*Significance

Identification of proteins modulated by nickel, cobalt and cadmium in the cyanobacterium *Synechocystis* sp. PCC 6803

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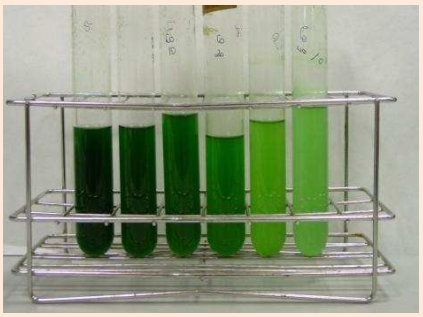
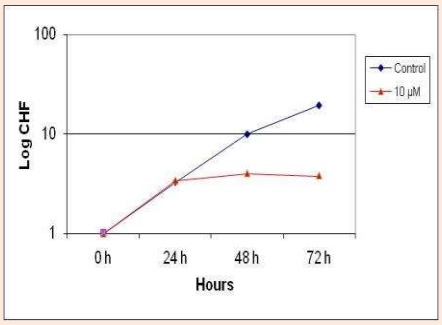
Significance: This is the first report on the proteomic profile of *Synechocystis* sp. PCC 6803 wild type and mutant strains for the identification of proteins affected by the heavy metals Ni, Co and Cd. We have identified proteins commonly responsive to all three metals and also chaperones specifically modulated by each metal. Our data also supports previous studies that suggest the existence of additional sensor systems for Co.

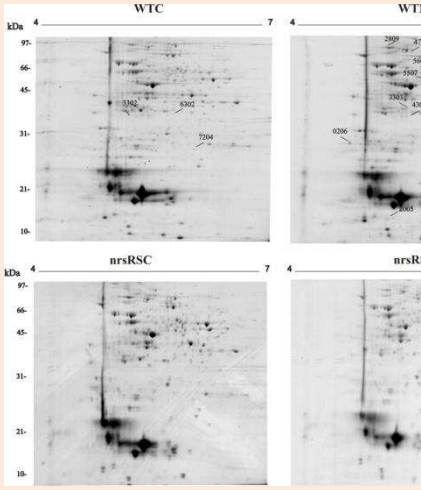
*Graphical Abstract (for review)

Effect of metal on growth



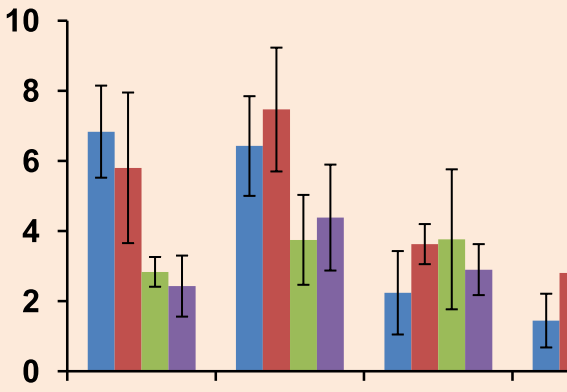


Differential proteo



Protein identification and validation

Spot	Gene ID	Protein description	NCBI Accession #	Fold change[a] WTNi/WTC	Fold change[a] nrsRSNi/nrsRSC
0206	slr0793	cation efflux system protein involved in nickel and cobalt tolerance	NP_442628	exclusive to WTNi	-
2005	sll1514	Chaperone (hspA)	NP_440316	exclusive to WTNi	-
2809[b]	slr1463	elongation factor G	NP_442851	1.75	-1.11
	sll1553	phenylalanyl-tRNA synthetase subunit beta	NP_441710	1.75	-1.11
3302	slr0513	periplasmic iron-binding protein	NP_442521	-4.69	-4.15
3303	sll1578	phycocyanin α subunit	NP_440551	-1.53	-1.51
4303[b]	slr0244	hypothetical protein	NP_440027	-1.85	-1.25
		phycocyanin associated linker protein	AB058393	-1.85	-1.25
4702	sll1987	catalase HPI	NP_441295	-1.8	2.64
5301	sll1260	30S ribosomal protein S2	NP_441467	1.63	-1.33
5507	slr1133	argininosuccinate lyase	NP_440604	2.35	-1.26
5607	sll1435	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B	P74215	2.77	3.2
6302	slr1643	ferredoxin-NADP oxidoreductase	NP_441779	1.62	1.66
6603	slr1322	putative modulator of DNA gyrase	NP_441393	2.10	1.08
6605	slr1550	lysyl-tRNA synthetase	NP_440803	-1.66	Exclusive to nrsRSC
6608	slr0118	thiamine biosynthesis protein ThiC	NP_442586	2.11	1.10
7204	slr1161	hypothetical protein	NP_441650	-1.75	1.08
7407	slr0536	uroporphyrinogen decarboxylase	NP_442753	-1.57	1
7601	slr1945	phosphoglyceromutase	NP_441933	-1.75	-2.0
8502	slr1165	sulfate adenylyltransferase	NP_441655	1.95	1.02
8511	slr1176	glucose-1-phosphate adenylyltransferase	NP_443010	-1.9	1.44
8601	slr0009	ribulose bisophosphate carboxylase	NP_442120	-2.0	-2.68



Highlights

- First report of proteomic profile of *Synechocystis* sp. PCC 6803 wild type and mutant strains in response to excess Ni, Co and Cd.

- Identification of proteins commonly responsive to all three metals.

- Identification of chaperones specifically modulated by Ni and Co.

- Support for the suggestion of the existence of other sensory systems for Co.

Proteomic pattern alterations of the cyanobacterium *Synechocystis* sp. PCC 6803 in response to cadmium, nickel and cobalt

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Abstract

Cyanobacteria represent the largest and most diverse group of prokaryotes capable of performing oxygenic photosynthesis and are frequently found in environments contaminated with heavy metals. Several studies have been performed in these organisms in order to better understand the effects of metals such as Zn, Cd, Cu, Ni and Co. In *Synechocystis* sp. PCC 6803, genes involved in Ni, Co, Cu and Zn resistance have been reported. However, proteomic studies for the identification of proteins modulated by heavy metals have not been carried out. In the present work, we have analyzed the proteomic pattern alterations of the cyanobacterium *Synechocystis* sp. PCC 6803 in response to Ni, Co and Cd in order to identify the metabolic processes affected by these metals. We show that some proteins are commonly regulated in response to the different metal ions, including ribulose1,5-bisphosphate carboxylase and the periplasmic iron-binding protein FutA2, while others, such as chaperones, were specifically induced by each metal. We also show that the main processes affected by the metals are carbon metabolism and photosynthesis, since heavy metals affect proteins required for the correct functioning of these activities.

Significance: This is the first report on the proteomic profile of *Synechocystis* sp. PCC 6803 wild type and mutant strains for the identification of proteins affected by the heavy metals Ni, Co and Cd. We have identified proteins commonly responsive to all three metals and also chaperones specifically modulated by each metal. Our data also supports previous studies that suggest the existence of additional sensor systems for Co.

Key-words: metal resistance, 2-DE, mass spectrometry, cyanobacteria

Introduction

Environmental contamination has been an aspect of major concern in recent years. The occurrence of heavy metals in the biosphere is a natural phenomenon as they are elements that cannot be destroyed or degraded. Differently from organic pollutants, heavy metals tend to accumulate in the environment, especially in marine and lake sediments [1, 2]. One of the approaches to control the presence of heavy metals involves the use of microorganisms to remediate these contaminants [3].

Cyanobacteria represent the largest and most diverse group of prokaryotes capable of performing oxygenic photosynthesis and are frequently found in environments contaminated with heavy metals. Several studies have been performed with these organisms in order to better understand the effects of metals such as Cr, Zn, among others [4-8]. It has been shown that cyanobacteria can perform a passive accumulation of these metals and also remove them from the environment [9-11]. It was observed that polysaccharides and carboxyl groups present in the biomass of these organisms are responsible for metal ion ligation [12, 13]. These studies demonstrate the potential use of cyanobacteria for biorremediation of contaminated areas.

Metal resistance systems have been characterized in different microorganisms and recent progress has been made in the identification of the genes and proteins involved [14-21].The main resistance mechanism that bacteria use to overcome the toxicity of transition metal ions is the efflux of these metals out of the cytoplasm. In cyanobacteria, proteins that sense metal ions and regulate metal efflux pumps have been characterized in *Anabaena* sp. PCC 7120 [22] and *Osciliatoria brevis* [23, 24]. In *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) substrate binding proteins, which seem to help load metal ions onto the correct ATP binding cassette (ABC)-type importers have been described for Mn, Zn and Fe [25-30]. Furthermore, *Synechocystis*

genes involved in Ni, Co, Cu and Zn resistance have also been reported and are grouped in a gene cluster [16, 31-34]. Moreover, exopolysaccharides (EPS) have been implicated in metal resistance; it was shown that the most resistant isolate of *Synechocystis* sp. (BASO670) produced the highest levels of EPS [35]. Recently, Jittawuttipoka et al. [36] also showed that EPS protects cyanobacteria against salt and metal stresses.

Synechocystis has been considered an attractive model for functional genomic analyses, including proteomic studies, since the genome of this cyanobacterium has been sequenced [37] and is available at CyanoBase (www.kazusa.or.jp/cyano/Synechocystis/index.html). Most proteomic analyses in *Synechocystis* have focused on the search for proteins present in the external membrane [38] and thylakoids [39-42], as well as proteins involved in heterotrophy [43] and salt-stress [44]. However, proteomic studies for the identification of proteins modulated by heavy metals have not been reported so far.

