposed to phenol. The addition of Cr(VI) induced qualitative and quantitative proteome variations related to NM and CM, stress, fatty acids and LPS metabolism (FA/LPS), as well as transcription and translation processes (T&T).

Master gels for the three analyzed conditions are presented in Fig. 2.

3.3. Proteomic variations in *A. guillouiae* SFC 500-1A exposed to phenol

3.3.1. Phenol degradation, carbon assimilation and energetic processes

Phenol-exposition caused an increase in the relative abundance of proteins related to phenol degradation, such as phenol hydroxylase, catechol 1,2-dioxygenase, muconate cycloisomerase, muconolactone isomerase and 3-oxoadipate CoA-transferase (Table 2.1). Phenol hydroxylase catalyzes the initial reaction in phenol biotransformation to catechol, which is then cleaved by catechol 1,2-dioxygenase to cis, cis-muconate. These results suggest that phenol degradation in this strain occurs through the β-ketoadipate pathway, since catechol 1,2-dioxygenase, together with muconate cycloisomerase and muconolactone isomerase have been described as key enzymes for the degradation of aromatic compounds to β-ketoadipate. Furthermore, 3-oxoadipate CoA-transferase is essential for the degradation products to reach the TCA cycle [18, 19].

In addition, a decrease in the intracellular concentration of two key enzymes of the TCA cycle (2-oxoglutarate dehydrogenase and succinyl-CoA synthetase) was observed while the relative abundance of the isocitrate lyase was increased (Table 2.2). Isocitrate lyase is involved in the bypass of TCA cycle to glyoxylate cycle, an anaplerotic pathway to synthesize cellular precursors [20]. Such bypass is not usual in bacteria that carry out phenol degradation through β-ketoadipate formation, but it has been described in phenol-degrading bacteria through meta-fission pathway [21, 22]. A similar behavior was reported in *Acinetobacter* sp. DW-1 grown on a mixture of phenol and acetate and in *A. oleivorans* DR1 during hexadecane degradation [19, 23]. Given that glyoxylate shunt constitutes a shortcut for providing high biomass yield avoiding the CO_2-releasing steps [24], it is likely that *A. guillouiae SFC* 500-1A needs to synthesize cellular precursors at a rapid rate during phenol detoxification.

The high abundance of an electron transfer flavoprotein and the catalytic subunit (β) of the ATP synthase protein suggest an active energetic metabolism when this strain is grown in the presence of phenol (Table 2.3). Furthermore, the induction of enzymes associated to B vitamins synthesis (pyridoxine 5'-phosphate oxidase and 6,7-dimethyl-8-ribityllumazine

synthase) would indicate the need of cofactors for redox reactions during phenol oxidation and carbohydrates catabolism [25].

3.3.2. Metabolism of fatty acids

It is known that one of the main cellular targets of phenol toxicity is the phospholipid bilayer. For instance, phenol increases membrane fluidity, changes protein-lipid ratio and destabilizes its functioning. As a way to alleviate such effect, some bacterial cells are able to reorganize the fatty acids composition of their membrane in order to increase its rigidity [26].

In this context, the exposure of *A. guillouiae* SFC 500-1A to phenol caused an increase in the abundance of proteins associated to fatty acids biosynthesis and degradation (Table 2.4.1). For example, acyl-CoA synthetase is involved in fatty acid activation for the further β-oxidation, while acetyl-CoA carboxylase catalyzes the carboxylation of acetyl-CoA to malonyl-CoA, a precursor of fatty acids synthesis. Moreover, methylisocitrate lyase (MICL) was among the most strongly expressed enzymes in the presence of phenol (Table 2.4.2). MICL and 2-methylisocitrate dehydratase belong to the methylcitrate cycle, which is crucial for the clearance of toxic propionyl-CoA formed during β-oxidation of odd-chain and branched-chain fatty acids [27]. These results suggest a possible membrane restructuring in *A. guillouiae* SFC 500-1A exposed to phenol, reducing the levels of unsaturated branched-chain fatty acids that destabilize the lipid bilayer. Adjusting the saturation degree of fatty acids has played a major role in the homeostasis of cytoplasmic membrane viscosity in other *Acinetobacter* strains exposed to toxic substrates [28, 29].

Phenol led to a decrease in relative concentration of succinyl-CoA:3-ketoacid-coenzyme A transferase (SCOT) and 3-hydroxybutirate dehydrogenase (HBDH), enzymes involved in degradation of polyhydroxyalkanoates (PHA), but there was no evidence about induction of PHA synthesis (Table 2.4.3). A similar behavior has been observed in other bacteria under stress situations. Apparently, the release of acetoacetyl-CoA from PHA granules increases oxidative stress due to generation of NADH and $FADH_2$, which are utilized by the electron transport chain. Therefore, the inhibition of PHA depolymerization would operate as bacterial antioxidant response [30]. This finding is important from a biotechnological perspective considering that *A. guillouiae* SFC 500-1A could be a PHA producer, although this potential does not appear to be stimulated by phenol.

3.3.3. Membrane proteins and transporters

The content of the outer membrane protein Omp38 and the porin OprB was increased up to 2.7-fold after phenol exposure (Table 2.5).

