Metallomics

PAPER

Synergistic gold–copper detoxification at the core of gold biomineralisation in *Cupriavidus metallidurans*†

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L. Bütof,[a] N. Wiesemann,[a] M. Herzberg,[a] M. Altzschner,[b] A. Holleitner,[b] F. Reith[c] and D. H. Nies *[a]

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The bacterium *Cupriavidus metallidurans* is capable of reducing toxic Au(I/III)-complexes into metallic gold (Au) nano-particles, thereby mediating the (trans)formation of Au nuggets in Earth surface environments. In this study we describe a novel detoxification pathway, which prevents synergistic copper (Cu)/Au-toxicity. Gold-complexes and Cu-ions exert cooperative toxicity, because cellular uptake of Au(I/III)-complexes blocks Cu(I) export from the cytoplasm by the Cu-efflux pump CupA. Using a combination of micro-analytical and biochemical methods we show that inducible resistance to these Cu/Au mixtures is mediated by the periplasmic Cu(I)-oxidase CopA, which functions as an oxygen-consuming Au(I)-oxidase. With high Au-complex loads the enzymatic activity of CopA detoxifies the reduction pathway of Au(III)-complexes *via* Au(I)-intermediates to Au(0) nanoparticles in the periplasm. Thereby the concentration of highly toxic Au(I) in the cytoplasm is diminished, while allowing direct reduction of Au(III) to Au nanoparticles in the periplasm. This permits *C. metallidurans* to thrive in Au-rich environments and biomineralise metallic Au.

Significance to metallomics

The metallophilic bacterium *C. metallidurans* can live in the presence of a mixture of toxic transition metal ions, which occur also in auriferous soils. Here, Cu ions and Au complexes exert synergistic toxicity because Au(I) inhibits CupA, the main efflux system for surplus cytoplasmic Cu(I). Using the copper-containing periplasmic Au(I) and Cu(I) oxidase CopA, the ions are oxidized to Au(III) and Cu(II), respectively, which prevents their uptake into the cytoplasm and the subsequent synergistic toxicity, and which allows direct reduction of Au(III) to periplasmic Au(0) nanoparticles without using the toxic Au(I) intermediate.

Introduction

A biogeochemical cycle of Au exists, and archaea as well as bacteria are involved in every step, from the formation of primary Au deposits to its solubilisation, dispersion and re-concentration as secondary gold under surface conditions.[1] Because mobile Au(I/III)-complexes are very toxic,[2] cells interacting with these compounds, *e.g.*, in biofilms on Au nugget surfaces,[1] need Au resistance systems for their protection. Although bacteria resistant to a range of transition metals were isolated and their molecular resistance mechanism elucidated, to date there are only few examples for Au resistance.[3] Outside of the cells, Au-complexes may be sequestered by chalkophores, siderophores and other metallophores, which induce reduction of the Au-complexes to metallic Au and consequently detoxification.[4,5] Archaea and bacteria containing an Fe(III)-reductase can precipitate and actively reduce Au-complexes.[6,7] In the periplasm of Gram-negative bacteria, Au(I) may be exported by transenvelope efflux systems to protect this compartment against redox stress.[8] Thereby, Au(I) appears to be the species of Au that enters the cytoplasm, where it causes oxidative stress.[9,10] Here, Au(I) binds to CueR/CupR-type regulators of the MerR family of regulators, which control expression of genes for Cu-exporting P-type ATPases and other enzymes involved in Cu resistance.[11] CueR from *Escherichia coli*, GolS from *Salmonella* and CupR from *Cupriavidus metallidurans* are activated by Au-complexes,[12–14] leading to synthesis of the CopA, GolT and CupA efflux pumps, respectively. While GolT is able to export Au ions,[15] CopA is not[16] and CupA is inhibited by ions of this precious metal.[17]

[a] *Molecular Microbiology, Institute for Biology/Microbiology, Martin-Luther-University Halle-Wittenberg, Kurt-Mothes-Str. 3, 06120 Halle (Saale), Germany. E-mail: d.nies@mikrobiologie.uni-halle.de; Tel: +49-345-5526352*

[b] *Walter Schottky Institut and Physik-Department, Technical University Munich, Garching, Germany*

