Title: Whole Genome Transcriptional Analysis of Heavy Metal Stresses in
Caulobacter crescentus

Running title: Transcriptional response to heavy metals in bacteria

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1   **ABSTRACT**

2   The bacterium *Caulobacter crescentus* and related stalk bacterial species are known for

3   their distinctive ability to live in low nutrient environments, a characteristic of most heavy metal

4   contaminated sites. *Caulobacter crescentus* is a model organism for studying cell cycle

5   regulation with well developed genetics. We have identified the pathways responding to heavy

6   metal toxicity in *C. crescentus* to provide insights for possible application of *Caulobacter* to

7   environmental restoration. We exposed *C. crescentus* cells to four heavy metals (chromium,

8   cadmium, selenium and uranium) and analyzed genome wide transcriptional activities post

9   exposure using a Affymetrix GeneChip microarray. *C. crescentus* showed surprisingly high

10   tolerance to uranium, a possible mechanism for which may be formation of extracellular

11   calcium-uranium-phosphate precipitates. The principal response to these metals was protection

12   against oxidative stress (up-regulation of manganese-dependent superoxide dismutase, *sod*A).

13   Glutathione S-transferase, thioredoxin, glutaredoxins and DNA repair enzymes responded most

14   strongly to cadmium and chromate. The cadmium and chromium stress response also focused on

15   reducing the intracellular metal concentration, with multiple efflux pumps employed to remove

16   cadmium while a sulfate transporter was down-regulated to reduce non-specific uptake of

17   chromium. Membrane proteins were also up-regulated in response to most of the metals tested. A

18   two-component signal transduction system involved in the uranium response was identified.

19   Several differentially regulated transcripts from regions previously not known to encode proteins

20   were identified, demonstrating the advantage of evaluating the transcriptome using whole

21   genome microarrays.

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INTRODUCTION

Potentially hazardous levels of heavy metals have dispersed into subsurface sediment and groundwater in a number of metal contaminated sites and represent a challenge for environmental restoration. Effective bioremediation of these sites requires knowledge of genetic pathways for resistance and biotransformation by component organisms within a microbial community. However, a comprehensive understanding of bacterial mechanisms of heavy metal toxicity and resistance has yet to be achieved. While many metals are essential to microbial function, heavy metals, i.e. most of those with a density above $5g/cm^3$, have toxic effects on cellular metabolism (46). The majority of heavy metals are transition elements with incompletely filled d orbitals providing heavy metal cations which can form complex compounds with redox activity (46, 70). Therefore, it is important to the health of the organism that the intracellular concentrations of heavy metal ions are tightly controlled. However, due to their structural and valence similarities to non-toxic metals, heavy metals are often transported into the cytoplasm through constitutively expressed non-specific transport systems (46). As such, heavy metals invariably find their way into the cell. Once inside the cell, toxic effects of heavy metals include non-specific intracellular complexation, with thiol groups being particularly vulnerable (53). Interactions of these non-specific complexes with molecular oxygen leads to the formation of reactive oxygen species such as H_2O_2, resulting in oxidative stress within the cell (23). In addition to oxidative stress, complexation of sulfhydryl groups with heavy metal cations results in reduced activity of sensitive enzymes (16). Previous studies identified several examples in which heavy metals were toxic to cellular processes: 1) heavy metal cations, (for example, cadmium) tend to bind to SH groups (46). In Gram negative bacteria, heavy metal cations can bind to glutathione. The resulting product (bisglutathione complexes) tends to react with

molecular oxygen to form oxidized bisglutathione, releasing the metal cation and hydrogen

peroxide. The bisglutathione must be reduced in an NADPH-dependent reaction with the

released metal ion beginning another cycle of binding and bisglutathione oxidation (23, 46),

resulting in considerable oxidative stress; 2) some metal ions structurally mimic physiologically

important molecules, for example, chromate resembles sulfate, arsenate resembles phosphate,

thus interfere with physiological processes in which those molecules are required (16); 3) some

metals, for example, chromate, are reduced intracellularly, by both enzymatic and non-enzymatic

mechanisms with the reduction process producing reactive oxygen species. This process may

inadvertently be a major factor in causing damages to many cellular components, including DNA

and proteins (12, 58, 65).

Resistant bacteria possess a number of strategies to withstand elevated concentrations of

heavy metals. Many resistance mechanisms revolve around removing the heavy metal or

decreasing its toxicity (70). Alternatively, the concentration of metal entering the cytoplasm may

be decreased through active (extracellular precipitation) and passive (native biosorption)

processes (30, 35). Metal-chelating proteins have been reported as a means of resistance mainly

in eukaryotes and also in some limited examples of prokaryotes (70). The major bacterial

resistance mechanisms includes: 1) Active efflux. 2) Transformation of the heavy metal ion to a

less toxic form, for example, Cr(VI) can be reduced to Cr(III) (12). 3) Precipitation, either inter-

or extracellular (35, 64, 66, 67).

Caulobacter spp. is extremely ubiquitous and is able to survive in low nutrient

environments (51). It has been found in freshwater, seawater, soil (51), ground water (37), waste

water (36), deep sea sediment (38), deep subsurface mine gold mine (19) and has been noted for

its ability to survive in broad environmental habitats where contamination may be present (8,

1   48). In addition, *Caulobacter crescentus* has been shown to form high-density biofilms with the

2   potential for use in bioreactors for bioremediation (60) and has been used as a model organism to

3   study cell cycle control (32, 40, 41). Previous knowledge of this organism including its genome

4   sequence (45) has provided new and extremely valuable tools to study genome wide response to

5   heavy metal stress. Both oligonucleotide cDNA microarrays and Affymetrix GeneChip

6   microarrays have been used to study cell cycle regulation (31, unpublished data by McGrath and

7   McAdams). In this paper we use a *Caulobacter* Affymetrix GeneChip[©] microarray (*Caulobacter*

8   chip) to study *C. crescentus* transcriptional response to heavy metal stress. This chip was

9   designed by the McAdams laboratory at Stanford University in collaboration with Affymetrix. A

10   complete description of all features of the chip will be published separately. In our work

11   reported here, only the gene expression assay features were used which are based on 9 optimally

12   selected 25mer match/mismatch probe pairs per predicted open reading frame.

13   **MATERIALS AND METHODS**

14   **Bacterial strains, media and growth**

15   The *Caulobacter crescentus* strain CB15N was used in this study. Cultures were

16   maintained on PYE (peptone-yeast extract) agar plates containing 0.2% (wt/vol) Bacto Peptone

17   (Difco) , 0.1% (wt/vol) yeast extract (Difco), 1 mM MgSO$_4$, and 0.5 mM CaCl$_2$ and 1.5%

18   (wt/vol) agar (Difco) . The knockout mutants were maintained with 10 µg/ml tetracycline on

19   solid medium (PYE agar), with tetracycline (5 µg/ml) being used in the overnight culture but not

20   in the culture for transcriptional analysis. Liquid culture used for transcriptional response studies

21   was M2 minimal salts medium (6.1 mM Na$_2$HPO$_4$, 3.9 mM KH$_2$PO$_4$, 9.3 mM NH$_4$Cl, 0.5 mM

22   MgSO$_4$, 10 µM FeSO$_4$ [EDTA chelate; Sigma Chemical Co.], 0.5 mM CaCl$_2$) with 0.2% (wt/vol)

23   glucose as the sole carbon source (M2G). Overnight culture was inoculated with colonies from

1 PYE plates and was grown at 30°C and shaken continuously at 225 rpm. *E. coli* K12 and

2 *Pseudomonas putida* KT2440 were used as reference bacteria to uranium tolerance. Both were

3 grown in M2G medium (identical conditions to that of *C. crescentus*) described above.

4 **Toxic metal effect on growth, survival and morphology**

5 Metal stock solutions were prepared by dissolving the compounds (Sigma-Aldrich) in

6 water to 10,000 ppm with the exception of the uranyl nitrate stock solution which was 100 mM

7 (23,800 ppm). All metal stocks were sterilized by filtration through a 0.2 μm membrane.

8 Overnight cultures were diluted in fresh M2G medium with various concentrations of cadmium

9 sulfate ($CdSO_4$), sodium selenite (Na_2SeO_3), potassium chromate (K_2CrO_4), potassium

10 dichromate ($K_2Cr_2O_7$) and uranyl nitrate ($UO_2(NO_3)_2 \cdot 6H_2O$). Growth was followed

11 spectrophotometrically ($OD_{600 \, nm}$). One metal concentration was selected to be used for each set

12 of microarray experiments, based on the following requirements for each metal: 1) the stressed

13 conditions only slightly affected growth (increased doubling time by 15-30 min); 2) the addition

14 of the heavy metal compound did not result in precipitation with salts or cause other obvious

15 changes in the medium; 3) the metal concentration was above a level considered toxic or close to

16 those conditions used commonly in other studies. While not all these criteria were fully satisfied,

17 the final concentrations used in this study were a compromise of the factors described above and

18 are reported in the results.