Omp38 is an alternative name used to refer to OmpA, a family of monomeric proteins located in the outer membrane with a wide range of functions [31]. Among them, it is to be highlighted the emulsification of aromatic compounds, which increases their bioavailability and allow their incorporation into bacterial cells [32]. Therefore, this outer protein may be involved in phenol uptake by the strain SFC 500-1A, as it was already observed in other *Acinetobacter* strains [33].

OprB is an outer membrane porin with high affinity for glucose that is also able to non-specifically bind other compounds with hydroxyl groups [34]. In some pseudomonads, for example, OprB was involved in phenol uptake and its disruption altered the microbial behavior in presence of the contaminant [35, 36]. This background along with the results obtained from our proteomic study suggested that both membrane proteins may play some role in phenol uptake by this strain.

3.3.4. Nitrogen metabolism

The 2DE profiles of the phenol-stressed strain showed a decreased abundance of the nitrogen regulatory protein P-II and glutamine synthetase (Table 2.6.1). In general, P-II protein is up regulated during stationary phase and nutrient-limiting conditions, favoring the ammonium acquisition and assimilation into glutamine by glutamine synthase [37]. The down regulation of P-II has been described in another *Acinetobacter* strain as a mechanism to balance the uptake of nitrogen and carbon in order to avoid wasting of metabolic energy under stress [38].

The increased concentration of some enzymes responsible for amino acids metabolism also suggests no nitrogen limitation in *A. guillouiae* SFC 500-1A under the tested conditions (Table 2.6.2). Among them, the enzyme imidazole glycerol phosphate synthase plays a central role in normal nitrogen metabolism through histidine biosynthesis [39]. Additionally, D-alanine-D-alanine ligase and dihydrodipicolinate reductase are involved in the synthesis of peptidoglycan precursors (alanine and meso-diaminopimelate). Peptidoglycan is essential for cell wall stabilization in gram-negative bacteria and helps to decrease cell permeability to hydrophobic compounds. Modification of bacterial envelope composition caused by exposure to aromatic compounds is well documented [40, 41].

3.3.5. Transcription and translation processes

In cells of *A. guillouiae* SFC 500-1A grown on phenol, the expression levels of proteins involved in synthesis processes were variable. For example, the concentration of 30S ribosomal protein S1 and RNA polymerase (Table 2.7), as well as various enzymes involved in the amino acids metabolism (Table 2.6.2) was increased, but the abundance of 50S ribosomal protein L9 and

transcription termination factor NusA was decreased. A similar behavior was observed in strains of *A. baumannii* exposed to antimicrobial agents, which has been related to the typical increase in generation time and lag phase observed when bacteria are exposed to toxic chemicals [38, 42].

3.3.6. Stress response

Heat shock proteins, starvation proteins and molecular chaperones are commonly induced in bacteria in response to environmental stress [43]. Aromatic compounds such as phenol and organic solvents are known to be toxic to bacterial cells, as they can damage the cell membrane through lipids peroxidation and proteins release [44-46]. Thus, the strong expression of the peroxidase AhpC, the osmotically inducible protein OsmC and the chaperone DnaK may be a possible defense strategy of *A. guillouiae* SFC 500-1A against phenol toxicity (Table 2.8).

The alkyl hydroperoxide reductase AhpC is one of the best-characterized enzymes able to detoxify organic hydroperoxides in bacteria. It has been reported that AhpC acts as a key antioxidant protein involved in the survival of *Acinetobacter* strains exposed to different stressors [47, 48]. Recently, the combined activity of AhpC with the osmotically inducible protein OsmC has been demonstrated in other microorganisms as a defense mechanism against organic hydroperoxides [49].

Moreover, the induction of DnaK protein reflects the presence of misfolded proteins in the cytoplasm of *A. guillouiae*. This Hsp-70 chaperone plays an important role in the protection of newly formed proteins in bacteria under stress conditions, such as exposition to aromatic compounds [45, 47].

Following phenol treatment, the content of the AraC transcriptional regulator was around 50-fold increased (Table 2.9), suggesting the essential role of this protein in the response of *A. guillouiae* SFC 500-1A to phenol. In this sense, many transcriptional regulators for aromatics degradation in bacteria belong to AraC family [18]. They have also been involved in the resistance to oxidative stress agents, antibiotics and organic solvents [50].

3.3.7. Remediation of aromatic compounds different from phenol

The abundance of quercetin 2,3-dioxygenase and nitroreductases, which are involved in catabolism of flavonoids and nitroaromatic compounds, was increased after phenol addition (Table 2.10). Such enzymatic induction by structural analogues has been already detected in aromatic-degrading microorganisms and is usually employed as an acclimation strategy [51].

As it is well known, quercetin 2,3-dioxygenase catalyzes the breakdown of some flavonoids to generate protocatechuate [52]. Flavonoids are important compounds for plant-microorganism associations and cellular signaling. Bacteria able to degrade flavonoids have been pointed as good candidates for rhizosphere colonization and, consequently, may be suitable for assisted phytoremediation [53]. In addition, nitroreductases have a central role in the activation of nitroaromatic and nitroheterocyclic compounds for their further bioremediation, with influence on the environment and human health and significant biotechnological and medical potential [54].

Thus, this proteomic study demonstrated the capability of *A. guillouiae* SFC 500-1A for degrading phenol through the β-ketoadipate pathway, and also suggested its potential for bioremediation of nitro-substituted phenols and some kind of polycyclic compounds.

