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Metallomics of two microorganisms relevant to heavy metal bioremediation reveal fundamental differences in metal assimilation and utilization

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Although as many as half of all proteins are thought to require a metal cofactor, the metalloproteomes of microorganisms remain relatively unexplored. Microorganisms from different environments are likely to vary greatly in the metals that they assimilate, not just among the metals with well-characterized roles but also those lacking any known function. Herein we investigated the metal utilization of two microorganisms that were isolated from very similar environments and are of interest because of potential roles in the immobilization of heavy metals, such as uranium and chromium. The metals assimilated and their concentrations in the cytoplasm of *Desulfovibrio vulgaris* strain Hildenborough (DvH) and *Enterobacter cloacae* strain Hanford (EcH) varied dramatically, with a larger number of metals present in *Enterobacter*. For example, a total of 9 and 19 metals were assimilated into their cytoplasmic fractions, respectively, and DvH did not assimilate significant amounts of zinc or copper whereas EcH assimilated both. However, bioinformatic analysis of their genome sequences revealed a comparable number of predicted metalloproteins, 813 in DvH and 953 in EcH. These allowed some rationalization of the types of metal assimilated in some cases (Fe, Cu, Mo, W, V) but not in others (Zn, Nd, Ce, Pr, Dy, Hf and Th). It was also shown that U binds an unknown soluble protein in EcH but this incorporation was the result of extracellular U binding to cytoplasmic components after cell lysis.

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Introduction

Metal ions are essential for all forms of life and efforts to comprehensively characterize the entire metalloproteome of organisms is a dynamic and emerging field of research.[1] It is estimated that one third to one half of all proteins require one or more metal ions to function properly, yet relatively few metalloproteins have been extensively characterized. Except for well characterized protein families, it is not possible to predict metalloproteins on a genome-wide scale because the sets of amino acid residues in a given protein that bind a particular metal site are extremely diverse.[2] In addition, studies of recombinantly-expressed proteins can be misleading since this approach often leads to the incorporation of a metal that is different than that found in the native protein produced in its natural host.[3] The need to study native proteins to reliably determine the true biologically-relevant metal is often difficult in that many proteins are expressed at a low level and most organisms do not have genetic systems allowing homologous over-production of putative novel metalloproteins for further characterization.[4]

In contrast to their essential roles in biological pathways, even less is known about the toxicity of certain metals to various microorganisms at the molecular level.[5,6] The study of organisms that can tolerate high concentrations of various metals is important not only to our mechanistic understanding of selective incorporation and immobilization, but also for efforts to harness these abilities for bioremediation. The most commonly encountered metals and metalloids causing adverse health effects in humans are lead, mercury, arsenic and cadmium.[7] Uranium and chromium are also acutely toxic elements and are present at elevated levels in some contaminated environments. Efforts are underway to study the microbial

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communities present at such sites to better understand the diversity and interactions among species in these environments.[8–10] In particular, a great deal of attention has been focused on the role of sulfate-reducing bacteria from the genus *Desulfovibrio* in the realm of metal bioremediation (including for Cr, U, Cu, Mn and Ni:[11–27]). *D. vulgaris* strain Hildenborough (DvH) and related species have been shown to immobilize metals (Cr, U) through specific reduction by bifunctional enzymes[14,17,25] and through the non-specific reaction of metals with sulfide, the product of dissimilatory sulfate reduction. One of the goals of the research described herein was to assess the metalloproteome of the model bioremediator, DvH by determining the metals that it assimilates and comparing these data with metalloproteins predicted from its genome sequence.

The *Enterobacter cloacae* complex (ECC) is a group of closely related, yet genetically diverse organisms that are environmentally ubiquitous.[28] ECC organisms have been studied extensively as causative agents of human and plant infections.[29–32] In addition to *Desulfovibrio* spp., members of the ECC have been investigated for their use in metal bioremediation including immobilization *via* exopolysaccharide production and intracellular accumulation.[33–38] A second goal of the current project was, therefore, to assess the metalloproteome of an *Enterobacter cloacae* strain isolated from a contaminated site in Hanford, WA, and to determine the metals that it assimilates from laboratory culture. For this we also obtained a draft genome sequence of *E. cloacae* strain Hanford (EcH) to allow comparative bioinformatics analyses of predicted *versus* assimilated metals.

An experimental methodology to determine the metals that are assimilated by microorganisms was recently developed and applied to three microorganisms from very different environments.[4] These were *Escherichia coli*, a member of the bacterial family *Enterobacteriaceae*, and two members of the archaea, *Pyrococcus furiosus*, a neutrophilic hyperthermophile, and *Sulfolobus solfataricus*, an acidophilic hyperthermophile. *E. coli* is found in the gut of humans and other animals and is a facultative anaerobe, *P. furiosus* is found in shallow marine hydrothermal vents and is an obligate anaerobe, and *S. solfataricus* was isolated from volcanic hot springs and is an obligate aerobe. The results of this study showed the incorporation of many unexpected metals by all three organisms and revealed a great diversity in the types and amounts of metals incorporated. For example, *P. furiosus* was found to contain vanadium, lead and uranium, *E. coli* incorporated vanadium, arsenic, lead, cadmium and uranium, while *S. solfataricus* contained barium, lead, tin, antimony and thallium. All of these metals were present as contaminants in the standard laboratory growth media used to cultivate these organisms.