[c] *The University of Adelaide, School of Biological Sciences, Adelaide, South Australia 5005, Australia*

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The β-proteobacterium *Cupriavidus metallidurans* can grow in the presence of highly concentrated mixtures of transition metals, such as Zn, Ni, Cr and Cu.[18–20] Gold-rich environments, such as weathering Au deposits, auriferous soils and sediments as well as biofilm layers on Au nugget surfaces, contain highly toxic mobile Au-complexes in addition to a challenging assortment of transition metals.[17,21,22] While *C. metallidurans* is able to fend off the threat caused by other transition metals using its powerful, plasmid-located metal resistance determinants,[19] Cu-ions and Au-complexes exert a synergistic toxicity that cannot be mollified by the plasmid-encoded systems.[17] Recent research has shown that Au(III)-complexes are rapidly reduced to intermediate Au(I)-species by *C. metallidurans* cells and Au(I)-species are subsequently imported into the bacterial cytoplasm,[9,10,23] where they exert toxic effects.[9,13]

In the cytoplasm the Cu-exporting P-type ATPase CupA is a major contributor to Cu resistance in *C. metallidurans*.[17] Note: this efflux pump was named "CupA" because the name "CopA" had been previously assigned to the periplasmic Cu-oxidase, an ortholog of the long-known *Pseudomonas* enzyme[24,25] and CueO from *Escherichia coli*.[26] Since the Cu(I)-export ability of CupA is inhibited by Au(I)-complexes reaching the cytoplasm,[17] Au(I)-mediated inhibition of CupA-dependent Cu(I)-export in *C. metallidurans* leads to increased accumulation of cell-bound Cu-ions and to synergistic Cu/Au toxicity. Synergistic Cu/Au toxicity is also accompanied by increased accumulation of Au in form of periplasmic Au nanoparticles.[17]

Because Cu(I) can no longer be exported in the presence of toxic cytoplasmic Au(I), *C. metallidurans* had to evolve a mechanism to survive synergistic Cu/Au toxicity in Au-rich environments. This could occur by preventing Cu(I) and Au(I) accumulation in the cytoplasm. As an efflux pathway for cytoplasmic Au has not been identified in *C. metallidurans*, import of Au(I) into the cytoplasm could be decreased, thus mediating Au resistance. Indeed, pre-incubation of *C. metallidurans* with Cu-ions results in increased resistance to Cu/Au mixtures, indicating the presence of such an inducible Cu/Au resistance system.[17]

The two native plasmids of *C. metallidurans* with their powerful metal resistance determinants were not required for this inducible Cu/Au resistance. Consequently, the responsible determinant must be located on one of the two chromosomes.[17] The chromosomal *copABCD* determinant of *C. metallidurans* is up-regulated in the presence of Au-complexes[9] and required for full Au-resistance.[10] The gene product of *copA* belongs to the CueO/CopA/PcoA group of oxidases of the cupredoxin superfamily,[27,28] an important class of enzymes, which reduce molecular oxygen to water with concomitant one-electron oxidation of their specific substrates.[29] The periplasmic CopA protein is widespread across the bacterial world, and oxidises highly toxic Cu(I) to less toxic Cu(II).[27] The genes *copBCD* encode helper proteins needed to insert, *i.e.*, metallate, a Cu cluster into the CopA protein, because Cu-ions also form the active centres of these Cu-dependent oxidases.[27,28,30,31] Copper(II) is reduced to Cu(I) in the periplasm upon contact with reduced components of the respiratory chain,[32] and as a Na(I) analogue much more rapidly imported in the cytoplasm than Cu(II).[33,34] Oxidation of Cu(I) to Cu(II) by CueO/CopA/PcoA-like enzymes therefore decreases Cu uptake into the cytoplasm.[33,34]

Since bacterial communities associated with Au deposits are significantly enriched in CopA,[35] the enzyme may be required to decrease the uptake of Au(I) into the cytoplasm. The redox potential of the $Au(III)Cl_4^-/Au(0)$ is E° = +1002 mV,[36] close to that of the molecular O_2/H_2O couple, E° = +1229 mV.[36] Instead of molecular O_2, CopA might use $Au(III)Cl_4^-$ as electron acceptor to oxidise Cu(I) back to Cu(II) (E° = +153 mV) with a ΔE° = +849 mV. If CopA is such an Au(III)-dependent Cu(I)-oxidase, CopA would be central to formation of Au nanoparticles by *C. metallidurans* when growing in Au-rich environments.[37–39] However, although Au is mainly accumulated as periplasmic Au nanoparticles, CopA is not essential for this process,[17] falsifying this hypothesis.