In the present work, we have analyzed the proteomic alterations of *Synechocystis* in response to Ni, Co and Cd. Metal concentrations that inhibited growth but maintained cells viable were used in an attempt to better understand the metabolic processes involved in the homeostasis of these metals. We show that some proteins were commonly regulated in response to the different metal ions, including those related to carbon metabolism and photosynthesis, while others, such as chaperones, were specifically induced by each metal.

Materials and Methods

1. *Synechocystis* sp. PCC 6803 growth conditions

The wild type cyanobacterium *Synechocystis* sp. PCC 6803 (WT) and the mutant strains nrsRS (lacking the Ni sensors *nrsR* (sll0797) and *nrsS* (sll0798)) [32] and corR

(lacking the Co sensor CoR (sll0794)) [31], were grown photo-autotrophically at 30º C under continuous illumination with white light (50 μE/m^2s) in liquid BG11 medium [45] supplemented with 1 g of $NaHCO_3$ per liter (BG11C) and bubbled with 1% v/v CO_2 in air. At the mid-exponential phase, when the culture reached a chlorophyll concentration of 3-4 μg/mL [31], different concentrations of $NiSO_4$, $CoCl_2$ and $CdCl_2$ were added in order to determine the maximum amount of metal tolerated, which resulted in growth inhibition. A μmolar range from 5 to 50 μM was tested for all metal ions.

2. Preparation of *Synechocystis* sp. PCC 6803 soluble cytosolic fraction

Samples of the growing cultures were harvested at 6, 24 and 48 h after the addition of the corresponding metal and broken with glass beads as previously described [46] using a buffer containing 25 mM HEPES-NaOH (pH 7.0), 15 mM $CaCl_2$, 5 mM $MgCl_2$, 15% v/v glycerol and 1 mM PMSF. The cellular debris were removed by centrifugation at 3000 *g* for 5 min and total membranes were thereafter pelleted by centrifugation at 16000 *g* for 20 min, yielding a supernatant containing most cytosolic proteins. All steps of this procedure were carried out at 4º C and the samples were stored at -20 ºC. Protein concentration determination was performed using the Bradford Reagent (BioRad), according to the manufacturer's instructions.

3. Bidimensional electrophoresis (2-DE)

Isoelectric focusing was conducted using 11-cm immobilized pH gradient (IPG) strips with a pH range of 4–7 and a Protean IEF Cell electrophoresis system (BioRad). Strips containing approximately 250 μg of protein were rehydrated with 2% (v/v) CHAPS, 8 M urea, 40 mM dithiothreitol (DTT) and 2% IPG buffer. Second dimension

analysis was performed by SDS-PAGE using 12% polyacrylamide gels and the molecular mass marker “Low range SDS-PAGE standard” (Bio-Rad). Three biological and five technical replications of each condition were performed. Protein spots were visualized after Comassie brilliant blue (CBB) staining.

4. Image analysis

The 2D gel images were analyzed by laser densitometry scanning with a GS-800 calibrated densitometer (Bio-Rad) and three high quality gels (each representing a biological replicate) from each condition were analyzed using the PDQuest software version 7.0 (Bio-Rad). Automatically detected spots were checked and some of them were manually added or removed. Following the detection procedure, the normalization step was carried out to attribute a common spot identity for the same spots derived from different images using the reference gel construct. A synthetic gel from each condition was constructed by using the mean value of volume percentage of each protein spot present in the three biological replicates. Missing values were estimated based on the minimum detectable spot according to the PDQuest software’s (Bio-Rad) instructions. The synthetic gels were overlapped using the molecular marker as well as several protein spots present in all profiles as landmarks. The proteins were accepted as having been differentially expressed when they displayed a fold change of at least 1.5 and differences were significant in Student’s t-test at a significance level of 95%. Image analysis was performed on the set of reproducible spots from each stage and in order to obtain consensus results, only spots present in at least two out of three replicates were considered for the differential expression analysis.

5. Trypsin digestion and mass spectrometry analysis

CBB-stained proteins were excised from the gels, destained, dried and rehydrated in 100 µL 50 mM NH_4HCO_3. A total of 15 µL of 0.1 mg/mL trypsin in 1 mM HCl were added and digestion was performed overnight at 37ºC. Peptides were extracted with 20 µL 0.5% TFA and 0.5 µL of each sample were applied onto the MALDI plate. MALDI-TOF MS spectra were acquired on an Autoflex apparatus (Bruker Daltonics). External calibration was performed using Peptide Calibration Standard (Bruker Daltonics) and the trypsin auto-digestion products of *m/z* values 842.5094 and 2211.1046 were used for internal calibration. Proteins were identified as the highest ranked result by searching the databases NCBInr or MSDB, including all species, using the MASCOT search engine (Matrix Science, London, UK). The mass tolerance was of 100 ppm and one missed cleavage was allowed. Carbamidomethylation of cysteines, oxidation of methionine, and acrylamide-modified cysteines were considered for PMF searches. For accepting the identification, the cutoff value for the Probability Based Mowse score calculated by MASCOT (at $p < 0.05$) was used. For MS/MS data, the peptide mass tolerance was of 0.5 Da, MS/MS ion mass tolerance of 0.5 Da, allowance of 1 missed cleavage, and charge state +1.

6. RNA isolation and Northern blot hybridization.

Total RNA was isolated from 25 mL samples of cultures at the mid-exponential growth phase (3 to 4 µg of chlorophyll/mL). Extractions were performed by vortexing cells in the presence of phenol-chloroform and acid-washed baked glass beads (0.25 to 0.3 mm diameter; Braun) as previously described [47].

For Northern blot analyses, 5 µg of total RNA was loaded per lane and electrophoresed in 1.2% agarose denaturing formaldehyde gels. Transfer to nylon membranes (Hybond N-Plus; Amersham), prehybridization, hybridization, and washes

were in accordance with Amersham instruction manuals. Probes for Northern blot hybridization were PCR synthesized using the following primer pairs: groSF and groSR, Gpx1F and Gpx1R, NRSBF and NRSBR, HspAF and HspAR, futA2F and futA2R (Table 1). DNA probes were ^{32}P labeled with a random-primer kit (GE Healthcare) using (α-^{32}P) dCTP (3000 Ci/mmol). All of the filters were stripped and reprobed with a *Hin*dIII-*Bam*HI 580-bp probe from plasmid pAV1100 that contains the constitutively expressed RNase P RNA gene (*rnpB*) from *Synechocystis* sp. strain PCC 6803 [48]. To determine counts per minute of radioactive areas in Northern blot hybridizations, an Instant Imager Electronic Autoradiography apparatus (Packard Instrument Company) was used. Radioactive signals of three independent experiments for each strain were quantified and averaged. RNA levels were normalized with the *rnpB* signal and expressed as fold change of treated vs non-treated.

Results and Discussion

Determination of minimal inhibitory metal concentrations

In the present work, we have analyzed the effects of three heavy metals (Ni, Co and Cd) on the protein expression by *Synechocystis*. We have used metal concentrations that inhibited growth but maintained cells viable in order to investigate the metabolic processes involved in the homeostasis of these metals. As a first step, the highest metal concentration tolerated by the cyanobacterium in liquid culture was screened. For that, cells were grown until mid-exponential phase, exposed to increasing concentrations of metal and used for chlorophyll content measures to monitor growth. For Ni, the

concentrations of 5, 10, 20, 30, 40 and 50 µM $NiSO_4$ were tested and the results showed that *Synechocystis* stopped growth at 40 µM $NiSO_4$, however, cells were still viable (see Supplementary Fig. 1). These results indicate that at this concentration, *Synechocystis* activates mechanisms for Ni homeostasis in order to maintain cell survival. We also analyzed the mutant strain nrsRS, which lacks *nrsRS* genes coding for a two-component system that detects extracellular Ni and activates transcription of the *nrsBACD* Ni resistance operon [32]. This mutant strain presented a lower tolerance to Ni exposure on plates [32]. We observed that the nrsRS strain could tolerate only 20 µM $NiSO_4$ (see Supplementary Fig. 1), half of the amount tolerated by the wild type strain, which reflects the inability to activate the expression of the *nrsBACD* operon required for Ni resistance.

In the case of Co, concentrations of 5, 10, 20, 30 and 40 µM were tested for the wild type and 5, 10 and 15 µM for the corR mutant strain, which lacks the Co-sensing transcription factor CorR [31].This transcriptional factor activates the expression of *corT* in response to an excess of Co [31, 33] and in response to $vitB_{12}$ (cyanocobalamin, a Co containing vitamin) metabolism [33]. WT was able to grow in the presence of up to 20 µM $CoCl_2$, while corR mutant strain tolerated only 10 µM, in agreement with the sensitivity previously described for this strain [31, 33] (see Supplementary Fig. 2). Finally we also analyzed the growth of the WT strain in the presence of $CdCl_2$ using final concentrations of 5, 15, 20 and 30 µM and determined that it could tolerate up to15 µM $CdCl_2$ (see Supplementary Fig. 3).

Once the metal concentration tolerated by each strain was determined, samples were collected at 6, 24 and 48 h after the addition of maximum tolerated concentrations of the corresponding metal. The SDS-PAGE protein profiles of the WT in the presence of the corresponding metal were compared to the control condition (a parallel culture

without metal added). The results obtained revealed a more pronounced alteration in the profiles at 48 h after the addition of the metal (data not shown). Therefore, all further analyses using two-dimensional electrophoresis were performed using the 48 h samples.

