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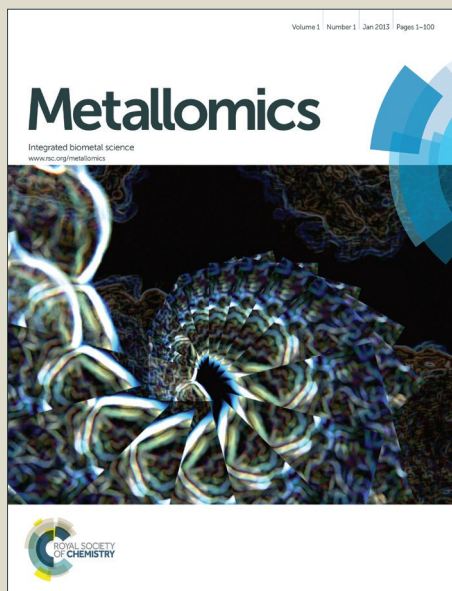
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Proteomic responses to gold(III)-toxicity in the bacterium *Cupriavidus metallidurans* CH34

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1

1   Running Title: Gold(III)-toxicity in *Cupriavidus metallidurans* CH34

2

3   Abbreviations:

4   BN-PAGE: blue-native polyacrylamide gel electrophoresis

5   IPS: Internal pooled standard

6   LA-ICP-MS: Laser ablation inductively coupled plasma mass spectrometry

7   PCA: Principal component analysis

8   PHA: Poly-hydroxyalkanoates

9   PHB: Poly-hydroxybutyrate

10   rpm: revolutions per minute

11   RT: room-temperature

12   SD: standard deviation

13

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2

Abstract

The metal-resistant β-Proteobacterium *Cupriavidus metallidurans* drives gold (Au) biomineralisation and the (trans)formation of Au nuggets largely via unknown biochemical processes, ultimately leading to the reductive precipitation of mobile, toxic Au(I/III)-complexes. In this study proteomic responses of *C. metallidurans* CH34 to mobile, toxic Au(III)-chloride are investigated. Cells were grown in the presence of 10 and 50 μM Au(III)-chloride, 50 μM Cu(II)-chloride and without additional metals. Differentially expressed proteins were detected by difference gel electrophoresis and identified by liquid chromatography coupled mass spectrometry. Proteins that were more abundant in the presence of Au(III)-chloride are involved in a range of important cellular functions, *e.g.*, metabolic activities, transcriptional regulation, efflux and metal transport. To identify Au-binding proteins, protein extracts were separated by native 2D gel electrophoresis and Au in protein spots was detected by laser absorption inductively coupled plasma mass spectrometry. A chaperon protein commonly understood to bind copper (Cu), CupC, was identified and shown to bind Au. This indicates that it forms part of a multi-metal detoxification system and suggests that similar/shared detoxification pathways for Au and Cu exist. Overall, this means that *C. metallidurans* CH34 is able to mollify the toxic effects of cytoplasmic Au(III) by sequestering this Au-species. This effect may in the future be used to develop CupC-based biosensing capabilities for the in-field detection of Au in exploration samples.

Significance to Metallomics

The bacterium *Cupriavidus metallidurans* CH34 is known to survive on the surface of and (trans)form natural gold grains. It reacts to the toxicity induced by mobile gold-complexes using a range of proteomic responses. Specifically, *C. metallidurans* overexpresses the chaperone protein CupC, which binds cytoplasmic gold, forms part of a multi-metal detoxification system to export

3

1 gold, likely using ATPase metal efflux proteins. To support gold detoxification, proteins involved in

2 oxidative stress reduction, energy metabolism and lipid formation are produced.

3

4

1.   Introduction

The metallophilic β-Proteobacterium *Cupriavidus metallidurans* CH34 was first isolated from a decantation tank at a zinc factory in Belgium.[1] Since then it has been detected in variety of metal-rich environments, *e.g.*, industrial sites around the world as well as the cooling water of the Russian Space Station.[2,3] *C. metallidurans* has been shown to be highly adaptive to changing environmental conditions, possibly because its genome readily incorporates and expresses foreign genes.[4] Heavy metal resistance genes have been acquired, re-combined and re-arranged from a range of sources; hence, the genome of *C. metallidurans* CH34 has undergone many alterations, which have allowed it to inhabit a wide range of environments containing high concentrations of heavy metals.[4,5] It harbours a genetic system with at least 25 *loci* for heavy metal resistance located in two large circular chromosomes and two megaplasmids.[4,5] Therefore, it has become a model organism to study microbial responses to heavy metals and its extreme tolerance to a range of metals including Ag(I), AsO-, Au(I), Au(III), Bi(III), Co(II), Cd(II), CrO_4^{2-}, Cs(I), Cu(I), Cu(II), $HAsO_4^{2-}$, Hg(II), Ni(II), Pb(II), SeO_3^{2-}, SeO_4^{2-}, Sr(II), Ti(I) and Zn(II) has been assessed in a range of studies.[4,5]

Advances in microscopic, micro-analytical and molecular techniques have facilitated an evolution in our understanding of how microorganisms interact with metals in the environment. With regards to Au, the element is no longer thought of as a stable and inert substance under Earth surface conditions. Rather, microorganisms have been shown to play a fundamental role in the solubilisation, transportation and re-concentration of Au in Earth surface environments.[6,7] Understanding the role *C. metallidurans* plays in the biogeochemical cycling of Au and Au nugget (trans)formation is of particular interest.[8,9] *C. metallidurans* has been shown to mobilise Au[10] and dominate biofilm communities inhabiting the surfaces of Au grains from several Australian sites.[9,11] In quartz-sand-packed column experiments, columns inoculated with *C. metallidurans* retained >99

5

1 wt% of percolating highly toxic Au(I)-thiosulfate (Minimal Inhibitory Concentration (MIC) = 0.5 μM),

2 in comparison to <30 wt.% retained in sterilised or abiotic controls.[12] Here the formation of

3 intracellular Au nanoparticles and extracellular micro-nuggets was also observed.[12] To survive such

4 close associations with Au, *C. metallidurans* CH34 needs to be able to detoxify Au-complexes.

5 By grouping the treatment of toxic metals into the same transcriptional network, organisms

6 are able to detoxify their environment without energetically expensive metal specific resistance

7 systems.[13,14] For example, in *C. metallidurans*, cellular uptake of Au(I/III)-complexes co-regulates a

8 range of metal export pathways, especially those for Cu, which may be co-utilised for detoxification

9 and export.[15] However, the interpretation of the molecular mechanisms involved in Au(I/III)-

10 detoxification in *C. metallidurans* is complex and is as yet not fully understood. Earlier studies have

11 shown that the *cupRAC* cluster encoding a regulatory protein (CupR), a heavy metal translocation

12 P-type ATPase (CupA) and a heavy metal chaperone protein (CupC), was strongly up-regulated in

13 Au-complexes amended cells.[8,16] Modified *C. metallidurans* cells, containing an engineered plasmid

14 carrying the red fluorescent protein (*rfp*) gene under the control of the CupR-upregulated promoter

15 *cupC,* showed specific up-regulation of this *cupR(rev)-PcupA/R(rev)-PcupC-rfp* cluster with Au(III)-

16 complexes but not Cu-ions, when induced with 50 μM of the metals.[17] This led to the development

17 of a highly-selective fluorescence-based whole-cell biosensor for Au-complexes.[17] In another study

18 addition of Au(III)-complexes to the supernatant of a *C. metallidurans* culture, which had expressed

19 truncated CupC, resulted in the formation of Au nanoparticles; note: truncated CupC contained its

20 metal-binding motif and was fused to secretion carrier Rmet_3428, a homolog of known secretion-

21 carrier OsmY found in *Escherichia coli.*[18] However, the majority of studies investigating the

22 response of *C. metallidurans* to Au(I/III) have relied on transcriptomics.[8,13,15] Transcriptomics uses

23 the amount of mRNA in a cell as an indicator of cellular function, but mRNA abundance is not

24 necessarily indicative of protein abundance or cellular function. Therefore, it is important to study all

25 levels of molecular expression and activity to gain a complete view of microbial responses to

6

environmental changes. There have only been a limited number of studies on proteomic changes that occur within *C. metallidurans, e.g.*, studies on the effect of Cu[14], Pb[19] and microgravity[20]. The study investigating the effect of Pb on the proteome of *C. metallidurans* clearly demonstrated one of the advantages of using proteomic techniques to investigate molecular responses of microorganisms to stress.[19]

In the current study we describe the proteomic response of *C. metallidurans* CH34 to Au(III) compared to control cells amended with Cu(II) and in media without additional metals. Two Au(III)-concentration, *i.e.*, 10 and 50 µM, were chosen to assess a possible dosage effect of Au. Similar to the study by Tseng *et al.*[17], a Cu^{2+}-control concentration equal to the highest Au(III)-concentration was chosen, because a central-aim of this study was to explore for Au-binding proteins with the potential for later use in protein-based biosensors. These need to function specifically to the analyte, *i.e.*, Au(III), even if considerable concentration of a potentially cross-regulating/cross-binding metal, *i.e.*, Cu^{2+}, is also present in the solution. To effective analyse the experiments we used techniques that allowed protein isoforms to be detected and proteins to remain intact during separation. As proteins remained intact during separation, spatially resolved laser absorption inductively coupled plasma mass spectrometry (LA-ICP-MS) was used to detect any proteins that directly bind Au.

