1 Shotgun proteomics analysis of nanoparticle-synthesising *Desulfovibrio alaskensis* in 2 response to platinum and palladium

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13 Abstract

14 Platinum and palladium are much sought-after metals of global critical importance in terms
15 of abundance and availability. At the nano-scale these metals are of even higher value due to
16 their catalytic abilities for industrial applications. *Desulfovibrio alaskensis* is able to capture
17 ionic forms of both of these metals, reduce them, and synthesize elemental nanoparticles.
18 Despite this ability very little is known about the biological pathways involved in the
19 formation of these nanoparticles. Proteomic analysis of *D. alaskensis* in response to platinum
20 and palladium has highlighted those proteins involved in both the reductive pathways and the
21 wider stress-response system. A core set of 13 proteins was found in both treatments and
22 consisted of proteins involved in metal transport and reduction. There were also 7 proteins
23 specific to either platinum or palladium. Over-expression of one of these platinum-specific
24 genes, a NiFe hydrogenase small subunit (Dde_2137), resulted in the formation of larger
25 nanoparticles. This study improves our understanding of the pathways involved in the metal
26 resistance mechanism of *Desulfovibrio* and informs how we can tailor the bacterium for
27 nanoparticle production, enhancing its application as a bioremediation tool and as a way to
28 capture contaminant metals from the environment.

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30 Importance

31 Bacteria, in particularly *D. alaskensis*, represent a biological and greener way to capture high
32 value metals such as platinum group metals from environmental and industrial waste streams.
33 The recovery of these metals in nanoparticle forms adds extra value to this process as they
34 can be used in a variety of different industrial applications as they have exceptional catalytic
35 capabilities. *D. alaskensis* ability to do this, has been widely reported, though very little is
36 understood about the underlying protein and genetic components. It is by understanding the
37 biological basis of this capability that we can further improve and adapt this bacterium to be
38 better at bioremediation and to control its ability to do so.

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42 Introduction

43 There is an increasing demand for expensive platinum group metals. Amongst other
 44 applications, they are used for automotive catalytic converters from which platinum and
 45 palladium escape via the exhaust and are therefore a source of environmental pollution (1).
 46 Although heavy metal ions are toxic to most bacteria, highly-resistant strains are available
 47 that absorb them and reduce them to the metallic state. This results in the formation of
 48 nanoparticles that can be harvested to provide a source of these valuable metals.
 49 Furthermore, small nanoparticles from bacteria have important biotechnological applications,
 50 for example, as chemical catalysts. Not only does biological recovery from environmental
 51 sources offer an attractive way to recover them from dilute solutions, but also it provides a
 52 mechanism for decontaminating polluted environments. Prokaryotes have been used to
 53 remediate metal ions from solution for decades: mostly high value metals such as gold,
 54 platinum and silver have been targeted (2, 3).

55 The potential uses of nanoparticles are expanding, from cancer treatment using platinum or
 56 arsenic nanoparticles (4, 5) to the use of silver nanoparticles as antimicrobials (6), palladium
 57 nanoparticles as catalysts in fuel cells (7) and to treat water contaminated with
 58 pharmaceuticals (8). Tailoring these nanoparticles to their implementation for industrial or
 59 medical use is vital but so far there has been little standardisation with regards to the size of
 60 nanoparticles formed (9). As different sized nanoparticles have different catalytic properties,
 61 their use has been limited. Bacteria potentially represent a genetically programmable method
 62 for the customisation of nanoparticle size and morphology for specific purposes. As well as
 63 this bioremediation by bacteria involves mild conditions and ambient temperatures, thus
 64 avoiding the disadvantages of chemical recovery that requires the use of high temperatures or
 65 hazardous chemicals.

66 Anaerobic, sulphate reducing bacteria of the genus *Desulfovibrio* are amongst the most
 67 widely used bacteria to remediate metal ions. They reduce multi-valent metal ions to zero-
 68 valent or bi-elemental nanoparticles. The list of known metals that it can process is ever
 69 increasing and currently consists of chromium, magnesium, iron, technetium, uranium, nickel
 70 (10-15), and also includes platinum and palladium: palladium has been the most studied. The
 71 *Desulfovibrio* sp. are ideally suited for further analysis as they are already highly tolerant to
 72 platinum and palladium ions, and can survive up to 2 and 5 mM respectively (15). They
 73 reduce these metals at incredible speed, with up to 100% of Pd²⁺ being converted to Pd(0)
 74 within 5 minutes (7).

