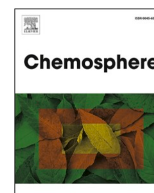
Chemosphere 339 (2023) 139657

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere



From biomolecules to biogeochemistry: Exploring the interaction of an indigenous bacterium with gold

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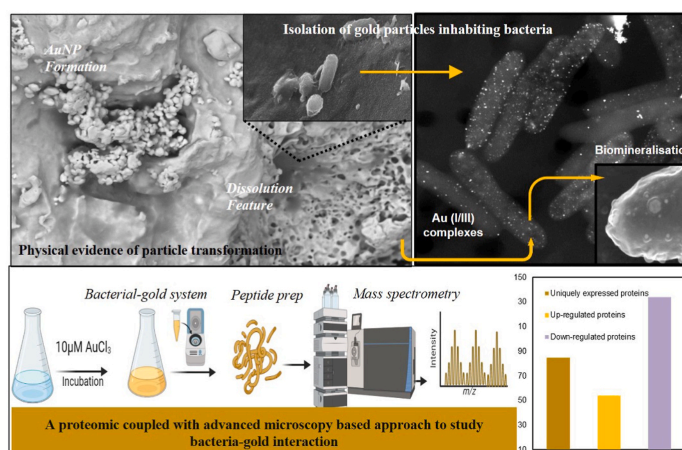
HIGHLIGHTS

- Proteomic response of an indigenous bacterium to cytotoxic gold is elucidated.
- The bacterium *Serratia proteamaculans* was isolated from natural gold particles.
- Oxidative stress & cell-damage repairing proteins were up-regulated to deal with toxicity soluble Au.
- Up-regulation of S-bearing-radical proteins was found in bacteria-Au experiments.
- Possible metabolic pathway for bacteria-mediated Au biomineralisation is proposed.

GRAPHICAL ABSTRACT



ARTICLE INFO

Handling Editor: Lena Q. Ma

Keywords:
Microbe-mineral interaction
Gold
Copper
Serratia proteamaculans
Gold-associated proteins
Biogeochemistry

ABSTRACT

Specialised microbial communities colonise the surface of gold particles in soils/sediments, and catalyse gold dissolution and re-precipitation, thereby contributing to the environmental mobility and toxicity of this 'inert' precious metal. We assessed the proteomic and physiological response of *Serratia proteamaculans,* the first metabolically active bacterium enriched and isolated directly from natural gold particles, when exposed to toxic levels of soluble Au^{3+} (10 µM). The results were compared to a metal-free blank, and to cultures exposed to similarly toxic levels of soluble Cu^{2+} (0.1 mM); Cu was chosen for comparison because it is closely associated with Au in nature due to similar geochemical properties. A total of 273 proteins were detected from the cells that experienced the oxidative effects of soluble Au, of which 139 (51%) were upregulated with either sole expression (31%) or had synthesis levels greater than the Au-free control (20%). The majority (54%) of upregulated proteins

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https://doi.org/10.1016/j.chemosphere.2023.139657
Received 28 May 2023; Received in revised form 23 July 2023; Accepted 24 July 2023
Available online 3 August 2023
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were functionally different from up-regulated proteins in the bacteria-copper treatment. These proteins were related to broad functions involving metabolism and biogenesis, followed by cellular process and signalling, indicating significant specificity for Au. This proteomic study revealed that the bacterium upregulates the synthesis of various proteins related to oxidative stress response (e.g., Monothiol-Glutaredoxin, Thiol Peroxidase, etc.) and cellular damage repair, which leads to the formation of metallic gold nanoparticles less toxic than ionic gold. Therefore, indigenous bacteria may mediate the toxicity of Au through two different yet simultaneous processes: i) repairing cellular components by replenishing damaged proteins and ii) neutralising reactive oxygen species (ROS) by up-regulating the synthesis of antioxidants. By connecting the fields of molecular bacteriology and environmental biogeochemistry, this study is the first step towards the development of biotechnologies based on indigenous bacteria applied to gold bio-recovery and bioremediation of contaminated environments.

1. Introduction

Although gold is relatively sparse in the Earth's crust (~4 ppb; Haynes et al., 2016), gold particles are common in sediments as well as some mining waste, including large river ecosystems affected by artisanal and small-scale gold mining (Casso-Hartmann et al., 2022; Yoshimura et al., 2021). Gold is perceived as an 'inert' metal, but over the past 20 years, it has been revealed that specialised microbial communities colonise the surface of these particles and catalyse gold dissolution and re-precipitation, thereby contributing to the environmental mobility and toxicity of this precious metal (Gadd, 2010; Reith et al., 2016; Sanyal et al., 2019b), both directly (gold toxicity) as well as indirectly (e.g., release of mercury from gold-mercury alloys).

Collectively, the processes that influence the transformations and mobility of gold in the environment are referred to as the *gold biogeochemical cycle* (Southam et al., 2009). Bio-mediated reduction-oxidation (redox) reactions govern gold dissolution; recrystallisation from coarse-grained to nano-particulate; and hence dispersion/mobility (Bohu et al., 2019; Brugger et al., 2013; Falconer and Craw, 2009). Microbes through their diverse metabolic activities drive the biogeochemical cycling of essential elements and micronutrients, such as carbon, sulfur, nitrogen, iron, and copper; cycling of these elements plays important roles in environmental mobility of gold (Sanyal et al., 2019b). Hence, bacteria contribute both directly and indirectly to the biogeochemical cycling of gold. These contributions have been interpreted from the structural and chemical characterisation of gold particles in association with (remnant) bacteria detected on the surface of these particles, in environments ranging from subtropical to subarctic (Reith et al., 2013, 2018). In general, gold particle transformation includes macroscale and microscale changes to the structure and chemistry of the affected particles. The gradual re-shaping of angular particles to more rounded and nugget-like morphologies is primarily attributed to physical movement within a given environment (Craw, 1992). In contrast, biogeochemical particle transformation usually occurs on the microscale and involves the formation of dissolution textures and the precipitation of chemically pure (compared to the parent particle) gold nanoparticles on the surface of gold particles. These secondary gold structures occur at the interface where (bio)geochemical processes proceed (Shuster et al., 2017).

It has been suggested that gold dissolution is controlled by conditions within local microenvironments at the surface of the gold particles or within topographically low regions (polymorphic layers) that contain secondary gold structures (Reith et al., 2010; Shuster et al., 2017). In addition to spatial heterogeneity, these gold mobilisation processes are thought to occur as punctuated "events" reflecting changes in geochemical conditions, affected by external factors such as rainfall (Fairbrother et al., 2012; Reith et al., 2012), or self-mediated by *in-situ* microbial communities (Reith and McPhail, 2006; Sanyal et al., 2019a). Anthropogenic activities such as artisanal and small-scale gold mining using mercury also have a direct effect on the gold particles and microbiota residing within gold-bearing soils/sediments, and thereby could impact the natural biogeochemical cycling of gold (Sanyal et al., 2020a).

Microbial communities on the surface of gold particles differ markedly from the communities in the host soils or sediments (Reith et al., 2006), and it has been inferred that dissolution of gold from particles during biogeochemical transformation is one of the major processes that defines the micro-environment around gold particles, selecting for metal-tolerant bacteria (Sanyal and Shuster, 2021; Sanyal et al., 2019a). Molecular studies involving known metalophilic bacteria (culture collection or type-strains) *Cupriavidus metallidurans* CH34 and *Delftia acidovorans* 2167 demonstrated that these bacteria use their unique biochemical mechanisms to deal with the toxic effects of soluble gold (Bütof et al., 2018; Johnston et al., 2013; Pontel et al., 2007; Reith et al., 2009; Wiesemann et al., 2017; Zammit et al., 2016). However, these bacteria were not isolated from gold particles or gold-bearing soils. The present study demonstrates bacterial interactions with soluble gold using the bacterium *Serratia proteamaculans strain D2,* the first organism to be isolated from the surface of environmental gold particles undergoing (bio)transformation.