Alternatively, the substrate-specificity of the periplasmic O_2- and Cu-dependent oxidase CopA may include Au(I) in addition to Cu(I). CopA would re-oxidise Au(I) back to $Au(III)Cl_4^-$ using molecular O_2 as electron acceptor, a reaction with ΔE° = +518 mV.[36] This also would decrease import of Au(I) into the cytoplasm.[9,10,23] At the same time, the direct reduction from Au(III) to Au(0) (E° = +1002 mV[36]) would still be possible with reduced components of the respiratory chain serving as electron donators reminiscent to the reduction of Cu(II).[32] This direct reaction would not require CopA and the function of CopA in this scenario would be to shift the reduction of Au(III) to Au(0) from a pathway involving the toxic Au(I) intermediate to the direct Au(III)/Au(0) reaction. To test this hypothesis, we first demonstrated the role of *copABCD* in the inducible resistance to Cu/Au mixtures. Secondly, we studied the Au-transforming abilities of CopA.

Results

The *copABCD* determinant is required for inducible Cu/Au resistance

As published[10] deletion of *copABCD* resulted in decreased Au resistance in *C. metallidurans* strain AE104. While the parent strain AE104 displayed increased resistance to Cu/Au mixtures when pre-incubated with 100 μM Cu(II)-chloride,[17] pre-incubation of the Cu-sensitive Δ*cop* strain with 50 μM $CuCl_2$ did not increase resistance to a Cu/Au mixture compared to untreated cells (Fig. 1). This indicates that *copABCD* is required for inducible resistance to Cu/Au mixtures.

Without presence of the respective other metal, the number of Cu and Au atoms in strain AE104 and its Δ*cop* mutant was similar and approximated 100 000 Cu and 50 000 Au atoms per cell (Table 1 and Table S1, ESI†). Presence of both metals increased Cu content in AE104 cells 1.7-fold and Au content 3.5-fold,[17] and in Δ*cop* cells Cu content 2.5-fold and Au-content 2.3-fold (Table 1 and Table S1, ESI†). In Δ*cop* cells grown in a Cu/Au mixture, Cu content had significantly increased (2-fold), whereas Au content decreased (1.7-fold) compared to the parent strain. Therefore, the Cop system is required to decrease the cellular Cu content and to increase the Au content in the presence of the Cu/Au mixture. Pre-incubation of the cells in the presence of Au showed no effect on these numbers (Table 1 and Table S1, ESI†).

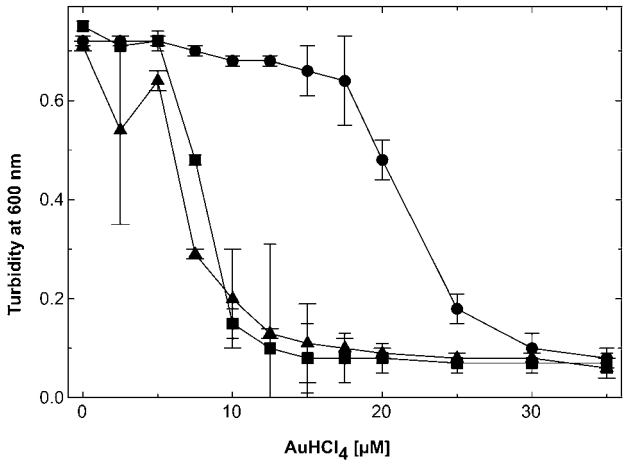

Fig. 1 Gold resistance of *C. metallidurans* of the Δ*copABCD* mutant of strain AE104. Cells were cultivated with increasing Au concentrations in the presence or absence of $CuCl_2$ in a 96 well plate in Tris-buffered mineral salts medium for 20 h with shaking at 30 °C. The pre- and main culture additionally contained copper or not: no Cu additions in pre- and main culture (closed circles, ●), 50 μM Cu only in the main culture (closed squares, ■), 50 μM Cu in both cultures (closed triangles, ▲). The optical density (O.D.) at 600 nm was determined in a TECAN multiple plate reader. Deviations shown, $n \geq 3$.