In the present study we sought to compare metal incorporation by two microorganisms that inhabit similar soil environments, the bioremediators, DvH and EcH. It was anticipated that the results would give a new perspective on species-specific metal metabolism and bioremediation, and guide future research into identifying the specific pathways and enzymes involved in metal reduction and sequestration.

Materials & methods

Isolation

EcH was isolated from an enriched consortia resulting from lactate amended bioreactors that were inoculated with groundwater from a well located in the chromium contaminated 100 H area at Hanford, WA, USA, using established procedures.[8] The enriched consortia were then serially diluted and plated for colony isolation. All work was conducted anaerobically. Picked colonies were grown in the same liquid medium as used in the bioreactors. Use of fructose rather than lactate (each 30 mM) as the carbon source resulted in more rapid and more robust growth and this was used in the standard growth medium for EcH.

Growth

Both organisms were grown under strictly anaerobic conditions using argon as the head space gas. DvH was grown in a modified LS4D lactate sulfate medium as described[15] except that the trace metal (6.25 ml l^{-1} instead of 12.5 ml l^{-1}) and PIPES (5 mM instead of 30 mM) contents were reduced. DvH was grown in a 600-liter metal (stainless steel) fermentor and in duplicate 5-liter glass fermentors with no wetted metal parts. EcH was grown in a 600-liter fermentor in the CCM medium previously described[15] except sulfate was omitted and 30 mM fructose replaced lactate as the carbon source. Growth of EcH in Cr and U supplemented media were performed in 4-liter sealed glass bottles using the same fructose CCM medium. In all cases cells were harvested in the mid-log phase (a Sharples centrifuge was used for the 600-liter scale) and the resulting cell mass was flash frozen with liquid nitrogen and stored at −80 °C. Aliquots (50 ml) of cell culture at the end of growth were transferred to 50 ml falcon tubes and centrifuged for 15 min at 2800 × *g* at 4 °C. A sample (15 ml) of this cell-free medium supernatant was transferred into acid-washed 15 ml falcon tubes and stored at −20 °C prior to metal analysis as described below.

Triplicate washing of U-supplemented cells

U-supplemented EcH cells were washed three times by centrifuging (7000 × *g*, 10 min), removing the supernatant, and resuspending in 1× base salts (EcH fructose CCM media lacking vitamins, fructose and trace minerals). Washed-cytoplasmic extracts were prepared for both unwashed and triplicate washed cells as described below.

Preparation of washed-cytoplasmic extracts

All steps were carried out under anaerobic conditions and all glassware was acid-washed. Approximately 10 g (wet weight) of DvH and EcH frozen cells were separately transferred into an anaerobic chamber (Coy Laboratory Products, Grass Lake MI) and resuspended in 30 ml of anaerobic cell lysis/wash buffer (50 mM Tris HCl, pH 8.0). The cell suspensions were loaded into a pre-cooled (4 °C) 35 ml French Press cell (pre-washed four times in anaerobic lysis/wash buffer) and lysed under a flow of argon using the "high" setting and a pressure of 14 000–16 000 psi (800–1000 setting). The cell extracts were collected on ice under a flow of argon and the complete process was repeated.

The resulting whole cell lysates were centrifuged at 140 000 × *g* for 75 min at 4 °C. The supernatant, representing the soluble cytoplasmic fraction (S100) was collected and a 2 ml sample was removed and placed into a buffer-washed Amicon Ultra-4 centrifugal filter device (3 kDa cutoff), which was centrifuged at 4300 × *g* at 4 °C until the volume was reduced to 0.5 ml. The flow-through (FT) was retained for metal analysis. Anaerobic lysis/wash buffer (1.5 ml) was added to the retained S100 sample and this was re-centrifuged and the flow-through of the once-washed S100 (W1) was collected for metal analysis. This procedure was repeated and second (W2) and third (W3) washes were collected for metal analysis along with the final retained S100 sample. This was made up to 2 ml with buffer yielding a 256-fold buffer-exchanged, washed S100 sample (S100W).

Metal analysis

To minimize contamination by extraneous metals in reagents and containers, all solutions were made up using glass-distilled deionized water (gddH_2O) that was distilled and collected without contacting metal. All glass and plastic vessels were pre-washed for 16 h in HNO_3 (2%) and rinsed twice in gddH_2O. Trace-metal grade HNO_3 (Sigma-Aldrich 7697-37-2) was used to acidify samples. All buffers used were analyzed by ICP-MS separately as controls for the biological samples. A detailed description of how biological samples were prepared and metal analyses were carried out has been published.[4,39] Briefly, soluble samples were diluted in a 2% v/v solution of high-purity trace metal grade HNO_3 in glass distilled deionized water and incubated at 37 °C for 90 min to denature protein and release bound metals. Cell membrane samples were dried overnight at 100 °C, dissolved in 70% trace metal grade HNO_3 and boiled dry before dilution. Samples were centrifuged (2400 × *g*) for 10 min at 25 °C before analysis using an Agilent 7500ce octopole ICP-MS with a collision/reaction cell and Cetac ASX-520 autosampler. All samples were prepared in 15 ml acid-washed polypropylene tubes. All media and S100 samples were analyzed in triplicate, and chromatographic fractions were analyzed in duplicate. Quantitation was performed in Full-Quant mode *via* in-line addition of internal standard (inorganic ventures IV-ICPMS-71D) and the use of an external calibration (inorganic ventures IV-ICPMS-71A, CCS-5, CMS-2) with 0, 0.1, 0.25, 0.5, 0.75, 1, 5, 10, and 50 ppb for each element analyzed.