75 Only fragmentary information is available at the genetic level concerning *Desulfovibrio*
 76 genes required for heavy metal ion reduction. One exception is *cycA* from *D. vulgaris*
 77 Hildenborough, which encodes a tetraheme cytochrome *c*₃ involved in uranium reduction *in*
 78 *vitro* (16). The NiFe hydrogenases of *D. fructosovorans* have been shown to be involved in
 79 technetium reduction (17). They similarly have a role, along with a Fe-containing
 80 hydrogenase, in Pd(0) deposition and clustering in both the periplasm (18) and on cellular
 81 membranes (19), while more recently have been implicated in having a roll in palladium
 82 deposition in *Shewanella* (20). While important for the anaerobic reduction of palladium by
 83 *Desulfovibrio* sp., they are not required for the aerobic reduction of palladium by *E. coli* (21).
 84 Large gaps remain in our knowledge of the proteins involved in metal ion reduction. As the
 85 particles formed differ between growing and non-growing cultures, in many previous studies
 86 washed bacteria were incubated in the presence of the target metal in buffer rather than in
 87 substrates required for growth. We therefore designed experiments to identify changes in the
 88 proteome of *D. alaskensis* strain G20 during incubation with Pt⁴⁺ or Pd²⁺ solutions under
 89 conditions used in previous studies both by ourselves and many other research teams. One of

the genes implicated in Pt^{4+} reduction was cloned into an expression vector in a proof of principle experiment designed to demonstrate that over-production of the gene product results in changes in the physical properties of the nanoparticles formed.

Experimental Details

Growth of bacterial strains. *D. alaskensis* G20 was purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) and grown in Postgate Medium C (PGMC) using lactate as a carbon source (22). Cultures were grown and manipulated in an anaerobic chamber (Don Whitley) at 30°C in an atmosphere of 10% CO_2 , 10% H_2 in N_2 . For strains carrying the pMO9075 plasmid the medium was supplemented with 100 µg/ml spectinomycin (Cambridge Bioscience).

Platinum and palladium treatment. Bacteria were grown in Postgate Medium C to an OD_{600} of 1.0 and centrifuged for 10 min at 4000 rpm. The cell pellets were washed three times in 10 mM MOPS (pH 7.0) and re-suspended finally in MOPS. Solutions of PtCl_4 or Na_2PdCl_4 were then added to a final concentration of 2 mM. The cells in the presence of palladium and platinum and an unsupplemented control suspension were incubated anaerobically at 30°C. After 2 h, samples were centrifuged as before and the supernatant was removed. The pellets were either snap-frozen and stored at -80°C or used immediately for TEM analysis.

Proteomics analysis: trypsin digestion, LC-MS and data analysis. Cells were disrupted in 8 M urea and the total protein was assayed using a Bradford kit (Biorad, UK). One milligram of protein per sample was digested with trypsin as described previously (23). Briefly, samples were diluted to a final concentration of 2 M urea with 25 mM ammonium bicarbonate and 5 mM DTT. After incubation for 30 min at room temperature, iodoacetamide was added to a final concentration of 12.5 mM. Trypsin (10 µg; Worthington) was added and the sample was digested overnight at room temperature. The samples were cleaned with 25 mg of Bond Elut LMS (Agilent Technologies). Peptides eluted with acetonitrile were aliquoted, dried under low pressure and stored at -20°C until analysis. Prior to LC-MS analysis, 4 µg of the samples were reconstituted in 12 µl of loading buffer (0.05% Trifluoroacetic acid in water) and analysed by capillary-HPLC-MS/MS on an on-line system consisting of a 1200 binary HPLC micro-pump system (Agilent Technologies) coupled to a hybrid LTQ-Orbitrap XL instrument (Thermo-Fisher) on a 140 minute gradient.

MS/MS data were analysed using MASCOT Versions 2.4 (Matrix Science Ltd, UK) against the 3258 sequences in the *D. alaskensis* G20 genome from <http://genome.ornl.gov/microbial/ddes> (24). The Mascot search parameters were two missed-cut. Allowances were made for variable methionine oxidation and fixed cysteine carbamidomethylation in all searches. The precursor mass tolerance was fixed at 7 ppm and MS/MS tolerance to 0.4 amu. The significance threshold was set at 0.05 and an additional cut off peptide score of 20. Progenesis (Nonlinear Dynamics, UK) was used for label-free quantitation. Only peptides that were not shared between different proteins were used for quantification. Data for a sub-set of MS/MS peaks with positive charges of 2, 3, or 4 were extracted from each LC-MS run and the median global ion intensities were extracted for normalization. The abundance of each protein was calculated from the sum of the intensities of unique peptides with positive charges of 2, 3, or 4. Because the method of detection can generate a significant amount of near zero measurements for which a standard log transformation is not ideal, the calculated protein abundances were transformed using an ArcSinH function. The within group means were calculated to evaluate the fold change and