2.   Materials and methods

2.1.   Experimental design and statistical procedures

Four biological replicates in each of four experimental groups: 10 µM Au(III) stress, 50 µM Au(III) stress, 50 µM Cu(II) stress and an unamended control, producing a total of 16 samples that were analysed by Differential-in-Gel-Electrophoresis (DIGE). The number of biological replicates per group was chosen based on the decision to include a balanced dye-swap design where two

1 biological replicates in each group were labelled with Cy3 and the remaining two with Cy5. After

2 image analysis using DeCyder 7.0 (GE Healthcare, Little Chalfont, United Kingdom), the spot

3 volume data was exported using the DeCyder 7.0 XML toolbox (GE Healthcare) and the individual

4 spot volume data was standardised by the corresponding spot volume of the internal pooled

5 standard (IPS) channel.[21] The resulting data was log-2 transformed to produce a normal

6 distribution of the standardised spot volumes. For statistical testing, a two-tailed Student's t-test

7 was applied, with a level of significance $\alpha = 0.05$. A t-test was chosen as the interpretation of the

8 resulting p-value is straight forward, being the probability of measuring an extreme distribution of

9 the data points under the assumption of the null hypothesis (H_0: $\mu_1 = \mu_2$). The p-value is used as

10 evidence of two measured distributions not having equal means, which is interpreted as the

11 difference in protein expression between the two tested groups. The false discovery rate was

12 estimated using q-values. False discovery rate can be interpreted as the proportion of proteins

13 exhibiting a p-value below the significance level expected to be type-I errors (Table 1). Assessment

14 of protein expression change was undertaken by calculating the distance in multiples of standard

15 deviations (SD) of each group of measured spot volumes, with 3 SD being set as the cut-off. These

16 protein spots were assigned as the class of primary interest. Principal component analysis (PCA)

17 was used to visualise these high-dimensional data by projecting them into a lower dimensional

18 subspace while preserving the maximum possible amount of variance, and a biplot was used to

19 interpret these principle components in terms of the original variables – *i.e.*, the protein spots with

20 the highest loadings in the respective principal component, contributing the most to these

21 differences.

22

23 **2.2.   Microbial strains and growth conditions**

1   *C. metallidurans* CH34 was obtained from the Deutsche Sammlung von Mikroorganismen und

2   Zellkulturen GmbH and grown in Tris-buffered mineral salts medium[1] containing 2 g L^{-1} sodium

3   gluconate at 30°C with shaking at 120 revolutions per minute (rpm). *C. metallidurans* CH34 was

4   initially grown on solid media and a single colony was picked and grown in liquid media, until the

5   exponential growth phase (OD_{600} 0.55 ± 0.05). In 150 mL Erlenmeyer flasks, 10 μL of culture was

6   added to 40 mL of fresh medium and grown to the beginning of the exponential phase (OD_{600} 0.15

7   ± 0.05). At this stage each set of quadruplicate cultures had either 10 μM of Au(III), 50 μM of Au(III)

8   (in the form of $AuCl_4$) or 50 μM of Cu(II) (in the form of $CuCl_2$) added. Au(III) was chosen for this

9   study as this form has been shown to be more toxic than Au(I) to *C. metallidurans* CH34 cells.[8,15]

10 Another quadruplicate set of cultures had no additional metals added, serving as a control. Cultures

11 were left to grow until the end of the exponential phase (OD_{600} 0.55 ± 0.05) and the cells were

12 harvested by centrifugation at 10,000 rpm for 10 min at 4°C. Cells were then washed three times

13 with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) and stored at -80°C. An

14 independent set of biological triplicate cultures was grown under the same conditions as described

15 above for analysis by native 2D gel electrophoresis.

16

17 ### 2.3.   Protein extraction and purification

18 Washed cells were suspended in 250 μL of Isoelectric Focussing (IEF) sample buffer (7 M urea

19 (Merck, Darmstadt, Germany), 2 M thiourea (GE Healthcare), 30 mM Tris (Merck), 4 % CHAPS

20 (Roche Diagnostics, Basel, Switzerland), 1 % protease inhibitor cocktail (Sigma-Aldrich, St. Louis,

21 Missouri, USA), 1.1 % Pefabloc® SC (PSC) protector reagent (Roche Diagnostics), pH 8.5). Cells

22 were lysed using an ultrasonic probe (B-30, Branson Danbury, Connecticut, USA); with 30 pulses

23 of sonication (40 % duty cycle), subsequently cooled in ice water for two min, and sonicated for

24 another 30 pulses. Samples were then centrifuged at 20,000 x *g* at 15°C for 60 min and the

1 supernatant was collected and stored at -80°C. The concentration of protein was determined using

2 the EZQ® protein quantification assay (Life Technologies, Carlsbad, California, USA) against an

3 ovalbumin standard curve according to the manufacturer's protocol.

4

5 2.4. Differential in Gel Electrophoresis (DIGE)

6 *2.4.1. Protein labelling*

7 Three 200 pM μL^{-1} solutions of three CyDyes (Cy2, Cy3 and Cy5; GE Healthcare) in anhydrous

8 dimethylformamide were made and stored under Ar in 1 mL aliquots at -80°C. For protein labelling,

9 100 µg of protein from each sample was added to 1 μL of either the Cy3 or Cy5 labelling solutions.

10 IPS was made consisting of 50 µg of protein pooled from each sample and this was labelled with

11 Cy2. Labelling solutions were incubated in the dark at room temperature (RT) for 30 min, upon

12 which the labelling reaction was stopped with the addition of 1 μL of 1 M lysine and incubated in the

13 dark at RT for 10 min. After labelling, 1,4-dithiothreitol (DTT, Roche Diagnostics; 0.1 g dissolved in

14 17 µL H_2O) was added to the solution to a volume of 2 % (v/v) and incubated on ice for 60 min in

15 the dark, then Pharmalyte 3-10 (GE Healthcare) was then added to a volume of 2 % (v/v) and

16 samples were stored at -80°C.

17 *2.4.2. Isoelectric focusing (IEF)*

18 Eight IPG strips spanning a 240 mm non-linear pH 3 - 11 range (GE Healthcare) were rehydrated

19 overnight at RT in 450 μL rehydration buffer (6 M urea, 2 M thiourea, 1% CHAPS, 0.5% pH 3 - 11

20 NL carrier ampholytes (GE Healthcare) and 200 mM 2,2'-dithiodiethanol (Sigma-Aldrich). Samples

21 were applied by anodal cup loading. IEF was performed in the dark on an IPGphor II (GE

22 Healthcare) at 20°C using a six step program with the current limited to 50 μA per strip; step one:

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1   150 V for 1 h; step two: 300 V for 1 h; step three: 600 V for 2.5 h; step four: 600-8000 V gradient

2   over 1.5 h; step five: 8000 V for 27 kVh.

3   *2.4.3. SDS-PAGE*

4   Following IEF, strips were equilibrated for 15 min in a proprietary equilibration buffer (Serva

5   Electrophoresis GmbH, Heidelberg, Germany) containing 6M urea and 10 mg mL^{-1} DTT (Roche

6   Diagnostics). After equilibration, the solution was removed and exchanged with equilibration buffer

7   containing 40 mg mL^{-1} iodoacetamide (GE Healthcare) instead of DTT. Separation in the second

8   dimension was carried out using 18 x 25 cm^2 2DGel DALT NF flatbed pre-cast polyacrylamide gels

9   (T=12.5%; Serva). Electrophoresis was performed at 7 mA per gel for 1 h, 13 mA per gel for 1 h,

10   then the IPG strip was removed and electrophoresis continued at 40 mA per gel for 3 h and 50 min.

11   *2.4.4. Image analysis*

12   Gels were scanned using an Ettan DIGE Imager (GE Healthcare) at 100 μm resolution, with the

13   following exposure times; Cy2: 2.50 s; Cy3: 0.45 s; and, Cy5: 0.50 s. ImageQuantTL (GE

14   Healthcare) software was used to orientate gel images and subsequent image analysis was carried

15   out using DeCyder 2D software version 7 (GE Healthcare). The spot detection algorithm was set to

16   an estimate of 5,000 spots. Detected spots were excluded if they exhibited a slope >1.1, an area <

17   300, a volume < 30,000, a peak height < 80 or > 65,534 (to exclude saturated signals). Normalised

18   spot volumes were exported using XML toolbox sub-program of DeCyder 7.0. Data was

19   standardised by the spot volume of the corresponding spot in the Cy2 (IPS) channel. Standardised

20   data was log-2 transformed and statistical significance was tested using a two-tailed Student's t-

21   tests, with significance level α = 0.05. PCA and biplot was conducted on the standardised, log-2

22   transformed data using R. Post-hoc power calculation was performed using Piface (v1.64)[22] and

23   resulted that a fold-change of 1.7 or 1.6 (as applicable, Table 1) was detected with a probability of

11

1 80 %. The false discovery rate at the significance level was determined using q-values (Table 1) as

2 described in Penno *et al.*[23] Proteins were fixed into the gel using 40 % (v/v) ethanol, 10% (v/v)

3 acetic acid overnight at RT and visualised using Coomassie Brilliant Blue G250. Spots from DIGE

4 gels analysed by mass spectrometry were picked using an Ettan Spot Picker (GE Healthcare). All

5 image files, exported DeCyder analysis file and picking lists can be downloaded from

6 ProteomeXchange PXD005034.

7

2.5.   Native two dimensional-polyacrylamide gel electrophoresis

9 Three hundred µg of protein extracts from each of the three biological replicates from the three

10 conditions (Control, 50 µM Cu(III) and 50 µM Au(III)) were separated using native IEF in 11cm IPG

11 pH 3-10NL (Bio-Rad, Hercules, California, USA) in combination with blue native-polyacrylamide gel

12 electrophoresis (BN-PAGE) according to the protocol outlined in.[24] Potential protein complexes

13 were visualised using Ag staining.[25] Eleven protein spots, which were only visible in all three

14 replicates from cells grown with 50 µM Au(III) (as in comparison with all replicates of the two other

15 conditions), were chosen for further identification by MS.