19 For morphology and motility observations, a sample was taken from mid-log phase

20 culture ($OD_{600 \, nm}$=0.3-0.4) as t_0. Metal stock was added to the final required concentration to be

21 used in microarray experiments. After 30 minutes, another sample was taken as t_1. A final

22 sample was taken 3-4 hours post stress as t_2. Ten microliters of each sample was examined under

23 100X phase-contrast light microscopy. Fifty microliters of each sample was used to determine

1   colony forming units. Bacterial membrane integrity was assessed using a Live/Dead BacLight

2   Bacterial Viability kit (Molecular Probes, OR) according to the manufacturer's recommended

3   protocol.

4   **RNA extraction**

5   An overnight culture of *C. crescentus* CB15N was diluted into fresh M2G medium.

6   When the culture reached exponential growth ($OD_{600\ nm}$ just over 0.3), 10 ml culture was

7   removed (as non-stressed control). Heavy metal stock was added and incubation was continued

8   for another 30 minutes. After this period, a further 10 ml of sample was removed (as the stressed

9   sample). Immediately after the samples were collected, they were centrifuged at 10,000g for 5

10   minutes and supernatant removed. The cell pellets were frozen with liquid nitrogen and stored at

11   -80 ^{0}C. The RNA extraction protocol was described previously (18). Briefly, total RNA was

12   extracted with Trizol reagent (Invitrogen) and any contaminating DNA digested with DNaseI.

13   The RNA samples were further purified with acid-phenol:chloroform:isoamylalchohol

14   (125:24:1, pH 4.5)(Ambion) extraction followed by salt-ethanol precipitation. RNA quantity was

15   determined by $OD_{260\ nm}$ and quality was determined by 2% (wt/vol) agarose gel electrophoresis

16   and by $OD_{260\ nm}/OD_{280\ nm}$ ratio.

17   **Affymetrix GeneChip RNA Expression Analysis**

18   Procedures for sample preparation and array processing are described fully in the

19   Affymetrix GeneChip® Expression Analysis Technical Manual, prokaryotic sample and array

20   processing (http://www.affymetrix.com/support/technical/manuals.affx) and briefly described

21   here. Transcripts of three genes (*Bacillus subtilis dab*, *phe* and *thr*) were added to the total RNA

22   as spike-in controls to monitor labeling, hybridization and staining efficiency. To generate the

23   spike-in control RNA, the plasmids containing *B. subtilis phe*, *thr* and *dap* genes were purified

1   from strains ATCC87483, ATCC 87484 and ATCC87486, respectively. Linear template DNA

2   was generated by digesting the plasmid with restriction enzyme *Not*I and sense RNA produced

3   subsequently by *in vitro* transcription using T3 RNA polymerase (MEGAscript™ T3 kit,

4   Ambion).

5   Total RNA (12 µg) was primed with random primer (Invitrogen) and cDNA was

6   synthesized with reverse transcriptase (superscript II, Invitrogen). The resulting cDNA was

7   fragmented with DNaseI (Amersham) and biotin labeled using the Enzo BioArray terminal

8   Labeling kit (Affymetrix). Biotin-labeled samples were hybridized onto the *Caulobacter*

9   microarray at 50°C overnight and chip washing and staining followed standard Affymetrix

10   GeneChip protocols (with stringent washing at 50°C). The high density chip was scanned using

11   an Affymetrix Scan3000 scanner.

12   **Microarray data analysis and Identifying differentially expressed transcripts under heavy**

13   **metal stress**

14   The *Caulobacter* Affymetrix chip was used to assay gene expression levels for all 3767

15   genes (45). For analysis of the protein coding region, probe-sets consisting of multiple (typically

16   9) 25-mer oligonucleotide probe pairs covering the gene were used for transcriptional

17   interrogation. These probe-sets were analyzed using the MAS5 statistical algorithm (3) for

18   background adjustment and scaling in GCOS software (Affymetrix). Briefly, data from a

19   minimum of three independent experiments were included as biological replicates in each

20   comparison. Global scaling of all probe-sets to a target signal intensity of 500 was applied to

21   each chip (all microarray data were available at:

22   http://greengenes.lbl.gov/Download/Caulobacter_metal_stress_supplemental_microarray_data/).

23   The dataset was normalized using the spike-in controls mentioned above. For each comparison

the *t* test was performed on the data. For a probe-set (gene) to be considered up-regulated under

metal stress in these studies, it had to meet the following criteria: 1) The gene must be called

"present" by GCOS software for every experiment which is under metal stress. 2) The average

difference score (signal) for this gene had to be equal to or greater than 200, eliminating very low

expression levels requiring more sophisticated analyses. 3) The P value of the student's t test

must be less than 0.01, ensuring that the difference between the two conditions (non-stressed and

stressed) is significant at a 99% confidence interval. 4) The ratio of the average signals from

stressed culture versus the average signals from non-stressed culture must show at least two fold

differences in expression.

      The probes for predicted small protein regions were tiled every fifteen nucleotides on

both strands. Since the boundary of these predicted proteins may be inaccurate, it is possible that

some of the probes in the default probe-set do not belong to a single transcript. It is inappropriate

to use any of the existing software to obtain probe-set values. At present, available microarray

analysis programs do not allow dynamic definition of probe-sets, thus identification of

transcripts driven by experimental data is not possible. To analyze these regions, we opted to

examine expression of these regions on a probe-by-probe basis. The background adjustment and

normalization were performed using custom scripts developed by our lab. Probe-level data were

examined in which individual probes meeting the following criteria were selected: 1) the

average difference score (signal) for a probe was equal to or greater than 200. 2) The P value of

the student's t test was less than 0.015. 3) The average signals from stressed culture versus the

average signals from non-stressed culture were at least two-fold different to be considered for

further analysis. Probes selected using these criteria were assembled in order to find continuous

regions of up-regulation. The criteria defining a probe-set for the small protein regions were as

1	follows: 1) the maximum number of non-passing probes within a probe-set (i.e. those not

2	meeting the individual probe criteria listed above) should not exceed 3; 2) the minimum length

3	of assembled transcript should be >100 bp. Probe-set boundaries were also corrected according

4	to probe behavior. For probe-sets of interest, manual inspections were performed for final

5	evaluation. This approach permitted monitoring of the transcriptional activities without bias.

6	**Gene Annotation and metabolic pathway analysis**

7	Initial *Caulobacter crescentus* open reading frame annotations were taken from

8	GeneBank accession number NC_002696 (45). COG (clusters of orthologous gene) descriptions

9	(69) were used if it described the functions more clearly. If a clearly definitive annotation of

10	function was not found, BLAST was performed against all bacterial genomes and Pfam analysis

11	was run to identify any domains. The identification of pathways involved in metabolism was

12	aided by the use of BioCyc (http://biocyc.org). Other analyses, such as operon prediction and

13	gene ontology were improved through the use of the VIMSS (Virtual Institute for Microbial

14	Stress and Survival) database (http://www.microbesonline.org/).

15	**Transmission electron microscopic (TEM) observation.**

16	The samples were fixed in 4% paraformaldehyde in 0.1 M cacodylate buffer at pH 7.4

17	(Electron Microscopy Sciences) for 1 h at room temperature. The fixed samples were dehydrated

18	with a graded series of ethanol and t-butyl alcohol. The samples were freeze-dried and mounted

19	on 200-mesh copper grids for characterization with a HF2000 field-emission transmission

20	electron microscope (Hitachi, Inc.), which was equipped with energy-dispersive x-ray

21	spectroscopy (EDX). TEM was operated at an accelerating voltage of 200 kV. The abiotic

22	sample was prepared by adding uranium stock solution to the sterile growth medium followed by

23	centrifugation at 10,000 g for 1 min.