the ArcSinH transformed data were then used to calculate the p-values using one-way ANOVA. Proteins were considered to be differentially expressed only if at least two peptides were detected with an absolute ratio of at least 1.5-fold more abundant or 0.667 less abundant and a probability of false discovery of $p < 0.05$, as illustrated in **Figure 1**. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD004457 and 10.6019/PXD004457 (25). The data are also available in the supplemental material. Functions of the proteins highlighted in this study were predicted using a combination of literature searches and cross-referencing with the KEGG (<http://www.kegg.jp/kegg/kegg2.html>), STRING (<http://string-db.org/>), Pfam (<http://pfam.xfam.org/>) and UniProt databases (<http://www.uniprot.org/>). The presence of platinum group metals in RNA preparations prevented us from using Quantitative Real-Time PCR to confirm at the transcription level changes in expression of genes for the proteins highlighted.

Construction of pMO-2137. The gene for the Dde_2137 subunit of the nickel-iron hydrogenase was cloned using a standard Phusion PCR protocol into the expression vector pMO9075 (26). Forward and reverse primers were dde_2137F 5'-CAGTACTCTGCAGATCTGATCCTTTTCATTGC-3' and dde_2137R 5'-TGCATGCCAATTCGTTTCGGTACCGGC-3'. Restriction sites for *ScaI* and *SphI* are underlined. The PCR product was digested with *ScaI* and *SphI* and ligated into pMO9075 using these restriction sites. The sequence was inserted downstream of the promoter of the kanamycin resistance gene in pMO9075, the expression from which has been shown to complement mutations in *Desulfovibrio* sp.

The pMO-2137 and empty pMO9075 plasmids were transformed into *D. alaskensis* made competent using an adaptation of Li and Krumholz's method (27). Briefly, cultures at an OD_{600nm} of 0.6-0.8 were centrifuged, washed twice in 400 mM sucrose, 1 mM MgCl₂, and finally resuspended in 1/10 of the original volume of sucrose solution. One microgram of the plasmid was then added. After 30 min on ice, the suspension was electroporated at 1.5 kV. After electroporation 1 ml of PGMC broth was added immediately to allow the cells to recover at 30°C. After 4 hours, cells were plated onto PGMC agar containing 100 µg/ml of spectinomycin. After 7 days at 30°C, transformants were purified and screened by plasmid isolation for the presence of the construct. For comparative analysis of nanoparticles produced by bacteria transformed with either the empty plasmid control or the plasmid carrying the extra copy of the dde_2137 gene, the OD_{600nm} of the cells were matched before the addition of platinum ions.

Transmission electron microscopy (TEM). Samples were drop cast onto a 200 mesh copper grid with a formvar coating (TAAB). The sample was allowed to settle for 5 minutes before excess liquid was removed using filter paper. The grid was then loaded into a JEOL JEM-1400 Plus electron microscope and imaged using a GATAN OneView Camera. The images were processed and the size of the nanoparticles was determined using ImageJ software (<http://imagej.nih.gov/ij/>).

Energy-dispersive X-ray spectroscopy. Samples prepared as above for conventional TEM were drop cast onto holey-carbon nickel grids (Agar Scientific). The samples were analysed using a JOEL JEM 2011 TEM fitted with an ISIS system and viewed at an accelerating voltage of 200 kV.

Results and Discussion

Overview of the proteomic data.

Transfer of a bacterial culture from a growth medium into buffer containing toxic levels of a heavy metal results in an almost total cessation of growth. Amongst many other consequences, this results in ribosome degradation and therefore decreased demand for stable RNA and ribosomal proteins (**Table 1**). The degradation products can then be recycled for the synthesis of components beneficial to the stress response. Conversely, incubation in a buffer in the absence of growth nutrients inevitably limits the ability of bacteria to respond to stress. Any increased protein synthesis is therefore likely to be limited to synthesis of those proteins that increase survival of the stress imposed. These were expected to be proteins involved in the reduction of the specific metal ions, in transporting them out of the cell, or in a more general response to stress. A total of 1061 proteins from the *D. alaskensis* G20 potential proteome were identified by shotgun label-free proteomics, representing 32% of the global proteome (See supplemental material for complete list). In terms of coverage this is in with good keeping with other proteomic studies with metal trails in bacteria, including those of *Desulfovibrio* sp. (28, 29).