Serratia proteamaculans strain D2 was initially isolated from gold particles recovered from Donnybrook, a historic gold-mining site located inWestern Australia. Gold mining from deep underground workings stopped in 1935, thus residual gold particles in mullock heaps underwent biogeochemical transformation for a minimum of nearly 88 years at near-surface (<50 cm depth), warm-temperate climate conditions. The average annual temperature is 22.6 °C and annual rainfall is 972.5 L m^{-2} (Sanyal et al., 2019a, 2020b). A diverse range of bacteria was detected from the gold particles, and a small fraction of this population was viable and included *Serratia proteamaculans* (Sanyal et al., 2020b). When exposed to Au^{3+} chloride, *S. proteamaculans* reduced more than 80% of soluble gold to elemental gold nanoparticles (AuNPs) within 4 h. For details see Sanyal et al. (2020b). Genomic analysis revealed that this bacterium contained a diverse range of heavy-metal resistant genes and stress-tolerance genes, which could explain its resistance to gold cytotoxicity. Although *Serratia* spp. are ubiquitous in the environment and some metal-tolerant strains (e.g., Mn-resistant *S. marcescens*) have been isolated from mine water (Jurat-Fuentes and Jackson, 2012; Barboza et al., 2017), gold-tolerant *S. proteamaculans* could provide insight for the development of gold recovery biotechnologies.

Earlier studies provided textural evidence of particle transformation, as well as physiological and genomic perspectives for possible survival mechanisms by this bacterial isolate when subjected to soluble gold (Fig. 1) (Sanyal et al., 2019a, 2020b). From these previous results, we hypothesize that *S. proteamaculans* mediates the cytotoxicity of soluble gold by upregulating the expression of different cellular and metabolic genes. Therefore, the purpose of this study is to provide a proteomic perspective of how an indigenous environmental bacterium, *S. proteamaculans,* that belongs to a community of microbes inhabiting the surface of gold particles, responds to, or mediates, the cytotoxic effects of soluble gold. This study attempts to provide a bridge between biochemistry and geochemistry by using advanced proteomics to characterize the changes in protein expression in bacteria exposed to soluble gold. Cultured bacterial cells were treated with Au^{3+}-chloride at Minimum Inhibitory Concentrations (MIC) (Ta et al., 2014) to create an *in-vitro* laboratory-model representing the interactions between bacteria with ionic gold during particle dissolution events. In addition to

controlled experiments where no metal was added to the growth medium, a set of experiments was conducted by adding Cu^{2+}-chloride instead of Au^{3+} chloride at concentrations resulting in similar cytotoxicity to the cultures. Due to similar geochemical behaviour, Cu and Au are closely associated in nature (Brugger et al., 2016), and Cu provides a useful comparison as the proteomic response of bacteria to copper toxicity is well understood compared to that of gold (Monchy et al., 2006; Valenzuela et al., 2006; Zammit et al., 2016).

2. Materials and methods

2.1. Bacterial enrichment and growth phases

Serratia proteamaculans strain D2.2 was previously enriched, isolated, and purified from gold particles obtained from Donnybrook, Western Australia; as described in Sanyal et al. (2020b). For this study, *Serratia proteamaculans* was initially grown on solid Tris 15 Minimal Medium (TMM) agar plates and incubated for 48 h at 25 °C. After incubation, a single colony was picked and transferred to a Falcon tube containing 7.5 mL of freshly prepared liquid Tris Minimal Medium (TMM) and placed in a 25 °C shaking incubator (120 rpm) for 120 h. To establish relative growth phases, a second bacterial culture was prepared in parallel to the first. In doing so, growth was tracked by measuring optical density (OD), using a SpectraMax Plus 384 Spectrophotometer (Molecular Devices, USA) and analysed following the logistic growth model (Prism, GraphPad). Measurements were taken at 2 h intervals for the first 24 h of incubation followed by 12 h intervals for the remaining 96 h of incubation. An “exponential growth” phase was defined by increasing OD_{600nm} values between 0.13 and 0.57, whereas “stationary phase” was definedby OD_{600nm} values ≥ 0.57 (see supplementary Fig. S1).

2.2. Estimating the Minimum Inhibitory Concentrations (MIC) of Au^{3+} and Cu^{2+}

A 96-well plate method was used to estimate the MICs of $AuCl_3$ and $CuCl_2$ salts for *S. proteamaculans* (Wiesemann et al., 2017). Briefly, each well was filled with 130 μL of fresh TMM medium supplemented with different concentrations of $AuCl_3$ (10 μM intervals from 0 to 100 μM) or $CuCl_2$ (0.1 mM intervals from 0 to 1.0 mM). *S. proteamaculans* was grown in liquid TMM, as previously described, up to an early stationary growth phase. This bacterial culture was then diluted 1:20 in fresh liquid TMM and had an $OD_{590} = 0.05$. Aliquots (20 μL) of the diluted culture were inoculated into each well except those which were used for the abiotic controls (i.e., TMM only, TMM with Au^{3+} or Cu^{2+}). This plate method was performed in triplicate. The well plates were incubated for 72 h at 24 °C in a shaking incubator (180 rpm). Cell density was measured after incubation using the same spectrophotometer mentioned above. By plotting the cell densities (Fig. S2), the IC_{50} (i.e., the metal concentrations that led to turbidity reduction by half) were estimated to be 25 μM for Au^{3+} and 0.25 mM for Cu^{2+}. Therefore, Au^{3+} & Cu^{2+} concentrations of 10 μM and 0.1 mM were used, respectively, in bacteria-gold and bacteria-copper experiments to ensure a comparable proteomic response.

2.3. Bacteria-gold and bacteria-copper proteomic experiments

2.3.1. Experimental setup

An aliquot (1 mL) of S. proteamaculans grown to early stationary growth phase was transferred to 12 sterile Erlenmeyer flasks each containing 49 mL of fresh TMM. All flasks were incubated at 25 °C in a shaking incubator (150 rpm) for 3 h. After incubation, soluble $AuCl_3$ was added to four flasks each containing a final concentration of 10 μM Au^{3+}. Similarly, soluble $CuCl_2$ was added to four flasks so that each flask contained a final concentration of 0.1 mM Cu^{2+}. The remaining four flasks were used as control systems (i.e., a pure culture of *S. proteamaculans* grown in TMM without any added metals). All bacterial-metal systems and the controls were performed in parallel and incubated under the same conditions previously described. Growth was tracked over time from two replicates of each bacteria-metal systems and control. In doing so, 5 mL aliquots were sampled, and OD was measured using the same spectrophotometer. At the end of the exponential phase (i.e., $OD_{600nm} \simeq 0.53$; Fig. S1), cells were harvested from

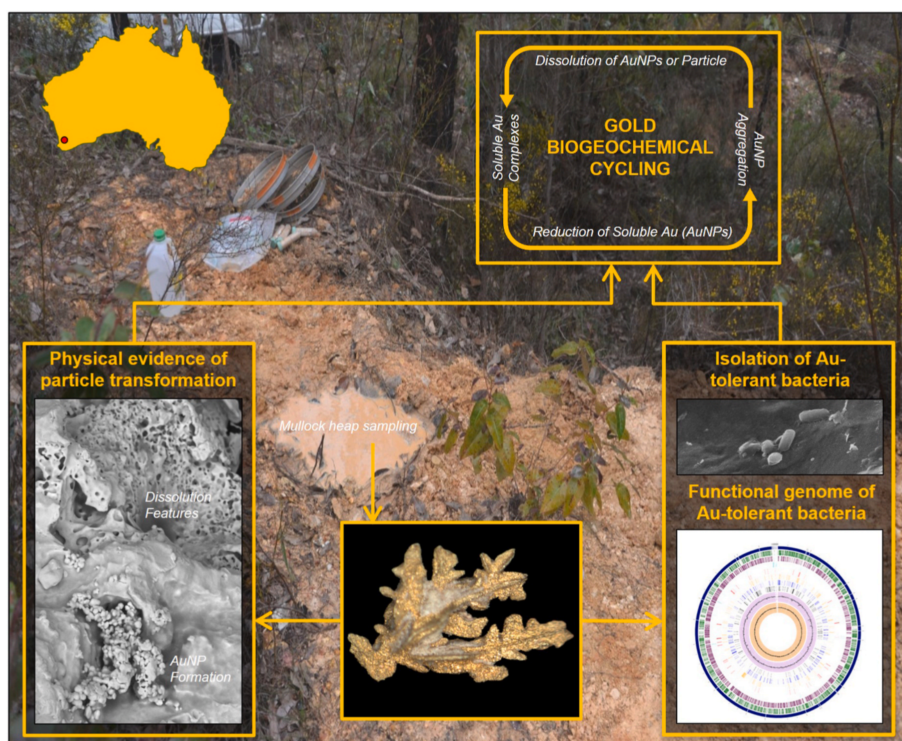


Fig. 1. A comprehensive schematic diagram highlighting the foundations for this present study. Gold (Au) particles were obtained from mullock heaps located in an abandoned mine in Donnybrook, Australia, undergoing natural restoration. Particle surfaces appeared transformed with physical structures attributed to past biogeochemical gold dissolution and gold nanoparticle precipitation. Gold-tolerant bacteria, such as *Serratia proteamaculans*, were enriched and isolated from particles. These bacteria contained many heavy-metal and stress-tolerance genes, thereby contributing to particle transformation and thus gold biogeochemical cycling.