Genome sequencing

Enterobacter cloacae strain Hanford genomic DNA was sequenced essentially as described previously,[40] except that an Illumina MiSeq sequencing instrument[41] and CLC Genomics Workbench (version 6.0.2) were used in this study. After trimming and filtering for quality, 4 850 091 paired-reads with an average length of 249.2 bp were assembled into a draft genome sequence. Draft genome sequences were annotated at Oak Ridge National Laboratory using an automated annotation pipeline, based on the Prodigal gene prediction algorithm.[42] Genome files, metabolic reconstructions and annotations are available at http://genome.ornl.gov/microbial/guest/hudson_lab_ludwigii_CLC/. The entire DNA sequence data set has been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database (WGS accession: ATCK01000000, BioProject accession: PRJNA206261).

Genome comparison

The total number of metalloproteins encoded in the genomes of DvH and EcH were predicted as previously described.[43] Briefly, the predicted amino acid sequences of all protein-encoding genes annotated in the genomes of DvH (RefSeq NC_002937.3 and NC_005863.1) and EcH were analyzed using EBI's iprscan tool (version 4.8; database release 41), a standalone version of InterPro.[44] The resulting InterPro ID (IPR) to protein matches were entered into a relational database for each organism. The abstract for each IPR entry was parsed from the accompanying interpro.xml.gz file and entered into the corresponding database. The IPR abstracts were searched using regular expression patterns relating to metal ions, metal cofactors and metal binding domains and the results were used to classify which IPR entries may encompass proteins that utilize specific metals. The dataset formed the InterPro Metalloprotein Database (IPMD) for each organism and these were utilized to predict likely metalloproteins encoded in each genome.

HPLC-ICPMS

A sample (24 μl) was injected onto BioRad Bio-Silect 250 SEC column at a flow rate of 1.5 ml min^{-1} (25 mM Tris buffer, pH 8 75 mM NaCl) using an Agilent 1200 HPLC connected to an Agilent 7500ce ICP-MS. Absorbance at 280 nm was monitored and uranium data was collected in time resolved analysis mode without quantitation.

Cytoplasmic fractionation

A sample (9.7 ml) of the EcH cytoplasmic fraction (S100) was diluted 1 : 1 with anaerobic 50 mM Tris/HCL buffer pH 8.0 (buffer A) and loaded onto a pre-packed 5 ml DEAE-Sepharose FF column pre-equilibrated with buffer A using an ÄKTA Purifier automated liquid chromatography system (GE Healthcare). Unbound protein was washed from the column with buffer A and bound protein was eluted using a linear gradient from 0–500 mM NaCl in buffer A. A total of 80 fractions (1 ml) were collected and their metal contents were determined as described above. Protein was estimated using the Bradford method using bovine serum albumin as the standard.[45]

Results and discussion

Metal content of the media during growth of DvH and EcH

DvH and EcH were each grown in a 600-liter stainless steel fermentor and DvH was also grown in a 5-liter glass fermentor. A total of 9 elements (Fe, W, Mn, Co, Zn, Cu, Mo, Se and Ni) were specifically added to the growth media for DvH and for EcH. Of the 44 elements analyzed, 18 were found in all three media prior to inoculation (V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ga, As, Rb, Sr, Mo, Sb, Ba, Hf, W and Th), of which 10 (V, Cr, Sr, Ga, As, Rb, Sb, Ba, Hf, Th) were not specifically added to any medium

and arise as contaminants from other media components. Two elements, Cd and Lu, were found in the two DvH media but not in the EcH medium, while Nd and Tm were found only in the EcH medium. The medium in the metal fermentor for DvH also contained in detectable amounts Rh, Sn, Cs, La, Ce, Pr, Sm, Eu, Gd, Ho, Tl and U to give a total metal count of 32. Differences were observed between the two types of fermentors used for DvH as the glass fermentor medium uniquely contained Pd, Ta, and Pb, to give a total metal count of 23. The total number of detectable metals in the EcH medium was 20.

To assess the changes in available extracellular metals throughout growth of DvH and EcH, samples of the cultures were removed periodically, the cellular material was removed by centrifugation and the metal content of the supernatant was determined. The concentrations of several metals decreased during the growth of both organisms, with the effect being much more pronounced for DvH, even though the growth rates and cell yields of the two organisms were comparable. For example, in the 600-liter fermentor, DvH and EcH exhibited doubling times of 3.0 and 1.5 h^{-1}, respectively, and cell yields of 350 g and 725 g (wet weight), respectively. The initial concentrations and percent decrease of several metals in the media at the end of growth is summarized in Table 1. Of these metals, ten of them were decreased to less than 70% of their initial values by DvH in both the glass and metal fermentors (Zn, Fe, Co, Mo, Cu, Sb, As, Pb, Sn, Cd), while only Fe decreased by a comparable amount in the EcH growth. Decreases in metal concentrations over the course of DvH growth were similar for the metal and glass fermentors.

The decrease in concentrations of metals during the DvH growth is perhaps unsurprising given the production of large amounts of sulfide by this sulfate-reducing microorganism since most transition metals, such as Fe, Cu, Co, Mo and Ni, readily form insoluble sulfide complexes. However, low availability of essential metals might limit growth of the organism towards the end of the growth phase. Only the soluble metals in the growth media were analyzed here, and this approach does not distinguish between metals lost by extracellular precipitation or from assimilation into the cell. Nevertheless, this approach does show that several metals, such as Cd, Pb, Sb, Cu and Ba, show a dramatic reduction in concentration during growth of DvH and are decreased to low or sub-nM concentrations. Pb, Cu and Sb are not assimilated, indicating that DvH does not utilize these metals, possibly because they form insoluble sulfide complexes in the medium that are not accessible. On the other hand, for EcH, all metals other than Fe were available at the end of growth at more than 50% of their initial concentrations, and so they are unlikely to be limiting growth.