Using a significance threshold of 1.5-fold change and a p-value below 0.05, the abundance of 46 proteins decreased during incubation in the presence of palladium and platinum ions of which 24 were in the platinum dataset, 22 were in the palladium dataset, with 20 proteins found in both datasets (**Figure 1**). Only 20 proteins were found to be more abundant in cells treated with palladium compared with untreated cells. Similarly, 20 proteins were also more abundant in cells treated with platinum, thirteen of which were also induced during incubation with palladium ions. Consequently, 7 of the more abundant proteins were unique to each dataset (**Figure 2**).

Proteins less abundant in both the platinum and palladium datasets.

As expected, proteins found in lower amounts in both datasets are predominantly those involved in RNA transcription and translation. These include both large and small ribosomal subunit proteins (L32, L29, S187, S12) as well as transcription regulators of the TraR/DskA and MucR families (**Table 2**). One hypothetical protein identified (Dde_0492) is also predicted to be involved in valyl-tRNA synthesis while another (Dde_1811), of unknown function, is only conserved among the *Desulfovibrio* sp. and SRB deltaproteobacteria. The least abundant protein relative to the control was the periplasmic Fe hydrogenase small subunit (Dde_2280), which together with Dde_2274 are potential hydrogenases not involved in the reduction of either platinum or palladium. This suggests that there might be a separate hydrogenase and other electron transfer proteins that are specific for platinum and palladium reduction. Also in the less-abundant dataset are proteins involved in energy production and metabolism such as oxidoreductases (Dde_0584, Dde_1113, Dde_3240, Dde_2272 and Dde_1638), one ATPase subunit (Dde_0990) and the prokaryotic molybdopterin-containing oxidoreductase (Dde_2274).

Platinum specific proteins. Proteins involved in cell integrity and maintenance as well as those required for growth dominated the list that were less abundant following incubation with Pt⁴⁺ ions. Proteins such as Dde_1413, Dde_0453, Dde_1368, Dde_2153, Dde_3632, Dde_1689 and Dde_1131 are predicted to be involved in membrane biogenesis and integrity, which is consistent with decreased synthesis of new membranes due to cessation of growth. This list also includes other proteins with a common role: Dde_3488, Dde_3490, Dde_3567, Dde_2763, Dde_3070, Dde_3703 and Dde_2744 are all predicted to be involved in amino acid synthesis. Again this supports that

due to the limited media in which the NPs are produced (MOPS, (3-(N-morpholino)propanesulfonic acid) buffer) there is limited new synthesis of new proteins. Other less abundant proteins Dde_2018, Dde_0445, Dde_2669 and Dde_2670 are required for iron or haem uptake, possibly as a risk reduction strategy due to their general metal binding and transportation ability.

Palladium specific proteins. The palladium dataset is dominated by ribosomal subunit proteins both small (Dde_2233, Dde_2608, Dde_2253, Dde_2232, Dde_1133) and large (Dde_2637, Dde_1095, Dde_2254, Dde_2607, Dde_2255). There are also other proteins involved in transcription and translation such as the rRNA E-loop binding protein (Dde_2127), the response regulator and CheY-like protein (Dde_0327), the translation initiation factor (Dde_3162) and the MucR transcriptional regulator (Dde_0346). Decreased levels of these proteins would have a global effect on transcription and translation and silencing their expression would arrest the cell cycle. The next largest group that are less abundant specifically in Pd²⁺-treated bacteria are proteins required to provide metabolites for energy production and growth. The proteins Dde_1111 to 1114 (with Dde_1113 found down regulated following both platinum and palladium specific treatment) are products of a group of contiguous genes encoding part of the membrane bound Qmo electron transport complex. The operon is vital to the growth of *D. vulgaris* Hildenborough on lactate-sulphate media (26, 30). Down-regulation of this operon, would make sense as the cells have gone from a lactate-sulphate containing growth medium to a very minimal one.

Proteins more abundant following incubation with Pt⁴⁺ or Pd²⁺ ions.

Three of the proteins of increased abundance in both datasets included a ferredoxin and a molybdoprotein, MopB, encoded by adjacent genes on the chromosome, and the rhodanese Dde_0806. Evidence that these proteins are required for metal ion reduction includes a report that a *mopB* mutant is unable to grow with hydrogen or formate as the electron donor (31), and that a rhodanese mutant is also unable to use hydrogen as an electron donor (32). More recently it was shown that molybdoproteins are required for Pd⁴⁺ reduction by *E. coli* (21).