the remaining two replicates of each bacterial-metal system and the control. Specifically, the systems and controls were separately centrifuged (10,000 rpm for 10 min at 4 °C) to form bacterial pellets, and the supernatant was removed. The pellets were washed in phosphate-buffered saline (PBS, pH = 7.2) and vortexed to resuspend bacteria homogeneously in solution. Bacteria were re-pelleted by centrifugation and the PBS supernatant was removed. This washing procedure was performed a total of three times to ensure that all spent growth medium and any cellular debris was removed (Fig. 2B).

2.3.2. Protein extraction and peptide preparation

Immediately after the final wash, cells were lysed to extract proteins. In doing so, pellets were individually resuspended in 100 μL of 20%$_{(aq)}$ trichloroacetic acid and vortexed to homogenise cells in solution. These cell suspensions were transferred to separate Bioruptor® sonication tubes (Diagenenode, Australia) and sonicated at high power for 10 min (Bioruptor®). After sonication, ultrapure water (100 μL) and 100% cold acetone (600 μL) were added to each sample (i.e., extractions from 2 control systems and 2 bacteria-gold system and 2 bacteria-copper system) and incubated in a −20 °C freezer overnight. After incubation, all samples were centrifuged (20,000×g for 20 min at −9 °C) in a pre-cooled rotor. The supernatants were removed, and each pellet was resuspended in 200 μL of solution containing 1.0%$_{(aq)}$ sodium dodecyl sulfate (SDS), 50 mM Tris (pH = 8.0), and 50 mM dithiothreitol (DTT).

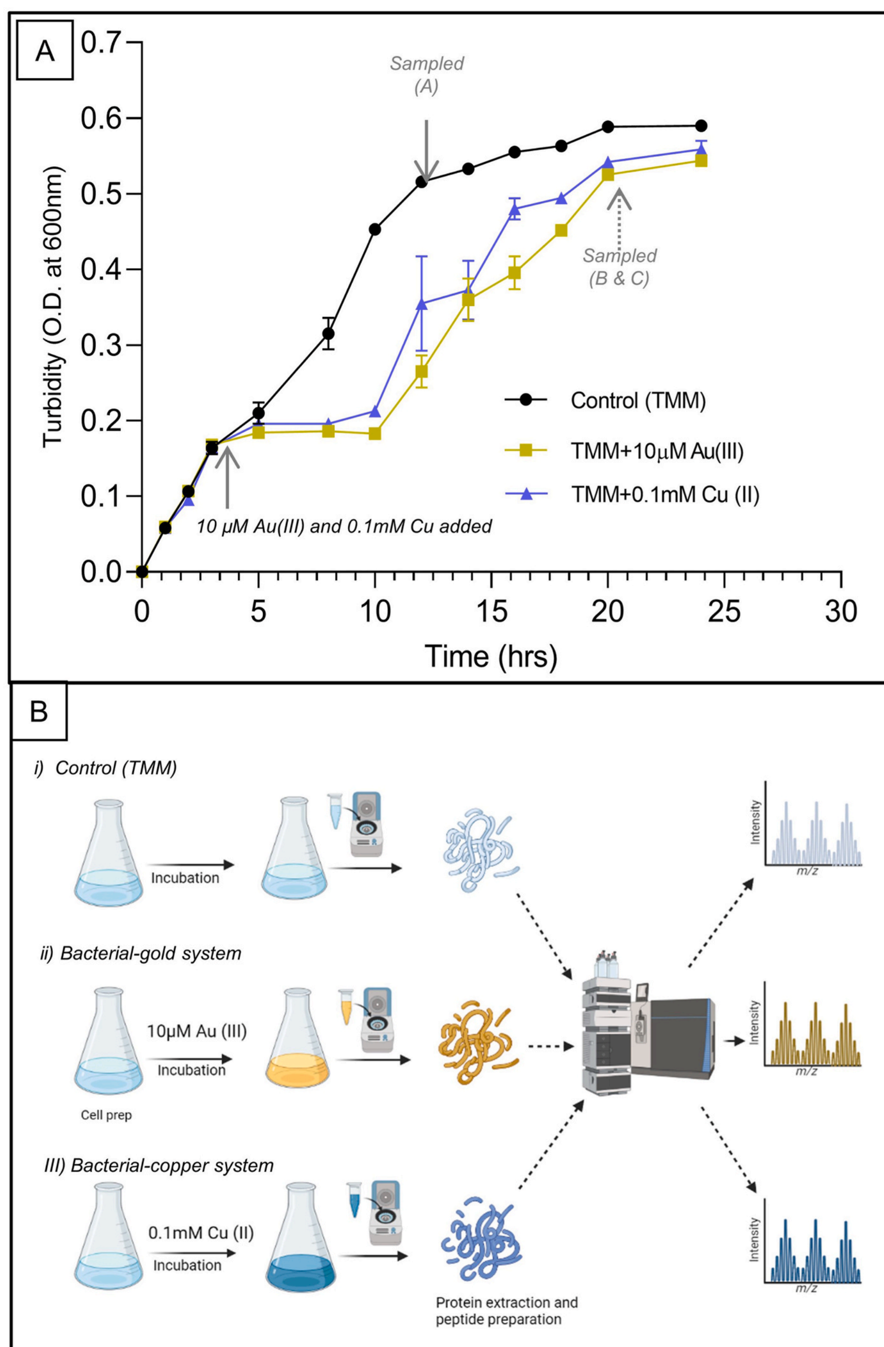


Fig. 2. (**A**). The growth curves of *S. proteamaculans* grown in TMM. After 3 h of incubation, soluble Au and Cu was added, to form the bacteria-gold/copper systems. The control systems and bacteria-gold/copper systems were sampled at the end of exponential phase, which occurred at 12 and 20 h, respectively. **2(B)**. A schematic showing the steps of this proteomic experiments.

The suspension was sonicated for 1 min, vortexed, and placed in a 56 °C incubator for 30 min to aid the recovery of insoluble proteins. All samples were centrifuged (10,000×*g* for 5 min) to remove insoluble debris and processed using a modified in-solution filter-aided sample preparation (FASP) protocol (Wiśniewski et al., 2009). Briefly, the samples were re-solubilised in a solution containing 8 M urea, $1.0\%_{(aq)}$ SDS, 100 mM ammonium bicarbonate, and $1.0\%_{(aq)}$ protease inhibitor cocktail (Millipore Sigma) and incubated with 1 M DTT at 30 °C for 1 h, followed by centrifugation (13,000×*g* for 5 min). Protein concentration was measured using the EZQ™ Protein Quantitation Kit (ThermoFisher, Scientific). To digest the samples, ca. 100 µg of each sample was alkylated with 55 mM iodoacetamide (IAA) in a Vivacon 500 spin column (30 kD molecular weight cut-off) for 20 min in the dark. Sequencing-grade trypsin (5 µg) dissolved in 10 mM ammonium bicarbonate was added and incubated overnight at 37 °C. Digested samples were then eluted through the spin filters, reduced to ca. 1 µL using a vacuum centrifuge, and resuspended in $3.0\%_{(aq)}$ acetonitrile (ACN). The peptide concentration was measured on a NanoDrop™ 2000/2000c Spectrophotometer (Thermo Fisher Scientific) at a wavelength of 205 nm. Samples were then acidified using trifluoroacetic acid to a final concentration of $0.1\%_{(v/v)}$.