3.4. Proteomic variations in *A. guillouiae* SFC 500-1A exposed to phenol plus Cr(VI)

3.4.1. Phenol oxidation and carbon assimilation

The increased content of enzymes from the β-ketoadipate pathway proved that *A. guillouiae* SFC 500-1A is able to metabolize phenol even in the presence of Cr(VI) (Table 2.1). Nevertheless, the differences observed in the abundance of these proteins between treatments could explain the negative effect of Cr(VI) on phenol degradation efficiency previously reported for this strain [9].

The addition of Cr(VI) also affected the central pathways of carbon assimilation. On the one hand, the level of the TCA enzymes 2-oxoglutarate dehydrogenase and succinyl-CoA synthetase decreased. On the other hand, unlike that observed in the presence of phenol, the glyoxylate shunt seems not to have occurred (Table 2.2). It is possible that the lesser rate of phenol degradation generated a lower concentration of acetyl-CoA and succinate to be incorporated into assimilatory pathways. These changes could also be a strategy carried out by this strain to decrease the flux through the electron transport chain and mitigate the oxidative damage caused by the combination of both pollutants, as it has been observed in other bacteria under stress conditions [30, 55]. The induction of succinate dehydrogenase (SQR) is framed in this scenario. In this sense, Yankovskaya et al. [56] found that SQR prevents the reactive oxygen species (ROS) formation through succinate oxidation and flavins reduction in *E.coli* under aerobic conditions.

3.4.2. Cr(VI) metabolism and stress-associated response

It has been previously demonstrated that *A. guillouiae* SFC 500-1A is able to reduce Cr(VI) to Cr(III) through soluble NADH dependent chromate reductases [9]. However, these enzymes have not yet been identified. Our proteomic study revealed that its exposure to phenol

plus Cr(VI) caused a significant increase in cellular concentration of enzymes involved in redox reactions, such as ferredoxin-NADP reductase (Fpr), dihydrolipoamide dehydrogenase (DLD) and alkyl hydroperoxide reductase (AhpC) (Table 2.8). These enzymes may be implicated in Cr(VI) reduction and stress mitigation and could help to elucidate what happens in the strain during the simultaneous bioremediation process.

In certain bacteria, Fpr and DLD proteins carry out a two-step Cr(VI) reduction in which the electron transfer from NADPH to Cr(VI) is mediated by FAD [57- 59]. Therefore, a possible role of these flavoproteins in the Cr(VI) reducing potential of *A. guillouiae* SFC 500-1A may be proposed. The possible overproduction of riboflavin caused by an increase in the cellular concentration of 6,7-dimethyl-8-ribityllumazine synthase and riboflavin synthase (Table 2.3) could also be related to Cr(VI) reduction to Cr(III). Riboflavin is a FAD precursor crucial for Cr(VI) tolerance and detoxification in different microorganisms [60-62].

It is well known that Fpr and DLD proteins produce the transient radical Cr(V), which cycles redox and creates ROS, damaging macromolecules and altering cellular processes [57, 58]. In this regard, the increased abundance of RecA recombinase and HscA chaperone in cells of *A. guillouiae* SFC 500-1A would indicate damage to DNA and proteins and demonstrates the effort made by the strain to protect these macromolecules (Table 2.8). RecA belongs to SOS system, which can repair some DNA injuries produced by redox-active intermediates Cr(V/IV) and Cr(III)-DNA adducts [63]. Meanwhile, HscA is a specialized chaperone involved in [Fe-S] proteins refolding and maturation under different stress conditions [64, 65]. As it can be deduced from Table 2.7, DNA transcription and translation processes were also affected in this strain like in other Cr(VI)-exposed bacteria [66].

The alkyl hydroperoxide reductase AhpC and various enzymes involved in cysteine metabolism were also found to increase after exposition to phenol and Cr(VI) (Table 2.6.2.3). Both AhpC and cysteine probably contribute to offset the cell damage generated during the co-remediation process considering that AhpC is a thiol-specific antioxidant and cysteine constitutes an efficient reducing agent. The involvement of AhpC in ROS scavenging and bacterial protection against multiple abiotic stresses is well documented [67] and the up-regulation of cysteine biosynthesis constitutes a defense mechanism in other chromate-stressed microorganisms [62]. Furthermore, a relation has been established between the levels and the redox state of cysteinyl groups of certain molecules and the activity of AhpC in cellular response to stress [68].

The abundance of several proteins related with PHA synthesis and mobilization was also modified in response to phenol and Cr(VI) (Table 2.4.3). This is the case of protein 3-ketoacyl-ACP reductase, a supplier of 3-hydroxyacyl-CoA for the synthesis of PHA [69], whose cell

content increased 2.4 fold, while the relative abundance of SCOT and HBDH (involved in their degradation) was low, in agreement with the results obtained in the phenol treatment. These results suggest that PHA accumulation occurred when *A. guillouiae* SFC 500-1A was exposed to both pollutants, as reported for other Cr(VI)-resistant bacteria [37]. PHA synthesis and the inhibition of their degradation are important defense mechanisms under oxidative stress conditions. Additionally, it has been demonstrated that the polymer endows bacteria with enhanced survival, competition abilities and tolerance to toxic substances [70].