Table 1 Metal content of *the* Δ*copABCD* mutant[a]

Addition to the		1000 atoms per cell	
Pre-culture	Main culture	Copper	Gold
None	None	12 ± 3	3 ± 3
None	Au	*3 ± 1*	46 ± 4
	Cu	112 ± 27	0 ± 0
	Cu/Au	**283 ± 64**	**104 ± 6**
Cu	Au	62 ± 9	119 ± 37
	Cu	97 ± 32	2 ± 1
	Cu/Au	**149 ± 9**	**201 ± 11**
Au	Au	11 ± 5	64 ± 18
	Cu	82 ± 4	8 ± 2
	Cu/Au	**194 ± 15**	**116 ± 4**

[a] Cells of the Δ*copABCD* mutant of *C. metallidurans* strain AE104 were pre-incubated for 24 h in the presence of 2.5 μM Au(III)-chloride, 100 μM Cu(II), or without addition, and diluted 50-fold into the main culture. At a turbidity of 100 Klett units, 2.5 μM Au(III)-chloride, 100 μM Cu(II)-chloride, both metals or none of them were added, incubation was continued with shaking at 30 °C until 150 Klett units were reached (about 160 min). The cells were harvested, washed, and the metal content was determined by ICP-MS. Deviations of three independent experiments indicated. Table S1 (ESI) gives the comparison with the published[17] data for the parent strain AE104.

As published, pre-incubation with Cu(II) induced a lower Cu and Au content in Cu/Au-grown AE104 cells (ESI,† Table S1[17]). In contrast, Δ*cop* cells contained twice as many Cu and Au atoms when grown in the Cu/Au-mixture compared to AE104 cells (Table 1 and Table S1, ESI†). This is despite being cultivated in a Cu-amended pre-culture: Cu-pre-treated Δ*cop* cells grown in a Cu/Au mixture contained the same number of Cu and Au atoms (about 140 000 Cu, 190 000 Au) than not induced AE104 parent cells under the same conditions (ESI,† Table S1). This indicated that the Cop system was responsible for the Cu-inducible resistance of strain AE104 to Cu/Au mixtures. Cop mediated this resistance by decreasing the accumulation of either metal by half.

CopA is a Au(I) oxidase

To directly assess the effect of CopA on Au(I/III)-complexes, CopA from *C. metallidurans* was produced in *E. coli* and purified. CopA contained 0.69 mol Cu and 0.56 mol Zn, 1.3 mol Mg and traces of other metals per mol of protein, instead of four mol Cu in a fully metallated active centre.[30,40] Recent evidence indicates that the CopA ortholog CueO of *E. coli* is transported in an undermetallated state from the cytoplasm to the periplasm,[41] explaining why CopA was not fully metallated in *E. coli* cells.

Activity of CopA was tested using fluorescent oxygen nanoprobe particles (Fig. 2 and Fig. S1, ESI†); these are quenched by the presence of molecular O_2 and fluoresce when O_2 is consumed.[42] CopA indeed acted as Cu-dependent Au(I)-oxidase, and hence the first bacterial Cu(I)-oxidase described to be involved in Au transformation. Oxygen consumption by CopA, measured as un-quenching of the particles, was only demonstrated in the presence of Au(I)-thiosulfate and Cu(II), but not in any of the controls (Fig. 2 and Fig. S1, ESI†).