Proteins responsive to Ni

Ni is a highly toxic metal at high concentrations, known to inhibit processes such as respiration and photosynthesis. On the other hand, Ni is an essential metal for the catalytic activity of several enzymes. Therefore, the maintenance of a precise homeostasis through sensing and transport systems is of extreme importance for cell survival. It is known that the uptake of Ni in microbial organisms is mediated by non-specific transport systems as well as by high affinity specific systems such as multicomponent ABC (ATP-binding cassette) transport systems [49] and one-component permeases [50-52]. Transcription factors that repress and/or activate specific genes in response to nickel have also been identified [53, 54]. Export systems are equally important for metal homeostasis and have also been reported in microorganisms. The Cnr (cobalt, nickel) and the Ncc (nickel, cobalt, cadmium) systems, for example, are membrane protein complexes involved in Ni efflux [55-58]. In *Synechocystis,* a nickel resistance operon (*nrsBACD*) formed by four open reading frames (ORFs) was described [31]. López-Maury et al. [32] showed that a two-component signal transduction system controls the Ni-dependent induction of the *nrsBACD* operon and is involved in Ni sensing. Although genes directly involved in Ni sensing and transport have been reported, little is known about the metabolic processes affected by Ni exposure.

In this study, we have investigated the global protein changes that occur in *Synechocystis* in the presence of 40 μM $NiSO_4$, a concentration that inhibited growth.

The results obtained showed that the main targets of Ni excess were proteins related to photosynthesis and carbon metabolism (Figure 1 and Table 2), which are in agreement with other studies on heavy metal effects in plants and microorganisms [59, 60]. Several of these proteins were down-regulated including glucose-1-phosphate adenylyltransferase, also known as ADP-glucose pyrophosphorylase or AGP, ribulose bisphosphate carboxylase large subunit, phycocyanin and uroporphyrinogen decarboxylase, among others.

The periplasmic iron-binding protein (FutA2) was also clearly down-regulated by Ni exposure (Figure 1 and Table 2, spot 3302). This protein is involved in Fe and Cu uptake and may also be implicated in the transport of other metals since it is able to alter the periplasmic pools of Mn, Co and Zn [20]. However, the role of this protein in Ni homeostasis has not been reported so far.

Furthermore, we have also identified an HspA chaperone (spot 2005), which was specifically up-regulated by Ni (Figure 1 and Table 2). HspA has been primarily associated to thermotolerance [61-63], however additional roles in salt and oxidative stress responses have also been reported [64-66]. Sakthivel et al. [66] suggested that HspA stabilizes membrane proteins and protects photosystems and phycobilisomes from oxidative damage. Schauer et al [67] reported that *Helicobacter pilori* possesses a particular HspA, which contains a unique His-rich C-terminal extension and was able to bind Ni *in vitro*. The HspA reported in the present study does not present this Ni binding domain. It is possible that the up-regulation of this chaperone observed in our study could be related to the protection of the cells against the oxidative stress damage caused by excess of Ni. Further studies are needed in order to verify the specific induction of this chaperone by Ni and to determine the function of this protein in Ni tolerance.

Another protein exclusively induced by Ni was the cation efflux system protein NrsB (spot 0206), which is involved in Ni and Co tolerance [31]. This protein is encoded by *nrsB* which is part of *nrsBACD* operon regulated by the NrsRS two-component system [32]. The up-regulation of the NrsB protein in the presence of Ni observed in our study (Figure 2) was expected, since it has been demonstrated that the *nrsBACD* operon is highly induced in response to Ni [31, 32].

The mutant strain nrsRS [32] was also analyzed and its protein profile determined in the presence of 20 μM $NiSO_4$. The proteins identified in the WT strain were searched in the nrsRS profile in order to verify the behavior of these proteins in the mutant strain. As expected, the NrsB protein was exclusively expressed in the WT in response to Ni but not in the nrsRS protein profile. HspA, which was specifically induced by Ni in the WT, was also absent in the protein profile of the nrsRS strain. Interestingly, when the nrsRS mutant protein profile was compared to that of the wild type, an opposite expression pattern for several proteins could be observed. The induction of translation related proteins in the WT strain (elongation factor G, 30S ribosomal protein) could be reflecting that WT cells are able to respond to high levels of Ni by increasing translation. In contrast, the nrsRS strain repressed translation-related processes and induced catalase and AGP (glucose-1-phosphate adenylyltransferase). This could reflect an inability to respond to Ni, which causes an increase in oxidative stress, growth impairment and therefore the accumulation of fixed carbon into glycogen. These results indicate that the two-component signal transduction system composed by the *nrsS* and *nrsR* genes may be the main sensor system for Ni although another Ni sensing system has been recently characterized [68]. This system contains a new protein named InrS (internal nickel-responsive sensor), which is involved in Ni homeostasis by repressing the *nrsD* gene, the last gene of the *nrsBACD* operon, in the

absence of Ni. However, it seems that InrS alone is not able to protect the cells from excess levels of Ni, since in our study the nrsRS strain showed a lower tolerance to Ni exposure when compared to the WT.

Ni excess also down-regulated lysyl-tRNA synthetase (spot 6605) both in the WT and the nrsRS strains. Aminoacyl-tRNA synthetases (aaRS) are enzymes that catalyze the correct attachment of amino acids to their cognate tRNAs primarily during protein synthesis [69, 70]. It has been shown that tRNAs are implicated in cellular functions that go beyond protein biosynthesis, such as transfer of amino acids to membrane lipids and proteins and as intermediates for antimicrobial molecule synthesis [71]. It has also been reported that the levels of lysyl-tRNA synthetase decrease in response to heat shock, oxidative stress or ethanol stress in bacteria [72, 73]. Although different aaRS have been associated to other functions, including stress conditions, their direct relation with metal stress response has not been reported.

In order to further investigate the differential expression observed in this study, Northern blot analysis was performed. The results obtained for *hspA* (Figure 2) revealed an increased abundance of mRNA was observed after exposure to Ni. These results are consistent with the proteomic results, which show an increase in HspA protein at 48h. Unexpectedly, in the nrsRS strain, *hspA* mRNA was also induced at higher levels but only at the beginning of the stress treatment (6 h after Ni stress). At later time, *hspA* expression is highly reduced in this strain, which could explain the failure to detect this protein at 48 h after Ni addition. The early expression of *hspA* in the nrsRS strain may be due to a strong Ni stress in this strain lacking the Ni homeostasis mechanism. *nrsB* expression was also highly induced by Ni with a maximum expression after 6 h in the WT strain and this induction was completely abolished in the nrsRS strain (Figure 2).

Northern blot analysis was also performed for the *futA2* gene, which was down-regulated in response to Ni. The results showed repression of *futA2* mRNA in the WT exposed to Ni (Figure 3), which is in agreement with the 2-DE analysis. Further studies need to be performed in order to verify if this gene could be involved in Ni homeostasis.

Protein expression altered by Co

Co is essential as a trace element, however, above critical concentrations it can be toxic to the cell. Co is the central metal cofactor in the corrin ring of vitamin B_{12} and also plays important biological roles. As for Ni, Co uptake is mediated by nonspecific transport systems and by high-affinity specific systems. A gene (*nhlF*) from *Rhodococcus rhodochrous* J1 that mediates Co transport into the cell in an energy-dependent manner has been identified [74]. Co export and resistance operons have also been described including *czc* (for Cd, Zn, and Co resistance) [75], *cnr* (for Co and Ni resistance) [57], and *ncc* (for Ni, Co, and Cd resistance) [58]. In *Synechocystis,* a gene cluster composed of nine open reading frames (ORFs) involved in Ni, Co and Zn sensing and tolerance was described [31, 33]. However, information on the metabolic processes responsive to high concentrations of Co is still limited.

In this study, the analyses of the 2D maps of *Synechocystis* in response to Co were compared to the control condition (without Co). The results revealed that, as in the case of Ni stress, several proteins were down-regulated in the presence of Co (Figure 4 and Table 3). Several enzymes involved in carbon metabolism showed reduced intensity including phosphoglycerate kinase (spot 3502), glyceraldehyde-3-phosphate dehydrogenase 1 (spot 7404), ribulose 1,5 bisphosphate carboxylase (spots 7704 and 8703) and AGP (glucose-1-phosphate adenylyltransferase; spot 9603). It is possible that

the stress caused by the presence of this metal lead to a reduction in the central carbon metabolism as a protection mechanism.

As reported for Ni, the periplasmic iron-binding protein, FutA2 was again clearly down-regulated by Co exposure (Figure 4; spot 3406). As mentioned earlier, *Synechocystis futA2* mutant cells showed Co accumulation in the periplasmic space, indicating that FutA2 may be involved in its transport inside the cell [20].

The chaperonin GroES (spot 6003) was specifically induced by Co exposure (Figure 4 and Table 3). This chaperonin is different from HspA, the chaperone induced by Ni. Interestingly, Hongo et al. [76] reported a novel Co/Mn-dependent ATP/ADPase activity in thermostable chaperonins from *Pyrococcus furiosus*, *Pyrococcus horikoshii*, *Methanococcus jannaschii* and *Thermoplasma acidophilum* and suggested that this function may be a general feature of these chaperonins. Although the dependence on Co for a specific chaperonin activity was reported, the effect of high Co levels in the induction of chaperonins has not been shown. It would be interesting to further investigate the induction and the specificity of these chaperones in order to understand their role in Co and Ni resistance.