16

2.6.   Liquid Chromatography-Mass Spectrometry (LC-MS)

18 All samples were analysed by either Orbitrap (Thermo-Fisher, Waltham, MA, USA) or amaZon 3D

19 iontrap (Bruker Daltonics, Bremen, Germany) MS. Coomassie-stained spots were destained using

20 30 % (v/v) acetonitrile (Merck) until they appeared blank, in case of silver-stained spots, proteins

21 were destained using rapid fixer (Agfa-Gevaert, Mortsel, Belgium) before reduction and alkylation.[26]

22 Proteins were digested overnight at 37°C using 100 ng sequencing grade Trypsin (Promega,

23 Madison, USA). For Orbitrap analysis, peptides were separated on a nano-flow Ultimate 3000

12

1 HPLC system (Dionex, Sunnyvale, USA). Peptides were loaded onto a Acclaim PepMap 100 C18

2 column (3 µm particle size, 75 µM inner diameter, 2 cm length, Thermo-Fischer) and desalted for 5

3 min in buffer A (2 % (v/v) acetonitrile, 0.1 % formic acid) and 5 µL min^{-1} flow rate. Peptides were

4 using a two buffer system (buffer A and buffer B (80 % (v/v) acetonitrile, 0.1 % (v/v) formic acid))

5 separated on a Acclaim PepMap RSLC C18 column (2 µM particle size, 75 µM inner diameter, 15

6 cm length, Thermo-Fischer) using a linear gradient from 5 % buffer B to 45 % buffer B over 29 min,

7 followed by a 90 % buffer B wash for 13 min and column equilibration in 5 % buffer B for 18 min at

8 a flow rate of 300 nL min^{-1}. Column oven temperature was set to 60°C. Peptides were injected in to

9 the Orbitrap via a nano spray ESI source. Orbitrap was set to Nth order double play mode in

10 positive ion mode, scanning between 300-2000 m/z with a set resolution of 60,000. The top 6

11 intense m/z features were subjected to CID MS/MS (minimum signal intensity: 5,000, isolation

12 width: 3.00, normalised collision energy: 35.0). Samples analysed by the amaZon 3D iontrap were

13 separated on an Agilent 1100 nano-flow HPLC (Agilent, Santa Clara, USA). Desalting of peptide

14 mixture was carried out by loading onto a Acclaim PepMap 100 column (same specifications as

15 above) and washing with 100 % buffer C (0.1 % (v/v) formic acid) for 4 min at 5 µL min^{-1}. Peptides

16 were separated on a Acclaim PepMap RSLC C18 column (same specifications as above) using a

17 two buffer system (buffer C and buffer D (90 % (v/v) acetonitrile, 0.1 % formic acid) in a linear

18 gradient from 5 % to 45 % buffer B over 17 min, followed by a 5 min 95% buffer B and a 3 minute

19 equilibration step with 5 % buffer B. AmaZon Iontrap was set for enhanced resolution scan in

20 positive mode between 300-200 m/z and 3 most ionisable features were chosen for CID

21 fragmentation.

22   Orbitrap and amazon Iontrap raw data was converted to mzXML format using Proteowizard

23 software[27] and submitted to Comet version 2016.2[28]. Processed data was searched against NCBI

24 database of human, porcine trypsin and *C. metallidurans* CH34 (28,243 sequences), downloaded

13

1   on 25/07/2016. Search parameters applied for Comet search were: precursor mass tolerance of

2   either 20 ppm (Orbitrap) or 0.4 Da (ion-trap MS), fragment bin size was set to 1.005, trypsin was

3   set as protease (no cleavage after proline), a maximum of two missed cleavages was allowed,

4   while fixed modification was set as carbamidomethyl (C) and variable modification as oxidation (M).

5   Proteins were considered identified if two peptides with an expectation below 0.05 using the comet

6   scoring algorithm were detected. In case of spots picked from DIGE gels, if more than one protein

7   was identified per cut-out gel spot, the protein with the largest number expressed as product of

8   unique sequences and protein coverage was chosen over the other proteins. In case these number

9   was similar among the identified proteins, all these proteins are reported. As Comet could not

10 provide details to the matched fragment ions, a MASCOT (version 2.3.01, Matrix Sciences, UK)

11 search of the MS data of Spot 9 and the designated protein spot corresponding to the Au signal on

12 the native 2D gels was performed for easy visualisation (see Supplement S1). For this, 3D ion trap

13 raw data was converted to mgf by Proteowizard software. Parameter deviating from the comet

14 search, using MASCOT were as follows: 0.4 Da fragment mass tolerance, re-scoring using

15 percolator. All mzXML & pep.xml files, exported MASCOT xml and processed results files and

16 Comet search parameters can be downloaded from ProteomeXchange PXD004199.

17

18 2.7.   Native 2D Western-blot

19 Gel for n2D-PAGE was cast and electrophoresis was performed according to the protocol

20 described above. Three hundred µg protein extract from *C. metallidurans CH34* cells grown in 50

21 µM Au(III) was separated by native IEF. Before BN-PAGE as second dimension, 100 µg protein

22 extract of *C. metallidurans CH34* cells grown in 50 µM Au(III) was mixed with 1.8 µl 5 % (w/v)

23 Coomassie G-250 (Sigma-Aldrich) in H_2O and filled up to a volume of 10 µL with Equilibration

24 Buffer.[24] This mixture was pipetted onto a filter paper (3 mm CHR, Whatman, Maidstone, United

14

1 Kingdom) and placed onto the upper edge of the gel next to the equilibrated IPG strip, serving as

2 primary marker lane for subsequent LA-ICP-MS. After BN-PAGE, proteins were transferred by a

3 Criterion wet-blotting system (Bio-rad) to a PVDF membrane (Merck) using the buffer system

4 described in Wittig et al.[29] with the following settings: 400 mA for 2 h at RT, limited to 70 V.

5

2.8. Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS)

7 The PVDF membranes from the native 2D WB was backed onto quartz slides. LA-ICP-MS mapping

8 was conducted using a Resonetics M-50-LR 193 nm Excimer laser coupled to an Agilent 7700cx

9 Quadrupole ICP-MS housed at Adelaide Microscopy (Table S1). Ablation was carried out in a two-

10 volume ablation cell designed by Laurin Technic Pty using UHP He (0.7 L min^{-1}) as a carrier gas.

11 Immediately upon exiting the cell, the aerosol cell was mixed with Ar (0.93 L min^{-1}) and passed into

12 the torch. The ICP-MS was optimised to maximise sensitivity on isotopes of the mass range of

13 interest, while keeping production of molecular oxide species (i.e., $^{232}Th^{16}O/^{232}Th$) and doubly

14 charged ion species (i.e., $^{140}Ce^{2+}/^{140}Ce^{+}$) as low as possible, and usually <0.2 %.

15 Analysis was performed by ablating sets of parallel line rasters in a grid across the samples.

16 A beam size of 300 μm and a scan speed of 150 μm s^{-1} were chosen which resulted in the desired

17 sensitivity of elements of interest, and adequate spatial resolution for the study. A laser repetition of

18 10 Hz was used at a constant energy output of 80 mJ, resulting in an energy density of ~4 J cm^{-2} at

19 the target. Using these beam conditions depth of ablation during mapping was around 10 μm. A set

20 of 8 elements (^{13}C, ^{29}Si, ^{31}P, ^{34}S, ^{60}Ni, ^{65}Cu, ^{66}Zn, ^{197}Au) were analysed with dwell time for all

21 masses set to 0.003 s, resulting in a total sweep time was ~0.07 s. A 30 s background acquisition

22 was acquired at the start of every raster, and to allow for cell wash-out, gas stabilisation, and

23 computing processing, a delay of 15 s was used after each line. Identical rasters were done on

24 NIST SRM 610 at the start and end of a mapping run. Upon detection of Au in the primary marker

15

1 lane, the laser ablation was continued in perpendicular direction to pin-point the gold signal to a

2 specific protein signal in the n2D-PAGE part of the blot. After LA-ICP-MS the blot was stained using

3 Coomassie R250. The resulting protein pattern and the locus of Au was compared to silvers

4 stained native 2D gels and the corresponding spot was identified by mass spectrometry as

5 described above.

6

2.9.   Structural and interaction analyses of proteins

8 A search of the NCBI database (blast.st-va.ncbi.nlm.nih.gov/Blast.cgi)[30] returned Cu chaperon

9 proteins that only consisted of amino acids 68-133, therefore only these amino acids were used for

10 further analysis. A model of this protein was made using the SWISS-MODEL program

11 (swissmodel.expasy.org/)[31] with the *C. metallidurans* CH34 genome (NCBI Accession: PRJDB279).