Five other proteins encode putative ABC-type transportation proteins, Dde_0155, Dde_0186, Dde_0258, and an efflux pump, Dde_3627 and Dde_1010, which potentially encodes an outer membrane porin. Also in this group is a member of the TolC family of type I secretion systems (Dde_1415) (**Table 1**). The remaining five proteins common to both datasets all play more general roles in the response to stress. They include Dde_0194, which is an alpha-beta barrel protein, and Dde_1442, an ATP-dependent peptidase likely to be involved in the degradation of proteins that have misfolded due to the presence of the heavy metals (33). Also in this group is Dde_3216, a protein predicted to be involved in lipid metabolism and repair: such proteins would have a role in managing the effects heavy metals or nanoparticles have on lipid membranes. Lipid peroxidation in particular is one of the ways in which bacterial treatment with TiO₂ nanoparticles (33) and copper ions/nanoparticles act as antimicrobials (34). Finally Dde_2342 is glyceraldehyde-3-phosphate dehydrogenase, which understandably would play a critical role in glycolysis as the cells metabolise endogenous reserves for ATP production.

Proteins accumulated only in response to exposure to Pt⁴⁺ ions.

Some proteins accumulated in the presence of Pt ions but not Pd ions and vice versa. Included in the platinum-specific dataset are Dde_2137, which is the small subunit of

the NiFe hydrogenase, and Dde_2138, which a c₃-type cytochrome that is believed to donate electrons to the NiFe hydrogenase active site. The NiFe hydrogenase subunit is transported into the periplasm by the twin arginine targeting TAT pathway. Both of these proteins were shown to be required for metal ion reduction by *D. fructosovorans* (17). The NiFe hydrogenase genes are flanked by two genes encoding the large and small subunits of an alternative NiFeSe hydrogenase and maturation proteins, but none of these other proteins were more abundant following incubation with Pt⁴⁺. Another ferredoxin, Dde_2642, also accumulated during incubation with Pt⁴⁺ ions. This protein is thought to be involved in metal-binding and electron transfer in the cytoplasm. Whether it is able to shuttle electrons to the platinum ions in *Desulfovibrio* is unknown.

An outer membrane efflux protein, Dde_3267, accumulated only in the platinum-treated bacteria. This could be involved either in the export of platinum ions out of the cell to limit their cytotoxic effect, or even in the efflux of the nanoparticle itself once it has been formed.

The ability of platinum ions to cause DNA lesions that inhibit cell division is the basis for their chemotherapeutic use in drugs such as cisplatin. The single strand binding (SSB) protein (Dde_1545) accumulated in bacteria incubated with Pt⁴⁺ ions (35). Binding of Pt is prevented by the binding of SSB to single stranded DNA. Two other potential stress response proteins accumulated during incubation with Pt⁴⁺ ions. One was rubrerythrin (Dde_1214) that due to its ability to protect *D. vulgaris* against hydrogen peroxide damage is suggested to protect against cytoplasmic oxidative stress (36, 37), it was also previously found to be down-regulated when using nitrate as electron acceptor (38). The other is a methyl-accepting chemotaxis sensory transducer (Dde_1279), which contains a PAS domain at the conserved-C-terminus that is likely to be part of a signal response to promote motility in the presence of platinum.

Proteins accumulated only in response to exposure to Pd²⁺ ions.

No obvious candidates for redox proteins required for Pd²⁺ reduction accumulated specifically in response to incubation with Pd²⁺ ions. Although carbon monoxide reductase (Dde_3028), the hybrid cluster protein (Hcp) (Dde_2640) and a quinone-linked NAD(P)H dehydrogenase (Dde_1618) were more abundant than in the control, these increases were just over 1.5-fold. The Hcp in particular has been implicated in response to nitric oxide stress in both *D. vulgaris* (39) and *E. coli* (40). The four other proteins that increased significantly included a peptidase that might be involved in a stress response, but again the increases relative to the control were only about two-fold. The Dde_2200 protein is a 1-deoxy-D-xylulose-5-phosphate synthase involved in the metabolism of pyruvate, a key intermediate in various metabolic pathways. Since the cells are in buffer (with palladium), it is likely that this would increase metabolism of what little pyruvate is available. Similarly Dde_1699 and Dde_0444, both of which are zinc-containing peptidases, are also present and are likely to be degrading proteins that have misfolded due to the presence of the palladium/oxidative stress involved in metal reduction (41). Clearly, however, most of the proteins induced in response to exposure to Pd²⁺ were those also induced in the presence of Pt⁴⁺ ions.