1   **RESULTS AND DISCUSSION**

2   **Heavy metal affect on growth and survival**

3   Relatively low concentrations of cadmium sulfate, potassium chromate and potassium

4   dichromate were sufficient to slow *C. crescentus* growth (supplemental figure 1, 2, 3). For

5   microarray experiments, we defined the stressed condition as 6 μM cadmium sulfate, 40 μM

6   potassium chromate and 27 μM potassium dichromate. Under these conditions, the doubling

7   times for the cells were 2.5 hours under cadmium stress and over 3 hours under chromium stress

8   compared to non-stressed cells which had doubling times of 2 hours. Selenite stress was apparent

9   at a concentration of 300 μM sodium selenite (doubling time increased by approximately 10 - 15

10   min). For uranium stress, a concentration 200μM uranyl nitrate was used. The relevance of these

11   metal concentrations to those in contaminated and uncontaminated groundwater is summarized

12   in Table 1. Surprisingly, at the 200 μM is uranium concentration used in this study (a

13   concentration close to the highest observed at the NABIR Field Research Center, Table 1),

14   *Caulobacter* growth rate was not significantly affected (supplemental figure 4) and it was not

15   until a concentration of 1 mM uranium that *Caulobacter* growth slowed (doubling time slowed

16   from 120 min to 180 min). Under these conditions (1 mM) visible precipitation was noted in the

17   medium, hence this concentration was not used for microarray analysis. Under the same

18   conditions (1 mM uranyl nitrate), growth of *E. coli* K-12 was completely stopped and the growth

19   of *Pseudomonas putida* KT2440 was drastically reduced (Figure 1). Growth of *E. coli* was

20   significantly affected at 500 μM uranium, while growth of *P. putida* was initially inhibited at 800

21   μM uranium, although the *P. putida* culture did grow to higher density after more than 20 hours.

22   We believe this is the first study to identify *C. crescentus* as a uranium-tolerant bacterium.

23   However, due to precipitation at higher concentrations of uranium, it was not possible to

10

1   definitively determine the absolute concentration of *Caulobacter* uranium tolerance. The

2   comparison to the reference bacteria (*E. coli* K12 and *Pseudomonas putida* KT2440) was

3   performed using the identical medium preparation and growth protocol, demonstrating that *C.*

4   *crescentus* is relatively more tolerant, is valid, but relative. A previous study showed that *E. coli*

5   was killed at approximately 333 μM of uranium at pH 4 (66), however it is not immediately

6   comparable to our data as uranium is known to be more soluble and more toxic at acidic pH.

7   Microscopic examination after exposure to the various metals did not indicate significant

8   changes in cell morphology with the exception of some loss in motility after 3-4 hours of

9   exposure to chromate or dichromate. Membrane integrity test (by BacLight kit) at 30 minutes

10   post chromium exposure did not reveal any obvious membrane damage at this point. No

11   significant change in viability (CFU) was noted in stressed compared to non-stressed cells (data

12   not shown).

13   **Differentially regulated genes common to multiple heavy metals**

14   Transcriptional response to cadmium, chromate, dichromate and uranium shared 4 up-

15   regulated transcripts (Table 2), however, the only gene with known function was the superoxide

16   dismutase with Mn as its cofactor (*sod*A). The greatest induction of this gene occurred under

17   cadmium and chromium stress, with induction under uranium stress being lower. Superoxide

18   dismutases are known to remove superoxide radicals which may be generated upon exposure to

19   heavy metals (22, 23, 46, 63). While *Caulobacter crescentus* has three superoxide dismutase

20   genes with different cofactors, *sod*A (CC1777, Mn), *sod*B (CC3557, Fe) and *sod*C (CC1579, Cu-

21   Zn), the major gene involved in heavy metal response appears to be *sod*A. Under non-stressed

22   conditions, it was at background levels, but was up-regulated 19, 14, 9 and 3 fold in cadmium,

23   chromate, dichromate and uranium stress respectively. Transcription of *sod*B on the other hand

1   was constitutive and was only two fold up-regulated under cadmium stress. Since *sod*B was only

2   induced under cadmium stress, when *sod*A was already substantially upregulated (19 fold), it is

3   possible that *sod*B was up-regulated as a compensation mechanism under substantial oxidative

4   stress. Transcription of *sod*C was also constitutive under non-stressed conditions but did not

5   increase above the 2 fold threshold under any of the metal stress conditions. The difference in the

6   regulation of the three enzymes may reflect their subtle differences in response to various

7   oxidative stressors. It is known that *C. crescentus sod*A(Mn) and *sod*B(Fe) are cytosolic and

8   *sod*C(Cu-Zn) is periplasmic (55). The induction of *sod*A and *sod*B but not *sod*C suggests that the

9   oxidative stress imposed by the metals is intracellular rather than extracellular, which is

10   consistent with the view that the oxidative stress originated from the reactive oxygen species

11   generated by the interaction of metals and cellular components. The different scale of induction

12   of *sod*A with various metals probably reflects the variable oxidative potentials of the metals

13   inside the cells. Chromium and cadmium are well known to induce oxidative stress in cells,

14   while uranium in our system seemed to provoke a smaller response, although the precise

15   intracellular quantity of metals is not directly measured in our system. As such it is not possible

16   to determine whether the variable oxidative response is a function of metal properties or if it is a

17   dose response.

18   Four genes (CC3254, CC3255, CC3256 and CC3257) consecutively located on the

19   chromosome were commonly up-regulated under cadmium, chromate, dichromate and selenite

20   stress (supplemental data). The last three of the genes were suggested to be in an operon. Since

21   two (CC3254, CC3257) out of the four genes were predicted to be membrane proteins, this

22   general response is likely to be associated with membrane protein function, however protein

23   sequences did not show any similarity to typical efflux pumps or transporters.

1   We identified total of 59 genes which were down-regulated at least 5 fold under

2   cadmium, chromium or uranium stress although we did not observe any genes commonly down-

3   regulated more than 5 fold. The most significant finding was the down-regulation of a sulfate

4   transporter under chromium stress (supplemental data). Since chromate structurally resembles

5   sulfate, and chromate typically enters the cell by the sulfate uptake system (47), we believe a

6   response intended to reduce chromium entering into the cell. Similarly, dichromate stress

7   provoked the down-regulation of the same transporter despite its structural differences. The

8   reason may be rooted in chromate and dichromate equilibrium. Chromate and dichromate

9   equilibrium is concentration and pH dependent ($K = 10^{-2.2}$). Under our experimental conditions

10   (pH 6-7 and concentration below 0.05 mM), significant amount of chromate will be present (44)

11   in the dichromate solution, thus eliciting a transcriptional response to the chromate stress.

12   **Differential gene expression under cadmium stress**

13   A total of 144 genes were up-regulated at least 2 fold under cadmium stress

14   (supplemental data). Several groups of annotated genes (Table 3) are discussed here.

15   The expression data demonstrated that one major detoxification mechanism appears to be

16   active efflux employing a translocation P-type ATPase to lower the intracellular cadmium ion

17   concentration. This has previously been recognized as a detoxification mechanism in other

18   bacteria (46, 57). There were two groups of efflux proteins involved that were physically

19   clustered at different chromosomal locations. The main cluster (cluster one) was from CC2721 to

20   CC2727 and expression of all the genes in this cluster were at background levels under non-

21   stressed conditions but specifically induced under cadmium stress, in fact the majority of the

22   genes were up-regulated over 10 fold. CC2721 is an outer membrane efflux protein and CC2722

23   overlaps CC2721, presumably in the same operon. CC2722 contains a HlyD family domain,

1   which is involved in the export of proteins not requiring cleavage of N-terminal signal peptides.

2   CC2726 is a cation transporting P-type ATPase, which has previously been reported to be

3   primarily responsible for translocating cadmium ions (and other closely-related divalent heavy

4   metals such as cobalt, mercury, lead and zinc) across membranes (6). CC2724 is highly

5   homologous to *ncc*A (Nickel-cobalt-cadmium resistance protein) and *czc*A (heavy metal efflux

6   pump for divalent cations – cadmium, zinc and cobalt). While CC2723, CC2725 and CC2727

7   were also up-regulated over 10 fold on the same chromosomal cluster, their functions are not yet

8   known.

9   Another cluster (cluster two) of efflux pumps, CC3195, CC3196 and CC3197 were also

10   specifically up-regulated under cadmium stress, however, they were transcribed at basal but

11   clearly above background level under non-stressed condition. The increase of these transcripts

12   was moderate, about 3 fold under cadmium stress. It is possible that this group of proteins acted

13   as 'patrol' agents, responding to stresses if needed. On the other hand, proteins in cluster one

14   were truly specifically induced by cadmium (expression was at background level under non-

15   stressed conditions). It is not been experimentally demonstrated whether the two groups of

16   transporters have subtle differences in functions. If the differential transcriptional patterns cannot

17   be explained by functional requirement, this may be another example of supplementary induction

18   similar to that observed with superoxide dismutase. In this scenario, it appears that the main

19   system is specifically induced, and if it alone is not sufficient to manage the effects of

20   intracellular stressors, a supplementary mechanism is also induced. At this point, it is not clear

21   whether any of these efflux pumps work synergistically as essential components of one system,

22   or simultaneously to increase total output.