12 The following templates were selected from a search of the Protein DataBank (pdb.org)[32] and used

13 to construct a model: 2rml.1.A, a copper transporting P-type ATPase CopC; 2rog.1.A and 2roe.1.A,

14 both heavy metal binding proteins.[33-36] STRING (Search Tool for Retrieval of Interacting Genes/

15 Proteins; http://string-db.org) was used to visualise predicted protein-protein interactions for the Au-

16 binding protein detected.[37]

17

3.   Results and discussion

3.1.   DIGE-analyses of differentially expressed proteins

20 In total 2,196 protein spots were detected by DeCyder (Fig. 1). Of the 1,597 proteins spots

21 detected in at least two samples, of each of the four experimental groups, 59 exhibited a very

22 stringent (p-value < 0.001) expression pattern of up-regulation in both Au groups compared to both

23 Cu and unamended control groups (Table S2). Principle component analysis of the DIGE data

16

1 (using the 864 protein spots detected in all 16 samples) showed that the first principle component

2 produces a clear separation of Au-amended cells to unamended cells and Cu-controls, with the fifth

3 principle component separating the 10 µM from the 50 µM Au group (Fig. 2 A ,B). This

4 demonstrates a specific proteomic response of *C. metallidurans* to increasing Au(III)-stress under

5 these conditions.

6   We were most interested in proteins that exhibited up-regulation in both Au concentrations in

7 comparison to both controls; 183 protein spots exhibited this pattern. Of these 183 proteins, 60

8 were also differentially abundant between the Cu-amended experiment and the unamended

9 control, leaving 123 spots that were uniquely more abundant in both Au groups with no statistically

10 significant change in expression between Cu and the control (p-value >0.05; Fig. 3A). Forty two of

11 the 123 protein spots in greater abundance exhibited a very stringent expression pattern and were

12 three times the standard deviation from the mean (Table S2). Twenty-nine of these proteins,

13 showing the highest degree of differential expression were successfully identified by MS (Table 2A,

14 Table S3).

15   These proteins are involved in a range of metabolic activities, transcriptional regulators,

16 efflux proteins, and transporters (Fig. 3B; Table 2, Table S3). Specifically, a predicted DNA-binding

17 transcriptional repressor was identified (spot 1237; Table 2). The sequence of this DeoR family

18 protein contains a region that has been shown to regulate carbohydrate transport and metabolism

19 in *Corynebacterium glutamicum* ATCC 13032.[38] A protein disaggregation chaperon was identified

20 (spot 259), this protein disaggregates miss folded and aggregated proteins.[39] DNA gyrase subunit

21 B was identified from spot 256, this protein contains a metal (Mg^{2+}) binding site. Other notable

22 proteins were identified such as a DNA-binding transcriptional repressor (spot 1237). Five proteins

23 spots demonstrated a dosage response between 50 µM Au and 10 µM Au (spot 586, 609, 863, 977

24 and 1681; Fig. 3A; Table S2). The identified metabolic proteins provide an indication that cells are

17

1 adjusting to increased stress, they are: i) the ABC-type sugar periplasmic transporter involved in

2 carbohydrate transport and metabolism, ii) glyceraldehyde-3-phosphate dehydrogenase involved in

3 a range of metabolic functions, iii) acetyl-CoA acetyltransferase, and iv) sn-glycerol-3-phosphate

4 dehydrogenase (FAD/NAD(P)-binding protein) involved in linking carbohydrate and lipid

5 metabolism (Table 2, Table S3).[40-42] The greater abundance of metabolism proteins (Fig. 3B)

6 suggests that cells are increasing metabolic rates and energy production so that biochemical

7 mechanisms to deal with elevated Au-stress can be activated. Oxidative stress from Au(III)-

8 complexes causes cell membrane damage, hence the overexpression of sn-glycerol-3-phosphate

9 dehydrogenase suggests that *C. metallidurans* CH34 is repairing oxidative damage to the cell

10 membrane.[43]

3.2. Native 2D electrophoretic analysis of differentially expressed protein complexes

13 Blue native 2D electrophoresis was performed to explore the effect of Au(III)-stress on

14 *C. metallidurans* CH34 protein complexes and protein-protein interactions (PPI), compared to Cu-

15 and unamended controls. Eleven protein spots were detected, which were only present when

16 *C. metallidurans* CH34 was grown in the presence of Au(III)-chloride (Fig. 4). These protein

17 complexes and PPI were scattered over a pI range from 3-10 and exhibit molecular weights up to

18 approximately 800 kDa. Excised proteins were identified using MS (Table 3, Table S4). Proteins

19 involved in cell membrane signalling, as identified using both protein separation methods, indicate

20 that cells are undergoing stress (Table 2 and 3). Spot 1 was identified as a signal peptide protein

21 that contains conserved regions of a periplasmic or secreted lipoprotein. Other proteins known to

22 be involved in managing oxidative stress were also identified, these include a lipid hydro-peroxide

23 peroxidase (Spot 5) capable of catalysing oxidation reactions and protein from the OhrB, OsmC

24 family (Spots 9, 10 and 11). Spot 6 was identified as alkyl hydro-peroxidase, this protein catalyses

18

the reduction of peroxides, which are likely formed when Au-complexes reach the cytoplasm.[8] This shows that Au(III) induces strong oxidative stress in the cells and confirms the results of earlier transcriptomic studies, in which the genes for these proteins were also strongly expressed.[8]

(R)-3-hydroxybutyryl-CoA dehydrogenase PhaB (Spot 3) was also identified. (R)-3-hydroxybutyryl-CoA dehydrogenase is an enzyme that catalyses the NADPH-dependent reduction of acetoacetyl-CoA, an intermediate of polyhydroxyalkanoates (PHA) synthetic pathways.[44] Poly-hydroxybutyrate (PHB) is the best known PHA and has been implicated in the intracellular storage/resistance to heavy metals.[45] In the closely related β-Proteobacterium *Burkholderia cepacia*, polyhydroxybutyrate (PHB) production occurs when cells are grown in the presence of 0.5–5mM Au(I)-thiolates, the polyhydroxybutyrate granules are associated with the storage and passivation of these Au-complexes.[46] Another hypothetical protein was identified (spot 4), however the function of this protein is unknown. Other proteins identified were largely associated with energy metabolism. Spots 2 was identified as malate synthase. Malate synthase works together with isocitrate lyase in the glyoxylate cycle to bypass two oxidative steps of Krebs cycle and permit C incorporation from acetate or fatty acids in many microorganisms.[47] Malate dehydrogenase (Spots 5, 5a) is an enzyme that reversibly catalyses the oxidation of malate to oxaloacetate using the reduction of NAD^+ to NADH.[48] This reaction is part of many metabolic pathways, including the citric acid cycle.[48] This strong up-regulation of metabolic genes involved in energy generation, which was also observed in DIGE separation (this study) and in an earlier transcriptomic study,[8] suggests that cells increase metabolic energy generation to enable the production of stress-coping proteins.

3.3.   Identification of Au-binding proteins

1 La-ICP-MS on native 2D Western Blot was used to identify Au-binding proteins and yielded one

2 spot containing a high counts for Au, while the count rate for other elements, especially the tested

3 metals, remained at background levels (Fig. 5). Four proteins were identified by MS at the spot

4 where Au was detected: i) 3-demethylubiquinone-9 3-methyltransferase (accession number:

5 93357244), ii) a putative glyoxalase/blemycin resistance protein (499835771), iii) a hypothetical

6 protein (657067637), and iv) a Cu chaperone CupC (93356308) (Table 4, Table S5). The Cu

7 chaperone protein, CupC, was used for further analysis, as this was the only protein to contain a

8 metal binding domain that could be responsible for binding Au-complexes.[18]

9 A model of the Cu chaperon protein was constructed based on two related Cu-binding

10 proteins: a 'multispecies' copper chaperone, heavy metal binding (modular protein) of an organism

11 described solely as *Cupriavidus* (WP_00864966) which had a 100% homology between amino

12 acids 68-133 with the Cu chaperon protein identified in this study, and a copper chaperone from

13 *Cupriavidus* sp. BIS7 (WP_019451883); the resulting protein model is shown in Fig. 6.

14 Computational simulation analysis of the protein model recognised eight ligand hits: $C_2H_3O_2^-$,

15 Ca(II), Cl$^-$, Cu(II), Cu(I), $C_3H_8O_3$, Na and Zn(II), while Au(I) and Au(III) ligands were not predicted to

16 bind to the protein model. However, the presence of Cu(I)-binding sites demonstrates that the

17 protein CupC contains metal-binding sites that are suitable for Au(I)-binding, due to chemical

18 similarity Au complexes are predicted to bind to Cu-binding sites.[4] In addition, a study by Ouyang *et*

19 *al.,*[18] showed that Au nanoparticles were formed in the presence of truncated CupC fused to the

20 secretion carrier Rmet_3428, suggesting the binding of Au to a metal-binding motif in CupC.

21 Overall, this highlights the limitations of computer-based protein modelling, which relies on the

22 utilisation of suitable datasets that is not available for the Au-complex studied.