Increase in nanoparticle sizes due to increased synthesis of the NiFe hydrogenase.

A major aim of the proteomics experiments was to investigate whether proteins could be identified that when over-expressed might facilitate the controlled production of metal nanoparticles of a specific size. Given the previous demonstration that a mutant defective in NiFe hydrogenase synthesis was also defective in nanoparticle production, the observation that this enzyme accumulated during Pt⁴⁺ reduction was especially significant. We therefore

designed experiments to determine whether over-expression of the NiFe hydrogenase would result in changes in the physical properties of the nanoparticles formed. The gene encoding the NiFe hydrogenase protein Dde_2137 was cloned into the *D. alaskensis* plasmid pMO9075 to give pMO-2137. Introduction of this plasmid to *D. alaskensis* G20 significantly increased the size of the Pt nanoparticles when cells were exposed to platinum ions ($p < 0.005$) (**Figure 4**). On average the size increased from 117 nm for the empty plasmid control to 324 nm for the strain carrying the pMO-2137. Increased production of the NiFe protein also increased the range of nanoparticle sizes formed (**Figure 5** and **Figure 6**). Many of the larger particles were free in the buffer, not attached to the cell. Though larger nanoparticles were formed, the transformants were not more resistant to platinum treatment. We therefore suggest that the increased amount of Dde_2137 leads to increased deposition of platinum nanoparticles on the cell surface. This leads to an increased deposition rates and, in turn, an increased localised turnover rate of the platinum ions by the autocatalytic abilities of the platinum nanoparticles thus leading overall to larger nanoparticles. This supports previous work which concluded that the NiFe hydrogenase is required for nanoparticle synthesis in *Desulfovibrio* (18) and also their deposition on the outer membranes in *Shewanella* (20). Samples of nanoparticles produced by *Desulfovibrio* transformed with either pMO-2137 or the empty vector control were analysed using EDS (Energy-dispersive spectroscopy). In both cases the nanoparticles were shown to be entirely made of platinum with some regions containing very regular atomic lattices (**Figure 7**).

Conclusions

Despite years of use of *D. alaskensis* for metal ion bioremediation, this paper to our knowledge presents the first shotgun proteomic analysis of bacteria synthesizing palladium and platinum nanoparticles. Although various strains of *Desulfovibrio* were used for these previous studies, only a few of the proteins reported here have previously been implicated in metal reduction and nanoparticle production.

We have identified some proteins that accumulate during both palladium or platinum reduction and others that respond to one of the metals, but not the other. This implies that there are separate pathways for reduction of each metal, but also an underlying core pathway that features in the reduction of both. One possible reason for this is that Pt^{4+} ions are reduced via Pt^{2+} and that the divalent Pd^{2+} and Pt^{2+} cations are reduced by a common pathway. Identification of both groups of these proteins presents the opportunity not only of increasing the removal of metals from platinum and palladium-containing solutions, but also for increasing the specificity of the process. Genetic manipulation to control the expression of the genes for these target proteins might enable increase metal specificity, efficiency and even metal resistance to be achieved. In proof of principle experiments to test these ideas, we have also shown the feasibility of genetically engineering *D. alaskensis* to alter nanoparticle sizes. One aim of future work will be to develop the ability to produce nanoparticles tailored for specific needs as well as the application of what we have engineered to environmental samples.

Conflicts of interest

There are no conflicts of interest to declare.

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Table 1. Proteins significantly more abundant (>1.5 fold) in the presence of platinum and palladium and their overlap between datasets.

Platinum and Palladium			Platinum Only			Palladium Only		
Protein ID	Predicted Function	Avg. Fold	Protein ID	Predicted Function	Avg. Fold	Protein ID	Predicted Function	Avg. Fold

		Change
Metal Sequestering/Transport		
Dde_0155	Molybdenum ABC transporter	3.17
Dde_0186	ABC-type transporter, periplasmic subunit family 3	2.85
Dde_0258	Extracellular ligand-binding receptor	2.19
Export Mechanism		
Dde_3627	Outer membrane efflux protein	5.62
Dde_1415	Type I secretion outer membrane protein, TolC family	2.78
Cytochromes/Hydrogenases		
Dde_2934	4Fe-4S ferredoxin iron-sulfur binding domain-containing protein	4.00
Dde_2933	Molybdopterin oxidoreductase	3.48
Other		
Dde_0806	Rhodanese-like protein	11.71
Dde_1442	Signal peptide peptidase SppA, 36K type	11.02
Dde_3216	Phosphatidylserine decarboxylase related protein	9.53
Dde_0194	Stress responsive alpha-beta barrel domain-containing protein	4.87
Dde_1010	Hypothetical protein Dde_1010	3.06
Dde_2342	Glyceraldehyde-3-phosphate dehydrogenase, type I	2.11