Presence of Cu(II) in the buffer solution was essential for enzymatic activity of CopA. The standard half-cell redox potential at pH = 0 of the Au(III)/Au(I) couple is 1.401 V and that of the Cu(II)/Cu(I) couple 0.153 V,[36] so that the given reduction of Cu(II) by Au(I) would have a potential of −1248 mV, calculated into a free energy of ΔG_o = +241 kJ mol^{-1}. Consequently, chemical reduction of Cu(II) by Au(I) followed by enzymatic O_2-consuming re-oxidation of Cu(I) by CopA should not be possible and could not serve as explanation for the Cu dependence of the Au(I)-dependent O_2 consumption by CopA. To rule this possibility out experimentally, CopA was loaded for 10 min on ice with a ten-fold higher amount of Cu(II), de-salted using a spin column, and subsequently its activity was tested (ESI,† Fig. S1B). The resulting CopA preparation did not contain

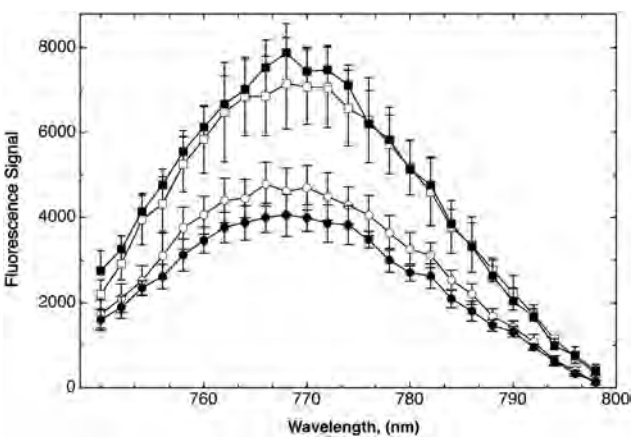

Fig. 2 CopA is a Cu-dependent Au(I) oxidase. Oxygen nanoprobe particles (0.1 g L^{-1}) that are quenched by molecular oxygen[42] were incubated for 10 min aerobically (open circles, ○, n = 7), anaerobically (open squares, □, n = 3), with CopA, Au(III)Cl_4^- plus Cu(II)Cl_2 (closed circles, ●, n = 5) or CopA, Au(I) plus Cu(II)Cl_2 (black squares, ■, n = 5), deviation bars shown, for the black squares only the upper part.

a measurable Cu or other metal content as determined by ICP-MS analysis (data not shown). While the major fraction of the enzyme had been largely inactivated by the loading and de-salting procedure, a minor fraction was still active. With this residual enzyme activity, O_2 consumption occurred significantly only in the presence of Au(I), but not of Au(III), and presence of additional Cu(II) no longer increased O_2 consumption (ESI,† Fig. S1B). Enzymatic activity of CopA was activated in the presence of Cu, probably by full metallation of the active site, but also rapidly inactivated again in the absence of Cu. Copper had a catalytic function in the enzyme but Cu(II)-dependent oxidation of Au(I) could be ruled out experimentally and for thermodynamic reasons.

This suggests that only fully metallated CopA was able to oxidise Au(I) to Au(III) to keep it from entering the cytoplasm, reminiscent of the detoxifying action of CopA with Cu(I).[28] Moreover, fully metallated CopA was very unstable *in vitro*, which might explain why the homolog CueO is *in vivo* synthesized and exported in *E. coli* in the under-metallated form.[41] Together, this demonstrated that CopA, as the only active enzyme of the *cop* determinant, indeed is able to mediate inducible resistance to Cu/Au mixtures by decreasing cellular accumulation of both metals: CopA is an O_2- and Cu-dependent Cu(I) and Au(I)-oxidase, rather than a Au(III)-dependent Cu(I) oxidase.

Effect of CopA on Au nanoparticle formation

To study the effect of CopA on Au nanoparticle formation we developed a new *in vitro* system. Since the redox potential of the Au(III)/Au(0) couple is more positive than that of the O_2/H_2O couple,[36] free Au(III)-ions are unstable in aqueous solution under standard conditions. However, Au(III) can be stabilised in aqueous solutions by complexing ligands, *e.g.*, halogenides, with chloride being the most important Au(III) ligand in natural environments.[22] A HEPES (4-(2-hydroxyethyl)-piperazin-1-ethanesulfonic acid) buffer with a sulfonic acid residue acted as Lewis base to sequester Au(III)-ions and subsequently allowed its reduction to Au nanoparticles with water as reducing agent. Water acted as substitute here of the (more) reducing environment of the *C. metallidurans* cell. Formation of Au nanoparticles was assessed using UV-VIS spectroscopy, single particle ICP-MS (spICP-MS) and helium ion microscopy (Fig. 3–5). Commercial citrate-stabilised Au nanoparticles served as positive control for all three analytical methods (ESI,† Fig. S2–S5).