23 An earlier study using transcriptomic approaches had shown a strong up-regulation of the

24 entire metal resistance and efflux operon *cupRAC* (Rmet 3523-3525).[8] Although the increased

25 transcriptional expression of *cupR* and *cupA* have been shown the resulting proteins were not

20

1 identified in this study as differentially more abundant or Au-containing in Au amended cells. This

2 may be due to experimental and analytical setup, *e.g.*, very strict criteria were employed to target

3 proteins to be identified by mass spectrometry, which may have excluded them, or masking

4 through co-migrating proteins. Another possibility might that the use of a 3-11 immobilised pH

5 gradient CupAR were not properly resolved and their signals where overlain by co-migrating

6 proteins. While *cupA*, the gene encoding the *C. metallidurans* ortholog of the Cu efflux pump used

7 by *S. enterica* for Au export and detoxification, was strongly expressed, Wiesemann *et al.*[15] have

8 shown that it is likely not involved in the direct Au efflux, and may have another role in Au

9 detoxification.[15] STRING analysis of CupC environment has shown that CupC may interact

10 with/bind to a range metal-transporting ATPases, *i.e.*, CadA, RdxI and CtpA1, which may also be

11 involved in Au-efflux, resistance and biomineralisation (Fig. 6); note setting for STRING analysis

12 allow experimental evidence to be transferred from other organisms.[49,50] This suggest that CupA

13 may not be Au binding and/or that Au may be transferred to other efflux pumps. Upregulation of

14 *cupR* in *C. metallidurans* suggests that CupR may act as Au-sensing regulator, similar to its

15 homologs GolS in *Salmonella* and CueR in *E. coli*.[51,52] In addition, binding of Au-complexes to

16 CupR has been demonstrated by Jian *et al.*,[16] and Tseng *et al.*[17] The affinity of CueR for copper is

17 in the zeptomolar range,[53] and that of CupR for Au(I) might be even higher.[16,17] Therefore, only

18 very few Au-complexes may be required in the cytoplasm for up-regulation of genes by CupR,

19 which appears to lead to a highly elevated production of the CupC. The majority of cytoplasmic Au-

20 complexes is then likely bound by the highly abundant metal-chaperone CupC. This, in combination

21 with using Western-blotting may explain why only highly abundant CupC-Au, but not CupR-Au, was

22 detected using LA-ICP-MS.

23

4. Conclusions

1 A combination of proteomic and micro-analytical techniques provided a detailed assessment of the

2 proteomic reactions triggered by mobile Au(III)-complexes, which may be employed by *C. metallidurans* CH34 to survive on the surface of Au grains. This study shows that Au-complexes

3 reach the cytoplasm. *C. metallidurans* CH34 uses chaperone CupC to bind Au and effect

4 detoxification by export, likely utilising ATPase metal efflux proteins otherwise utilised for a range of

5 metals. To support this detoxification and/or deal with Au-complex toxicity effects, proteins involved

6 in oxidative stress, energy metabolism and lipid formation are more abundant when

7 *C. metallidurans* CH34 was grown in the presence of Au(III). These include sn-glycerol-3-

8 phosphate dehydrogenase, glutathione peroxidase, glutathione S-transferase, lipid hydroperoxide

9 peroxide, the hypothetical protein Rmet_3428 and glyceraldehyde-3-phosphate dehydrogenase.

10 The greater abundance of proteins related to membrane lipids indicates that cell membrane

11 damage is occurring in the presence of metals and *C. metallidurans* CH34 is mitigating this

12 damage by increasing the production of membrane lipids. In addition, proteins like glutathione

13 peroxidase, glutathione S-transferase and lipid hydro-peroxide peroxide help to protect the cell

14 membrane from oxidative stress, which is caused by the accumulation of intracellular Au.

15

16

Acknowledgements

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References

1. Mergeay M, Nies D, Schlegel HG, Gerits J, Charles P, Van Gijsegem F. *Alcaligenes eutrophus* CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. *J. Bacteriol.* 1985; 162, 328-334

2. Mijnendonckx K, Provoost A, Ott CM, Venkateswaran K, Mahillon J, Leys N, et al. Characterisation of the survival ability of *Cupriavidus metallidurans* and *Ralstonia pickettii* from space-related environments. *Microb. Ecol.* 2013; 65, 347-360

3. Ott CM, Bruce RJ, Pierson DL. Microbial characterisation of free floating condensate aboard the Mir space station. *Microb. Ecol.* 2004; 47, 133-136

4. Janssen PJ, Van Houdt R, Moors H, Monsieurs P, Morin N, Michaux A, et al. The complete genome sequence of *Cupriavidus metallidurans* strain CH34, a master survivalist in harsh and anthropogenic environments. *PloS ONE* 2010; 5, e10433

5. Nies DH. The biological chemistry of the transition metal "transportome" of *Cupriavidus metallidurans*. *Metallomics*, 2016, 8, 481-507.

6. Southam G., Lengke MF, Fairbrother L, Reith F. The biogeochemistry of gold. *Elem.* 2009; 5, 303-307.

7. Rea MA, Zammit CM, Reith F. Bacterial biofilms on gold grains - implications for geomicrobial transformations of gold. *FEMS Microbiol. Ecol*. 2016; 92, fiw082.

8. Reith F, Etschmann B, Grosse C, Moors H, Benotmane MA, Monsieurs P, et al. Mechanisms of gold biomineralization in the bacterium *Cupriavidus metallidurans*. *Proc. Natl. Acad. Sci.* 2009; 106, 17757-17762

9. Reith F, Fairbrother L, Nolze G, Wilhelmi O, Clode PL, Gregg A, et al. Nanoparticle factories: Biofilms hold the key to gold dispersion and nugget formation. *Geology* 2010; 38, 843-846

10. Brugger J, Etschmann B, Grosse C, Plumridge C, Kaminski J, Paterson D, et al. Can biological toxicity drive the contrasting behaviour of platinum and gold in surface environments? *Chem. Geol.* 2013; 343, 99-110

11. Reith F, Rogers SL, McPhail DC, Webb D. Biomineralization of gold: biofilms on bacterioform gold. *Science* 2006; 313, 233-236

12. Fairbrother L, Etschmann B, Brugger J, Shapter J, Southam G, Reith F. Biomineralization of gold in biofilms of *Cupriavidus metallidurans*. *Environ. Sci. Technol.* 2013; 47, 2628-2635

13. Monsieurs P, Moors H, Van Houdt R, Janssen PJ, Janssen A, Coninx I, et al. Heavy metal resistance in *Cupriavidus metallidurans* CH34 is governed by an intricate transcriptional network. *Biometals* 2011; 24, 1133-1151

14. Monchy S, Benotmane MA, Wattiez R, van Aelst S, Auquier V, Borremans B, et al. Transcriptomic and proteomic analyses of the pMOL30-encoded copper resistance in *Cupriavidus metallidurans* strain CH34. *Microbiol.* 2006; 152, 1765-1776

15. Wiesemann N, Mohr J, Grosse C, Herzberg M, Hause G, Reith F, et al. Influence of copper resistance determinants on gold transformation by Cupriavidus metallidurans strain CH34. *J. Bacteriol.* 2013; 195, 2298-2308

16. Jian X, Wasinger EC, Lockhard JV, Chen LX, He C. Highly sensitive and selective gold(I) recognition by a metalloregulatory in *Ralstonia metallidurans*. *J. Am. Chem. Soc.* 2009; 131, 10869-10871.

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24

1   17.   Tseng H, Tsai Y, Yen J, Chen P, Yeh Y. A fluorescence-based microbial sensor for the

2   selective detection of gold. *Chem. Commun*. 2014; 50, 1735-1737.

3   18. Ouyang C, Lin Y, Tsi D, Yeh Y. Secretion of metal-binding proteins by a newly discovered

4   OsmY homolog in Cupriavidus metallidurans for the biogenic synthesis of metal nanoparticles.

5   *RSC Adv.* 2016; 6, 16798-16801.

6   19.   Taghavi S, Lesaulnier C, Monchy S, Wattiez R, Mergeay M, van der Lelie D. Lead(II)

7   resistance in *Cupriavidus metallidurans* CH34: interplay between plasmid and chromosomally-

8   located functions. *Antonie van Leeuwenhoek* 2009; 96, 171-182

9   20.   Leroy B, Rosier C, Erculisse V, Leys N, Mergeay M, Wattiez R. Differential proteomic

10   analysis using isotope-coded protein-labeling strategies: Comparison, improvements and

11   application to simulated microgravity effect on *Cupriavidus metallidurans* CH34. *Proteomics* 2010;

12   10, 2281-2291

13   21.   Alban A, David SO, Bjorkesten L, Andersson C, Sloge E, Lewis S, et al. A novel

14   experimental design for comparative two-dimensional gel analysis: two-dimensional difference gel

15   electrophoresis incorporating a pooled internal standard. *Proteomics* 2003; 3, 36-44

16   22.   Lenth RV. Statistical power calculations. *J Ani. Sci.* 2007; 85, E24-29

17   23.   Penno MA, Klingler-Hoffmann M, Brazzatti JA, Boussioutas A, Putoczki T, Ernst M, et al.

18   2D-DIGE analysis of sera from transgenic mouse models reveals novel candidate protein

19   biomarkers for human gastric cancer. *J. Proteomics* 2012; 77, 40-58.

20   24.   Weiland F, Zammit CM, Reith F, Hoffmann P. High resolution two-dimensional

21   electrophoresis of native proteins. *Electrophor.* 2014; 35, 1893-1902.

25

25. Blum H, Beier H, Gross H. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophor.* 1987; 8, 93-99

26. Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat. Protoc.* 2006; 1, 2856-2860.

27. Chambers MC, Maclean B, Burke R, Amodei D, Ruderman DL, Neumann S, et al. A cross-platform toolkit for mass spectrometry and proteomics. *Nat Biotech* 2012; 30, 918-920.

28. Eng JK, Jahan TA, Hoopmann MR. Comet: an open-source MS/MS sequence database search tool. *Proteomics* 2013; 13, 22-24.