		Change
Cytochromes/Hydrogenases		
Dde_2642	4Fe-4S ferredoxin iron-sulfur binding domain-containing protein	2.55
Dde_2137	Periplasmic (NiFe) hydrogenase, small subunit, isozyme 1	2.66
Dde_2138	Cytochrome-c3 hydrogenase	1.60
Other		
Dde_0168	ABC-type transporter, periplasmic subunit family 3	3.86
Dde_1279	Methyl-accepting chemotaxis sensory transducer with Pas/Pac sensor	2.23
Dde_1545	Single-strand binding protein	2.11
Dde_1214	Rubrrerythrin	1.94

		Change
Other		
Dde_0444	Peptidase M16 domain protein	2.17
Dde_2315	Hypothetical protein Dde_2315	2.12
Dde_3028	Carbon-monoxide dehydrogenase, catalytic subunit	1.68
Dde_1618	NAD(P)H dehydrogenase (quinone)	1.67
Dde_2641	Hybrid cluster protein	1.64
Dde_2200	1-deoxy-D-xylulose-5-phosphate synthase	1.63
Dde_1699	Processing peptidase	1.58

Table 2: Proteins significantly less abundant (<0.667 fold) in the presence of either platinum and palladium and their overlap between datasets.

Platinum and Palladium			Platinum Only			Palladium Only		
Protein ID	Annotation	Avg Fold Change	Protein ID	Annotation	Fold Change	Protein ID	Annotation	Fold Change
Energy Production/Metabolism			Energy Production/Metabolism			Energy Production/Metabolism		
Dde_2280	Periplasmic Fe hydrogenase small subunit	0.29	Dde_2018	HesB/YadR/YfhF-family protein	0.41	Dde_2273	Periplasmic (Sec) triheme cytochrome c	0.53
Dde_3667	Flavodoxin	0.35	Dde_0445	ABC transporter related protein	0.42	Dde_1114	Hypothetical protein Dde_1114	0.63
Dde_0584	NADH:quinone oxidoreductase subunit RnfE	0.45	Dde_2669	Ferrous iron transport protein B	0.53	Dde_2271	Inner membrane protein binds 2 heme b	0.63
Dde_1113	Quinone-interacting membrane-bound oxidoreductase	0.5	Dde_2670	Ferrous iron transporter component feoA	0.54	Dde_1111	Quinone-interacting membrane-bound oxidoreductase	0.64
Dde_3240	Protein of unknown function DUF224	0.55	Dde_1633	Gamma-glutamyl phosphate reductase	0.55	Dde_3604	D-lactate dehydrogenase (cytochrome)	0.65
Dde_2272	Hdr menaquinol oxidoreductase	0.56	Dde_2979	Carbonic anhydrase	0.57	Dde_2201	Polyprenyl synthetase	0.65
Dde_0990	H ⁺ transporting two-sector ATPase B/B' subunit	0.56	Dde_3520	Molybdate-transporting ATPase	0.58	Dde_3708	Transmembrane complex, integral membrane protein	0.65
Dde_1638	2-oxoglutarate synthase	0.58	Dde_2341	Fructose-1,6-bisphosphate aldolase, class II	0.66	Dde_1112	Quinone-interacting membrane-bound oxidoreductase	0.65
Dde_2274	Molybdopterin-containing oxidoreductase family, iron-sulfur binding subunit	0.59	Transcription/Translation			Transcription/Translation		
Transcription/Translation			Dde_1173	Protein of unknown function DUF306 Meta and HslJ	0.54	Dde_2637	Ribosomal protein L35	0.51
Dde_4007	50S ribosomal protein L32	0.31	Dde_2982	Ribosomal protein L31	0.66	Dde_1095	Ribosomal protein L19	0.52
Dde_0492	hypothetical protein Dde_0492	0.45	Cell Integrity/Maintenance			Dde_0327	Response regulator receiver protein	0.55
Dde_2249	ribosomal protein L29	0.47	Dde_1413	Organic solvent tolerance protein	0.01	Dde_0346	Transcriptional regulator, MucR family	0.55
Dde_2345	transcriptional regulator, TraR/DksA family	0.47	Dde_0453	MotA/TolQ/ExbB proton channel	0.28	Dde_0793	RNP-1 like RNA-binding protein	0.56
Dde_1411	ribosomal protein S18	0.53	Dde_1368	Outer membrane protein assembly complex, YaeT protein	0.42	Dde_2254	Ribosomal protein L2	0.57
Dde_2238	Ribosomal protein L15	0.56	Dde_2153	MotA/TolQ/ExbB proton channel	0.43	Dde_2233	30S ribosomal protein S11	0.57
Dde_2613	Transcriptional regulator, MucR family	0.57	Dde_1587	Hypothetical protein Dde_1587	0.52	Dde_2127	Ribosomal 5S rRNA E-loop binding protein Ctc/L25/TL5	0.58
Dde_2263	Ribosomal protein S12	0.6	Dde_3632	Tol-Pal system beta propeller repeat protein TolB	0.58	Dde_2608	Ribosomal protein S9	0.58
Cell Integrity/Maintenance			Dde_3641	Sulphate transporter	0.61	Dde_2607	Ribosomal protein L13	0.59
Dde_2145	Tol-pal system protein YbgF	0.46	Dde_1689	OmpA/MotB domain protein	0.61	Dde_2255	Ribosomal protein L25/L23	0.61
Other			Dde_2066	Thioredoxin reductase	0.63	Dde_2253	Ribosomal protein S19	0.63
Dde_0030	Hypothetical protein Dde_0030	0.44	Other			Dde_2232	Ribosomal protein S4	0.64
Dde_1811	Hypothetical protein Dde_1811	0.48	Dde_0448	ABC-type transporter, periplasmic subunit	0.24	Dde_1133	Ribosomal protein S2	0.64
			Dde_2440	Putative lipoprotein	0.37	Dde_3162	Translation initiation factor IF-2	0.66
			Dde_0493	Hypothetical protein Dde_0493	0.4	Dde_1175	RNP-1 like RNA-binding protein	0.66
			Dde_2152	Hypothetical protein Dde_2152	0.43	Dde_3166	Ribosomal protein S15	0.66
			Dde_0316	Hypothetical protein Dde_0316	0.51	Dde_1025	GrpE protein	0.66
						Dde_0507	Ribosomal protein S1	0.67
			Cell Integrity/Maintenance			Dde_2278	Biotin and thiamine synthesis associated	0.4
						Dde_2294	(Acyl-carrier-protein) S-malonyltransferase	0.59
			Other			Dde_1571	Response regulator receiver modulated CheB methyltransferase	0.54
						Dde_1694	Response regulator receiver protein	0.58