Metal content of the cytoplasmic fractions of DvH and EcH

The metals detected in the soluble cytoplasmic fractions of unwashed DvH and EcH cells are summarized in Table 2. We also determined whether the metals were strongly or weakly bound to cytoplasmic macromolecules, either by misincorporation or specific assimilation, of at least 3 kDa in size by washing the cytoplasmic extract with buffer three-times using a nominal 3 kDa filter. Fig. 1 shows an example of a metal (Co) that was strongly bound and one that was weakly bound (Rb) and readily removed by multiple washes. The amounts of metal bound in the cytoplasm of the two organisms are given in Fig. 2 as a side-by-side comparison. Both DvH and EcH assimilated six metals, Mn, Fe, Co, Ni, Mo and W, into soluble cytoplasmic macromolecular

Table 1 Metal content of the growth media for DvH and EcH and the decrease in concentration at the end of cell growth. DvH was grown in a metal and in a glass fermentor as indicated. The percent decrease is the concentration of each metal in the medium at the end of growth (mid-log phase) compared to the initial concentration prior to inoculation

	EcH		Glass DvH		Metal DvH	
	Conc. (nM)	Decrease (%)	Conc. (nM)	Decrease (%)	Conc. (nM)	Decrease (%)
Zn	2886.9	47.3	13712.2	87.9	8257.4	97.4
Fe	905.6	83.9	38548.0	91.1	17079.2	87.8
Mn	736.8	34.5	17712.6	8.2	14903.9	21.2
Co	521.0	33.3	8555.7	89.3	7138.3	98.3
Mo	302.6	39.4	1405.3	90.3	1110.0	93.5
Ni	187.2	32.5	2592.7	33.2	2141.5	61.9
W	165.9	42.2	186.4	21.9	244.0	18.2
Sr	162.5	33.8	256.7	9.9	116.5	10.6
Ba	143.4	38.4	77.4	21.5	103.1	64.2
Rb	140.8	34.6	293.5	6.4	98.6	24.5
Cu	104.9	56.0	806.9	97.9	78.7	100.0
Cr	49.1	39.2	51.4	14.8	81.0	18.6
V	40.1	31.3	9.5	0.3	16.5	33.5
Sb	3.6	40.8	5.4	100.0	3.4	71.1
As	2.5	51.1	8.7	23.1	6.9	72.3
Pb	ND	—	29.4	97.1	1.1	88.7
Sn	ND	—	ND	—	5.9	99.0
Cd	ND	—	48.9	98.4	2.5	76.0
Cs	ND	—	ND	—	8.0	26.0
U	ND	—	ND	—	0.6	28.1

Table 2 Metals strongly and weakly (–) bound to macromolecules (>3 kDa) in the cytoplasm of DvH and EcH. The concentrations of the metals (in nM) shown are before the samples were washed three times with buffer as described in the Materials and methods. Metals whose concentration decreased by more than 50% because of the washing procedure were designated weakly bound and are so indicated (–)

	EcH (nM)	DvH (nM)
Fe	3844.27	29717.59
Zn	1454.63	ND
Mn	433.27	640.61
Mo	169.54	71.04
Ni	109.41	156.78
Co	69.81	660.82
Cu	51.69	ND
Ba	18.84	6.60
W	11.60	133.81
Cr	9.36	ND
Ga	7.45	2.59
V	4.46	ND
Sr	2.65	4.21 (–)
Nd	2.15	ND
Rb	0.73 (–)	5.82 (–)
Sn	0.52	ND
Ce	0.30	ND
Dy	0.27	ND
Cd	0.12	0.90
Th	0.09	ND
Hf	0.09	ND
U	0.05	ND
Pr	0.02	ND

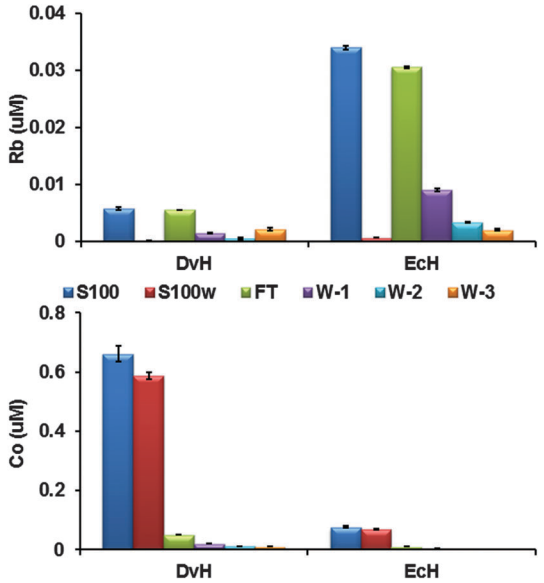

Fig. 1 Strongly and weakly bound metal in the cytoplasm of EcH and DvH. Cytoplasmic extracts were prepared (S100) and each was washed three times using a 3 kDa filter as described in the Materials and methods. The Co and Rb concentrations of the washed extract (S100W), the initial flow-through (FT), and the flow through after the three washes (W-1, -2 and -3).