In addition to intracellular response, it is known that cellular envelope and extracellular polymeric matrix play a key role in protection of Gram negative bacteria from toxic compounds. Capsular lipopolysaccharides (LPS) and exopolysaccharides (EPS) have been recognized as substantial factors for chromium tolerance [43, 71]. Therefore, the high concentration of the enzymes D-arabinose 5 phosphate isomerase and UDP-glucose 4-epimerase (Table 2.4.4) could be related to some kind of mechanism for external protection against the metal, since they are essential enzymes for extracellular glycolipids and glycoproteins synthesis. The abundance of OmpW was also significantly increased in the presence of phenol and Cr(VI) when compared to control and phenol-only treatments. Although the biological function of this protein remains largely uncharacterized, the participation of OmpW in bacterial protection against environmental stressors, resistance to antibiotics and virulence has been reported [72]. Similarly, the cellular concentration of AraC transcriptional regulator increased 1.4 times regarding only-phenol treated cells and was 65 times above than in control conditions. This result indicates that AraC protein may play crucial role defending the strain against phenol and Cr(VI) toxicity but also regulating in the bioremediation process.

Overall, the current results show that phenol and Cr(VI) significantly altered the proteome profile of *A. guillouiae* SFC 500-1A so that the bacterium could develop a proper response against these environmental pollutants.

4. CONCLUSIONS

The evaluation of proteome variations in *A. guillouiae* SFC 500-1A exposed to phenol and Cr(VI) served as a useful strategy for analyzing the bacterial response to these pollutants and designing a possible model to understand its bioremediation ability (Fig. 3).

After uptake, probably mediated by the OmpA-like porin Omp38 (Fig. 3.1), phenol is hydroxylated to catechol and further *ortho*-oxidated to β-ketoadipyl-CoA, a precursor of acetyl-CoA and succinate (Fig. 3.2). The assimilation of these phenol degradation products

would be associated to the bypass of TCA cycle to glyoxylate cycle (Fig. 3.3) to generate biomass and promote the cellular growth.

In spite of constituting a carbon source for many microorganisms, phenol is a strong inducer of cellular damage. In this strain, phenol toxicity would be associated to the oxidation of membrane fatty acids followed by hydroperoxides generation, damage to other structures and alteration of cellular processes. Restructuring the phospholipid composition seems to be a strategy of *A. guillouiae* SFC 500-1A to keep the membrane integrity (Fig. 3.4). Additionally, the activation of the methylcitrate cycle could be explained as a mechanism to prevent the accumulation of toxic propionyl-CoA generated during β-oxidation of odd-chain and branched-chain fatty acids (Fig. 3.5). The defense response would also include increasing the cellular concentration of hydroperoxide resistance enzymes (AhpC and OsmC) for stress mitigation and chaperone DnaK to protect newly formed proteins (Fig. 3.6).

The exposure to phenol and Cr(VI) caused more harm to the growth and energetic metabolism of the strain. Nevertheless, *A. guillouiae* SFC 500-1A was able to co-remediate these contaminants, oxidizing phenol through the β-ketoadipate pathway and removing Cr(VI) simultaneously by chromate reductases. Two flavoproteins, Fpr and DLD (Fig. 3.7), possibly associated with cofactors derived from riboflavin, could be involved in Cr(VI) reduction to Cr(III). The negative effect of both pollutants on cell growth may be related with the oxidative stress generated during the bioremediation process, strongly altering cellular processes such as transcription and translation and generating a significant damage to DNA and proteins. The assembly control of specialized [Fe-S] proteins by HscA chaperon and the activation of the SOS response that involves RecA protein for DNA repairing can be mentioned among the possible mechanisms displayed to alleviate the damage (Fig. 3.6). The oxidative stress mitigation together with the redox state regulation appears to be crucial for the survival of *A. guillouiae* SFC 500-1A exposed to both pollutants. Unlike the scenario with phenol-only treatment, in the presence of phenol and Cr(VI) the bacterium would display an active metabolism of cysteine (Fig. 3.6), a provider of thiol groups that are essential for maintaining an intracellular reducing environment. The increase in the polysaccharide matrix could also be mentioned as a bacterial response to Cr(VI), possibly involved in the extracellular protection from the metal (Fig. 3.8). Therefore, the ability of this strain to grow in the presence of phenol and remediate it efficiently could be explained as a successful balance between its assimilation and the stress mitigation. Moreover, the bacterium showed a metabolic machine adapted for phenol assimilation even in the presence of Cr(VI) and the enzymatic potential to simultaneously reduce Cr(VI) to Cr(III). The effort to mitigate the cellular stress was more evident during the co-remediation process, resulting in less growth and a reduced energy metabolism. It is likely

for the transcriptional regulator AraC, highly expressed in phenol exposed cells and even more in the presence of Cr(VI) (Fig. 3.9), to play some role in such bacterial response to contaminants. The implication of the porin OmpW in Cr(VI) resistance also remains to be clarified.

The results of this study are in agreement with previous findings that demonstrated the ability of this strain to co-remediate phenol and Cr(VI) and also provide information about potential target proteins and processes for improving the bioremediation of these pollutants. Additionally, they suggest other possible biotechnological skills of A*. guillouiae* SFC 500-1A, such as bioremediation of other phenolic compounds and synthesis of PHA, interesting to be addressed in future studies.