Unexpectedly, the comparison between the WT strain and the *corR* mutant strain in the presence of 20 μM $CoCl_2$ revealed a very similar expression pattern (Table 3); in other words, proteins up-regulated in the WT were also up-regulated in the *corR* mutant. This suggests the existence of other Co sensor systems in *Synechocystis*, which allowed the mutant strain to respond and adapt to the presence of Co. Although the *corR* mutant strain showed a similar protein pattern, the amount of Co tolerated was lower than that of the WT. The presence of an alternative system involved in Co tolerance has been suggested previously [31] and may account for the similarities found in the WT and *corR* mutant protein expression profiles. The only protein that showed an inverse

expression in response to Co in the WT (-1.95) when compared to the *corR* mutant (1.47) was identified as a seryl-tRNA synthetase. As discussed above in the response to Ni stress, it is possible that tRNA synthetases may play a role in metal response, however additional work would be required for confirmation.

Northern blot analysis was performed to further investigate the induction of *groES* and glutathione peroxidase (*gpx1*) in response to Co. The results obtained revealed that in the WT strain, *gpx1* mRNA was induced shortly after Co addition and remained high at 24 and 48 h after Co exposure (Figure 5). Likewise, in the *corR* mutant strain, higher levels of *gpx1* mRNA were detected at all sampling points after Co addition, when compared to the control condition. In the case of *groES,* its levels of expression remained almost constant during the time courses in both the WT and *corR* mutant strains. Regarding the *futA2* expression, its mRNA levels remained almost unchanged during the first 24h and reduced only at 48 h (Figure 3).

Protein expression in response to Cd

Cd toxicity has long been reported [77] since Cd is one of the most abundant toxic metals present in the environment and it is intensively spread out due to fossil-fuel burning and manufacturing of paints, batteries and screens [78]. Unlike Co and Ni, Cd has no known function in the cell, although it was shown that the addition of Cd to Zn-limited cultures enhanced growth in the phytoplankton species *Thalassiosira weissflogii* [79].

Houot et al. [80], by using a transcriptomic approach, showed that Cd triggers a reorganization of *Synechocystis* metabolism, under the control of the Slr1738 regulator. These authors reported that cells activated an ATP-sparing mechanism through down-regulation of cell metabolism in a way to limit Cd uptake and its poisoning

incorporation in place of the cognate metal cofactor of metalloenzymes. On the other hand, a breakdown of the photosynthetic machinery that impairs ATP production, releases different compounds that become available for the synthesis of Cd-toxicity protecting enzymes [80].

The effect of heavy metals on the photosynthetic process has been well documented and is reviewed in Aggarwal et al. [59]. Several studies have shown a stronger inhibition of PSII by heavy metals when compared to PSI [60, 81-83]. In our study, we have observed a contrasting situation: some photosynthesis proteins were down-regulated, such as the ferredoxin-NADP oxidoreductase, whereas others were up-regulated including the 12 kDa extrinsic protein of photosystem II (PsbU), whose expression increased 10 fold in treated cells, and a putative thylakoid lumen peptidyl-prolylcis-trans isomerase (sll0408), important for the stabilization of PSII (Figure 6 and Table 4). These two proteins have also been identified as up-regulated in response to Cd in *Synechococcus* sp WH8102, suggesting a conserved response in cyanobacteria [60].

Several proteins were up-regulated in the presence of Cd including ribosomal proteins, a cell division protein and a sulfolipid biosynthesis protein, required for sulfoquinovosyl diacyl glycerol biosynthesis [84], which is a typical constituent of photosynthetic membranes in plants and photosynthetic bacteria [85, 86]. Houot et al. [80] reported a down-regulation of protein synthesis genes, however the sampling was performed between 90 and 360 minutes of treatment and lethal doses of the metal were used. The results obtained in the present study suggest that at 48 h of Cd exposure, cells may have already adapted to the stress caused by Cd. In contrast, enzymes of carbon metabolism such as, phosphoribulokinase and ribulose bisphosphate carboxylase were down-regulated, while fructose 1,6 bisphosphatase was up-regulated. Similar results

were obtained by Houot et al. [80], who reported the down-regulation of most carbon metabolism genes in response to Cd excess.

Interestingly, several protein spots in the Cd-exposed *Synechocystis* protein profile (Figure 6) were identified as the periplasmic iron binding protein FutA2 and were also down-regulated, as observed in the response to the other metals used in this study. By contrast, Houot et al. [80] reported that Fe uptake genes were up-regulated by Cd exposure. It is known that Cd disturbs metal homeostasis and alters the expression of several metal transport genes [80], but further studies will have to be performed in order to better understand the relations of Fe uptake and other metal homeostasis.

As opposed to what was observed for Ni and Co, we did not find chaperones specifically induced by Cd. Houot et al. [80] reported that chaperone genes were induced as an early response to Cd (after 30 min), and our analyses were performed at a longer Cd exposure period.

In the Northern blot analysis of *futA2*, again a lack of correlation between the mRNA and protein levels were obtained (Figures 3 and 6; Table 4), as observed in the response to Co stress. An up-regulation of *futA2* mRNA was observed at 6 h, with an increase at 24 h after Cd addition (Figure 2), although a clear reduction in the protein spot intensity could be observed in the 2D map of the WT exposed to Cd (Figure 6). The lack of correlation between mRNA and protein levels has been well documented [61] and seems to be the case for *futA2* expression in response to Cd. It is probable that post-translational regulation occurs and could account for this difference as we have detected several spots corresponding to FutA2. Further analyses need to be performed in order to understand the regulation of *futA2* in response to Cd and Co.

Comparison of the proteins expressed in response to Ni, Co and Cd

A comparison of the proteins of *Synechocystis* responsive to the different metals identified in this study was performed based on the gene ID. The results obtained revealed that 7 proteins were commonly differential in response to the metals analyzed (Table 5). Most proteins showed a similar expression pattern, including ribulose1,5-bisphosphate carboxylase and periplasmic iron-binding protein, which were decreased upon exposure to all three metals. A hypothetical protein was also decreased in the response to Ni and Co. This protein could be an interesting candidate for future functional studies to determine its relation to metal stress.

Aspartyl/glutamyl-tRNA (Asn/Gln) amidotransferase subunit B showed an opposite expression pattern in Ni and Co treatments (Table 2 and Table 3). As discussed above, this protein is involved in protein biosynthesis and the direct relation of this protein to metal stress has not been reported so far. Similarly, the protein Ferredoxin-NADP oxidoreductase was increased in response to Ni and decreased after Cd exposure. This protein is involved in photosynthesis and oxidative stress protection and it seems that *Synechocystis* increases the abundance of this protein to control Ni stress effects. Cd is a more toxic metal to the cell and it may be more difficult for *Synechocystis* to cope with Cd stress.

We have also observed several proteins (with distinct gene IDs) responsive to the different metals with similar biological functions in the cell. For example, ribosomal proteins 30S and 50S were increased in response to Ni and Cd, respectively. Similarly, elongation factors G, Ts and Tu were increased by exposure to Ni (G) and Co (Ts and Tu). Generally, heavy metals suppress protein synthesis machinery in both prokaryotic [87] and eukaryotic organisms [88-90]. On the other hand, heavy metal tolerance involves the participation of proteins in the induction of transcription factors as well as protection and restoration of macromolecules [91]. It is possible that the increased

abundance of ribosomal proteins and elongation factors in *Synechocystis* may be related to the synthesis of proteins involved in Ni and Co transport and chelation as part of a metal resistance mechanism.

Glycolysis was also affected by the metals, especially by Co since most enzymes involved in this process were identified in Co exposed cultures. Glyceraldehyde-3-phosphate dehydrogenase 1, Glucose-1-phosphate adenylyltransferase and Glucose-6-phosphate isomerase were decreased in response to Co stress. The down-regulation of glycolytic enzymes has been observed previously in the response to heavy metals [92, 93].

Among the most important effects of heavy metal toxicity are oxidative stress [94] and reduced activity of sensitive enzymes [95]. Indeed, in *Synechocystis* most proteins were down-regulated in response to metal toxicity, including several enzymes involved in photosynthesis (Table 2, Table 3 and Table 4). One of the few proteins that showed up-regulation was glutathione peroxidase in the response to Co (Table 3). This protein is related to anti-oxidant activity and was 13 fold higher in the Co-exposed culture when compared to the non-exposed culture. This protein showed the highest fold difference when all three metal responses were compared. It seems that the exposure to Co resulted in a strong protective response against oxidative stress.

Conclusions

The results obtained in this study indicate that several proteins were affected after the addition of different metals (Ni, Co or Cd) to *Synechocystis* cultures. A general decline in proteins related to carbon metabolism and photosynthesis could be observed in response to all three metals. An interesting result obtained was the down-regulation

of FutA2 by all metals. This protein is primarily involved in the transport of Fe and Cu and seems to be essential for metal homeostasis in the cell. Another interesting result was the specific up-regulation of two different chaperones, one by Ni (HspA) and the other (GroES) by Co. The expression of these chaperones may be directly involved in the tolerance process by avoiding damage to proteins essential for cell survival. Although the up-regulation of chaperones during metal stresses is well known, the expression of HspA and GroES in response to Ni and Co, respectively, had not been reported earlier. The similarities observed in the protein expression pattern between the WT and the corR mutant strains in the presence of Co also allows us to suggest that there may be additional Co signaling systems. This is the first report of the global protein profile of *Synechocystis* in response to Ni, Co and Cd stress. Overall, the results obtained in this study give an insight into the proteins with altered abundance in response to heavy metals in *Synechocystis* and can provide basic information for future studies aiming at the elucidation of heavy metal resistance mechanisms in cyanobacteria.