Without CopA, addition of Au(III) to the HEPES system resulted in the formation of Au nanoparticles, as evidenced by an increase of absorption at 580 nm (Fig. 5) in comparison with the spectrum of citrate-stabilised commercial Au nanoparticles acting as positive control (ESI,† Fig. S2). The particles synthesised from Au(III) in HEPES, however, were unstable and hence not visible with either spICP-MS (ESI,† Fig. S3 and S4) or helium ion microscopy (Fig. 3A). Similar results were obtained in the presence of heat-inactivated CopA (Fig. 4A and 5). Addition of Au(I)-thiosulfate to HEPES never resulted in formation of Au nanoparticles (ESI,† Fig. S3 and S5) so that Au(I)-ions captured in an Au–S bond, which occurred also *in vivo* in *C. metallidurans*,[9] are indeed no efficient substrates for the formation of Au nanoparticles, at least under *in vitro* conditions in HEPES buffer.

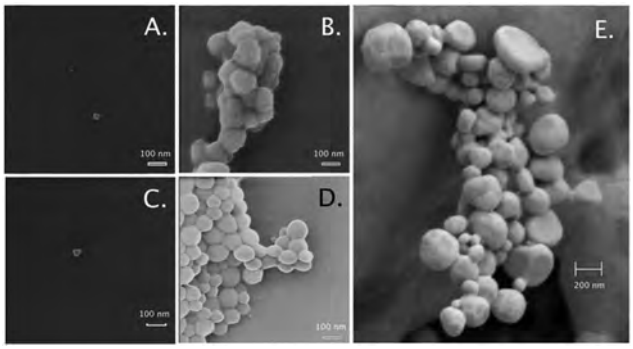

Fig. 3 Gold nanoparticles *in vivo* and *in vitro* using different microscopic techniques. Particles are shown by helium ion microscopy that are produced *in vitro* in 50 mM HEPES buffer pH 7 by 50 μM $Au(III)Cl_4^-$ alone (panel A), in the absence of 50 μM $Cu(II)Cl_2$ but presence of CopA (panel B), presence of copper only (panel C), or presence of 50 μM $Cu(II)Cl_2$ and CopA (panel D). See also Fig. S5 and S6 (ESI†). Panel E shows for comparison aggregates of nanometer-sized Au particles comprised of colloids from geomicrobial Au transformation (E).