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FIGURE CAPTIONS

Fig. 1. Number of identified protein spots whose abundance varied following *A. guillouiae* sp. SFC 500-1A exposure to phenol and phenol plus Cr(VI). Categories: PD (Phenol degradation), CM (Carbon metabolism), EP (Energetic processes), FA/LPS (Fatty acids and LPS metabolism), MP (Membrane proteins and transporters), NM (Nitrogen metabolism), T&T (DNA transcription and translation), Stress (Stress processes and antioxidant response), Sig&Chem (Signaling, regulation and chemiotaxis), AM (Metabolism of aromatic compounds).

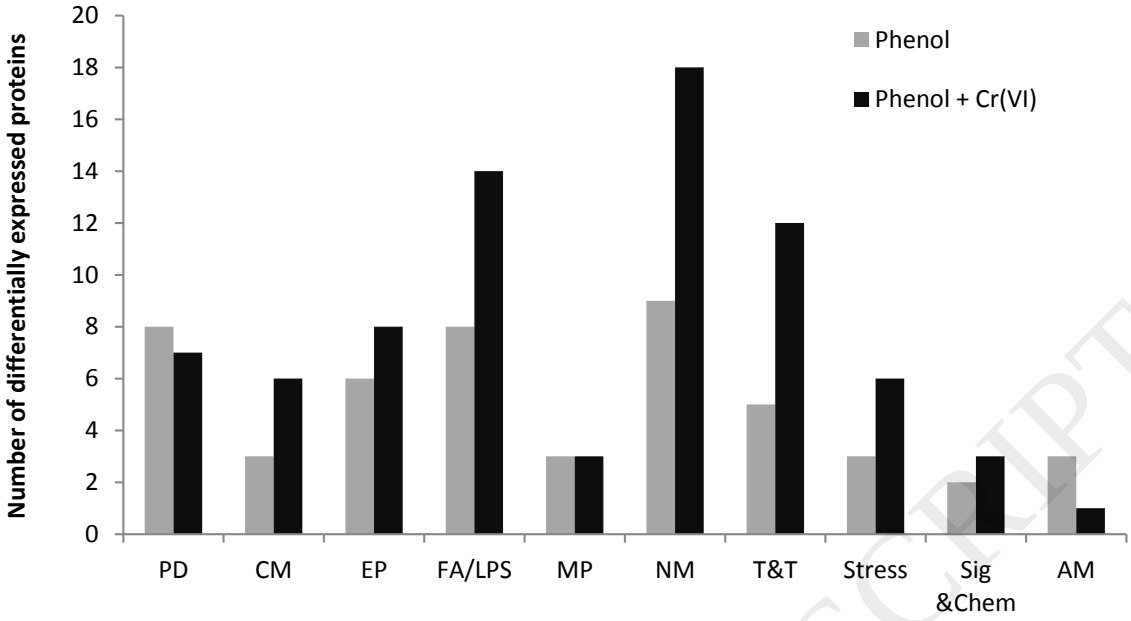

Fig. 2. Representative 2DE gels showing the proteome profile of *A. guillouiae* sp. SFC 500-1A in (A) control condition and in the presence of (B) phenol and (C) phenol plus Cr(VI). The identified protein spots whose relative abundance varied between the control condition and each treatment are indicated with numbers (corresponding to those reported in Table 2).

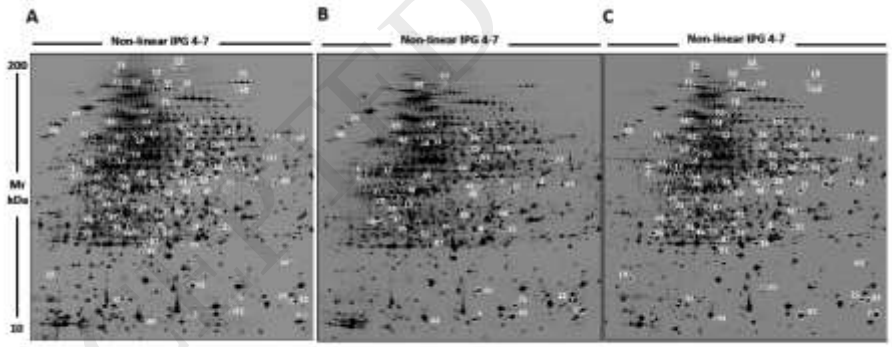

Fig. 3. Schematic representation of cellular changes in *A. guillouiae* SFC 500-1A following phenol or phenol plus Cr(VI) exposition. The increased and decreased proteins are marked by ↑ and ↓, respectively. Superscripts indicate the condition in which proteins

or processes registered variations: (P): Phenol, (P+C): Phenol plus Cr(VI), (P) (P+C): Both phenol and phenol plus Cr(VI). Protein abbreviations are included in Table 2. Numbers indicate: 1. Phenol uptake; 2. Phenol degradation through β-ketoadipate pathway; 3. Glyoxylate shunt; 4. Membrane restructuration; 5. Propionyl-CoA degradation by methylcitrate cycle; 6. Cellular defense against pollutants toxicity; 7. enzymatic reduction of Cr(VI) to Cr(III); 8. Production of polysaccharide matrix as extracellular response; 9. Transcriptional regulation by AraC protein.