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Figure legends

Figure 1. 2D maps of the WT and nrsRS mutant strains in response to Ni. WT and nrsRS mutant strains were cultured in the control condition (WTC and nrsRSC) and after 48 h exposure to 40 µM $NiSO_4$ (WTNi) and 20 µM $NiSO_4$ (nrsRSNi), respectively. Lines and numbers indicate the differentially expressed proteins identified by mass spectrometry.

Figure 2. Changes in *nrsB* and *hspA* expression in response to Ni.

A. Northen blot of *nrsB* and *hspA* in response to Ni in the WT and nrsRS strains. Total RNA was isolated from *Synechocystis* cells grown in BG11C medium not exposed or exposed for 1, 6, 24 and 48 h to 40 µM Ni for the WT strain and 20 µM $NiSO_4$ for the

nrsRS strain. Five micrograms of total RNA was denatured and separated by electrophoresis in a 1% agarose gel, blotted, and hybridized with probes for *nrsB* or *hspA* genes. The filters were stripped and re-hybridized with an *rnpB* gene probe as a control. A representative experiment is shown. **B.** Quantification of the mRNA levels of *nrsB* and *hspA* in response to Ni. Radioactive signals of three independent experiments for each strain were quantified and averaged. RNA levels were normalized with the rnpB signal and expressed as fold change of treated vs non-treated; sample error bars represent SE (Standard Error).

Figure 3. Changes in *futA2* expression in response to different metals in the media. **A.** Northern blot analysis of *futA2* expression in response to different metals in the media. Total RNA was isolated from *Synechocystis* cells grown in BG11C medium not exposed or exposed for 1, 6, 24 and 48 h to Ni, Co and Cd at the same concentrations used for the proteomic analysis. Five micrograms of total RNA was denatured and separated by electrophoresis in a 1% agarose gel, blotted, and hybridized with a probe for *futA2* gene. The filters were stripped and re-hybridized with an *rnpB* gene probe as a control. A representative experiment is shown. **B.** Quantification of the mRNA levels of *futA2* in response to different metals. Radioactive signals of three independent experiments for each strain were quantified and averaged. RNA levels were normalized with the rnpB signal and expressed as fold change of treated vs non-treated. sample error bars represent SE (Standard Error).

Figure 4. 2D maps of the WT and *CorR* mutant strains in response to cobalt. WT and c*orR* mutant strains were cultured in the control condition (WTC and CorRC) and after 48 h exposure to 20 μM (WTCo) and 10 μM $CoCl_2$ (CorRCo), respectively. Lines

and numbers indicate the differentially expressed proteins identified by mass spectrometry.

Figure 5. Changes in *gpx* and *groES* expression in response to Co.

A. Northen blot of *groES* and *gpx* in response to Co in the WT and corR strains. Total RNA was isolated from *Synechocystis* cells grown in BG11C medium not exposed or exposed for 1, 6, 24 and 48 h to 20 µM $CoCl_2$ for the WT strain and 10 µM $CoCl_2$ the corR strain. Five micrograms of total RNA was denatured and separated by electrophoresis in a 1% agarose gel, blotted, and hybridized with probes for *gpx* or *groES* genes. The filters were stripped and re-hybridized with an *rnpB* gene probe as a control. A representative experiment is shown. **B.** Quantification of the mRNA levels of *gpx* and *groES* in response to Co. Radioactive signals of three independent experiments for each strain were quantified and averaged. RNA levels were normalized with the *rnpB* signal and expressed as fold change of treated vs non-treated; samples error bars represent SE.

Figure 6. 2D maps of the WT strain in response to cadmium. WT was cultured in the control condition (WTC) and after 48 h exposure to 15 µM $CdCl_2$ (WTCd). Lines and numbers indicate the differentially expressed proteins identified by mass spectrometry.

Table 1. Primers used for amplification of probes for Northern blot analysis.

Gene symbol	Gene product	Forward primer	Reverse primer
groES	10 kDa chaperonin (groES)	groSF 5'-GCCAAAGAAAAACCCCAAAT-3'	groSR 5'-GCCGGCATACTTGGAATAGA-3'
gpx1	glutathione peroxidase-like NADPH peroxidase	Gpx1F 5'-GCTTCACTCCCCAATACCAA-3'	Gpx1R 5'-GGAAAACCAAGCACCGTAAA-3'
nrsB	Cation efflux system protein involved in nickel and cobalt tolerance	NRSBF 5'-ATCGGTGGGGGAAAAATTAG-3'	NRSBR 5'-GCCACAACTGGTTCTTTGGT-3'
hspA	Chaperone (hspA)	HspAF 5'-GGATACCCATAGCACCGAAA-3'	HspAR 5'-TGTGTTTTGGATTGCTCCAG-3'
futA2	Periplasmic iron-binding protein	futA2F 5'-GGGTACCCATTGATCCTGTG-3'	futA2R 5'-GAGCTGAATCGGGTTGGTAA-3'

Table 2. Proteins differentially expressed by *Synechocystis* 6803 WT and mutant strain nrsRS in response to 40 µM and 20 µM $NiSO_4$, respectively, identified by mass spectrometry.

Spot	Gene ID	Protein description	NCBI Accession #	Fold change[a] WTNi/WTC	Fold change[a] nrsRSNi/nrsRSC
0206	slr0793	Cation efflux system protein involved in nickel and cobalt tolerance	NP_442628	exclusive to WTNi	-
2005	sll1514	Chaperone (hspA)	NP_440316	exclusive to WTNi	-
2809[b]	slr1463	Elongation factor G	NP_442851	1.7	-1.1
	sll1553	Phenylalanyl-tRNA synthetase subunit beta	NP_441710	1.7	-1.1
3302	slr0513	Periplasmic iron-binding protein	NP_442521	-4.6	-4.1
3303	sll1578	Phycocyanin α-subunit	NP_440551	-1.5	-1.5
4303[b,c]	slr0244	Hypothetical protein	NP_440027	-1.8	-1.2
		Phycocyanin associated linker protein	AB058393	-1.8	-1.2
4702	sll1987	Catalase HPI	NP_441295	-1.8	2.6
5301	sll1260	30S ribosomal protein S2	NP_441467	1.6	-1.3
5507	slr1133	Argininosuccinate lyase	NP_440604	2.3	-1.2
5607	sll1435	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B	P74215	2.7	3.2
6302	slr1643	Ferredoxin-NADP oxidoreductase	NP_441779	1.6	1.6
6603	slr1322	Putative modulator of DNA gyrase	NP_441393	2.1	1.0
6605	slr1550	Lysyl-tRNA synthetase	NP_440803	-1.6	Exclusive to nrsRSC
6608	slr0118	Thiamine biosynthesis protein ThiC	NP_442586	2.1	1.1
7204	slr1161	Hypothetical protein	NP_441650	-1.7	1.0
7407	slr0536	Uroporphyrinogen decarboxylase	NP_442753	-1.5	1.0
7601[c]	slr1945	Phosphoglyceromutase	NP_441933	-1.7	-2.0
8502	slr1165	Sulfate adenylyltransferase	NP_441655	1.9	1.0
8511	slr1176	Glucose-1-phosphate adenylyltransferase	NP_443010	-1.9	1.4
8601	slr0009	Ribulose1,5-bisphosphate carboxylase	NP_442120	-2.0	-2.6

[a]The "-" signal indicates down-regulation.
[b]Mixture of proteins detected in Mascot search
[c]Spot present in two out of three gel replicates

Table 3. Proteins differentially expressed by *Synechocystis* 6803 WT and mutant strain CorR in response to 20 µM and 10 µM $CoCl_2$, respectively, identified by mass spectrometry.