1   Cadmium is known to cause oxidative stress by depleting glutathione and protein-bound

2   sulfhydryl groups, resulting in the production of reactive oxygen species. Consequently, it leads

3   to enhanced lipid peroxidation, DNA damage and altered calcium and sulfhydryl homeostasis

4   (63). We observed many groups of genes which were up-regulated to deal with this type of

5   stress. As described previously (**Differentially regulated genes common to multiple heavy**

6   **metals)**, the enzyme directly involved in response to oxygen stress is the superoxide dismutase

7   (Mn). Other proteins involved in removing toxic compounds and protecting thio-groups were

8   also up-regulated. Glutathione S-transferase has been shown to be induced by heavy metals in

9   plants (39). Glutathione (GSH) has two general functions: to remove toxic metabolites from the

10   cell and to maintain cellular sulfhydryl groups in their reduced form. Glutathione S-transferase

11   (GST) detoxifies xenobiotic compounds or products of oxidative stress by covalent linking of

12   glutathione to hydrophobic substrates (13, 17). *C. crescentus* GSTs CC1316 and CC2434 were

13   up-regulated in response to cadmium stress. Thioredoxin is a general protein disulfide reductase,

14   believed to serve as cellular antioxidant by reducing protein disulfide bonds produced by various

15   oxidants. In this study we noted three thioredoxin coding transcripts (CC0062, CC0110 and

16   CC3539) up-regulated under cadmium stress. Glutaredoxin is also known as thioltransferase; it is

17   a small protein of approximately one hundred amino-acid residues which functions as an electron

18   carrier in the glutathione-dependent synthesis of deoxyribonucleotides by the enzyme

19   ribonucleotide reductase. Like thioredoxin, which functions in a similar way, glutaredoxin

20   possesses an active center disulphide bond and exists in either a reduced or an oxidized form

21   where the two cysteine residues are linked in an intramolecular disulphide bond. It is not

22   surprising that *C. crescentus* glutaredoxin (CC2505) was up-regulated under cadmium stress,

23   while the other glutaredoxin (CC0829) was not, since CC0829 has more sequence homology to

1   *E. coli* glutaredoxin 3 (grx3), which has been shown to have narrower substrate specificity *in*

2   *vivo* (54). Peptide methionine sulfoxide reductase (CC0994 and CC1039) was also up-regulated

3   and together with thioredoxin, it can reverse the effects of oxidative damage on methionine

4   residues in proteins (34, 42). Under cadmium stress, both were up-regulated slightly over 2 fold.

5   Both the riboflavin biosynthesis pathway and GTP cyclohydrolase I (*fol*E) were up-

6   regulated (3.5 to 4 fold) under cadmium stress (Table 3). Their up-regulation may be due to

7   oxidative stress. Previous studies have shown that H_2O_2 induced expression of GTP

8   cyclohydrolase I mRNA in vascular endothelial cells (56) is extremely sensitive to oxidative

9   stress, and this enzyme is also one of the major targets of H_2O_2 in *E. coli* (33). There are several

10   possibilities for the induction of riboflavin biosynthesis pathway, for example, studies in *E. coli*

11   have demonstrated that GTP cyclohydrolase II is induced by redox-cycling agents and is

12   positively regulated by *sox*R and *sox*S (29), the global regulators for oxidative stress. Riboflavin

13   is a precursor of both FMN and FAD which are important coenzymes of several oxidoreductases

14   (1, 52). In addition, ribonucleotide reductase was up-regulated, and in *E. coli* at least, this

15   enzyme is activated by flavins (15). It provides precursors necessary for DNA synthesis by

16   catalyzing the reductive synthesis of deoxyribonucleotides from their corresponding

17   ribonucleotides. This reaction also replenishes the pool of reduced thioredoxin which also has a

18   role in protection against oxidative stress (54). Another example of an up-regulated enzyme with

19   FAD/FMN as cofactors is CC2129 which belongs to the NADH:flavin oxidoreductase family,

20   members of which are capable of reducing a range of alternative electron acceptors. It is also

21   possible that GTP cyclohydrolase II was up-regulated to protect genetic material against damage

22   from oxygen radicals. For example *E. coli* GTP cyclohydrolase II can hydrolyze 8-oxo-dGTP,

23   which is an oxidized form of dGTP, overproduction of this enzyme has been shown to reduce

16

1   mutation frequency of the *mut*T strain (mutT protein prevents A:T to C:G transversion by

2   hydrolyzing 8-oxo-dGTP) to almost normal levels (28).

3   Interestingly four genes resembling arsenic resistance system components were induced

4   by cadmium (Table 3). These genes have homologues in *Psedomonas putida* KT2440 (11), in

5   *Thiobacillus ferrooxidans* (10) and in the virulence plasmid pYV of *Yersinia enterocolitica* (43).

6   CC1503 is most similar to *ars*C, an arsenic reductase, responsible for arsenate reduction to

7   arsenite which is then translocated out of the cell by *ars*B using proton motive force. However,

8   while CC1504 would be the primary candidate for *ars*B according to its location and it also

9   contains a transmembrane domain, the sequence and the domain structure are different from

10   those of *ars*B. As such, its capacity to function in a similar manner to *ars*B or indeed its

11   involvement in response to cadmium stress is not clear. CC1505 is most similar to *ars*R which is

12   a transcriptional repressor with a helix-turn-helix DNA binding domain that is thought to

13   dissociate from DNA in the presence of metal ions (74). Sequence comparison shows that the

14   metal-binding site of *ars*R resembles that of *cad*C from *Staphylococcus aureas* plasmid pI258

15   (76) and from *Bacillus firmus* OF4 (7). Cadmium ion is thought to relieve repression by *cad*C

16   (20, 74). Therefore, it is possible the *ars*RBC operon in *C. crescentus* was up-regulated because

17   cadmium binds to *ars*R and release the repression. CC1506 is most similar to *ars*H, and was

18   highly up-regulated in *C. crescentus* cells under cadmium stress. Despite the fact that CC1506

19   contains a NADPH-dependent FMN reductase domain, its direct involvement in metal resistance

20   is not definitive. Likewise the detailed function of other *ars*H genes is unclear.

21   **Response to uranium stress**

22   Bacteria are known to possess several mechanisms for resistance to uranium that

23   frequently involve precipitation to reduce toxicity. For example, uranyl ions may be sequestered

1   intracellularly by complexation with phosphate granules, as in the case of *Arthrobacter* spp. (66)

2   and *Pseudomonas* spp. (64). In *Citrobacter* spp., inorganic phosphate is liberated from organic

3   forms resulting in precipitation of various uranyl phosphate crystal complexes outside the cell

4   (35). To investigate if such mechanisms were utilized as a uranium resistance mechanism by

5   *Caulobacter crescentus* we performed TEM and EDX analysis and demonstrated that *C.*

6   *crescentus* did not form any uranium-containing phosphate granules intracellularly. However,

7   TEM images of whole cells of *C. crescentus* revealed extracellular precipitates associated with

8   the cells (Figure. 2A). EDX spectra from cells and extracellular precipitates show that while

9   uranium is almost absent within cells (Figure. 2B), extracellular precipitates contain high

10   concentrations of uranium, phosphorus and calcium (Figure. 2C), suggesting that the

11   extracellular precipitates are composed mainly of these elements. The selected-area electron

12   diffraction (SAED) patterns of the extracellular precipitates indicated that the extracellular

13   precipitates are amorphous, although uranyl phosphate minerals are known to readily become

14   amorphous under the high vacuum of TEM (68). In order to examine whether the formation of

15   the extracellular precipitates was catalyzed by *C. crescentus* or was simply an abiotic

16   phenomenon, uranium-bearing precipitates formed in the same medium in the absence of *C.*

17   *crescentus* were also characterized (Figure. 2D). EDX analysis revealed that abiotic precipitates

18   were mainly composed of uranium and phosphorus with substantially lower calcium content than

19   the biological precipitates (Figure. 2E), indicating either direct or indirect biological involvement

20   in formation of the calcium-uranium-phosphate complex.