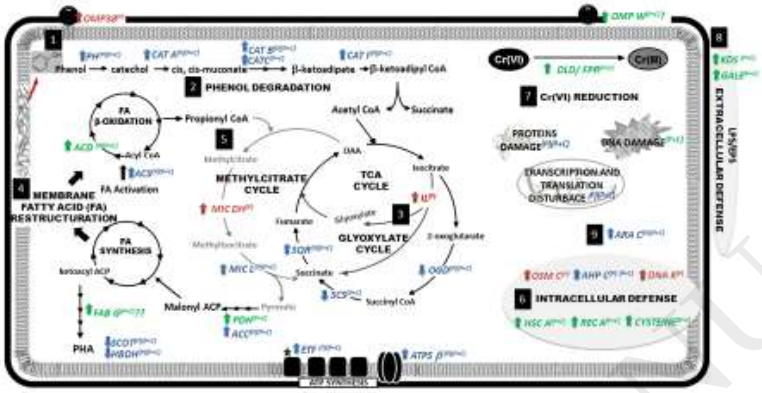

Table 1.

Bacterial growth and contaminants removal.

	Control	**Phenol[*1]**	**Phenol[*1] + Cr(VI) [*2]**
Initial cell count (CFU/ml)	2.8 x 10^8 (±0.3 x 10^8)	3.1 x 10^8 (±0.2 x 10^8)	3.0 x 10^8 (±0.3 x 10^8)
Final cell count (CFU/ml)	1.1 x 10^9 (±0.8 x 10^8)	1.7 x 10^9 (±0.9 x 10^8)	8.4 x 10^8 (±0.6 x 10^8)
Removed phenol (%)	-	60.8 (±2.3)	54.2 (±1.2)
Removed Cr(VI) (%)	-	-	38 (±3.9)

[*1] Initial concentration 300 mg/l

[*2] Initial concentration 10 mg/l

Table 2.
Differential abundance of proteins identified by MALDI-ToF MS in *A. guillouiae* SFC 500-1A exposed to phenol and phenol plus Cr(VI).

Spot	Protein name/classification	Abbreviation	Accession code	Theoretical		Mascot results			Average fold change*		
				pI	MW (KDa)	Score	Coverage	Matched peptides	Phenol/ Control	Ph+Cr(VI)/ Control	Ph+Cr(V)/ Phenol
	1 Phenol degradation										
1	Phenol hydroxilase (sub DMS)	PH(DMS)	gi|2605613	4.7	39.7	226	70	21	+6.6	+3.7	-1.7
2	Phenol hydroxilase (sub P5)	PH (P5)	gi|490859324	4.7	39.8	161	42	13	+6.5	+3.0	-2.1
3	Phenol hydroxilase	PH	gi|490859323	5.5	13.9	127	60	7	+8.4	ND	-4.1
4	Phenol hydroxilase (sub P1)	PH (P1)	gi|490859317	5.8	38.6	196	50	17	+12.3	+11.3	ND
5	Catechol 1,2-dioxygenase	CAT A	gi|490859513	4.9	34.6	166	64	16	+19.9	+11.2	-1.8
6	Muconate cycloisomerase 1	CAT B	gi|490859524	5.5	39.9	109	24	7	+17.7	+12.7	ND
7	Muconolactone delta-isomerase	CAT C	gi|490958629	5.7	11.4	101	48	7	+32.5	+32.9	ND
8	3-oxoadipate CoA-transferase (sub. A)	CAT I	gi|674995151	5.3	24.3	172	63	17	+26.9	+19.9	-1.3
	2 Carbon assimilation (TCA cycle, glyoxylate cycle, glycolysis)										
9	Isocitrate lyase	IL	gi|490994972	5.3	59.8	209	34	20	+2.0	ND	-2.8
10	2-oxoglutarate dehydrogenase (E1)	OGD 1	gi|490855238	5.8	106.1	364	44	39	ND	-4.5	-6.3
11	2-oxoglutarate dehydrogenase (E2)	OGD 2	gi|490855236	5.2	43.5	229	49	21	-1.4	-2.1	-1.5
12	Succinyl-CoA synthetase (sub. β)	SCS	gi|490855232	4.9	41.8	254	68	26	-1.9	-2.1	ND
13	Succinate dehydrogenase	SQR	gi|490855242	5.7	59.7	203	37	20	ND	+2.4	ND
14	Enolase	ENO	gi|490857888	4.9	46.2	237	48	18	ND	-2.2	-1.8
15	Aconitate hydratase 2	AH 2	gi|490858864	5.0	95.1	356	44	33	ND	-1.9	-2.0
	3 Energetic processes/biosynthesis of cofactors, prosthetic groups and carriers										
16	ATP synthase (sub β)	ATPS β	gi|490861379	5.1	50.3	173	50	17	+4	+3.2	ND
17	ATP synthase (sub β)	ATPS β	gi|490794371	5.1	50.3	105	32	10	ND	+3.1	+3.1
18	ATP synthase (sub α)	ATPS α	gi|490861381	5.2	55.6	226	39	21	-2.3	ND	ND
19	ATP synthase (sub δ)	ATPS δ	gi|490961892	4.6	19.3	93	44	5	ND	+1.8	+2.2
20	Electron transfer flavoprotein (sub α)	ETF	gi|490855183	4.9	31.4	248	89	20	+19	+6.8	-2.0
21	Pyridoxine 5'-phosphate oxidase	PDX H	gi|490856558	5.7	25.6	138	67	10	+2.2	+2.3	ND
22	6,7-dimethyl-8-ribityllumazine synthase	RIB 4	gi|490856707	5.8	16.4	189	75	14	+4.2	+3.4	ND
23	Riboflavin synthase (sub α)	RIB 5	gi|490863253	5.4	23.8	103	46	7	ND	+2.4	+1.7