29. Wittig I, Braun HP, Schagger H. Blue native PAGE. *Nat. Protoc.* 2006; 1, 418-428.

30. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 1997; 25, 3389-3402

31. Schwede T, Kopp J, Guex N, Peitsch MC. SWISS-MODEL: An automated protein homology-modeling server. *Nucleic Acids Res.* 2003; 31, 3381-3385

32. Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN, Bourne P E. The Protein Data Bank. *Nucleic Acids Res.* 2000; 28, 235-242

33. Biasini M, Bienert S, Waterhouse A, Arnold K, Studer G, Schmidt T, Kiefer F, Cassarino TG, Bertoni M, Bordoli L, Schwede T. SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic Acids Res.* 2014; 42, W252-W258

34. Arnold K, Bordoli L, Kopp J, Schwede T. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. *Bioinform* 2006; 22, 195-201

26

35. Kiefer F, Arnold K, Künzli M, Bordoli L, Schwede T. The SWISS-MODEL Repository and associated resources. *Nucleic Acids Res.* 2009; 37, D387-D392

36. Guex N, Peitsch MC, Schwede T. Automated comparative protein structure modeling with SWISS-MODEL and Swiss-PdbViewer: A historical perspective. *Electrophor.* 2009; 30, S162-S173

37. Franceschini A, Szklarczyk D, Frankild S, Kuhn M, Simonovic M, Roth A, et al. STRING v9.1: protein-protein interaction networks, with increased coverage and integration. *Nucleic Acids Res.* 2013; 41, D808-815

38. Gaigalat L, Schlüter J-P, Hartmann M, Mormann S, Tauch A, Pühler A, Kalinowski J. The DeoR-type transcriptional regulator SugR acts as a repressor for genes encoding the phosphoenolpyruvate: sugar phosphotransferase system (PTS) in *Corynebacterium glutamicum*. *BMC Mol. Biol.* 2007; 8, 104

39. Lee S, Sowa ME, Choi J-M, Tsai FTF. The ClpB/Hsp104 molecular chaperone—a protein disaggregating machine. *J Struct. Biol.* 2004; 146, 99-105

40. Gellert M. DNA Topoisomerases. *Ann. Rev. Biochem.* 1981; 50, 879-910

41. Iddar A, Valverde F, Assobhei O, Serrano A, Soukri A. Widespread occurrence of non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase among gram-positive bacteria. *Int. Microbiol.* 2005; 8, 251

42. Tomii K, Kanehisa M. A comparative analysis of ABC transporters in complete microbial genomes. *Genome Res.* 1998; 8, 1048-1059

43. Scoccianti V, Bucchini,] AE, Iacobucci M, Ruiz KB, Biondi S. Oxidative stress and antioxidant responses to increasing concentrations of trivalent chromium in the Andean crop species *Chenopodium quinoa* Willd. *Ecotoxicol. Environ. Saf.* 2016; 133, 25-35

44. Yeh JI, Chinte U, Du S. Structure of glycerol-3-phosphate dehydrogenase, an essential monotopic membrane enzyme involved in respiration and metabolism. *Proc. Natl. Acad. Sci.* 2008; 105, 3280-3285

45. Kim J, Chang JH, Kim EJ, Kim KJ. Crystal structure of (R)-3-hydroxybutyryl-CoA dehydrogenase PhaB from *Ralstonia eutropha*. *Biochem. Biophys. Res. Com.* 2014; 443, 783-788

46. Higham DP, Sadler PJ, Scawen MD. Gold-Resistant Bacteria: Excretion of a Cystine-Rich Protein by *Pseudomonas cepacia* induced by an antiarthritic drug. *J Inorg. Biochem*. 1986; 28, 253-261

47. Kornberg HL, Sadler JR. The metabolism of C2-compounds in micro-organisms. VIII. A dicarboxylic acid cycle as a route for the oxidation of glycollate by *Escherichia coli*. *Biochem. J.* 1961; 81, 503-513

48. Musrati RA, Kollárová M, Mernik N, Mikulásová D. Malate dehydrogenase: distribution, function and properties. *Gen. Physiol. Biophys*. 1998; 17*,* 193–210.

49 Tottey S, Rondet SA, Borrelly GP, Robinson PJ, Rich PR, Robinson NJ. A copper metallochaperone for photosynthesis and respiration reveals metal-specific targets, interaction with an importer, and alternative sites for copper acquisition. *J. Biol Chem.* 2002; 277, 5490-5497.

50 Radford DS, Kihlken MA, Borrelly GP, Harwood CR, Le Brun NE, Cavet JS. CopZ from *Bacillus subtilis* interacts in vivo with a copper exporting CPx-type ATPase CopA. *FEMS Microbiol Lett.* 2003; 220, 105-112.

51. Pontel LB, Audero ME, Espariz M, Checa SK, Soncini FC. GolS controls the response to gold by the hierarchical induction of Salmonella-specific genes that include a CBA efflux-coding operon. *Mol. Microbiol.* 2007; 66, 814-825

52. Stoyanov JV, Brown NL. The *Escherichia coli* copper-responsive copA promoter is activated by gold. *J Biol. Chem.* 2003; 278, 1407-1410

53. Changela A, Chen K, Xue Y, Holschen J, Outten CE, O'Halloran TV, et al. Molecular basis of metal-ion selectivity and zeptomolar sensitivity by CueR. *Science.* 2003; 301, 1383-1387

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Tables

Table 1 Statistical evaluation of differently expressed spots in DIGE experiments. Minimum fold-change indicates the minimum change required to achieve a power of 80 %.

Condition 1	Condition 2	Number of differently expressed spots	q-value at significance level α = 0.05[a]	Minimum fold-change[b]
unamended	10 μM Au(III)	684	0.0377	1.7
unamended	50 μM Au(III)	623	0.0479	1.7
unamended	50 μM Cu(II)	313	0.1392	1.6
10 μM Au(III)	50 μM Cu(II)	720	0.0396	1.6
10 μM Au(III)	50 μM Au(III)	169	0.3936	1.6
50 μM Au(III)	50 μM Cu(II)	668	0.0425	1.6

[a] q-value estimates the false discovery rate in the group of all proteins detected as differentially expressed at a significance level of α = 0.05.
[b] Minimum fold-change indicates the minimum change required to achieve a power of 80 %.

30

Table 2   Proteins identified from DIGE gels using LC-MS. Spot numbers correspond to Fig. 1. (A) Differentially abundant proteins between both Au amended conditions and both the Cu and unamended control conditions (p-value < 0.001 and at least 3 standard deviations from the mean). (B) Spot 2087 depicts the protein spots with the highest loading in PC1 vs. PC2 for Cu-amended biological replicates. Note: only the top protein identification in terms of number of unique significant peptides and sequence coverage is reported here, for complete list see Table S3.

Spot	NCBI Accession Number version	Locus	Taxonomy	Protein description	Unique peptides [a]	Sequence Coverage [%]	pl [b]	Molecular mass [kDa]
(A)								
209	ABF09369.1	Rmet_2492	C. metallidurans	aconitate hydratase 1	38	62.3	6.6	97944
218	ABF08082.1	Rmet_1196	C. metallidurans	pyruvate decarboxylase, E1 component of the pyruvate dehydrogenase complex	33	43.8	5.8	100668
219	ABF09369.1	Rmet_2492	C. metallidurans	aconitate hydratase 1	28	48.7	6.6	97944
256	ABF06889.1	Rmet_0003	C. metallidurans	DNA gyrase, subunit B	29	48.0	5.8	93227
	ABF09369.1	Rmet_2492	C. metallidurans	aconitate hydratase 1	26	44.1	6.6	97944
259	ABF08838.1	Rmet_1959	C. metallidurans	ATP-dependent Clp protease, ATP-binding subunit ClpB (protein disaggregation chaperone)	34	52.0	5.7	95942
562	ABF07136.1	Rmet_0250	C. metallidurans	C-terminal processing peptidase	21	43.9	9.0	58472
586	ABF09108.1	Rmet_2229	C. metallidurans	ABC-type sugar transporter, periplasmic component, probable sugar binding precursor	23	52.1	8.5	64793
593	ABF09108.1	Rmet_2229	C. metallidurans	ABC-type sugar transporter, periplasmic component, probable sugar binding precursor	28	61.9	8.5	64793
596	ABF09108.1	Rmet_2229	C. metallidurans	ABC-type sugar transporter, periplasmic component, probable sugar binding precursor	29	63.6	8.5	64793
597	ABF09108.1	Rmet_2229	C. metallidurans	ABC-type sugar transporter, periplasmic component, probable sugar binding precursor	36	71.2	8.5	64793
600	ABF09108.1	Rmet_2229	C. metallidurans	ABC-type sugar transporter, periplasmic component, probable sugar binding precursor	30	67.8	8.5	64793
605	ABF09108.1	Rmet_2229	C. metallidurans	ABC-type sugar transporter, periplasmic component, probable sugar binding precursor	3	7.8	8.5	64793
	ABF07136.1	Rmet_0250	C. metallidurans	C-terminal processing peptidase	2	7.3	9.0	58472
609	ABF09118.1	Rmet_2239	C. metallidurans	sn-glycerol-3-phosphate dehydrogenase, aerobic, FAD/NAD(P)-binding protein	28	73.3	6.4	59059
698	ABF09118.1	Rmet_2239	C. metallidurans	sn-glycerol-3-phosphate dehydrogenase, aerobic, FAD/NAD(P)-binding protein	13	35.7	6.4	59059
734	ABF10537.1	Rmet_3665	C. metallidurans	hypothetical protein Rmet_3665 (plasmid)	19	56.5	7.7	52335
778	ABF09014.1	Rmet_2135	C. metallidurans	transcription termination factor Rho	17	52.4	6.8	47329
863	ABF08240.1	Rmet_1357	C. metallidurans	acetyl-CoA acetyltransferase (Acetoacetyl-CoA thiolase)	26	82.4	7.7	40726
874	ABF07670.1	Rmet_0784	C. metallidurans	phosphoribosylglycinamide synthetase phosphoribosylamine-glycine ligase	35	82.5	5.5	44714
928	ABF09801.1	Rmet_2928	C. metallidurans	glycolate oxidase FAD binding subunit	22	81.2	6.7	39032
977	ABF07904.1	Rmet_1018	C. metallidurans	tyrosine aminotransferase, tyrosine-repressible, PLP-dependent	18	64.1	6.4	43143