2.3.3. Liquid chromatography - tandem mass spectrometry (LC-MS/MS)

Digested samples were analysed using an Ultimate 3000 nano-flow system (Thermo Fisher Scientific) coupled to a LTQ XL Orbitrap ETD mass spectrometer (Thermo Fisher Scientific). For each sample 1 µg of total protein was pre-concentrated onto a C18 trapping column (Acclaim PepMap100C1875 µm × 20 mm, Thermo-Fisher Scientific) for 10 min at a flow rate of 5 µL/min, using a buffer containing $2.0\%_{(v/v)}$ ACN in $0.1\%_{(v/v)}$ formic acid (buffer A). Peptides were then separated at a flow rate of 300 nL/min by using a 15 cm long C18 separation column (Acclaim PepMap100C18 75 µm × 15 cm, Thermo-Fisher Scientific) with a 65 min linear gradient of 5–45% buffer B ($80\%_{(v/v)}$ ACN in $0.1\%_{(v/v)}$ formic acid), followed by a 5 min wash with 90% buffer B, and a 15 min equilibration with 5% buffer B. Liquid chromatography and mass spectrometry data acquisition were controlled by Xcalibur version 2.1 (Thermo Fisher Scientific). The LTQ-Orbitrap instrument was operated in the positive ion mode with a normalised collision energy set to 35%. All mass spectrometry (MS) and tandem mass spectrometry (MS/MS) spectra were acquired in data-dependent mode with mass range of *m/z* 300 to 2000 at a resolution of 60,000 in the FT mode. The 10 most intense precursor ions were selected for MS/MS scan by collision-induced dissociation (CID) using a dynamic exclusion of 5 s, a minimum relative signal intensity of 1000, and ≥2 positive charge state. Note that all samples were analysed with technical quadruplicates. See Fig. 2B for a schematic overview of the methodology.

2.3.4. Data analysis

Mass spectrometry spectra were analysed and subjected to label-free quantification using the MaxQuant software (version 1.5.2.8) with the Andromeda search engine and the NCBI Genome dataset of *Serratia proteamaculans* (https://www.ncbi.nlm.nih.gov/nuccore/SRIA00000000). The search parameters included the maximum number of missed cleavages for trypsin set as two per peptide, carbamidomethylation of cysteine set as a fixed modification, and methionine oxidation considered as a variable modification. Label-free quantification was activated with a minimum ratio count of 2 and allowed a match between runs as well as unidentified features. The standard Orbitrap setting was used with the mass tolerances for MS and MS/MS set at 20 ppm and 0.5 Da, respectively.

Only proteins that were identified with more than 2 peptides and a minimum length of 7 amino acids were included. All expressed proteins were subcategorised based on function using a protein functional annotation database (www.uniport.org). In terms of synthesis levels, protein expression greater than the control (≥1.1 fold) or only expressed in the bacteria-gold and bacteria-copper systems were considered as up-regulated proteins. As such, protein expression with fold less than or equal to the control was considered down-regulated proteins.

2.4. Scanning electron microscopy (SEM)

When cells were harvested for protein extraction, an aliquot from a bacteria-gold system, which was used for tracking parallel growth, was sampled for scanning electron microscopy (SEM) analysis. Briefly, cells were fixed with $2.0\%_{(aq)}$ glutaraldehyde and prepared in the methods described for bacterial cells preparation by Shuster et al. (2019). Cells were characterised using a Helios NanoLab Scanning Electron Microscope (SEM) operating at 10 kV accelerating voltage. Micrographs were taken both in Secondary Electron (SE) and Back-Scattered Electrons (BSE) mode.

3. Results

3.1. Growth in the presence of soluble gold and copper

In the control system, growth of *Serratia proteamaculans* exhibited a characteristic sigmoidal growth pattern (Fig. 1S). After the addition of Au^{3+} and Cu^{2+} to the cultures, growth remained static for almost 7 h until it "re-started" after 10 h of incubation. Cells from both the control and the bacteria-gold/copper systems were sampled at $OD_{600nm} \simeq 0.53$, which was reached at 12 and 20 h, respectively (Fig. 2A). Microscopy analysis of cells from the bacteria-gold system confirmed that *S. proteamaculans* retained their rod-shape morphology (ca. 2.5 µm in length and ca. 0.75 µm in diameter) when grown in the presence of soluble gold (Fig. 3A). Gold nanoparticles appeared to nucleate either on the extracellular surfaces or near the cell boundary within the intracellular space (Fig. 3B and C).

3.2. Proteomic response to soluble gold, and comparison to copper

Proteomic analysis identified 1016 proteins from the control system (Table S1), which was approximately a quarter of the total number of proteins estimated from the genome of *Serratia proteamaculans* (https://www.ncbi.nlm.nih.gov/nuccore/SRIA00000000). A total of 273 proteins were detected and identified in the bacteria-gold system, similar to the amount in the bacteria-copper system (274). Hence, approximately 75% of the proteins expressed in the control system (i.e., no added Au or Cu) were not expressed in the bacteria-gold or bacteria-copper system. All proteins were subcategorised based on function and grouped into four broad categories: 1) metabolism and biogenesis 2) cellular processes and signalling 3) information storage and processing and 4) poorly characterised (Table 1).

In the bacteria-gold system, there were 139 up-regulated proteins; 61% of these proteins were solely expressed in the bacteria-gold system (Group A, Table 1 and S2) and 39% of proteins had fold changes greater than the control (Group B, Table 1 and S3). The remaining 134 proteins were down-regulated (Fig. 4A, Table S3). Overall, in the broad functional categories, there were 1.04 times more up-regulated proteins compared to down-regulated proteins (Fig. 4A, Table 1). The greatest number of up-regulated proteins were related to the broad cellular processes of metabolism and biogenesis; specifically, amino acid biosynthesis, organic and inorganic ion transport, and other metabolic functions. A smaller number of up-regulated proteins related to information storage and processing (transcription/translation) and cellular processes and signalling functions (cell wall/membrane/pili biogenesis). The respective number of up-regulated proteins varied within Groups A and B (Table 1). It is important to note that proteins solely expressed in the bacteria-gold system (Group A) had no associated fold change as the expression of these proteins were not detected in the control, albeit the corresponding genes were detected in the genome of the bacterium *Serratia proteamaculans* (https://www.ncbi.nlm.nih.gov/nuccore/SRIA00000000). Among up-regulated proteins with fold

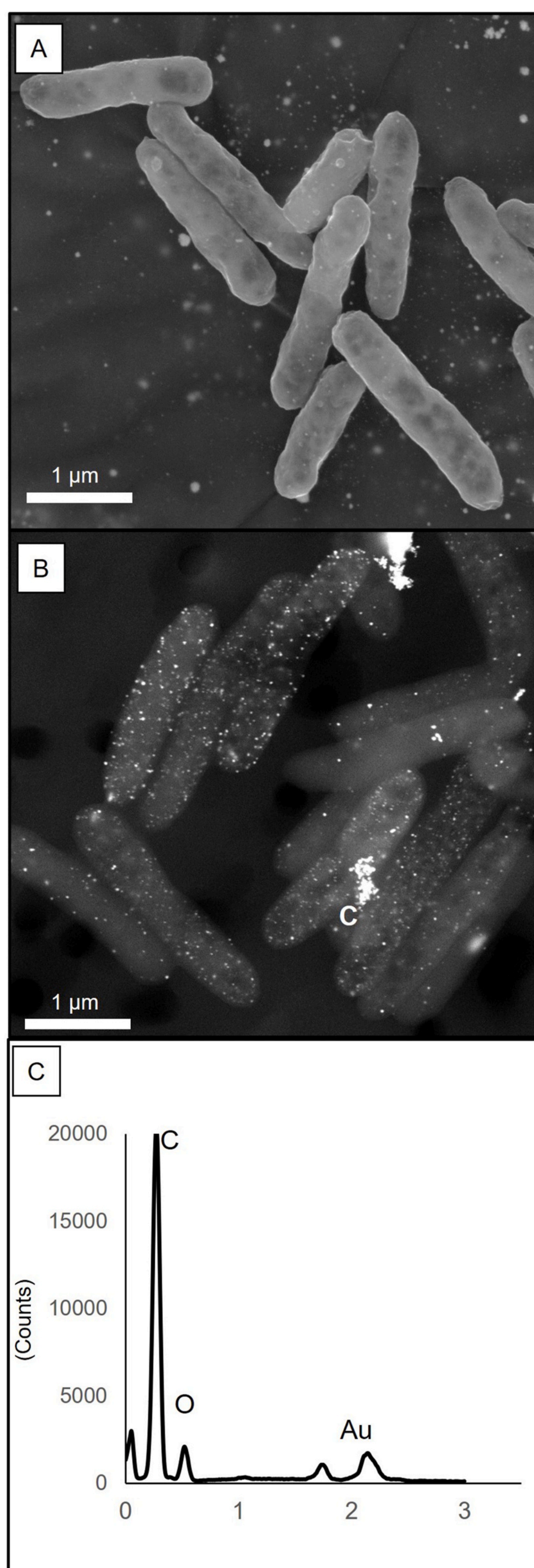


Fig. 3. **(A)** A high-resolution secondary electron (SE) scanning electron micrograph of *S. proteamaculans* cells grown in TMM **(B)** A high-resolution SE scanning electron micrograph of *S. proteamaculans* treated with 10 μM Au for 17 h. Soluble gold was reduced as nanoparticles that occurred either intracellularly or on the extracellular surface of rod-shaped cells. **(C)** A representative energy dispersive spectroscopy (EDS) spectrum of the gold nanoparticles embedded in/onto bacterial cells.