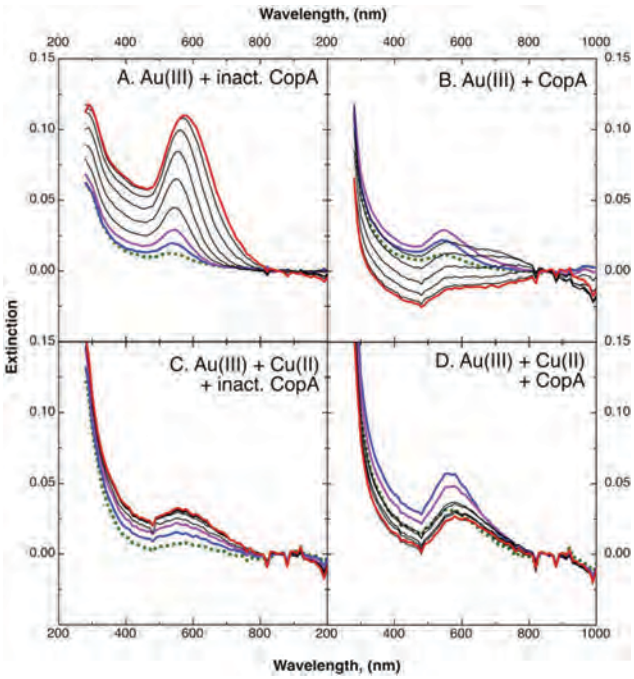

Fig. 4 Formation of aggregated Au nanoparticles by CopA. Time-dependent spectra of Au(III) in the presence of CopA. 50 μM Au(III) as chloride was incubated in 50 mM HEPES buffer (pH = 7.0) in the presence of heat-inactivated CopA (panel A), freshly isolated CopA (panel B), 50 μM Cu(II) chloride and heat-inactivated CopA (panel C), or the same concentration of Cu and freshly isolated CopA (panel D) in 96-well plates aerobically at 23.9 °C. A spectrum was determined between 280 nm and 1000 nm at 0 min (fat, green, dotted line), 7 min (blue line), 15 min (purple line), 20, 30, 35, 40, 50 min (black lines) and 60 min (dark red line). First, the spectrum of the buffer blank was subtracted. Secondly, from each spectrum the respective value at 850 nm was subtracted to move all spectra by parallel transformation to the 850 nm/0 point of the *x*-axis. Repetitions of experiments containing error bars are given in Fig. 5 in a time-dependent plot.

Addition of Cu(II) or CopA interfered with the synthesis of colloidal Au nanoparticles (Fig. 3B and C), but only the addition

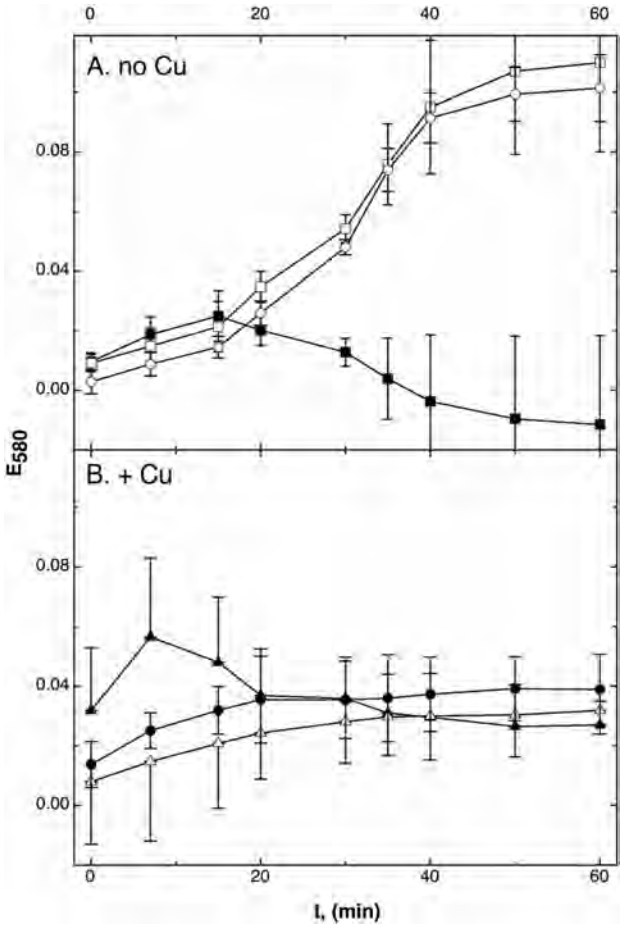

Fig. 5 Time-dependence of the extinction of Au(III) at 580 nm. Time-dependent spectra of Au(III) in the presence of CopA: 50 μM Au(III)-chloride was incubated in 50 mM HEPES buffer (pH = 7.0). The figure gives the extinction at 580 nm of the spectra as shown in Fig. 4. Panel A: Au(III) (open circles, ○), Au(III) plus heat-inactivated CopA (0.87 nM) (open squares, □), Au(III) plus CopA (0.87 nM) (closed squares, ■). Panel B: 50 μM Cu(II) plus Au(III) (closed circles, ●), Au(III) plus heat-inactivated CopA (0.87 nM) (open triangles, △), Au(III) plus CopA (0.87 nM) (closed triangles, ▲). n = 3, deviation bars shown, in panel B twice asymmetrically to avoid cluttering.