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1060	Q1LKG0.1	Rmet_2489	*C. metallidurans*	Malate dehydrogenase	19	71.3	6.2	35163
1221	ABF07285.1	Rmet_0399	*C. metallidurans*	glutamate and aspartate transporter subunit; periplasmic-binding component of ABC superfamily	14	51.8	9.0	32940
	ABF11455.1	Rmet_4593	*C. metallidurans*	polysaccharide deacetylase (plasmid)	12	57.1	6.3	32823
	ABF08097.1	Rmet_1211	*C. metallidurans*	Putative ABC transporter, periplasmic substrate-binding protein	12	53.9	9.3	33668
	ABF09341.1	Rmet_2464	*C. metallidurans*	acetyl-CoA carboxylase, beta (carboxyltranferase) subunit	13	47.2	6.5	31764
	ABF07038.1	Rmet_0152	*C. metallidurans*	conserved hypothetical protein	11	55.8	6.3	31058
1237	ABF09114.1	Rmet_2235	*C. metallidurans*	DNA-binding transcriptional repressor	18	79.5	5.3	28476
1417	ABF06992.1	Rmet_0106	*C. metallidurans*	acetyl-CoA acetyltransferase	25	89.3	6.7	41355
1429	ABF08241.1	Rmet_1358	*C. metallidurans*	Acetoacetyl-CoA reductase	11	58.9	6.7	26360
	ABF11607.1	Rmet_4745	*C. metallidurans*	Short-chain dehydrogenase/reductase SDR (plasmid)	11	51.2	7.7	26950
1433	ABF10099.1	Rmet_3227	*C. metallidurans*	stringent starvation protein A	10	66.5	6.5	23787
1672	ABF09807.1	Rmet_2934	*C. metallidurans*	glutathione peroxidase	7	64.0	6.1	18414
1681	ABF07968.1	Rmet_1082	*C. metallidurans*	peptidyl-prolyl cis-trans isomerase B (rotamase B)	4	30.7	5.4	18154
1933	ABF07344.1	Rmet_0458	*C. metallidurans*	universal stress protein, UspA family	4	39.6	6.4	15091
	Q1LQS5.1	Rmet_0615	*C. metallidurans*	10 kDa chaperonin	3	49.0	5.8	10436

(B)

2087	ABF12437.1	Rmet_5578	*C. metallidurans*	conserved hypothetical protein; putative signal peptide (plasmid)	2	17.0	8.5	9687
	ABF09813.1	Rmet_2940	*C. metallidurans*	DNA protection during starvation or oxydative stress transcription regulator protein; Metalloregulation DNA-binding stress protein	2	14.3	5.8	18047

a number of peptides above the MASCOT homology threshold; total peptides: Ssum of significant and non-significant peptides detected by mass spectrometry, assigned by MASCOT to respective protein

b pI: theoretical isoelectric point; ions score depicts the sum of all individual peptide ions scores relevant for the respective protein

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Table 3 Proteins identified from native 2D gel by LC-MS. Spot number corresponds to the numbers depicted in Fig. 4.

Spot	NCBI Accession Number Version	Locus	Taxonomy	Protein Description	Unique peptides[a]	Sequence coverage [%]	pI[b]	Molecular mass [kDA]
1	ABF10300.1	Rmet_3428	*C. metallidurans CH34*	conserved hypothetical protein; predicted periplasmic or secreted lipoprotein	6	26.4	9.38	29258
2	ABF08273.1	Rmet_1390	*C. metallidurans CH34*	malate synthase A	20	30.2	6.12	58841
3	ABF07098.1	Rmet_0212	*C. metallidurans CH34*	3-hydroxybutyryl-CoA dehydrogenase	7	26.8	5.87	30199
4	ABF09122.1	Rmet_2243	*C. metallidurans CH34*	conserved hypothetical protein	4	59.7	5.58	7298
5	Q1LKG0.1	Rmet_2489	*C. metallidurans CH34*	malate dehydrogenase	13	47.4	6.24	35163
5	ABF09940.1	Rmet_3068	*C. metallidurans CH34*	lipid hydro-peroxide peroxidase	8	56.6	5.83	17392
5	ABF10492.1	Rmet_3620	*C. metallidurans CH34*	multifunctional enzyme (serine-type endopeptidase / oxidoreductase) (degP / mucD-like) (plasmid)	2	6.8	6.16	50755
5A	Q1LKG0.1	Rmet_2489	*C. metallidurans CH34*	malate dehydrogenase	14	48.6	6.24	35163
5A	ABF09940.1	Rmet_3068	*C. metallidurans CH34*	lipid hydro-peroxide peroxidase	4	27.7	5.83	17392
5A	ABF10492.1	Rmet_3620	*C. metallidurans CH34*	multifunctional enzyme (serine-type endopeptidase / oxidoreductase) (degP / mucD-like) (plasmid)	2	6.8	6.16	50755
6	ABF10312.1	Rmet_3440	*C. metallidurans CH34*	conserved hypothetical protein; alkylhydroperoxidase AhpD core	4	32.9	6.72	16268
7	ABF08240.1	Rmet_1357	*C. metallidurans CH34*	acetyl-CoA acetyltransferase (Acetoacetyl-CoA thiolase)	4	10.9	7.65	40726
8	ABF11791.1	Rmet_4929	*C. metallidurans CH34*	glutathione S-transferase enzyme with thioredoxin-like domain protein (plasmid)	6	26.0	7.90	26701
8	ABF08466.1	Rmet_1583	*C. metallidurans CH34*	periplasmic L-asparaginase II	6	18.7	8.97	39691
8	ABF08240.1	Rmet_1357	*C. metallidurans CH34*	acetyl-CoA acetyltransferase (Acetoacetyl-CoA thiolase)	2	6.1	7.65	40726
9	ABF10490.1	Rmet_3618	*C. metallidurans CH34*	organic hydro-peroxide resistance protein OhrB, 0smC family (plasmid)	5	43.6	6.72	14509
10	ABF10490.1	Rmet_3618	*C. metallidurans CH34*	organic hydroperoxide resistance protein OhrB, 0smC family (plasmid)	7	45.7	6.72	14509
10	ABF09841.1	Rmet_2968	*C. metallidurans CH34*	2-Hydroxychromene-2-carboxylate isomerase	2	8.5	6.83	21809
11	ABF10490.1	Rmet_3618	*C. metallidurans CH34*	organic hydro-peroxide resistance protein OhrB, 0smC family (plasmid)	6	43.6	6.72	14509
11	ABF09499.1	Rmet_2622	*C. metallidurans CH34*	putative carboxymethylenebutenolidase (Dienelactone hydrolase)	2	7.2	7.68	31550

[a] number of peptides above the MASCOT homology threshold; total peptides: Ssum of significant and non-significant peptides detected by mass spectrometry, assigned by MASCOT to respective protein

[b] pI: theoretical isoelectric point; ions score depicts the sum of all individual peptide ions scores relevant for the respective protein
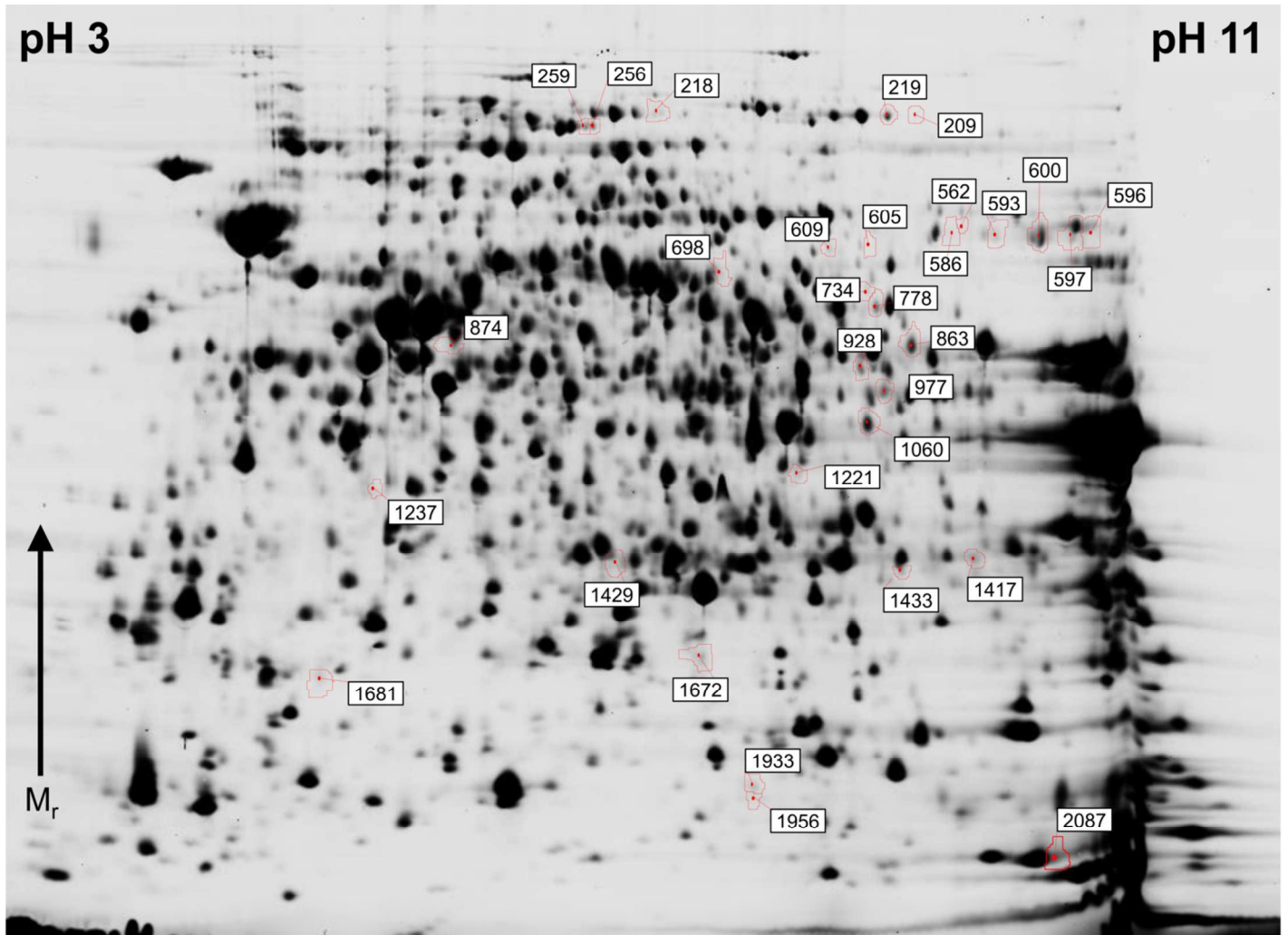
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Table 4 Proteins identified at the position where LA-ICP-MS detected Au, shown in Fig 5B.