changes (Group B), the greatest numbers were related to the functional subcategories including amino acid biosynthesis (11 proteins), transcription/translation (11 proteins), cell wall/membrane/pili biogenesis (8 proteins) and cellular stress response (6 proteins). These proteins constituted 66.7% of all up-regulated proteins within this group. From a synthesis perspective, up-regulated proteins related to the subcategories of cellular stress response (56.81 sum fold), amino acid biosynthesis (32.81 sum fold), cell wall/membrane/pili biogenesis (20.08 sum fold) and transcription/translation (17.73 sum fold) constituted the greatest (79% of the total) fold changes.

In the bacteria-copper system, a total of 187 proteins were up-regulated compared to the control; 62% of these proteins were uniquely expressed (Fig. 4A, Table 1 and S4) and 38% of proteins had fold changes greater than the control (Group B). The remaining 87 proteins were down-regulated compared to the control (Fig. 4A, Table 1 and S5). Overall, in the broad functional categories, there were 1.46 times more up-regulated proteins compared to down-regulated proteins (Table 1). A total of 172 proteins were detected both in the bacteria-gold and bacterial-copper systems, in which 140 proteins were expressed either with a synthesis level higher or lower than the controls (Table S6) and 32 proteins were found uniquely expressed in both treatments (Table S7).

4. Discussion

4.1. *A model for understanding bacteria-gold interactions in the environment*

Mine wastes resulting from historic mining at Donnybrook continue to undergo natural biogeochemical transformations since the cessation of industrial activity approximately 88 years ago. Therefore, gold particle transformation within these mullock heaps is an example of gold biogeochemical cycling accelerated by anthropogenic activity (Fig. 1 (Sanyal et al., 2019a);). The time required for gold particle transformation to occur depends on evolving conditions that promote or hinder particle dissolution. Conditions favourable to particle dissolution may occur infrequently over tens of decades (Shuster et al., 2017), but previous studies demonstrated that interactions between bacteria and soluble gold are near-instantaneous (Johnson and Fein, 2019; Kenney et al., 2012; Lengke and Southam, 2006; Tay et al., 2013). In the present study, *Serratia proteamaculans* represents a naturally occurring gold-tolerant bacterium living either on particle surfaces or within microenvironments surrounding particles (Sanyal et al., 2020b), providing the opportunity to study bacterial gold interactions that occur spontaneously in the environment. Therefore, this bacterial-gold system can be used as a model system to reveal how bacteria residing on gold particles, that are a widespread if volumetrically tiny component of soils and sediments, mediate the cytotoxic effects of soluble gold. The gold concentration used in this model was comparable to what could occur within microenvironments at the surface of gold particles when various geochemical factors promote particle transformation (Melchiorre et al., 2018; Rea et al., 2019). From a biochemical perspective, the bacteria-gold and bacteria-copper systems contained the sum of all possible metabolic responses, i.e., proteome response by *S. proteamaculans* prior to and during the exposure to soluble gold and copper. Most of these expressed proteins, however, likely result from the presence of soluble metal since this exposure was nearly six times longer than the initial 3 h incubation prior to exposure (Fig. 2A). It is important to note that each up-regulated protein can be considered important in terms of individual gene expression; however, many up-regulated proteins have the same or similar function in terms of mediating gold or copper cytotoxicity (Table 1). Additionally, it is possible that only a fraction of these up-regulated proteins may be expressed *in-situ*. For discussion purposes, selected up-regulated proteins will be used as examples highlighting broad functions to interpret *S. proteamaculans* growth in the presence of soluble gold – a model of bacterial survival

Table 1
The total number of detected proteins expressed by *Serratia proteamaculans* strain D2.2 when exposed to 10 μM Au(III) and 0.1 mM of Cu(II). These proteins were categorised by protein function and synthesis levels (i.e., up-/down-regulation). Note that up-regulated proteins include proteins solely detected in the bacterial-gold/copper system. (Group A) and proteins with fold changes greater than the control system (Group B).

BROAD FUNCTION CATEGORY Function Subcategory	Bacteria- Au System				Bacteria- Cu System			
	Total Proteins	Up Regulated Proteins		Down- Regulated Proteins	Total Proteins	Up Regulated Proteins		Down- Regulated Proteins
		Group A	Group B			Group A	Group B	
METABOLISM AND BIOSYNTHESIS								
Amino Acids Biosynthesis	40	10	11	19	30	9	14	7
Carbohydrate Metabolism	20	5	1	14	23	7	4	12
Lipid Biosynthesis and Metabolism	7	3	2	3	11	6	3	2
Nucleotide Biosynthesis	12	5	5	2	10	6	1	3
TCA Cycle, ATP synthesis	12	1	3	8	20	7	4	9
Organics & Inorganic Ion Transport	28	8	2	18	18	6	4	8
Others Metabolic Functions	17	8	3	6	18	12	3	3
Subtotal	*135*	*40*	*27*	*70*	*130*	*53*	*33*	*44*
CELLULAR PROCESSES AND SIGNALLING								
Cell Cycle Control/Cell Division	6	3	0	3	7	1	2	4
Cell Wall/Membrane/Pili Biogenesis	26	11	8	8	18	9	5	4
Cellular Stress Response	27	10	6	12	26	12	7	7
Chaperone, Protein Modification	12	5	0	7	15	7	2	6
Subtotal	*71*	*29*	*14*	*30*	*66*	*29*	*16*	*21*
INFORMATION STORAGE AND PROCESSING								
DNA Replication and Nucleic Damage Repair	8	3	2	4	8	4	2	2
Transcription/Translation	40	11	10	19	49	18	18	13
Subtotal	*47*	*14*	*12*	*23*	*57*	*22*	*20*	*15*
LIMITED CHARACTERISED								
Unknown Function	14	2	1	11	21	12	2	7
Subtotal	*14*	*2*	*1*	*11*	21	12	2	7
TOTAL	***273***	***85***	***54***	***134***	***274***	***116***	***71***	***87***

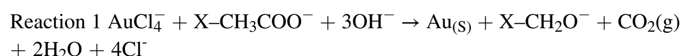
during particle dissolution/transformation. Where appropriate, we make a comparison with the effect of soluble Cu to highlight the results obtained for Au.

4.2. Proteomics perspective of indigenous bacterial interaction with gold

4.2.1. Gold biomineralisation

Soluble gold complexes, like other heavy-metal cations (Ag^{+}, Cd^{2+}, Hg^{2+}) as well as biologically essential trace elements like Zn^{2+}, Ni^{2+} and especially Cu^{2+} at higher concentrations, are highly cytotoxic and can cause cellular damage by "stripping" electrons from biomolecules (oxidative damage) (Bütof et al., 2018). This hinders the metabolic activity of bacteria and ultimately reduces cell viability (Nies, 1999). In our experiments, the deleterious effects of both Au and Cu was expressed via a "delay" in growth that lasted approximately 7 h, suggesting that the 10 μM Au^{3+} and 0.1 mM Cu^{2+} concentrations were both moderately toxic (i.e., sub-lethal) (Fig. 2A).