21   Based on the chemical composition, the precipitates are thought to be the uranyl phosphate

22   mineral autunite $Ca[(UO_2)_2(PO_4)]_2 \cdot 11H_2O$. Autunite is a major source of naturally occurring

23   secondary uranium ore and is known to persist under oxidizing conditions on a geological time-

1   scale (21). In contrast, the uranium-bearing precipitates formed abiotically in the uninoculated

2   control experiment appear to be chernikovite $H(UO_2)(PO_4) \cdot 4H_2O$. Interestingly, transcriptional

3   analysis did show a candidate (CC1295) which may involved in the uranium precipitation

4   process. CC1295 has a phytase domain. The protein with this domain was found to bind to Ca^{2+}

5   and organic phosphate (myo-inositol hexakisphosphate) (49). Further investigations are needed

6   to prove or disprove the hypothesis that the active site of this enzyme may facilitate the

7   precipitation of calcium-uranium-phosphate complexes by providing a nucleation site.

8   Uranium stress also induced transcription of 48 transcripts which were up-regulated at

9   least 2 fold (supplemental data), however, the response to uranium does not appear to overlap

10   substantially with other heavy metal stresses evaluated in this study. It is believed that uranium,

11   unlike cadmium and chromate, imposes less direct oxidative damage to cells, thus it is not

12   surprising that most of the commonly up regulated genes noted under cadmium and chromium

13   stress were not up regulated under uranium stress.

14   A large portion of the up-regulated genes were difficult to classify into pathways mainly

15   due to a lack of functional annotation (Table 4), although even with clearly annotated proteins,

16   such as two-component systems, their target(s) have not previously been identified. Two up

17   regulated two-component systems were identified: CC1293-CC1294 and CC1304-CC1305. They

18   are DNA-binding response regulators consisting of a CheY-like receiver domain and were up

19   regulated (7-9 fold) only under uranium stress. Both CC1293 and CC1304 have signal sensing

20   domains at the N-terminal and a DNA-binding domain at the C-terminal but no sigma factor

21   interaction domains were detected. CC1294 and CC1305 have histidine kinase domains at the C-

22   terminal and a HAMP membrane domain was also detected at the N-terminal of CC1294,

23   indicating it could be a membrane protein. The e-value of Pfam analysis for a membrane domain

1   in CC1305 is too low (0.27, compared to 6.6e-12 for CC1294) to believe it has a membrane

2   component, therefore, it may be cytoplasmic. The domain structure of the proteins suggests that

3   CC1294 and CC1305 receive their respective metabolic/environmental signals in the following

4   manner: the histidine kinase catalyzes ATP and transfers a phosphoryl group to the response

5   regulator (CC1293 or CC1304) resulting in activation of the DNA-binding domain that elicits the

6   specific response – activation or repression of the transcription of their targeted gene(s).

7   ΔCC1293 and ΔCC1294 are knockout mutants in which CC1293 and CC1294 were

8   replaced in-frame by tetracycline-resistant cassettes (59). Both mutants were generated using

9   strain CB15N, thus the results of the mutants should be directly comparable with other data in

10 this study. Sequencing of PCR amplification products confirmed the deletion and correct

11 chromosomal location, furthermore transcription of CC1293 or CC1294 was not detected in their

12 respective mutants. Compared to that of the wild type, the growth of ΔCC1294 was unaffected

13 under 200 μM uranyl nitrate stress, however growth did slow after 120 min of uranium stress at 1

14 mM. Growth of ΔCC1293 showed no significant difference from the wild type at any uranium

15 concentration tested. Expression of CC1293 decreased in the ΔCC1294 background, possibly

16 because CC1294 is closer to the transcriptional start of the operon and replacing it with a

17 tetracycline cassette negatively impacted the overall quantity of CC1293 message, despite this,

18 the regulation was not lost (Table 5). With the exception of four transcripts (Table 5) whose

19 functions are unknown, transcripts responding to uranium maintained the same regulation

20 patterns in the knockout mutants as in the wild type. Therefore it appears from growth and

21 microarray data that CC1293-CC1294 is not a master regulator of uranium response despite its

22 specific up-regulation under uranium stress.

1   In plant and human studies many antisense transcripts have been identified (24, 75) and

2   in this study the transcript that had the highest fold of induction specific to uranium stress came

3   from an antisense transcript of CC3302 (transcribed from the opposite strand of the predicted

4   CC3302). CC3302 was annotated as in the minus strand. The up regulated probe-set interrogates

5   the plus strand of the same region. Since the boundaries of this probe-set were arbitrary, we

6   examined the data at the probe-level and discovered that the first 3 probes of this probe-set and

7   the upstream region immediately adjacent to this probe-set were not transcribed. The total length

8   of the regulated transcript is about 420 bp, with an open reading frame containing two possible

9   start codons (ATG) of five amino acids apart (Figure. 3). The size of the possible protein would

10   be 112 or 117 amino acids. Further examples were detected of such regulated anti-sense

11   transcripts responding to uranium or other heavy metals, particularly chromium and cadmium

12   (Table 6). While the function of most anti-sense transcripts is not currently understood, these

13   may play important and previously overlooked roles in response to heavy metal stress. Detection

14   of such regulated transcripts is only possible using microarrays designed to cover the whole

15   genome without automatic assumption of previous (possibly incomplete) predictions.

16   **Differential gene expression under chromium stress**

17   Hexavalent chromium, Cr(VI), is found together with a variety of aromatic compounds in

18   a number of contaminated sites, including groundwater aquifers, lake and river sediments, and

19   soils. Both chromate resistance and toxicity can be related to Cr (VI) reduction. The central

20   mechanism of chromate toxicity is thought to be the reactive oxygen species that initial

21   intracellular chromate reduction generates (27, 58, 65). A common pathway for Cr(VI) reduction

22   to the less toxic Cr(III) is through an unstable Cr(V) intermediate (14) which is subject to redox

23   cycling, and as such can generate large amounts of reactive oxygen species which induce cellular

1   damage. While Cr(VI) reduction to Cr(V) occurs spontaneously by cellular components (61, 62)

2   only chromium resistant bacteria are capable of reducing Cr(V) to Cr (III) intracellularly, thus

3   minimizing the oxidative damage induced by Cr(V) as it occurs transiently within the cell (1, 2).

4   Although we present no direct evidence to discount intracellular chromate reduction in *C.*

5   *crescentus,* both the phenotypic data (the concentration of chromium severely reducing growth

6   was relatively low (50 µM) compared to that in efficient chromium reducing bacteria, e.g. 400

7   µM in *Pseudomonas putida*, (2)) and the transcriptional analysis in this study strongly suggest

8   that there was significant intracellular damage induced by oxidative stress, and therefore it is

9   unlikely that significant chromium reduction occurred.

10   In *C. crescentus*, it appears that the majority of up-regulated genes were in response to

11   chromium induced oxidative stress (Table 7). Both chromate and dichromate contain Cr (VI) and

12   subsequently a large portion of the up regulated genes (214) are common to both metal stresses,

13   however their chemical structures differ and this may account for the number of genes only up-

14   regulated two-fold upon exposure to one form chromium. Following chromate exposure 84

15   genes were specifically up-regulated while 23 were specifically up-regulated following

16   dichromate exposure (supplemental data).

17   Several genes which are known to be involved in response to oxidative stress were up-

18   regulated under either chromate or dichromate stress. The superoxide dismutase (Mn, *sod*A) had

19   9-14 fold induction while the other two SODs of different cofactors were not up-regulated more

20   than two fold. As with cadmium stress, glutathione S-transferase and thioredoxin were up-

21   regulated. However, under cadmium stress, two different glutathione S-transferases were up-

22   regulated (only 2-3 fold) and growth was less severely affected. Under chromium stress, one

23   glutathione S-transferase (CC2311) was up-regulated about 6 fold (Table 7), but unlike under

1   cadmium stress, glutathione synthetase (*gsh*B, CC0141) was not up-regulated. As the

2   physiological states of the cells were quite different between chromium and cadmium stresses

3   (substantially slower growth under chromium stress), this suggests that *C. crescentus* may

4   employ different processes to counteract oxidative stress depending on the physiological state of

5   the cell.

6   DNA damage by reactive oxygen species upon chromium exposure is well documented

7   (4, 73). Up-regulation of *rec*A is known to be induced by DNA breakage in *E. coli* and previous

8   studies have shown that chromate induced DNA damage strongly depends on the reactive

9   intermediates. Frequently, chromate causes DNA single strand breakage and 8-

10 hydroxydeoxyguanosine formation (correlated with hydroxyl radical as the DNA-damaging

11 species) (5, 26). In this study, DNA repair enzymes, such as CC2272 and CC2200 were up-

12 regulated (Table 7). This suggests that chromate induced damage in *C. crescentus* cells may

13 indeed be mediated by hydroxyl radicals generated through non-specific chromate reduction.