24	ATP-binding protein	PSTB	gi|490863632	5.4	56.5	258	51	22	+2.2	+1.7	-1.3
	4 Metabolism of fatty acids and lipooligosaccharides										
	4.1 Fatty acids synthesis/ oxidation										
25	Acyl-CoA synthetase	ACS	gi|674994296	5.5	61.8	108	24	10	+2.8	+2.0	ND
26	Acetyl-CoA carboxylase	ACC	gi|690996038	5.4	50.8	134	28	12	+2.0	+2.0	ND
27	Acetate kinase	ACK	gi|736601335	5.9	44.2	123	37	10	+2.6	+2.7	ND
28	Acyl-CoA dehydrogenase	ACD	gi|736607308	5.7	37.2	113	41	13	ND	+2.4	+2.8
29	Pyruvate dehydrogenase (sub. E1)	PDH	gi|493630557	5.4	102	138	15	13	ND	+2.2	+1.8
30	Pyruvate dehydrogenase (acetyl-transf)	PDH	gi|490856570	5.3	101	303	47	39	ND	+2.6	+2.1
31	Long-chain fatty acid-CoA ligase	ACSL	gi|490858943	5.6	60	244	57	23	ND	-2.5	-2.9
32	Acetyl-CoA carboxylase (biotin carrier)	ACC	gi|490857748	4.9	14.7	104	45	7	ND	-2.8	-2.1
	4.2 Methylcytrate cycle										
33	Methylisocitrate lyase	MICL	gi|490858658	4.9	32.6	123	36	12	+181	+68	-2.5
34	2-methylisocitrate dehydratase	MICDH	gi|490860929	5.1	95.9	206	29	21	+2	ND	-1.9
	4.3 PHA synthesis/degradation										
35	Succinyl-CoA:3-ketoacid-CoA transferase	SCOT	gi|490859391	4.9	25.3	116	54	10	-6.7	-3.4	ND
36	3-hydroxybutirate dehydrogenase	HBDH	gi|674995203	5.6	27.6	162	46	13	-2.9	-2.2	ND
37	3-ketoacyl-ACP reductase	FAB G	gi|490861494	4.8	27.1	143	53	11	-2.1	+2.4	+5.2
	4.4 Synthesis of capsular LPS and EPS										
38	D-arabinose 5-phosphate isomerase	KDS	gi|490862304	5.5	35.8	118	34	9	ND	+2.4	+1.5
39	UDP-glucose 4-epimerase	GALE	gi|490961831	5.1	37.3	252	67	22	ND	+3.6	+3.7
	5 Transmembrane proteins/ transporters										
40	Glucose-inducible porin	OPR B	gi|490861972	5.6	47.3	154	39	11	+2.3	ND	-1.8
41	Outer membrane protein 38	OMP 38	gi|490860976	5.7	37.2	182	45	14	+2.7	-5.2	-14
42	Family type VI secretion protein	EVP B	gi|490961779	5.0	55.5	10	47	17	-1.9	-2.2	ND
43	Outer membrane protein W precursor	OMP W	gi|514347513	5.6	21.9	126	39	8	ND	+6.3	+4.4
	6 Nitrogen metabolism										
	6.1 Nitrogen assimilation										
44	Nitrogen regulatory protein P-II	NRP PII	gi|490863245	5.4	12.2	202	83	13	-1.8	-3.3	-1.8
45	Glutamine synthetase	GLNS	gi|490862518	5.1	52.4	217	51	22	-5.3	-2.2	-2.0