Previous studies have characterised the capacity of various chemolithotrophic bacteria to reduce soluble gold either intracellularly or extracellularly (Lengke and Southam, 2006; Lengke et al., 2006; Shuster et al., 2014). At least some of this biomineralisation occurs via a passive mode. In this process, soluble Au initially sorbs onto the cell surfaces due to the electrostatic interaction between the net negative charge of extracellular surfaces and positively charged metal aqua ions (Gadd, 2004), or via complexing with S- or C-radicals on the cell surface (Lengke and Southam, 2006; Reith et al., 2009). Then, gold nanoparticle formation can occur via the oxidation of organic material that "donate" electrons to reduce soluble gold (Fig. 3B and C). From a geochemical perspective, this can be expressed by Reaction 1, which uses fatty acid as a model compound for organic material (Shuster et al., 2015), and the $AuCl_4^-$ complex as a model for soluble gold (Usher et al., 2009). If the barrier between the internal environment and the extracellular milieu is compromised, then cells will lyse and release intracellular material, thereby providing additional material that would act as reductants for soluble metals (Karthikeyan and Beveridge, 2002).


Reaction 1 $AuCl_4^- + X\text{–}CH_3COO^- + 3OH^- \rightarrow Au_{(S)} + X\text{–}CH_2O^- + CO_2(g) + 2H_2O + 4Cl^-$

4.2.2. Cellular structural repair

The extent of oxidative stress by toxic metals on bacteria is primarily dependent on the metal concentration, which is limited by its availability and solubility (Cabiscol Català et al., 2000). When bacteria are exposed to soluble gold, depending on the concentration, the cell walls or membranes may not always be completely compromised. Under these conditions, bacteria can up-regulate the expression of cell wall/-membrane biosynthetic proteins to repair any damage and maintain its structural rigidity (Gang et al., 2019; Ramos-Zúñiga et al., 2019). In this study, a total of 19 up-regulated proteins (73% of total detected proteins in this sub-category) in the bacteria-gold system were related to the function sub-category of *cell wall/membrane/pili biogenesis*; this is the largest number of proteins detected in the cellular processing and signalling broad functional category (Table 1). Similarly in the bacteria-copper system, a total of 14 proteins (78% of total detected proteins in this sub-category) belonging to same functional sub-category were also up-regulated. Of these up-regulated proteins, the expression of rod shape-determining protein MreB, was increased by 4.55-fold and 4.68-fold in the bacteria-gold and bacteria-copper systems, respectively (Fig. 4B, Table 2). This protein is responsible for maintaining the integrity (i.e., viability) of the cell wall by acting as a regulator for the biosynthesis of peptidoglycan (Figge et al., 2004). The SEM images illustrate that the *Serratia* sp. cells exposed to soluble gold retained their characteristic rod-shaped structure (Fig. 3B), which is likely one of the survival mechanisms of the bacterium during particle transformation. Interestingly, the undecaprenyl diphosphate synthase (UppS) protein was only expressed in the bacteria-gold system, and is known to play an important role in the biosynthesis of cell wall components such as peptidoglycan and lipopolysaccharide (Teng and Liang, 2012). In-terms

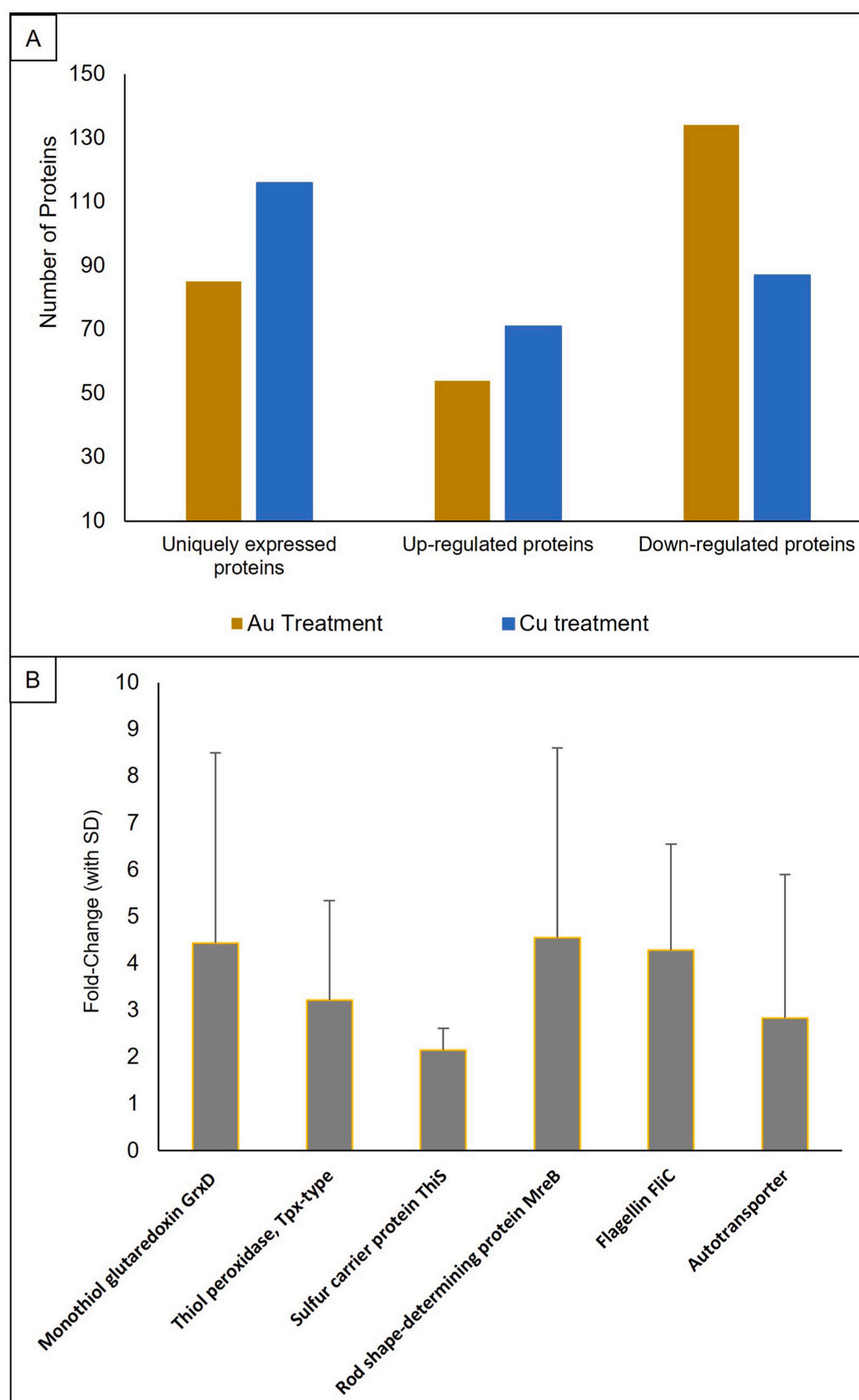


Fig. 4. (A) Bar charts showing the number of proteins expressed with different synthesis patterns (uniquely/solely expressed, up-regulated and down-regulated compare to control) in the Au and Cu treatments. **(B)** Bar-charts showing the fold-change of some important proteins up-regulated in bacteria-gold system.

of the bacteria-Au interaction, the cell wall/membranes are the first interception point. Therefore, the "oxidative stress" in the cell membrane could be greater compared to the other cellular components, which would cause the formation of various reactive oxygen species (ROS). For maintenance of the cellular homeostasis (i.e., viability), these ROS generated in the cell wall/membrane need to be neutralised (Ezraty et al., 2017). Interestingly, several proteins related to ROS neutralisation were up-regulated in the bacteria-gold treatment, such as monothiol glutaredoxine (Grx) (4.44 fold) and thiol peroxidase (Tpx) (3.22 fold) (Fig. 4). These proteins are known oxidoreductase that catalyse the reduction of oxidised membrane proteins (e.g., –SH, thiol containing proteins) from irreversible inactivation (Berndt et al., 2008).

Furthermore, the extracellular surface-fluid interface is the first line of interaction, where soluble Au^{3+} will initially interact with bacteria and could be reduced by stripping electrons from the cell wall/membrane to a less toxic form of gold (Fig. 3B and C). Ultimately this results in the formation of metallic gold nanoparticles, but *in-situ* spectroscopic studies indicate that the first step involves rapid formation of an

Table 2
A list of some selected up-regulated proteins detected in Bacteria-gold system.