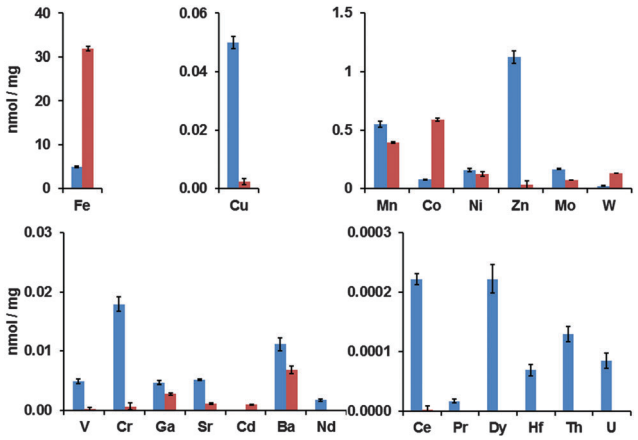

Fig. 2 Metals that were strongly-bound to macromolecules (>3 kDa) in cytoplasmic extracts (S100) of DvH and/or EcH. The cytoplasmic extracts were prepared and each was washed three times as described in the Materials and methods prior to metal analysis. Metal concentrations are expressed for EcH (blue) and DvH (red) in nmol mg^{-1} total protein.

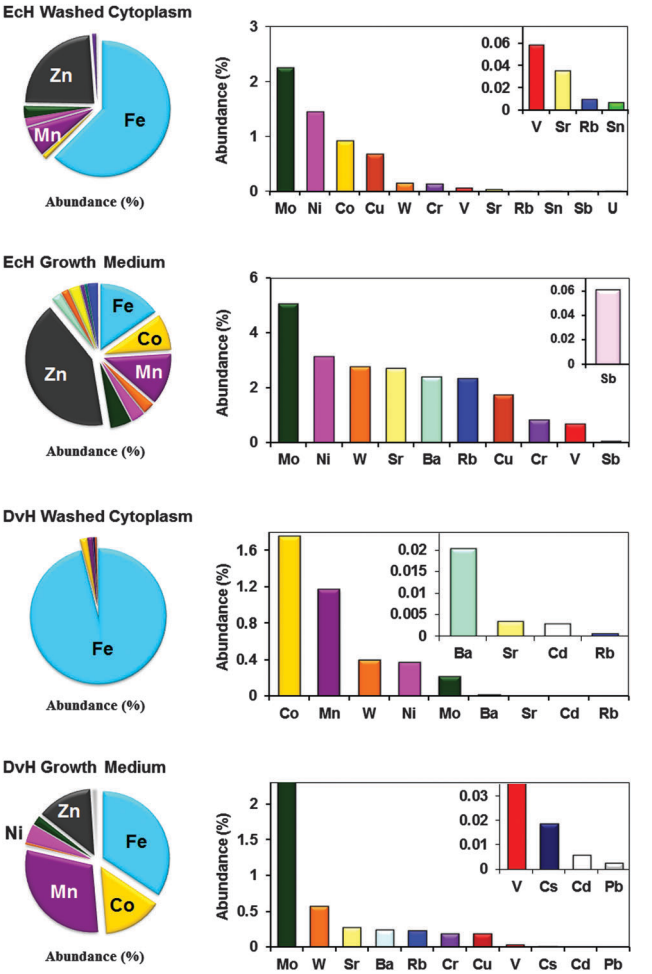

Fig. 3 Comparison of the metal compositions of the growth media and the cytoplasmic fractions of EcH (upper) and DvH (lower) grown in 600-liter cultures.

complexes above 3 kDa in size. As shown in Table 2 and Fig. 3, more than 95% of the metal incorporated by DvH, regardless of the fermentor vessel used, was Fe. This compares with 62% of the total for EcH, which also contained high relative amounts of Mn and Zn. For reasons that are not at all clear, even though Zn was specifically added to the growth medium, it was not taken up by DvH. The same was true for Cu, which was readily incorporated by EcH but not by DvH, although Cu has a particularly strong affinity for sulfide. In addition to those added to the growth media, several metals present as contaminants (not specifically added to the media) were taken up by one or both organisms. The cytoplasm of DvH contained tightly-associated Ga, Cd and Ba, while EcH contained V, Cr, Sr, Ce, Pr, Nd, Dy, Hf, Th, and U. Rb was found in the cytoplasm of both organisms but was weakly bound as it was removed by the cytoplasmic washing procedure.

In addition to the differential uptake of several metals by DvH and EcH, the overall metal compositions of their cytoplasmic fractions varied greatly (Fig. 3). For example, although Fe represented the majority of total cytoplasmic metal for both organisms, only Co and Mn were present in DvH at greater than 0.5% of the total, while EcH contained Zn (20%), Mn (7%), Mo, Co, Ni and Cu in significant quantities (>0.5%). A comparison of three organisms from very different environments, *P. furiosus*, *E. coli* and *S. solfataricus*, revealed species-specific metal incorporation. Of the three, only *P. furiosus* assimilated W, whereas all three contained Pb. In contrast both EcH and DvH assimilated W and neither contained Pb. While EcH incorporated both Cr and U, none of the three previously studied organisms incorporated Cr, while *P. furiosus* and *E. coli* contained U, which were likely misincorporated.[4] The results presented here demonstrate that distinct differences in species-specific metal incorporation also occur among species inhabiting similar environments, such as DvH and EcH.