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Figures

Figure 1. Volcano plot for the data generated in the shotgun proteomics. Red full circles are those proteins less abundant and green full circles are those that are more abundant, both of which are greater than 0.05 probability. Grey circles are proteins that are not significantly different across the three different datasets. Empty circles are those that are significant but are represented by a single peptide only.

Figure 2. A Venn Diagram showing the number of more/less abundant proteins in the proteomic study and there overlap between the two datasets.

Figure 3. Proposed mechanism of Pd and Pt nanoparticles synthesis in *D. alaskensis* G20 based on the proteomic data presented here for Palladium (A) and Platinum (B). Note the overlapping of proteins found in each of the individual metal datasets found in the ESI (Table 1). (C) Predicted production of increased size platinum nanoparticles due to increased levels of Dde_2137. Adapted and expanded from Capeness *et al*; 2015.

Figure 4. Electron micrographs of *D. alaskensis* incubated with 2 mM PtCl_4 for 120 minutes. A. Control containing the empty pMO9075 plasmid. B. containing the pMO-2137 plasmid. C. The nanoparticles from B. free in the media. Scale bar = 1 μm

Figure 5. Size distribution of the platinum nanoparticles produced by *D. alaskensis* containing the two different plasmids. The pMO9075 control and pMO9075 containing gene encoding Dde_2137. Error bars represent the standard error around the mean.

Figure 6. Frequency distribution of the sizes of the platinum nanoparticles produced by *D. alaskensis* containing either the control plasmid (pMO9075) or the one containing the gene dde_2137 encoding the small subunit of the NiFe hydrogenase (pMO-2137).

Figure 7. Samples of nanoparticles produced by *D. alaskensis* cells in the presence of platinum. (A) Cells containing the empty plasmid pMO9075 and attributed EDS spectra confirming the presence of only platinum, (B) cells containing the pMO-2137 plasmid expressing the small NiFe subunit, as before, the associated EDS spectrum shows only the presence of platinum. Note: the presence of nickel in the sample is due to the samples being mounted on a nickel grid.