Protein IDs (NCBI ID)	Proteins Name	Broad Functional categories: Functional Subcategory	Mol. weight [kDa]	Sequence length	Fold Change (* uniquely expressed)
4460	Rod shape-determining protein MreB	Cell wall biogenesis: cell shape	36.9	347	4.55#
3885	Undecaprenyl diphosphate synthase	Cell wall biogenesis	28.4	252	*
284	Sulfur carrier protein ThiS adenylyltransferase	Amino acid biosynthesis: Sulfur containing amino acid biosynthesis	26.54	249	2.15
3000	Flagellin FliC	Pili Biogenesis: Cell motility	43.06	413	4.30 (#)
748	Iron-sulfur cluster insertion protein ErpA	Chaperone, Protein Modification: Folding of Fe–S containing proteins	23.6	214	*(#)
2225	Monothiol glutaredoxin GrxD	Stress Response: Oxidative Stress response	13.2	115	4.44#
1555	Glutathione ABC transporter periplasmic binding protein	Stress Response: Oxidative Stress response	56.43	512	*
2680	Thiol peroxidase, Tpx-type	Stress Response: Oxidative Stress response	17.5	167	3.22
292	DNA-binding protein HU-alpha	Stress Response: Cellular response against DNA damage	18.3	165	*
2769	DNA-binding protein H-NS	Stress Response: Cellular response against DNA damage	17.7	158	*
292	Sulfate-binding protein Sbp	Organics & Inorganic IonTransport: Sulfate transport	56.0	517	*
2996	l-cystine ABC transporter (TcyJ)	Organics & Inorganic Ion Transport: sulfur containing amino acids	28.9	266	*
1066	Multidrug efflux system: inner-membrane proton/drug antiporter AcrB	Organics & Inorganic IonTransport Efflux pump	115.3	1048	*(#)
1951	Copper resistance protein CopC	Transport: Soluble Au resistance/Copper homeostasis	13.6	127	*
3692	Autotransporter	Organics & Inorganic IonTransport:	105.14	1011	2.84 (#)

#Also up-regulated in Bacteria-copper system; *Uniquely expressed in Bacteria-gold system.

intermediate Au^{+} -sulfide species (Lengke et al., 2006; Reith et al., 2009). Due to the high molecular weight, bacteria-mediated active efflux of soluble Au could be thermodynamically unfavourable if the conditions are not optimum (Lloyd, 2003). In that case, passive bacterial gold interactions via sorption and surface reduction would be more likely to take place and any damages caused by this biomineralisation process would require structural repair to ensure bacterial survival on gold particles. In contrast, copper is an essential micronutrient, and microbes have evolved various efficient biochemical copper handling pathways, which mainly involve active uptake, reduction and followed by efflux (Ladomersky and Petris, 2015). The proteomic data also shows that in the *organic & inorganics ion transpor*t sub-category, 55% of the detected proteins were upregulated in the bacteria-copper system, but only 33% in the bacteria-gold system, consistent with the differences in Au and Cu biogeochemistry.

4.2.3. Metabolic activity

Bacterially mediated precipitation of gold results in the concentration of the remaining soluble gold decreasing to a sub-lethal level. Thus, while it is reasonable to assume that a fraction of bacteria succumbed during gold biomineralisation via passive sorption (Fig. 3B), the cells that survived the initial exposure continued to grow, divide, and eventually reach a stationary phase comparable to the control system, albeit delayed (Figs. 2A & 3A). At sublethal concentrations, soluble metals can enter cells without causing death (Nies, 1999). In the bacteria-gold system, 52% of the detected proteins belonging to the category of *metabolism and biosynthesis* were down-regulated, which suggests a slight decrease in metabolic activity due to the damage caused by soluble gold. However, in the bacteria-copper system, 68% of the detected proteins belonging to same functional category were up-regulated, which highlights an increased metabolic activity. This supports the notion of well-developed cellular systems for copper handling compared to gold (Maertens et al., 2020; Wiesemann et al., 2013). Therefore, it is reasonable to suggest that in the event of mediating the toxicity of soluble Au, bacteria may first slow down their regular metabolic activities, and subsequently increase the synthesis of proteins related to cellular damage.

4.2.4. Intracellular repair

Gold has a geochemical affinity with sulfur-containing compounds. While Au^{3+} forms complexes with chloride and hydroxide in natural waters, Au^{+} form strong complexes with bisulfide (e.g., $Au(HS)_2^-$); this influences the environmental mobility of Au. In contrast, sulfide acts as a precipitating ligand for both Cu^{+} and Cu^{2+}, as demonstrated by the low solubility of copper sulfides such as covellite and chalcocite (Brugger et al., 2016). From a biochemical perspective, soluble Au also has a great chemical affinity for thiol (-SH) groups. A synchrotron speciation study revealed that in the metallophilic bacterium *C. metallidurans* CH34, the cellular Au accumulation is coupled to the formation of Au^{+}-S complexes (Reith et al., 2009), which may lead to the irreversible inactivation of –SH–group-containing proteins (Ortego et al., 2014; Wiesemann et al., 2017). We find that for *S. proteamaculans,* the proteins involved in S-compounds transport/uptake/biosynthesis, such as the ABC transporter substrate-binding (SbP), L-cystine ABC transporter (TcyJ) and iron-sulfur cluster insertion protein (ErpA), were uniquely expressed in the bacteria-gold system. Besides that, the sulfur carrier protein (ThiF) expression was also increased by 2.15-fold (Fig. 4B, Table 2). The up-regulation of proteins such as these suggests that *S. proteamaculans* could have been adjusting to the oxidative stress and increasing the biosynthesis of thiol-group-containing proteins to replenish those that were damaged by the reduction of Au^{3+} and formation of strong bonds with Au^{+} (Fig. 5). An experimental study by Krishnan et al. (2021) using soluble Au^{3+} and free thiols extracted from cell-free supernatant of the fungal species Candida parapsilosis ATCC 7330 showed the formation of polydisperse gold nanoparticles. Interestingly, the formation of polydisperse (and aggregated) gold nanoparticles by the cells of S. proteamaculans (Fig. 3B and C) and up-regulation of thiol group containing proteins during soluble Au stress corroborates Krishnan et al. (2021)'s findings.

Additionally, when a toxic-metal enters into a cell, it can form various other reactive oxygen species (ROS), which need to be reduced for cells to remain viable (Ezraty et al., 2017). Several proteins related to oxidative-stress neutralisation were expressed in the bacteria-gold treatment, such as the 4.44 folds increased monothiol glutaredoxine (Grx) and 3.22 folds increased Thiol peroxidase (Tpx) (Fig. 4). The latter protein is a small disulfide oxidoreductase that is known to catalyse the