Metal tolerance and assimilation

While U (0.6 nM) and Cr (81 nM) were detected in the growth medium for DvH in the metal fermentor (but not in the glass fermentor), neither element was present in the cytoplasmic extract (Table 1). In contrast, Cr (49 nM) was detected in the growth medium of EcH although U was not, but both metals were bound strongly to macromolecular complexes in the cytoplasmic fraction (Fig. 2 and 3). Since *Enterobacter* spp. have previously been shown to reduce both U(VI) and Cr(VI),[33,34] it was of great interest to determine the tolerance of EcH to elevated levels of these environmental contaminants and to find out to what extent they were incorporated or misincorporated into the cytoplasmic fraction. The growth of EcH was affected by concentrations of Cr (chromate) above 400 μM but not by U (uranyl acetate) up to 500 μM (Fig. 4).

The apparent cytoplasmic U concentration of EcH continued to increase dramatically with supplementation of the growth medium up to 0.5 μM, and this resulted in a 4500-fold increase (from 0.002 to 9.0 nmol mg^{-1}) in that measured in the absence of medium supplementation (Fig. 5). Only marginal increases in the amount of intracellular U were seen upon further addition of U to the medium above 0.5 μM (Fig. 5). However, when this approach was repeated with triple-washed U-supplemented cells, there was a dramatic reduction (>90% in cells grown with 0.5 μM U) in the U concentrations in the cytoplasmic fraction. To ascertain the nature of the U observed in the cytoplasmic fraction of unwashed EcH cells, anion-exchange chromatography was performed.

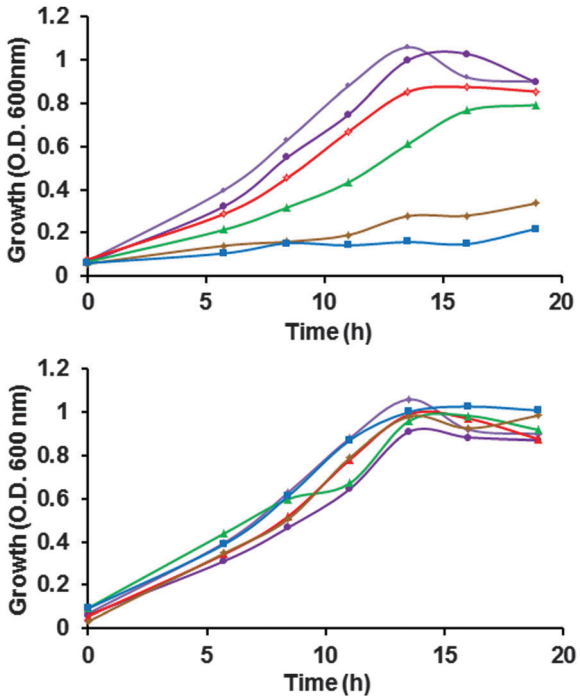

Fig. 4 Growth of EcH grown in media supplemented with dichromate (upper) or uranyl acetate (lower). The organism was grown under in the standard medium (triangle, purple) supplemented with 100 μM (circle, purple), 200 μM (diamond, red), 300 μM (triangle, green), 400 μM (diamond, brown), or 500 μM (square, blue) uranyl acetate or potassium dichromate.

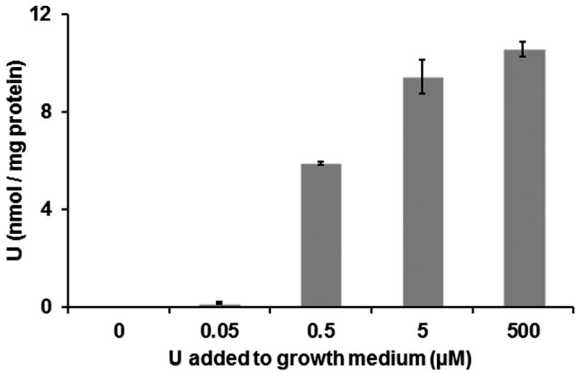

Fig. 5 Uranium in the cytoplasmic fraction of unwashed EcH cells grown in media supplemented with uranium. Cells were grown in media supplemented with the indicated concentration of uranium. The S-100 fractions were prepared and the sample was washed using a 3 kDa filter. The uranium concentration in the cytoplasm is expressed as nmol mg^{-1} of protein.

All procedures including preparing the extract and chromatographic separation were carried out under anaerobic conditions. The U eluted from the column in at least two overlapping peaks within the protein elution profile (data not shown), suggesting the presence of two U-binding, anionic proteins. Hence, it is apparent that while U may not be specifically taken up by EcH, this element can be incorporated into specific cytoplasmic proteins that are exposed to U after cell lysis.