Spot	Gene ID	Protein description	NCBI Accession #	Fold change[a] WTCo/WTC	Fold change[a] CorRCo/CorRC
2002	slr0923	Hypothetical protein	NP_442404	2.4	1.2
3406	slr0513	Periplasmic iron-binding protein	NP_442521	-4.8	-5.2
3502	slr0394	Phosphoglycerate kinase	NP_441843	-1.6	-1.4
4302	slr0244	Hypothetical protein (universal stress protein)	NP_440027	-3.1	-5.0
4504	slr2094	Fructose 1,6-bisphosphatase II	NP_441308	-1.5	-1.5
4707	slr0452	Dihydroxy-acid dehydratase	NP_442995	-2.5	-1.6
5302	sll1261	Elongation factor Ts	NP_441466	4.3	5.1
6003	slr2075	10 kDa chaperonin (groES)	Q05971	exclusive to WTCo	exclusive to CorRCo
6304	sll1261	Elongation factor Ts	NP_441466	2.1	4.5
6501	sll1099	Elongation factor Tu	NP_441641	2.6	1.3
6805	sll0529	Hypothetical protein (Thiamine pyrophosphate (TPP) enzyme family)	NP_442804	-1.5	-3.5
7203	slr1259	Hypothetical protein	NP_441173	3.6	1.8
7404[b]	slr1887	Porphobilinogen deaminase	NP_441025	-1.8	-1.3
	slr0884	Glyceraldehyde-3-phosphate dehydrogenase 1	P49433	-1.8	-1.3
7505	sll0018	Fructose-1,6-bisphosphate aldolase	NP_442114	2.9	1.4
7603	slr1703	Seryl-tRNAsynthetase	NP_440547	-1.9	1.4
7704[b]	sll1435	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B	P74215	-2.1	-1.1
	slr0009	Ribulose1,5-bisphosphate carboxylase	NP_442120	-2.1	-1.1
7706	slr1349	Glucose-6-phosphate isomerase	NP_441488	-2.6	-3.5
8208	sll1742	Transcription antitermination protein NusG	NP_440740	exclusive to WTCo	exclusive to CorRCo
8306[c]	slr1842	Cysteine synthase	P73410	-1.5	-1.6
8405	slr0884	Glyceraldehyde-3-phosphate dehydrogenase 1	P49433	-1.6	-1.3
8501	sll0018	Fructose-1,6-bisphosphate aldolase	NP_442114	2.0	1.1
8703	slr0009	Ribulose1,5-bisphosphate carboxylase	NP_442120	-2.3	-2.2
9103	slr1171	Glutathione peroxidase	NP_441664	13.3	exclusive to CorRCo
9603	slr1176	Glucose-1-phosphate adenylyltransferase	NP_443010	-2.5	-5.5
9701[c]	sll1750	Urease subunit alpha	NP_440403	-3.0	-5.0
9703	sll0735	Hypothetical protein	NP_442931	-3.2	-2.0

[a]the "-" signal indicates down-regulation.
[b]Mixture of proteins detected in Mascot search
[c]Spot present in two out of three gel replicates

Table 4. Proteins differentially expressed by *Synechocystis* 6803 in response to 15 μM $CdCl_2$, identified by mass spectrometry.

Spot	Gene ID	Protein description	NCBI Accession #	Fold change[a] WTCd/WTC
0101	sll1194	12 kDa extrinsic protein of photosystem II	BAA12221	10.3
0303	sll0408	Putative thylakoid lumen peptidyl-prolylcis-trans isomerase sll0408;	Q55118	exclusive to WTCd
1101	sll1746	50S ribosomal protein L7/L12	NP_440736	2.4
3203	slr0513	Periplasmic iron-binding protein	NP_442521	-2.0
3302	slr0513	Periplasmic iron-binding protein	NP_442521	-4.8
4301	sll1525	Phosphoribulokinase	NP_441778	-1.5
4305[b]	slr0513	Periplasmic iron-binding protein	NP_442521	-1.9
	sll1578	Phycocyanin α-subunit	NP_440551	-1.9
4805	sll1043	Polynucleotide Phosphorylase/polyadenylase	NP_439981	-2.2
6301	slr0952	Fructose-1,6-bisphosphatase	NP_441738	3.6
6601	slr0009	Ribulose 1,5-bisphosphate carboxylase	NP_442120	-6.3
7407	slr1020	Sulfolipid biosynthesis protein SqdB	NP_440474	-1.6
8503	slr1643	Ferredoxin-NADP oxidoreductase	NP_441779	-2.0
8602	slr0374	Cell division cycle protein	NP_442025	2.6

[a]the "-" signal indicates down-regulation.
[b]Mixture of proteins detected in Mascot search

Table 5. Proteins with overlapping differential expression in response to the different metals in *Synechocystis* 6803.

Gene ID	Protein description	Protein expression pattern		
		Ni	Co	Cd
sll1435	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B	up	down	-
sll1578	Phycocyanin α-subunit	down	-	down
slr0009	Ribulose1,5-bisphosphate carboxylase	down	down	down
slr0244	Hypothetical protein	down	down	-
slr0513	Periplasmic iron-binding protein	down	down	down
slr1176	Glucose-1-phosphate adenylyltransferase	down	down	-
slr1643	Ferredoxin-NADP oxidoreductase	up	-	down

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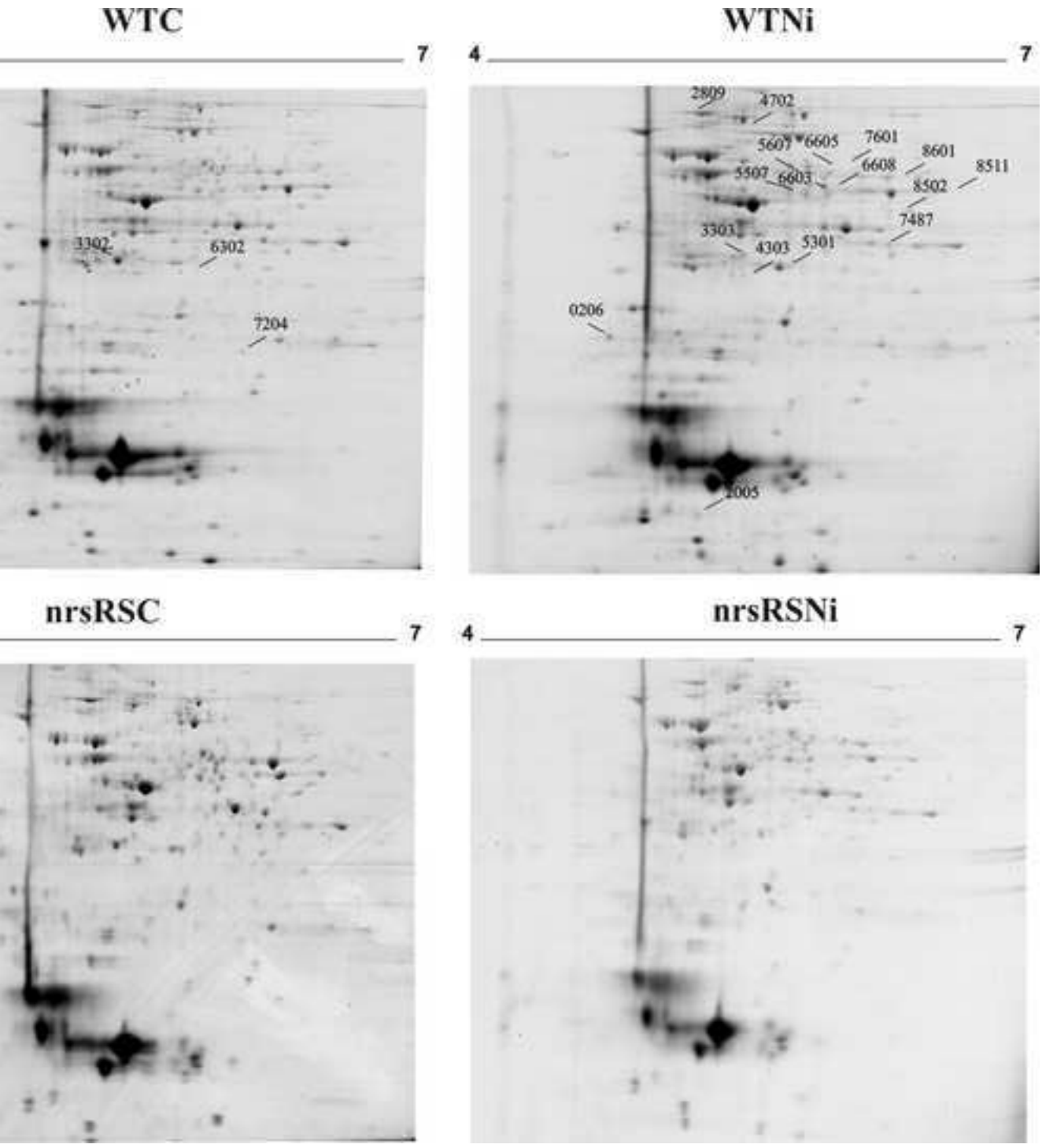
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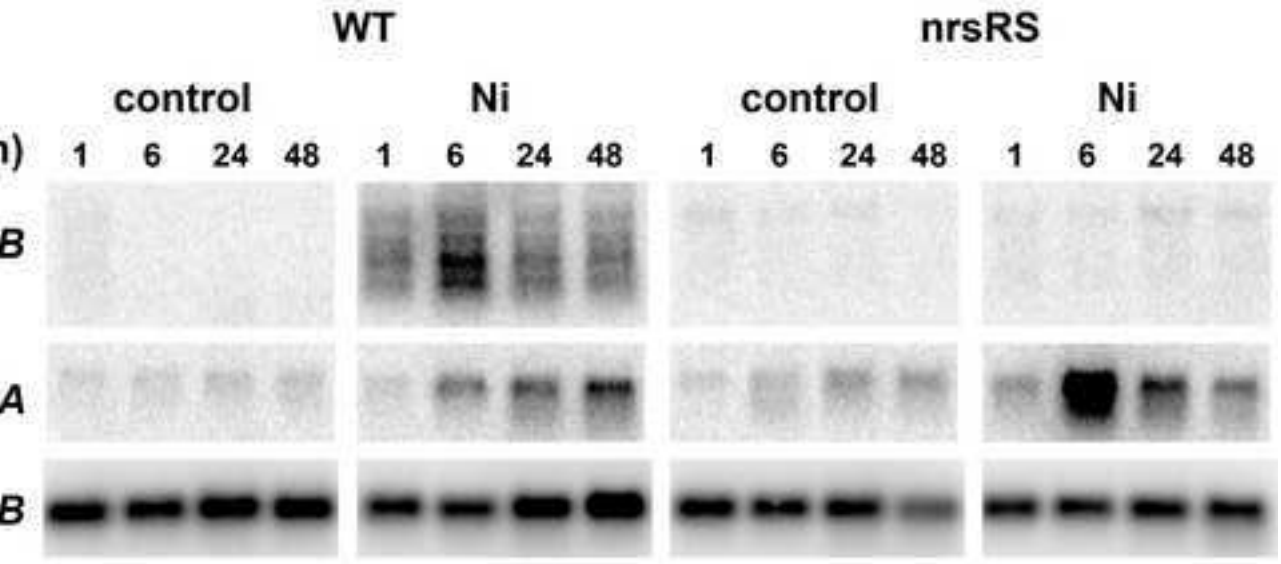
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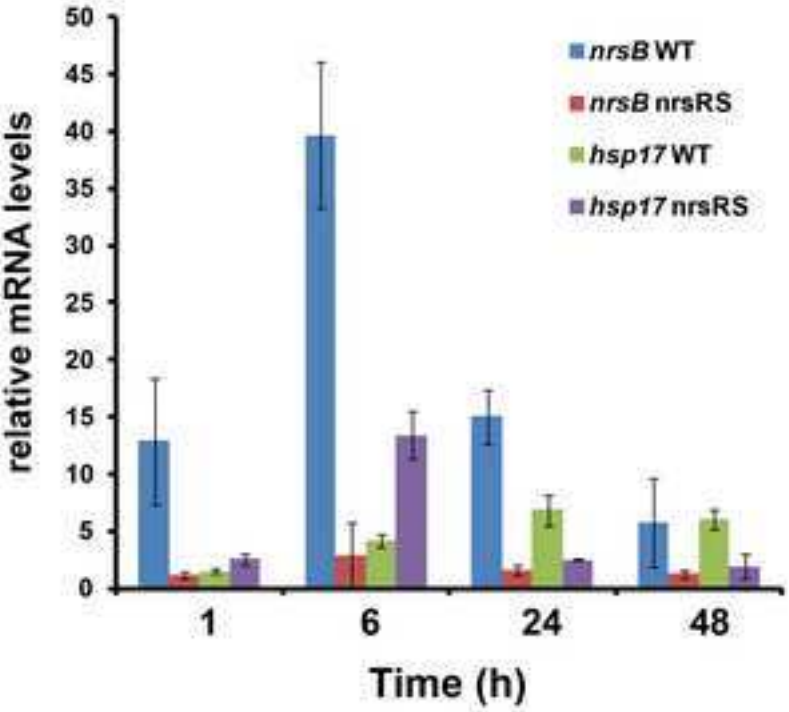
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nrsB nrsRS
hsp17 WT
hsp17 nrsRS