	6.2 Aminoacids metabolism										
46	D-alanine-D-alanine ligase	DDL	gi|490856584	4.9	33.6	171	53	13	+2.1	+1.6	-1.3
47	Dihydrodipicolinate reductase	DAP B	gi|490856795	5.5	28.5	165	44	11	+++	+++	-2.1
48	tRNA dimethylallyltransferase	MIAA	gi|490858824	5.5	36.2	133	35	9	+2.7	+2.7	ND
49	Branched-chain aminotransferase	BCAAS	gi|490861095	5.9	34.5	182	49	13	+2.6	+2.1	ND
	6.2.1 Aromatic amino acids										
50	Imidazole glycerol phosphate synthase	HIS F	gi|490954769	5.3	27.3	196	63	15	+9.3	+8.7	ND
51	Tyrosyl-tRNA synthetase	TYRS	gi|490856878	5.4	44.9	250	61	19	+1.5	+2.7	+1.7
52	Fumarylacetoacetase	FAA	gi|490855304	5.5	48.8	182	36	14	-2.2	-7.3	-3.5
53	DAHP synthase	DAHPS	gi|736607028	5.7	39.1	179	59	15	ND	+2.4	+2.2
54	4-hydroxyphenylpyruvate dioxygenase	HPPD	gi|490855316	4.9	40.1	276	58	31	ND	-2.1	-1.7
55	Histidine ammonia-lyase	HAL	gi|490859758	5.2	54.8	164	36	19	ND	-4.6	-3.0
	6.2.2 Glutamate										
56	Glutamine amidotransferase	GAT	gi|490855407	5.3	21	131	54	8	ND	+2.0	+1.8
57	Glutamate dehydrogenase	GDH	gi|490859749	5.7	46.5	143	43	13	ND	-3.3	-3.5
	6.2.3 Sulfur-containing amino acids										
58	S-adenosylmethionine synthase	MET K	gi|490857422	5.5	42.3	184	55	18	ND	+3.2	+4.0
59	Methionyl-tRNA formyltransferase	FMT	gi|736609637	5.3	34.7	119	42	10	ND	+2.7	ND
60	Homocysteine methyltransferase	HMT	gi|754745926	6.7	13.9	128	50	8	ND	+4.0	+2.2
61	Methionine synthase	MET E	gi|490957454	5.4	38.9	173	45	14	ND	-2.8	-1.8
	7 DNA transcription and translation /Nucleotides metabolism										
62	DNA-directed RNA polymerase (Sub. β)	RNA POL	gi|490856432	5.4	152	103	14	15	+3.8	+2.2	ND
63	DNA-directed RNA polymerase (Sub. β)	RNA POL	gi|497799882	5.3	152	138	12	16	ND	---	---
64	30S ribosomal protein S1	30S S1	gi|514347225	5.0	61.4	102	43	27	+2.2	+7.2	+3.2
65	50S ribosomal protein L9	50S L9	gi|490857291	5.7	15.7	155	68	11	-1.9	-40	-21
66	Transcription termination factor NusA	NUS A	gi|490856294	4.45	54.9	270	45	24	-2.4	-2.7	ND
67	Transcriptional regulator Crp	CRP	gi|490859097	4.97	26.7	217	69	18	ND	-2.3	-1.9
68	5'-nucleotidase surE	SUR E	gi|490859229	4.81	28.3	172	53	12	-2.2	-1.9	ND
69	RNA-binding protein	RBP	gi|490863328	5.69	87.2	505	60	44	ND	-7.0	-8.4
70	Elongation factor Tu	EF TU	gi|490861613	5.14	43.1	156	47	18	ND	-2.6	-2.2
71	Trigger factor	TF	gi|490864495	4.78	49.6	289	39	21	ND	-2.0	-1.6
72	Prolyl-tRNA synthetase	PROS	gi|736607424	5.05	63.1	271	53	27	ND	-2.2	ND
73	Phosphoribosyl formylglycinamidine synthase	FGAR	gi|490960834	5.00	140.1	142	20	17	ND	-2.4	-1.8

	8 Stress processes/Antioxidant response										
74	Chaperone protein DnaK	DNA K	gi|491176517	4.73	69.6	195	26	13	+2.8	ND	-3.1
75	Osmotically inducible protein C	OSM C	gi|490856544	5.91	14.9	104	49	6	+2.4	ND	ND
76	Peroxidase Ahp	AHP C	gi|736606013	5.40	23.9	133	40	8	+2.0	+3.0	+1.4
77	Ferredoxin-NADP reductase	FPR	gi|490859866	5.16	29.3	142	55	14	ND	+2.1	+2.5
78	Fe-S protein assembly chaperone HscA	HSC A	gi|490858594	5.25	67.8	106	42	19	ND	+4.0	+1.4
79	Recombinase RecA	REC A	gi|490857713	5.36	37.7	134	72	17	ND	+3.1	+1.7
80	Dihydrolipoamide dehydrogenase	DLD	gi|490864439	5.79	50.7	114	23	8	ND	+2.5	+2.1
81	Alkyl hydroperoxide reductase (Sub C)	AHP C	gi|490859781	5.03	20.8	141	51	9	ND	+2.5	+1.5
	9 Cell regulation and chemiotaxis										
82	AraC family transcriptional regulator	ARA C	gi|490856041	5,68	38.5	355	69	22	+46.0	+64.5	+1.4
83	Carbon storage regulator	SCR A	gi|490922332	5.89	9.9	114	56	11	ND	-3.6	-4.0
84	Chemotaxis protein CheY	CHE Y	gi|490860813	4.98	27	191	75	16	-1.4	-2.1	ND
	10 Metabolism of aromatic compounds										
85	Quercetin 2,3-dioxygenase	QR	gi|736602565	5.08	35.4	159	41	12	+2.4	ND	-1.6
86	Quercetin 2,3-dioxygenase	QR	gi|490856510	5.27	31.9	127	53	14	+6.6	ND	-3.0
87	Nitroreductase	NR	gi|736609117	5.22	21.8	310	64	20	+5.5	+2.3	-2.3

*Fold change is the ratio of protein abundance between the treatments. “+” and “–” indicate increased and decreased proteins, respectively. “+++” indicates the spots that only appeared in phenol and phenol plus Cr(VI) treatments, not in the control. “---“ indicates the spots that disappeared in phenol plus Cr(VI) treatment. ND: there were no significant differences in the relative abundance of a spot between two conditions.