Genome comparison and metalloprotein predictions

In order to predict potential metalloproteins in EcH, a draft genome sequence was obtained. The *de novo* assembly generated 63 DNA contigs with an average read depth of approximately 250×. The largest contig size was 802 220 bp with average of 76 729 bp. The estimated genome size was 4.8 Mb with a 54.5% G + C DNA content containing 4523 predicted protein-encoding genes. A multilocus analysis including 16s rRNA identified *Enterobacter cloacae* strain EcWSU1 as the most closely related fully sequenced ECC representative. EcWSU1 was isolated from onions grown in the Columbia River basin near Hanford, WA.[29,32]

The complete genome sequence of DvH has been published[46] and some properties of the genes annotated in the DvH and EcH genomes are summarized in Table 3. Over 77% of the genes have predicted rather than completely unknown functions. We recently published a computational framework for predicting

Table 3 Comparison of the genome sequences of DvH (complete) and EcH (draft). The DvH chromosome is 3.6 Mb (RefSeq NC_002937.3) with a 200 Kb megaplasmid (RefSeq NC_005863.1). The draft genome sequence of *Enterobacter cloacae* strain Hanford is 4.8 Mb in size (accession number to be added). The number of predicted protein-encoding genes is given, along with the number that match at least one InterPro entry and the number of those that match Domain of unknown function (DUF), Uncharacterized conserved protein (UCP), or Uncharacterized domain (UPF)

	Contigs	Nucleotides (Mb)	Genes	Genes with IPR match	IPR DUF, UCP, or UPF
DvH	2	3.8	3536	2706 (77%)	213
EcH	63	4.8	4523	4324 (96%)	438

Table 4 Genome-wide comparison of the predicted metalloproteomes of DvH and EcH. The number of genes with an IPMD match is given for each organism. Some genes have matches with multiple metals, and a total of 813 DvH and 953 EcH genes had a match to at least one metal

	DvH	EcH
Cadmium	19	22
Chromium	3	0
Cobalt	111	106
Copper	36	60
Iron	576	588
Lead	5	5
Manganese	17	31
Molybdenum	75	92
Nickel	64	72
Tungsten	14	19
Vanadium	7	7
Zinc	184	281
Uranium	0	0

metalloproteins on a genome-wide scale using the InterPro annotation database termed the IPMD.[43] Using this approach, 5559 of the current 24 356 InterPro entries (IPR) match genes in the DvH and EcH genomes. A total of 946 of these matched a key word related to one of 13 metals that were analyzed (Cd, Co, Cu, Cr, Fe, Pb, Mn, Mo, Ni, W, U, V and Zn). Of these 13, only U, not surprisingly, had no matches to any of the 24 356 IPR entries. Of the 946, 413 had matches in both organisms, while 384 were unique to EcH and 149 were unique to DvH. Overall, about 21% of genes in both genomes were covered by least one IPMD match, with a total of 813 in DvH and 953 in EcH (Table 4). On average, there are 3.9 genes per IPR and 2.9 IPRs per gene for DvH. Likewise, EcH has 3.8 genes per IPR and 2.9 IPR per gene indicating a balanced distribution in the analysis between IPRs and genes as well as between organisms. As expected, Fe and Zn matched the most InterPro entries with 525 and 265 hits respectively (Table 4).

Iron and zinc. Although Fe was overwhelmingly the predominant metal in the cytoplasmic fraction of DvH, the two organisms had a similar number of Fe IPMD matches, with 317 in DvH and 434 in EcH, corresponding to 576 genes in DvH and 588 in EcH. DvH has robust mechanisms for Fe uptake and its regulation including ferrous iron transport (Feo) and the ferric uptake regulator (Fur) systems and these are also present in the EcH genome. Transcriptomic studies of DvH have shown that Fe-uptake related genes are up-regulated under a variety of stresses,[47] as cultures transition into stationary phase and nutrients are depleted[15] and in high sulfide conditions.[48] The uptake and sequestration of large amounts of Fe by DvH is likely an adaptation to the limited availability of soluble Fe in sulfide-rich natural environments. Of the 434 Fe IPMD entries for EcH, 226 are also present in the DvH genome, highlighting the essential role of Fe metalloproteins in the core metabolism of EcH as well as DvH. From a bioinformatic perspective, a similar situation exists for Zn, where there are 243 matches for EcH and 150 matches for DvH, with 128 in common. Such data are inconsistent with the fact that Zn, which accounts for 24% of the total metal content of the cytoplasm of EcH, was present at an extremely low level in the cytoplasm of DvH. The low concentration of Zn in the cytoplasm of DvH remains a mystery, and suggests that the metal content of such protein should be confirmed in DvH, as some other metal may have taken over the function of Zn in some cases.

Molybdenum and tungsten. Although virtually all characterized organisms metabolize Mo, those that utilize the chemically-analogous element W are much less widespread.[49] Moreover, the InterPro entry IPR006655 (molybdopterin oxidoreductase) cannot be used to readily distinguish between Mo- and W-containing enzymes. Nevertheless, DvH has been shown experimentally to utilize W, as both W and Mo are incorporated into the active site of a formate dehydrogenase with a preference for W.[50] It is therefore perhaps not surprising that comparable amounts of W and Mo are found in the cytoplasm of DvH (Fig. 2). EcH also contains several genes that could potentially utilize W or Mo and while the relative proportion of W (to Mo) is lower than in DvH, its cytoplasmic content (Fig. 2) indicates that like DvH, EcH may also contain tungstoenzymes.