14   Studies of animal and plant cells have shown that chromium can cause membrane

15 damage through direct or oxidative stress-mediated interactions (14, 25, 50) and our

16 transcriptional data indicate that chromium exposure induces a membrane response. We

17 observed induction of two OmpA family proteins (*omp*A mutants exhibit increased sensitivity to

18 environmental stresses (72)), TonB-dependent outer membrane receptors and lipopolysaccharide

19 (LPS) biosynthesis (Table 7). Our data indicate that the TonB receptor family of proteins was

20 involved in the response to several different metals, such as chromium (Table 7), cadmium

21 (Table 3) and uranium (Table 4), and yet in most cases, different proteins in the family were up-

22 regulated under specific metal stress. However, it is not clear whether the up-regulated TonB

23 receptor gene interacts with TonB protein, since the expression of the predicted TonB protein

1   (CC2327) was suppressed and not activated under heavy metal stress. It is likely that the receptor

2   merely binds to the substrates (heavy metals) and communicates extracellular environmental

3   information.

4   We observed up-regulation of several genes and pathways which typically occur during

5   stationary phase or under nutrient-limiting conditions (Table 7), including acquisition of

6   ammonium, phosphate-starvation response, PHB biosynthesis (including genes in fatty acid

7   oxidation pathway which can provide precursors for synthesis of medium chain PHBs) and

8   energy/carbon utilization (PHB depolymerase and glucan glucohydrolase).

9   The cells appeared to exhibit an increased demand for ammonium and glutamate, since

10   ammonium transport, the glutamate synthase and the histidine degradation pathway (generating

11   ammonium and glutamate) were up-regulated (Table 7). Glutamate is one of the central amino

12   acids that links nitrogen (GS-GOGAT cycle) and carbon/energy metabolism (TCA cycle)

13   through α-keto-glutarate. There appeared to be an increased flow of glutamate to α-keto-glutarate

14   as indicated by the up-regulation of serine biosynthesis pathway. In addition, glutamate synthase

15   contains an iron-sulfur cluster and has close structural homology with glutamine

16   phosphoribosylpyrophosphate amidotransferase (71). This latter enzyme from *B. subtilis* is

17   known to be inactivated by O_2 in stationary phase (9). Although up-regulation of *ntr*BC and PII

18   usually indicates nitrogen limitation response, the key enzyme (glutamine synthetase) was not

19   up-regulated. It is possible that increased ammonium uptake was the first step towards

20   responding to nitrogen demand, and thus it seems plausible that simply obtaining more

21   ammonium from the environment would provide a more energy efficient response than

22   provoking a complete nitrogen starvation response. If this hypothesis is correct, our data suggest

23   that the three PII proteins of *C. crescentus* may be regulated separately by specific physiological

1 conditions and that *ntr*BC may be involved in activating the transcription of not only the

2 glutamine synthetase but other operons such as ammonium transport. Therefore, it may be that

3 nitrogen regulation in *C. crescentus* is more complicated than *E. coli* at least at the

4 transcriptional level.

5 **Differential gene expression under selenium stress**

6 The response to sodium selenite was mild compared to other metal stresses. Only 12

7 transcripts were up-regulated and at most by 4-5 fold (supplemental data). All of these were also

8 observed to be up-regulated under chromium or cadmium stresses and included membrane

9 components, glutathione S-transferases and transport proteins.

10 **CONCLUSIONS**

11 In this study, we investigated whole genome transcriptional response of *Caulobacter*

12 *crescentus* to the stress of several heavy metals, including chromium and uranium, which are

13 significant environmental contaminants and a current focus of bioremediation efforts. In addition

14 to the surprising finding that *C. crescentus* CB15N is tolerant to uranium, our studies combining

15 physiology observation, transcriptional measurement and imaging analysis, clearly showed that

16 *Caulobacter* formed a calcium-uranium-phosphate precipitate extracellularly, in contrast to the

17 intracellular sequestration mechanism of other resistant bacteria such as *Arthrobacter* spp. This

18 observation was consistent with a limited response to oxidative stress such as that seen with

19 other metals. The stress response strategy of lowering intracellular metal concentration was also

20 present in cadmium and chromium response. Efflux pumps were up-regulated under cadmium

21 stress. *C. crescentus* does not seem to have specific extrusion mechanism for chromium,

22 however, the cells down-regulated a sulfate transporter, which may reduce the uptake of

23 chromate.

1   In broader terms, cells exposed to cadmium share many up-regulated transcripts with

2   those under chromium stress. Most of those up regulated genes respond to oxidative stress, such

3   as superoxide dismutase, glutathione S-transferase, thioredoxin and glutaredoxin. However, on

4   closer inspection, the individual proteins up-regulated and the fold changes were specific to each

5   metal (for example, different sets of glutathione S-transferase were up-regulated under cadmium

6   and chromium stress), indicating the subtle difference of each metal stress and physiological

7   conditions. We also observed up-regulation of TonB-dependent outer membrane receptors which

8   may serve as sensors for environmental signals. While the detailed mechanisms of their

9   involvement is still not known, our results suggest that they may be an important member of the

10   response network.

11   We believe that not all the observed up- or down-regulation was a direct response to the

12   metal toxicity. This was particularly evident in the case of chromium and/or cadmium stress.

13   While the direct involvement of an arsenic reductase operon in cadmium response is unclear, the

14   likely mechanism is that cadmium binds to the repressor of the operon resulting in its up-

15   regulation. The response of *C. crescentus* under chromium stress was clearly different from that

16   of other oxidative stress (for example, cadmium), yet it may be complicated by secondary

17   responses. Future transcriptomics studies with varying concentrations of chromium, augmented

18   with proteomic analyses may help elucidate the complex response observed to this heavy metal

19   such as the role of cytochrome oxidases and the apparent nutrient-limitation response.

20   Our data have also clearly demonstrated the importance of interrogating the whole

21   genome on both strands. We have identified antisense transcripts which are differentially

22   regulated specific to metals, which, as either proteins or RNAs, may play an import part in the

23   response model.

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Table 1. Comparison of metal concentrations used for stress response experiments with concentrations found in uncontaminated (US EPA Maximum Contaminant Level Goals) and contaminated (NABIR Field Research Center, Oak Ridge, TN) groundwater.

Metal	US EPA (μM)[1]	NABIR FRC (μM)[2]	Stress used (μM)
Cadmium	0.04	39.59	6
Chromium	1.92	96.15	40-54[3]
Selenium	0.63	632.91	300
Uranium	0.13	289.92	200 - 1000[4]

[1] Data from US EPA website: http://www.epa.gov/safewater/hfacts.html

[2] Data from NABIR Field Research Center website: http://www.esd.ornl.gov/nabirfrc/. The highest value of recently sampled wells is reported.

[3] 27 μM potassium dichromate contains 54 μM chromium.

[4] A concentration of 200 μM uranyl nitrate was used for transcriptional analysis as significant precipitation was observed in the 1000 μM treatment.

Table 2. Genes up-regulated under cadmium, chromium and uranium stress

Gene	Fold change				Annotation
	cadmium	chromate	dichromate	uranium	
CC1777	18.9	14.1	8.6	2.9	Superoxide dismutase (cofactor: Mn^{2+}) (*sod*A)
CC3500	2.9	8.5	6.7	2.3	TonB-dependent outer membrane receptor
CC1532	3.2	3.8	3.6	2	Conserved hypothetical protein
CC3291	6.6	3.9	2.8	2.2	Hypothetical protein

Tables 3. Selected genes up-regulated under cadmium stress

Genes	Fold change	Annotation
Efflux pumps (Cluster I):		
CC2721	21.3	Outer membrane efflux protein
CC2722	36	Metal ion efflux membrane fusion protein, contains HlyD domain
CC2723	20	Hypothetical protein
CC2724	22.8	Homologous to *ncc*A and *czc*A
CC2725	8	Conserved hypothetical protein
CC2726	12.4	Cation transporting P-type ATPase
CC2727	6	Conserved hypothetical protein
Efflux pumps (Cluster II):		
CC3195	3.4	Outer membrane efflux protein
CC3196	2.6	Contains *Hly*D domain
CC3197	3	Cation/multidrug efflux pump, with AcrB/AcrD/AcrF domain
Protect against Oxidative stress:		
CC1777	18.9	Superoxide dismutase (cofactor: Mn^{2+}) (*sod*A)
CC3557	2.2	Superoxide dismutase (cofactor: Fe^{2+}) (*sod*B)
CC1316	3.2	Glutathione S-transferase