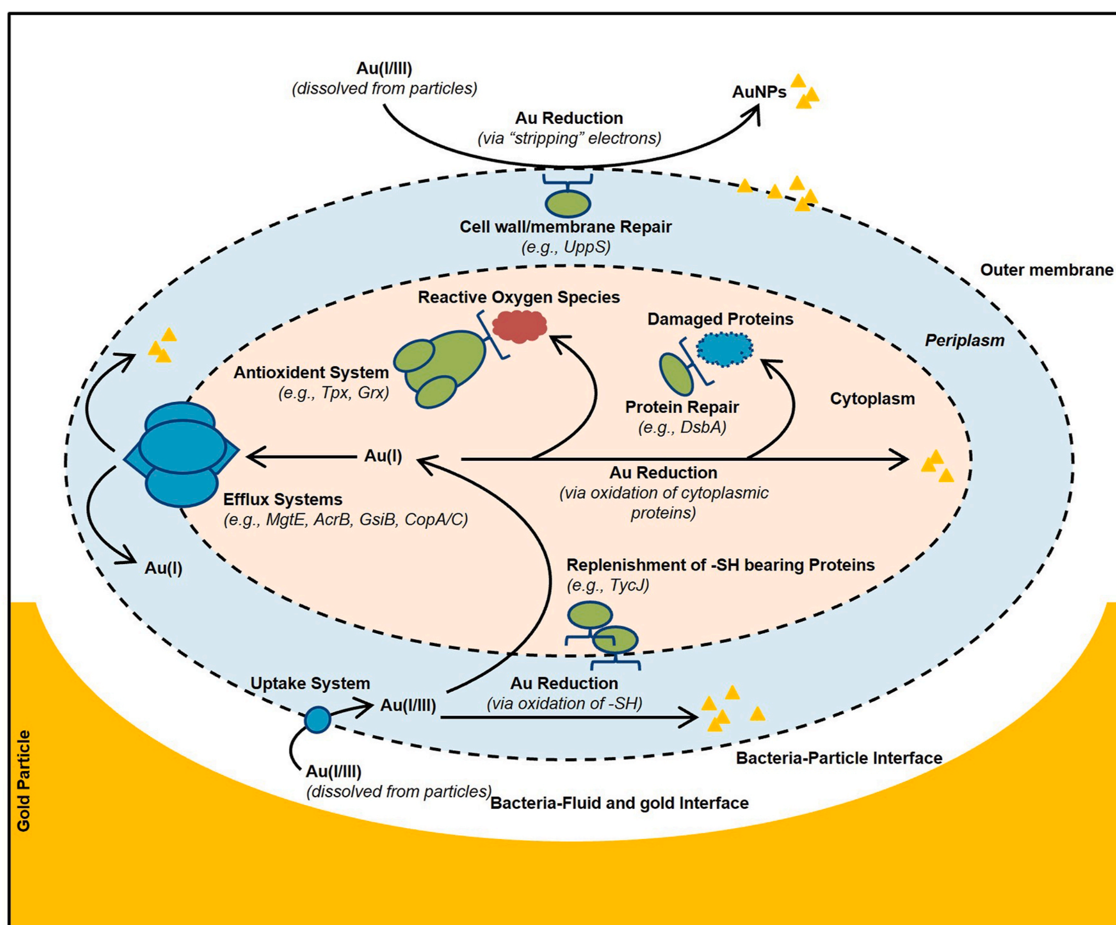


Fig. 5. A schematic diagram of a hypothetical gold-tolerant bacterium (e.g., *S. proteamaculans*) living on the surface of a gold particle undergoing dissolution (transformation). The proteomic response of the cell can vary when exposed to soluble gold. In general, the cytotoxicity of soluble gold is diminished by reducing gold complexes to gold nanoparticles (AuNPs) at the expense of the cell wall/membrane. Therefore, up-regulating proteins to repair damages and preserve the cells structural integrity. Soluble gold can also enter the intracellular environment whereby other proteins can become damaged and reactive oxygen species could be produced. As a response, the cell up-regulates proteins to repair any damages or replenish any "lost" proteins. Alternatively, cells could employ efflux systems to remove soluble gold out from the cytoplasm and into the periplasmic space where it could be reduced as gold nanoparticles.

reduction of disulfide bridges and "rescue" thiol groups from irreversible inactivation (Berndt et al., 2008). Contrary to Au, different oxidative stress tolerance proteins, e.g., Superoxide Dismutase and Glutathione Synthetase, were uniquely expressed in the Cu treatment, though the thiol peroxidase protein was upregulated in both the treatments. Similarly, the increased expression of DNA-binding proteins (HU-alpha and H-NS) in both systems also indicates the cellular response against the DNA damage resulting from severe oxidative stress from soluble Au and Cu. Therefore, oxidative stress tolerance induced by gold may involve two different yet simultaneous processes: repairing damaged cellular components and producing enzymes to remove reactive oxygen species.

4.2.5. *Efflux and up-regulation of metal-resistance genes*

Heavy-metal resistant bacteria can deploy cellular machineries to alleviate the oxidative effect of soluble Au, and the active reduction to nanoparticles in/outside the cellular environment can occur simultaneously depending on the concentration of soluble Au. In contrast, in response to the soluble Cu toxicity bacteria are likely using their well-developed efflux systems (e.g., Cop/Cus systems) (Orell et al., 2010). An earlier study showed that soluble Au can induce the expression of the Cu efflux system (Cop Cluster) in *C. metallidurans* (Wiesemann et al., 2013). In the present study, the up-regulation of the periplasmic copper-binding protein (CopC) was observed only in the bacteria-gold system. The protein CopC could contribute to bacterial Au resistance as it may act as a metallothionein-like protein, and possesses the ability to interact with Au^{3+}/Au^{+} as well as other heavy metals such as Ag^{+} (Li and Yang, 2014). Although CopC was not detected in the bacteria-copper system, the RNA chaperone protein ProQ that helps controlling cellular osmolarity was expressed in both metal systems but not in the metal-free control.

Relative to gold, copper is more common and soluble under Earth's surficial conditions and, therefore, has greater mobility and bioavailability (Orell et al., 2010). However, within microenvironments surrounding the gold particles, resident bacteria likely encounter toxic mobile gold complexes more than copper complexes. In such circumstances, along with other proteins, copper homeostasis systems can be co-utilised by the bacteria to mediate the toxicity of Au (Wiesemann et al., 2013). In this study, several other transporters were upregulated including the Mg/Co/Ni transporter (MgtE) and the multidrug efflux system permease subunit (AcrB), which is structurally similar to the copper efflux system membrane protein CusA (Kulathila et al., 2011). Additionally, upregulation of the glutathione ABC transporter periplasmic binding protein (GsiB) is consistent with the study by Wiesemann et al. (2017), who demonstrated that Au^{+} can be exported from the cell cytoplasm to the periplasmic space (Fig. 5). Therefore, it is reasonable to argue that the active cell-mediated response of bacteria against Au toxicity may vary from organism to organism. The response also depends on environmental factors such as pH, water- and nutrient availability, or concentrations of other metals, which all together control the mobility of gold on Earth's surficial environment.

5. Practical implications and future directions

This study provides valuable insights into the role of indigenous bacteria in the dissolution and re-precipitation (re-dox reaction) of gold from particles, as well as their impact on the environmental mobility and toxicity of gold. The identification of gold-responsive bacterial proteins and their mechanisms involved in the detoxification of soluble gold open ups the possibilities to engineer indigenous bacterial strains with enhanced gold tolerance and bioremediation capabilities. Further research could focus on developing biotechnologies that utilise these bacteria (i) to extract gold from low-grade ores or mining waste, (ii) for the efficient and eco-friendly removal of gold from contaminated soils, sediments, and wastewater and (iii) could be used as a bio-indicator to monitor or assess gold contamination levels in active or abandoned gold mines and potential risks in natural ecosystems.

6. Conclusion

The results indicate that bacterial resistance to soluble Au involves a combination of different cellular/biochemical responses mainly involving thiol replenishment, antioxidant/repair systems, efflux, and maintenance of cellular structural integrity. Collectively, these responses broadly reflect the structure-function relationship of how up-regulated proteins enabled *S. proteamaculans* to mediate the cytotoxic effects of soluble gold. This study highlights how gold-tolerant and viable bacteria could withstand the toxic effect of soluble gold derived from particle dissolution events. The number and extent of proteins expressed as a response to soluble gold "stress" in the model system investigated here would vary under *in-situ* conditions, especially in environments where natural biogeochemical processes have been impacted by anthropogenic activity. By connecting the fields of molecular bacteriology and environmental biogeochemistry, this study provides an understanding of bacterial contributions to gold particle transformation i.e., gold biogeochemistry. Moreover, these insights could contribute to the development of bioremediation strategies utilising indigenous bacteria for the recovery/removal of gold from contaminated soils, sediments, and wastewater, thereby aiding in environmental management and sustainable resource utilisation.

Credit authors statement

Santonu K. Sanyal: Conceptualisation, performing the experiments, data presentation, investigation. Writing the initial draft. **Tara Pukala**: Proteomic experiment design and method development **Parul Mittal:** Helping in Proteomic experiment, data analysis. **Frank Reith:** Conceptualisation, supervision and design of the methodology. **Joël Brugger:** Critical review data analysis and final editing. **Barbara Etschmann:** Critical review and Manuscript editing, **Jeremiah Shuster:** Conceptualisation, Microscopy, supervision, manuscript editing and project management.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

Proteomic analysis and electron microscopy analysis were performed at the Adelaide Proteomics Centre and Adelaide Microscopy, respectively, at the University of Adelaide. This research was funded by the Australian Research Council grant (ARC-FT100150200) awarded to the late Frank Reith (c/o JS). The support Frank provided to his research group and colleagues was inspiration to pursue this study and see it to fruition despite his lamentable absence. Therefore, this story is dedicated inrespect to him and his passion for gold geomicrobiology research – we miss you, Frank.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2023.139657.

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