Manganese, nickel, copper and vanadium. The EcH genome encodes nearly double the number of predicted Mn-containing proteins as that of DvH (Table 4) and has a significantly greater relative cytoplasmic abundance (Fig. 3). The presence of a high affinity Mn transporter (mntH) homolog in the genome of EcH but not DvH could be indicative of the greater role for Mn in the metabolism of EcH. DvH and EcH contain similar amounts of Ni in their cytoplasmic fractions (Fig. 2) and contain comparable numbers of genes that have IPMD matches (Table 4). In particular, both organisms possess Ni-containing hydrogenases, with those from DvH being extensively characterized.[51] Both organisms also encode copper-containing oxidoreductase systems. In DvH this includes oxygen-defensive mechanisms that are expressed even under anaerobic growth conditions. EcH, as an aerobe, is predicted to have nearly double the number of copper binding proteins as DvH (60 *vs.* 36) and the cytoplasmic concentration is more than 20-fold greater in EcH (Fig. 2) even under anaerobic growth conditions, when cytochrome *c* oxidase is not in use. The overwhelmingly greater cytoplasmic concentration in EcH may be explained both by a greater number of Cu-enzymes, but may also represent novel bioinorganic chemistry, as known uses for Cu are mostly specific to aerobic growth. DvH produces sulfide during growth, thus it is unsurprising that it would have limited use for Cu, as Cu forms highly insoluble copper sulfides.[52] The extent to which Cu availability in a sulfide-rich growth medium limits Cu assimilation by DvH is not known. Genome sequence analysis predicts only 7 V-related proteins in both EcH and DvH, however the cytoplasmic concentration is more than 20-fold greater in EcH than DvH. However, there are only a few examples of well-understood V biochemistry.

Uranium and chromium. No gene in either DvH or EcH match IPMD keywords relating to U since no IPMD entry is U-related. Only DvH had IPMD matches relating to Cr and these three IPRs (IPR003370, IPR014047, IPR018634) cover, with some overlap, only three genes: one on the chromosome (DVU0426) and two on the megaplasmid (DVUA0093, DVUA0094). The IPR entries suggest all three genes are related to chromate transport. In addition, a high-throughput system-biology approach to improve the functional annotation of hypothetical genes

identified DVUA0095 as Cr responsive, with a deletion mutant exhibiting increased Cr sensitivity.[53] The lack of reference to metal reductases reflects the limited knowledge about specific metal reduction mechanisms and the types of metalloproteins that might be involved. Proteins that have been implicated, such as cytochrome c_3 and periplasmic hydrogenases,[16,54] which are Fe-containing proteins, have other metabolic roles and are ubiquitous among a variety of related organisms that are not known to reduce U or Cr. Until specific chelators, reductases, or redox proteins that specifically impart the ability to reduce hexavalent metal ions are identified, bioinformatic analysis alone is unlikely to allow the prediction of such capability from genome sequences. Our observation described above regarding the abiotic misincorporation of U into soluble cytoplasmic proteins in EcH may have implications for toxicity involving homologous proteins, which could have an extremely high affinity for uranyl ions in a variety of other organisms.

Minor metals. As with U and Cr, the other metals that are present in the cytoplasm of EcH but not in DvH, such as Nd, Ce, Pr, Dy, Hf and Th, cannot be rationalized in terms of bioinformatic analysis since, with one exception, there are no known proteins that naturally contain these metals, and no structural or catalytic roles for them are present in the databases such as InterPro. The one recently described exception is the methanol dehydrogenase of *Methylacidiphilum fumariolicum* SolV which requires a lanthanide (La, Ce, Nd or Pr) cofactor.[55]

Conclusions

Metallomic investigation of two species of interest to bioremediation efforts, DvH and EcH, revealed fundamental differences in their abilities to incorporate metals. A greater diversity in the types of intracellular metal was seen in EcH. For example, it takes up V, Cu, Zn, Sn and U, but DvH does not. Cytoplasmic metal and bioinformatic analysis suggest that both EcH and DvH use W as well as Mo-containing oxidoreductases, and both are predicted to contain comparable numbers of Cu- and Zn-containing enzymes, yet the cytoplasmic concentrations of these metals in DvH were dramatically lower than those found in EcH. Neither organism accumulated Cr in their cytoplasmic fractions. Investigation of U tolerance and accumulation by EcH led to the observation that extracellular metal present in residual growth media or loosely bound to the membrane can bind strongly to cytoplasmic proteins when cells are broken for subsequent fractionation. The identity of the U-binding protein in EcH, and whether another biologically relevant metal was displaced, is currently under investigation. A significant amount of U (2.7% of dry weight from cells grown with 0.5 μM U) was found to be associated with the membrane fraction, even after triplicate washing. In addition to the known biologically relevant metals, some of those observed to be strongly bound to cytoplasmic macromolecules in DvH and EcH may be the result of misincorporation of extracellular metal. Further comprehensive studies are needed to firmly establish mechanisms of heavy metal tolerance and toxicity in these organisms.

Abbreviations

DvH	*Desulfovibrio vulgaris* Hildenborough
DEAE	Diethylaminoethyl anion exchange chromatography
DUF	Domain of unknown function
ECC	*Enterobacter cloacae* complex
EcH	*Enterobacter cloacae* strain Hanford
ICP-MS	Inductively couples plasma mass spectrometry
S100	Supernatant after centrifugation of a cell-free extract at 100 000 × *g*
S100W	S100 after washing three times using a 3 kDa filter
FT	Flow-through
UCP	Uncharacterized conserved protein
UPF	Uncharacterized domains

Authors contribution

ALM, FLP, WAL and MWA designed the research; WAL, BV and FLP carried out ICP-MS analyses; ALM, IMS, FLP and BV grew EcH and DvH in stainless steel fermenters and fractionated the biomass: IMS and BV performed growth studies of EcH and DvH; JG and TCH grew EcH and DvH in glass fermenters; DAE isolated and purified EcH from a natural environment; RAH and SDB determined and analyzed the genome sequence of EcH; WAL, BV and FLP carried out the metalloproteomic analyses of the DvH and EcH genomes; WAL, FLP, BV and MWA wrote the paper.

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