CC2434	2.2	Glutathione S-transferase
CC0062	2.4	Thioredoxin-like protein
CC0110	2	Thioredoxin
CC3539	2.3	Thioredoxin
CC2505	2.3	Glutaredoxin-related protein
CC0994	2.5	Peptide methionine sulfoxide reductase
CC1039	2.3	Peptide methionine sulfoxide reductase
CC0141	2.3	Glutathione synthetase
CC0885	3.6	Riboflavin biosynthesis protein (*rib*D)
CC0886	4.3	Riboflavin synthase, alpha subunit (*rib*E)
CC0887	4	GTP cyclohydrolase II (*rib*AB)
CC0888	3.6	Riboflavin synthase, beta subunit (*rib*H)
CC0459	4.1	GTP cyclohydrolase I (tetrahydrofolate biosythesis pathway)

Arsenic resistance pathway:

CC1503	4.8	Arsenic reductase (*ars*C)
CC1504	4.4	Transmembrane channel protein
CC1505	4.4	Transcriptional regulator (*ars*R)
CC1506	9.9	Arsenic resistant protein (*ars*H)

DNA repair:

CC1428	6	Deoxyribodipyrimidine photolyase, removes cyclobutane-type pyrimidine dimers in DNA
CC2590	2	Excinuclease ABC, subunit A

Others:

CC0260	2.7	Ribonucleotide reductase, alpha subunit
CC3492	2.2	Ribonucleotide reductase, beta subunit
CC2129	4.5	NADH:flavin oxidoreductase

Tables 4. Selected genes up-regulated under uranium stress

Genes	Fold change	Annotation
Protect against Oxidative stress:		
CC1777	2.9	Superoxide dismutase (cofactor: Mn^{2+}) (*sod*A)
Two-component signal transduction systems:		
CC1293	9.5	DNA-binding response regulator consisting of a CheY-like receiver domain
CC1294	6.6	Signal transduction histidine kinase
CC1304	9.7	DNA-binding response regulator consisting of a CheY-like receiver domain
CC1305	7.7	Signal transduction histidine kinase
ABC transporter:		
CC2090	3.8	Predicted permease component
CC2091	4.3	ABC transporter, ATPase component
CC2092	3.8	*Hly*D family secretion protein
Possible extracellular activities:		
CC1295	5.4	Possible phosphatase
CC0696	3.7	Similar to *gum*N (biosynthesis of extracellular polysaccharide)
CC0697	2.2	Similar to *gum*N (biosynthesis of extracellular polysaccharide)
Others:		

CC0411	3.1	Conserved hyperthetical protein
CC0412	3.8	Conserved hyperthetical protein
CC0815	7.9	TonB-dependent outer membrane receptor
CC3500	2.3	TonB-dependent outer membrane receptor
Cc1303	7.4	Hypothetical protein
CC1306	3.3	Unknown function. Contains a dihydrouridine synthase domain
CC1638	9.8	GMC (glucose-methanol-choline) family oxidoreductase
CC1891	8.6	Unknown function. Contains beta-helical repeats
CC2111	3.8	Conserved hyperthetical protein
CC2312	9.8	Predicted transcriptional regulator

Table 5. Altered Regulation to Uranium in ΔCC1293 and ΔCC1294 Mutants

	Wild type			ΔCC1293			ΔCC1294		
Gene	- U	+ U	p value	- U	+ U	p value	- U	+ U	p value
CC3291	995	3296	0.02	2500	5000	0.06	2300	2585	0.76
CC0139	460	1390	0.02	989	813	0.45	1013	1458	0.03
CC3446	1190	304	0.01	852	561	0.3	803	1107	0.1
CC2334	1481	229	0.004	1097	729	0.3	977	614	0.05
CC1293	482	6303	0.006	40	10	0.43	150	864	0.008
CC1294	946	8220	0.006	600	6500	0.004	91	20	0.38

The numbers in the –U or +U columns of this table are expression levels (average difference scores), each were an average of three independent experiments. The numbers in the "p value" column represent the results from *t* test. The genes listed in this table were differentially regulated under uranium stress in wild type (CB15N) background but the regulation was lost or altered when either CC1293 or CC1294 were deleted, except CC1293 and CC1294 were included to demonstrate the loss of the respective genes in the mutants. Evidently the regulation of CC1293-CC1294 operon is still maintained.

Table 6. Up-regulated antisense transcripts.

Corresponding genes	Metal stressor	Estimated fold change *	Possible ORF	Comments
CC1040 CC1041	chromate/ dichromate	5.3/5.7	No	There is no gap between the probes covering the two genes, thus it is likely to be one transcript. CC1040 and CC1041 were in one operon.
CC1127	uranium	7.8	Yes (116 aa)	
CC1416	uranium	8.4	maybe	A small ORF of 52 aa may exist.
CC2602	cadmium	4.8	Yes (135 aa)	
CC3553	chromate/ dichromate	3.1/3.7	No	Eight consecutive probes were differentially regulated. The transcript is predicted to be 200 bp.
CC3302	uranium	27.5	Yes. 112 or 117 aa	First three probes were not part of the transcript.

* The fold change was estimated from the responding probes. It is marginally different from the algorithms used to obtain probe-set values from all probes.

Tables 7. Selected genes up-regulated under chromium stress

Genes	Fold change		Annotation
	chromate	dichromate	
Protect against Oxidative stress:			
CC1777	14.1	8.6	Superoxide dismutase (cofactor: Mn^{2+}) (*sod*A)
CC1124	2	2.5	Glutathione S-transferase
CC2311	5.6	6.1	Glutathione S-transferase
CC0220	6.4	4.3	Thioredoxin-like
CC1039	9.3	6.5	Peptide methionine sulfoxide reductase
Outer-membrane response			
CC3500	8.5	6.7	TonB-dependent outer membrane receptor
CC0201	3.9	4.3	OmpA family protein; Outer membrane protein and related peptidoglycan-associated (lipo)proteins
CC0747	3.9	4.5	OmpA family protein; Outer membrane protein and related peptidoglycan-associated (lipo)proteins
Two-component signal transduction system:			
CC0247	2.6	2.2	DNA-binding response regulator consisting of a CheY-like receiver domain
CC0248	2.2	<2	Histidine kinase
DNA repair:			

CC2200	2.7	3	HNH Endonuclease
CC2272	3.3	2.8	EndoIII-related endonuclease
CC1087	4.1	3.5	*rec*A protein
CC1902	3.2	2.9	Repressor LexA SOS-response transcriptional Repressors (RecA-mediated autopeptidases)

Electron transport process/Cytochrome oxidases:

CC1770	2.7	2	Heme copper-type cytochrome c oxidase, subunit4
CC1771	2.8	2.3	Heme copper-type cytochrome c oxidase, subunit3
CC1772	3.8	3.1	Heme copper-type cytochrome c oxidase, subunit1
CC1773	5.8	4.1	Heme copper-type cytochrome c oxidase, subunit2
CC0762	2.8	2.3	Cytochrome d ubiquinol oxidase subunit 1
CC0763	3.6	3.6	Cytochrome d ubiquinol oxidase subunit 2
CC2269	2.7	3	Isoquinoline 1-oxidoreductase, beta subunit
CC2270	2.4	2.2	Isoquinoline 1-oxidoreductase, alpha subunit
CC0946	3.1	2.5	Cytochrome P450 family protein

Genes involved in metabolism:

Ammonium transport:

CC1338	5.1	3.6	Ammonium transporter. Ammonia permeases
CC1339	5.7	4.3	Nitrogen regulatory protein PII 2
CC1740	2.6	2.2	Nitrogen regulation protein NR(II) (*ntr*B). Signal transduction histidine kinase
CC1741	3.4	3	Nitrogen regulation protein NR(I) (*ntr*C). DNA-binding response regulator

Glutamate sysnthesis:

CC3606	4.1	4.1	Glutamate synthase, small subunit
CC3607	3	2.7	Glutamate synthase, large subunit

Phosphate starvation response:

CC2644	24.4	24.9	PhoH homolog. Phosphate starvation-inducible ATPase

PHB synthesis:

CC0510	6	4.7	Acetyl-CoA transferase (*phb*A)
CC0511	3.4	3.1	NADPH-dependent acetoacetyl-CoA reductase (*phb*B)
CC0947	2.7	2.3	Enoyl-CoA hydratase/isomerase (fatty acid oxidation)
CC2478	2.3	<2	Acyl-CoA dehydrogenase (fatty acid oxidation)
CC3087	2.5	2.3	Acyl-CoA dehydrogenase (fatty acid oxidation)

PHB and carbon/energy utilization:

CC0250	5.5	6.1	PHB depolymerase
CC0797	2.7	2.6	1,4-beta-D-glucan glucohydrolase D. Hydrolysis of the glycosidic bond between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate moiety

Histidine degradation:

CC0957	2.7	2.2	Urocanate hydratase (*hut*U)
CC0958	3.1	2.8	Formiminoglutamase (*hut*G)

CC0959	4.0	4.9	Histidine ammonia lyase (*hut*H)
CC0960	4.8	5.8	Imidazolonepropionase (*hut*I)
Serine biosynthesis:			
CC3215	2.6	2.1	D-3-phosphoglycerate dehydrogenase (*ser*A)
CC3216	3.1	3.2	Phosphoserine aminotransferase (*ser*C)
LPS synthesis:			
CC0118	3.6	3.7	Glucosamine--fructose-6-phosphate aminotransferase
CC1985	10.9	12.2	UDP-3-O-acyl-N-acetylglucosamine deacetylase (*lpx*C). Catalyzes the second step in lipopolysaccharide biosynthesis
Others:			
CC1506	2.8	2	Arsenic resistance protein (*ars*H)

Figure 1. Growth of *C. crescentus*, *E. coli* and *P. putida* at 1mM uranyl nitrate. Bacteria were grown in M2G medium. Uranyl nitrate was added at early growth phase as indicated in the legend:

──◆── : *C. crescentus* control, not exposed to uranyl nitrate; ──■── : *C. crescentus*, uranium was added at 135 minute to a final concentration of 1 mM uranyl nitrate; ──▲── : *E. coli* control, not exposed to uranium; ──✕── : *E. coli*, uranium was added at 135 minute to a final concentration of 1 mM uranyl nitrate; ──■── : *P. putida* control, not exposed to uranium; ──✳── : *P. putida*, uranium was added at 275 minute to a final concentration of 1 mM uranyl nitrate.

Figure 2. Transmission EM image of uranium precipitates formed in the presence and absence of *C. crescentus* CB15N. (A) Extracellular precipitates associated with the cells. (B) The EDX spectra shows uranium is almost absent in cells. (C) Cell associated extracellular precipitates are composed of uranium, calcium and phosphorus. EDX spectra were taken in the arear indicated by arrows. (D) Abiotic precipitates formed between uranium and culture medium are mostly composed of uranium and phosphorus. (E) The EDX spectrum from the abiotic precipitates shows calcium is nearly depleted in the uranyl-phosphate precipitates.

Figure 3. A possible ORF differentially expressed under uranium stress. This transcript was from the opposite strand of predicted hypothetical protein CC3302. the antisense transcript is up-regulated 27.5 fold under uranium stress. An ORF encoding 112 or 117 aa was predicted within the transcr
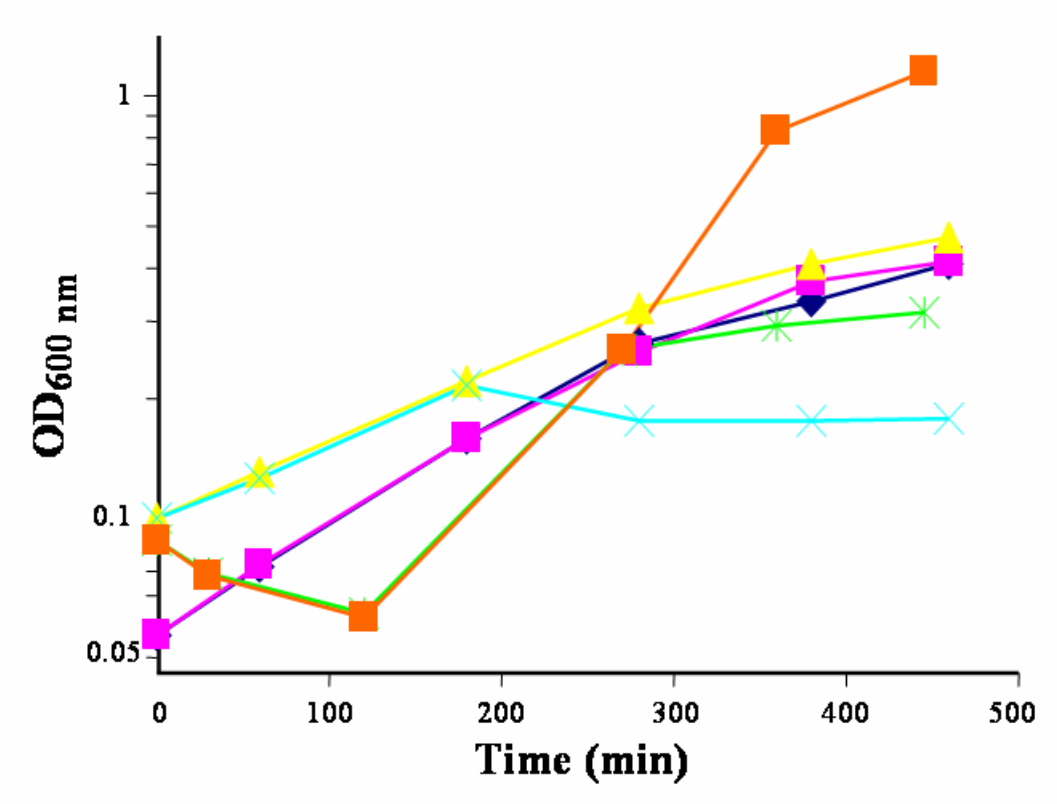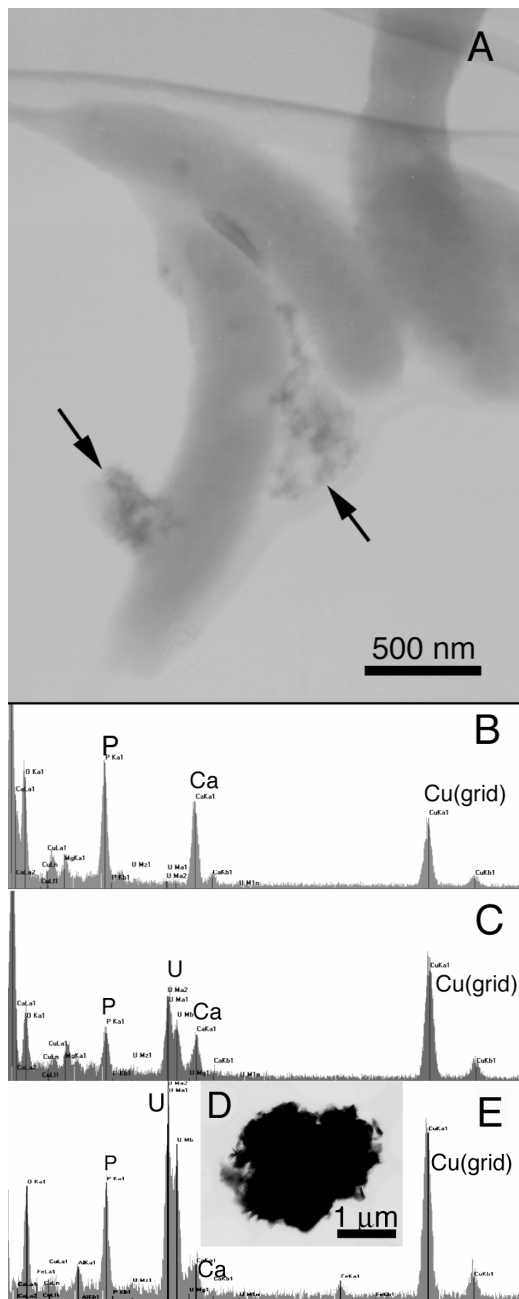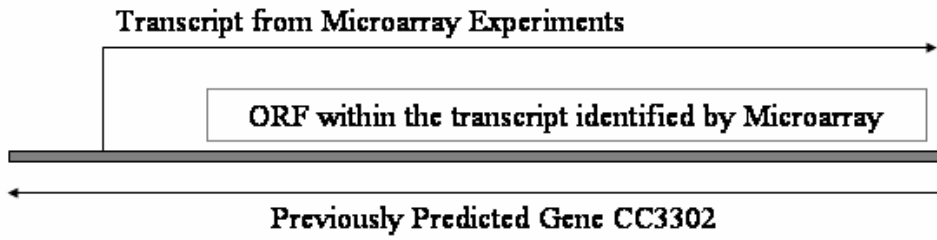
Figure 1

54

Figure 2

Figure 3

Transcript from Microarray Experiments

ORF within the transcript identified by Microarray

Previously Predicted Gene CC3302

Sequence of the new ORF:

MRKFIMSLTTVATLSLAAVPVLGLTQAANASESDPRVSIAVSDLNLS
NPAQAALFKARVQQAGETLCRAKLRNNTLDMSFGQCRVEVQREAERQ
LSKPQRKALIQAKRATTVELAAQ