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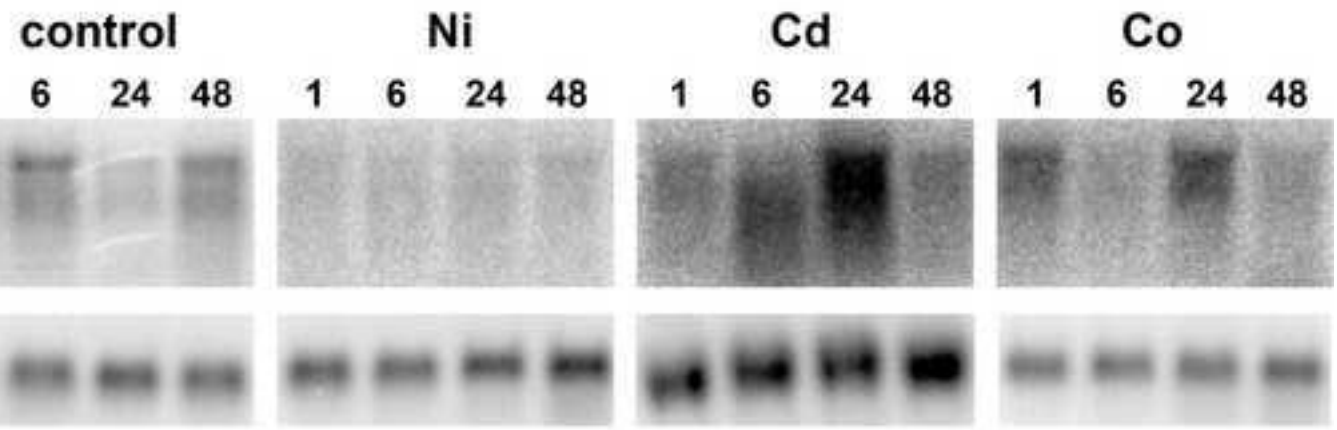
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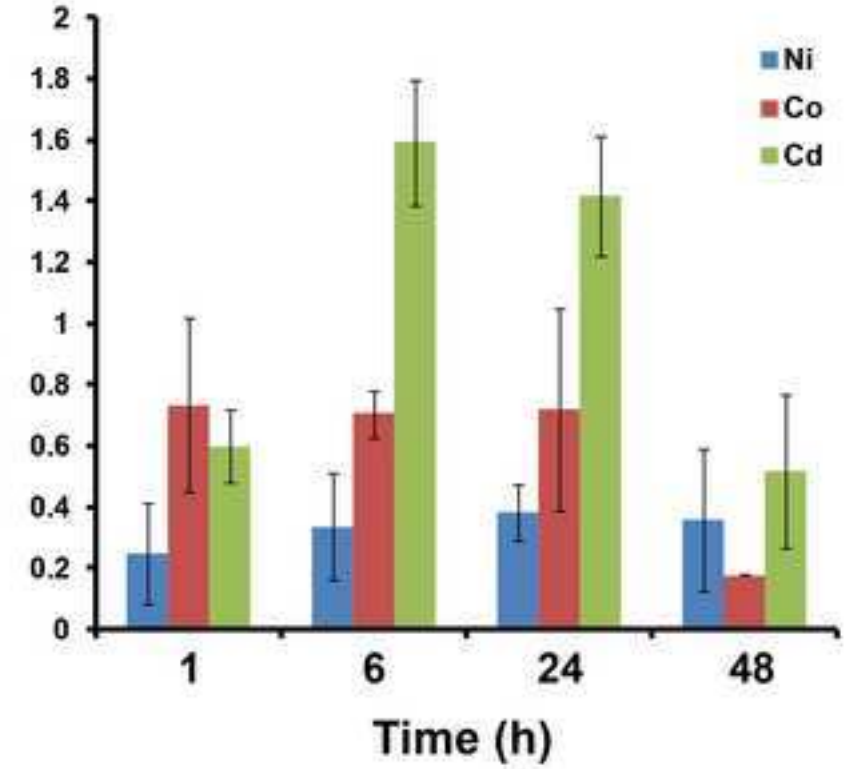
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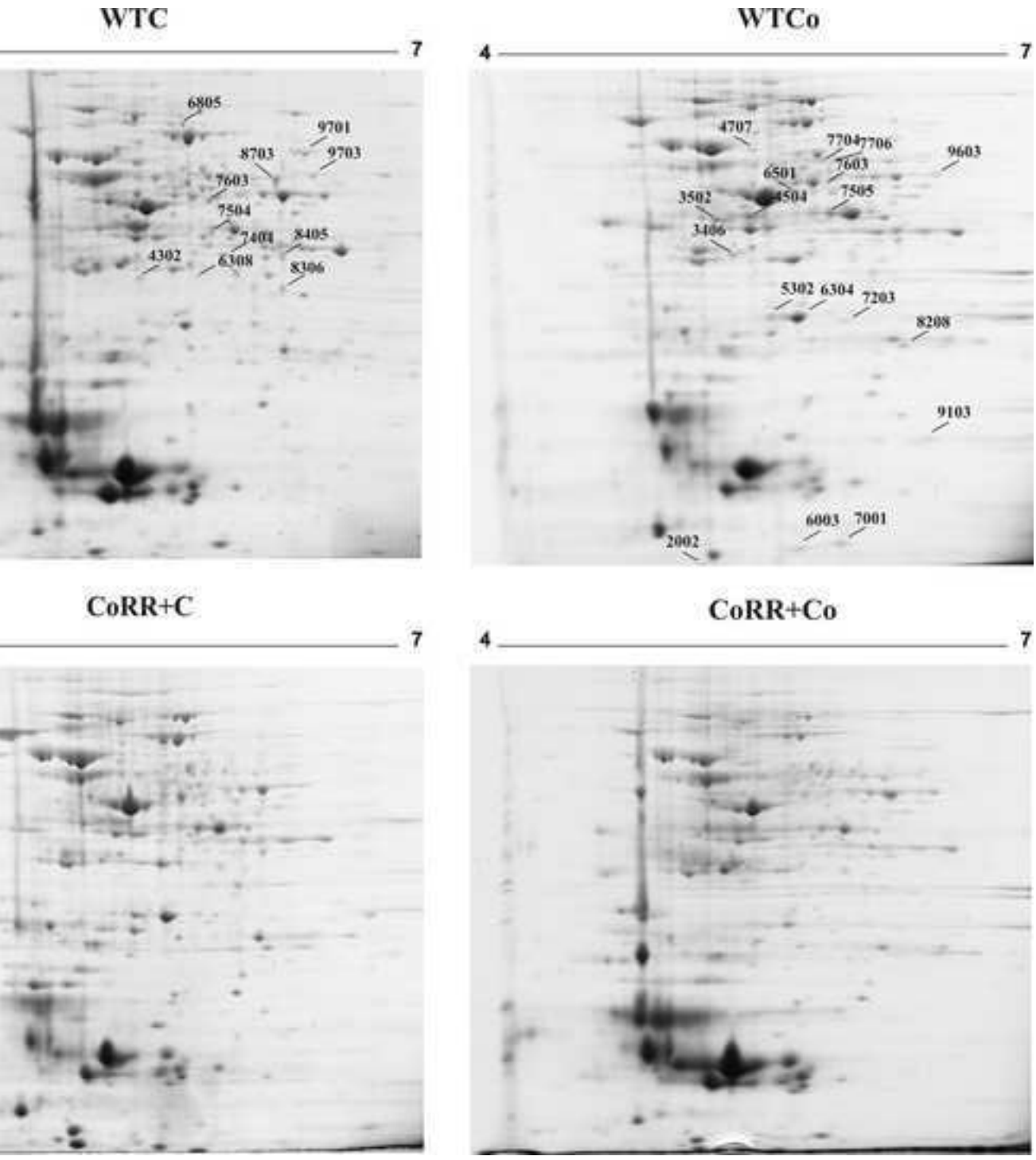
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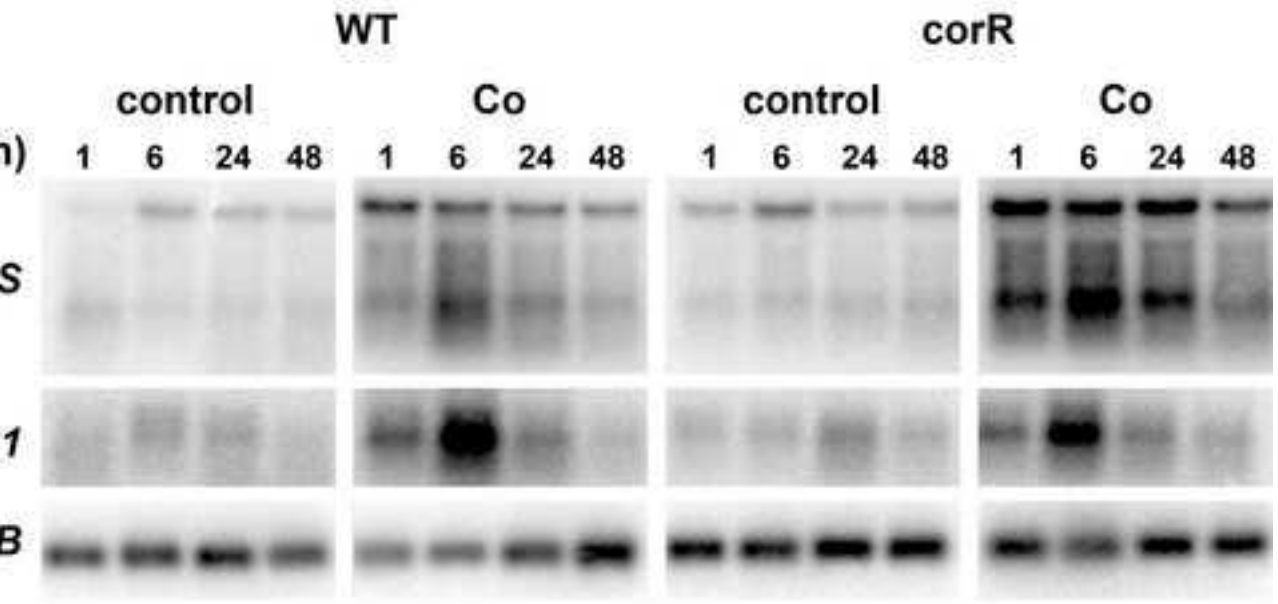
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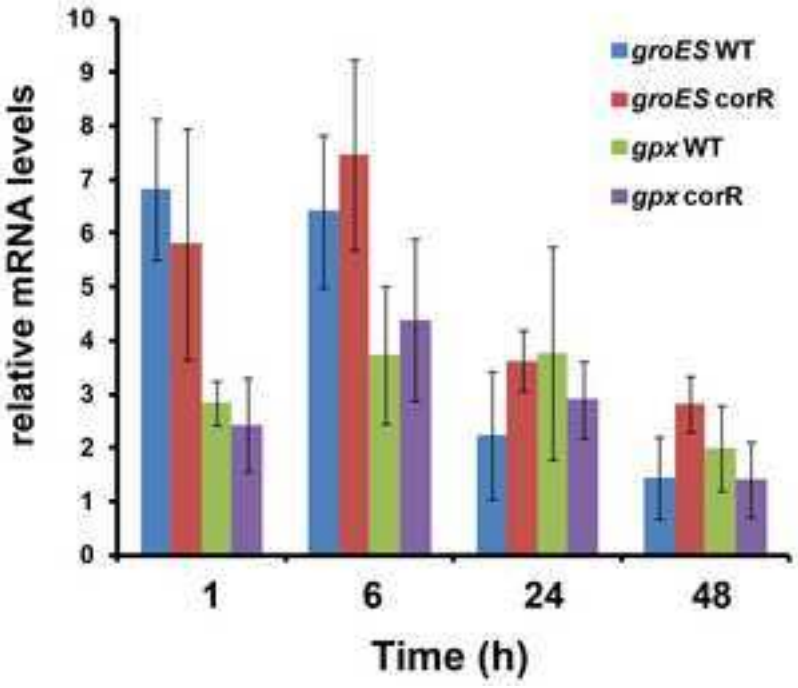
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Time (h)