NCBI Accession Number Version	Locus	Taxonomy	Protein description	Unique peptides[a]	Sequencec coverage [%]	pI[b]	Molecular mass [kDA]
ABF09276.1	Rmet_2399	*C. metallidurans CH34*	conserved hypothetical protein	3	34.6	5.7	11664
ABF10397.1	Rmet_3525	*C. metallidurans CH34*	copper chaperone, heavy metal ion binding (modular protein)	3	27.1	8.6	14773
ABF08652.1	Rmet_1773	*C. metallidurans CH34*	putative Glyoxalase/bleomycin resistance protein/dioxygenase	3	18.2	5.1	15767
ABF11332.1	Rmet_4467	*C. metallidurans CH34*	3-demethylubiquinone-9 3-methyltransferase (plasmid)	2	18.8	5.1	16113
ABF09855.1	Rmet_2982	*C. metallidurans CH34*	conserved hypothetical protein	2	11.1	5.0	15693
O33522.2	Rmet_2922	*C. metallidurans CH34*	Chaperone protein DnaK	2	3.5	4.9	69786

[a] number of peptides above the MASCOT homology threshold; total peptides: Ssum of significant and non-significant peptides detected by mass spectrometry, assigned by MASCOT to respective protein
[b] pI: theoretical isoelectric point; ions score depicts the sum of all individual peptide ions scores relevant for the respective protein

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Figure Legends

Figure 1   DIGE analysis of *C. metallidurans* CH34 grown in the presence of Au(III), Cu(II) and unamended. Representative DIGE gel, IPG 3-11NL, T=12.5%. Representative DIGE gel, numbers correspond to numbers in Table 2 and show differentially expressed proteins between *C. metallidurans* CH34 challenged with Au(III) compared to Cu(II) and the unamended cells.

Figure 2   Principal component (PC) analysis **(A)** PC *vs*. PC2 and **(B)** associated biplot and **(C)** PC5 *vs*. PC2 and **(D)** associated biplot of DIGE protein fingerprints of the four experimental groups. *C. metallidurans* CH34 grown with Au(III) separates from other conditions in PC1 demonstrating a specific proteomic response to Au(III)-stress; PC5 *vs*. PC2 separates all experimental groups.

Figure 3   **(A)** Analysis of the 152 proteins that were differentially regulated between Au(III), Cu(II) and the unamended control **(B)** Functional classification of 29 identified proteins showing the highest degree of differential expression between Au(III) and the Cu(II) / unamended controls.

Figure 4   **(A)** Representative native 2D gel, IPG 3-10NL, 300 µg native protein extract of *C. metallidurans* grown with 50 µM Au(III)-chloride; gel was Ag-stained. Numbers depict proteins identified by mass spectrometry (see Table 3). Insert exhibits gamma-adjusted area for better visualisation of Spot 1. **(B, C, D)** detailed view of regions of interest of native 2D gels across three replicate of Cu(II), unamened and Au(III) amended cells, respectively, showing protein spots 1-11 up-regulated in Au(III) amended extracts (D).

Figure 5   Results of LA-ICP-MS on native 2D Western Blot of a protein extract of *C. metallidurans* cells challenged with 50 µM Au(III). Shown analyses for Au, Cu and the C background **(A)** on the corresponding native IEF dimension blot **(B)**.
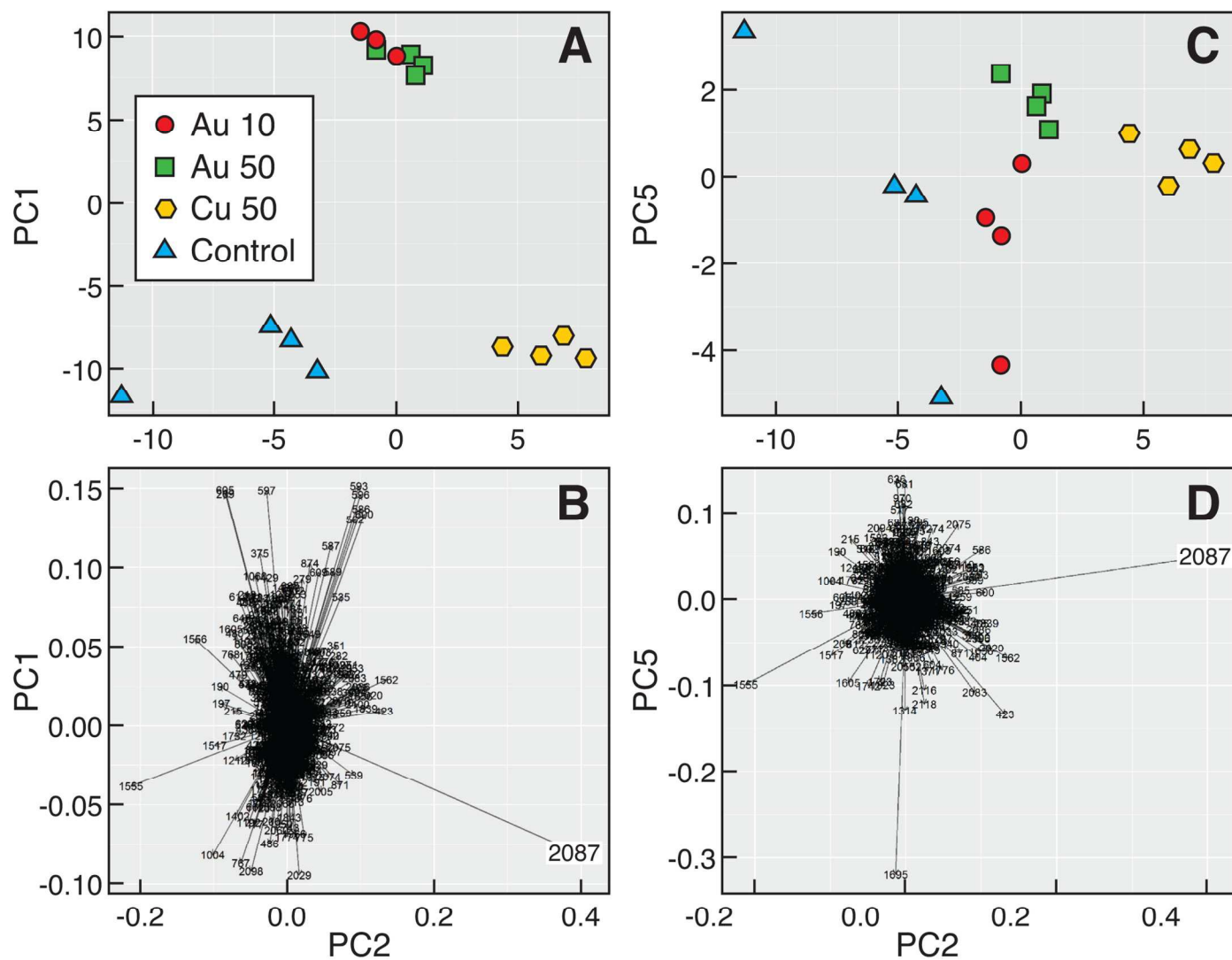
Figure 6   Network visualisation of the CupC environment using STRING V.9, shown are proteins interacting with CupC based on confidence settings of 0.9 **(A)**, indicating that CupC may bind to a number of ATPase, including the P-type ATPase CupA, the Pb/Cd-transporting ATPase CadA, the heavy metal transporting P-type ATPase and the metal transporter ATPase, CtpA1, as well as the DNA-binding transcriptional activator of copper-responsive regulon genes CupR. **(B)** Protein model of CupC from *C. metallidurans* CH34. Protein model of CupC constructed in SWISS-PROT modelled on CupC and two heavy metal binding proteins, *i.e*., 2rml.1.A, a copper transporting P-type ATPase CopC; 2rog.1.A and 2roe.1.A, both heavy metal binding proteins (31-34) ().

Figure 1

Figure 2

Figure 3

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Figure 4

Figure 5

Figure 6