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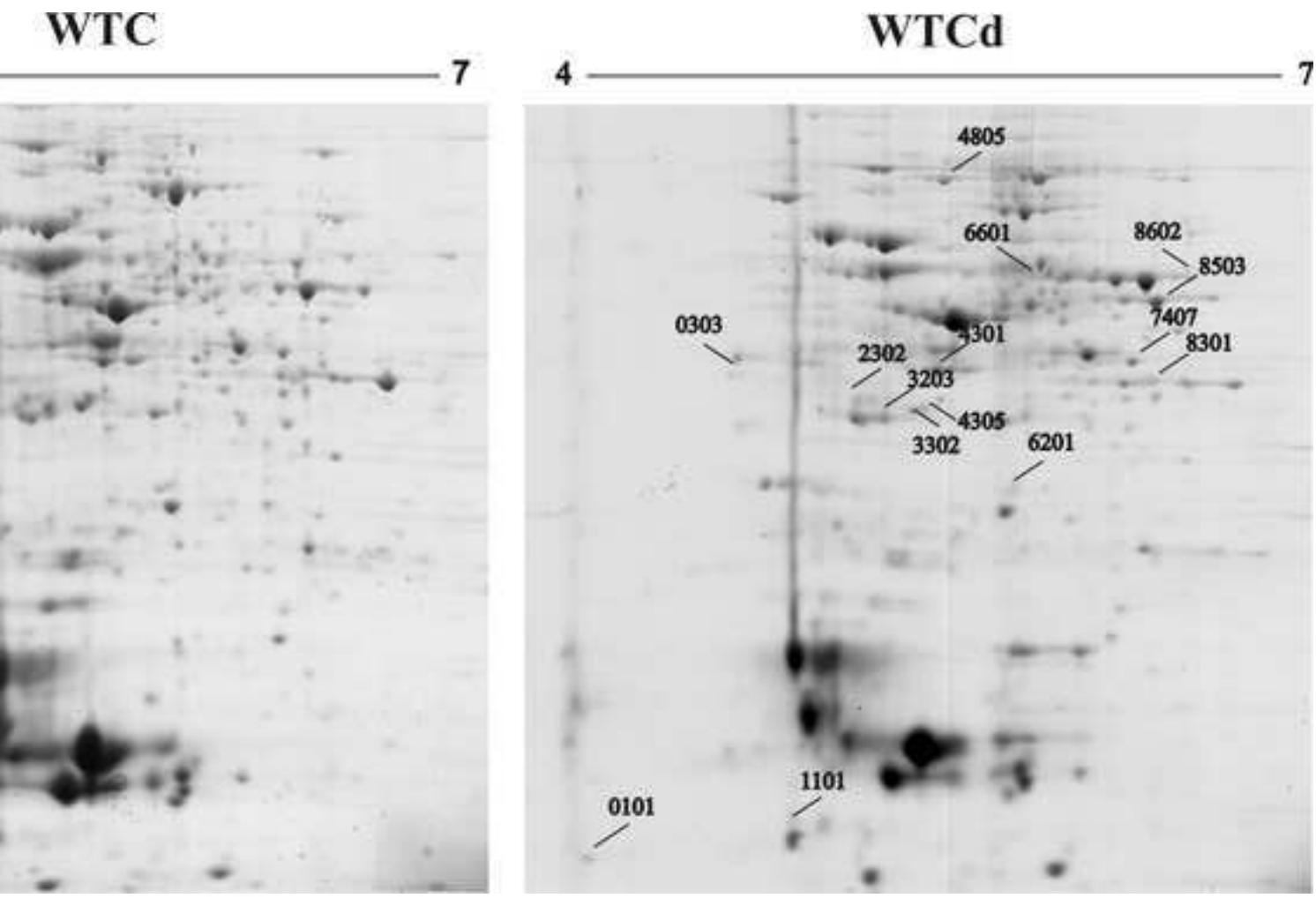
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