of both agents resulted in stable particles (Fig. 3D). Addition of Cu(II) alone inhibited the formation of Au nanoparticles (Fig. 5). Addition of CopA without Cu(II) mediated the formation of semi-stable larger Au aggregates without defined border (Fig. 3B and Fig. S5, ESI†). In contrast, addition of both CopA and Cu(II) to Au(III) in HEPES resulted in the formation of Au nanoparticles after a few minutes (Fig. 3). Stable Au nanoparticles occurred in large piles of smooth and round spheres with diameters of about 50 nm (Fig. 3D and Fig. S4D, S6, ESI†), while heat-inactivated CopA did not intervene (Fig. 4A and 5). Obviously, presence of other ions, Cu(II) or Au(I) intermediates not removed by CopA, interfered with the formation of Au nanoparticles. Thus, CopA re-oxidizes Au(I) back to Au(III), prevents uptake of Au(I) to the cytoplasm and allows efficient formation of Au nanoparticles by direct reduction of Au(III) to Au(0) (Fig. 6).

Discussion

A body of experimental evidence assigns CopA a triple function in resistance to Cu/Au mixtures (Fig. 6), these are: (I) decrease Cu accumulation by Cu(I) oxidation so that Au inhibition of Cu efflux by CupA is mollified; (ii) decrease cytoplasmic Au accumulation by Au(I) oxidation with the same effect; and (iii) remove Au(I) from Au(III) to Au(0) reduction pathways so that the toxic Au compounds are being transferred into stable Au nanoparticles as a sink for Au associated with *C. metallidurans* cells. The products of the chromosomal *cop* determinant were required for full Au resistance[10] and for Cu-inducible resistance to Cu/Au mixtures (Fig. 1). *C. metallidurans* cells cultivated in an Cu/Au mixture accumulated high amounts of both metals, leading to synergistic toxicity.[17] Copper was mainly located in the cytoplasm, Au in the periplasm as Au nanoparticles, which account for most of the cell-bound Au.[17] Presence of the Cop system influenced the number of Au atoms per cells grown in a Cu/Au mixture but the physiological conditions determined if Cop increased or decreased this number (Table 1). CopA acted

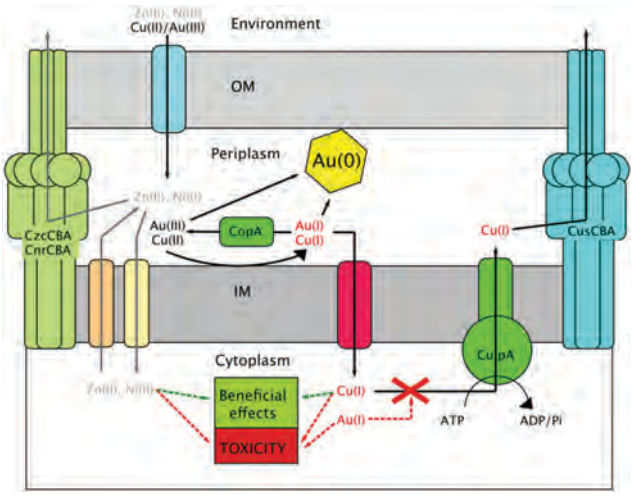

Fig. 6 Detoxification of Au-complexes as part of transition metal resistance in *C. metallidurans*. In the metal-rich environments *C. metallidurans* encounters cations of Zn, Ni (in grey) and Cu, but also Au(III)-complexes are imported into the periplasm of this Gram-negative bacterium by outer membrane (OM) porins (blue). Cations such as Zn(II) and Ni(II) are imported further on across the inner membrane (IM) into the cytoplasm,[43–45] exerting beneficial, but at high concentration also toxic effects. Surplus cytoplasmic Zn and Ni ions are exported back into the periplasm to counteract their toxic action by efflux systems (light orange) and from here further on to the outside by transenvelope efflux systems such as CzcCBA and CnrCBA (light green, left side).[19] In contrast to these ions, Cu(II) ions and Au(III) complexes are reduced upon contact with the *C. metallidurans* cell to Cu(I) and Au(I),[9,32,37] respectively, which are rapidly imported into the cytoplasm.[10,23] Cu(I) exerts its beneficial action mainly in the periplasm so that toxic and surplus Cu(I) is removed to the periplasm by the P-type ATPase CupA and further on to the outside by the transenvelope efflux system CusCBA (blue, right side). Efflux of Cu(I) by CupA is inhibited by cytoplasmic Au(I). *C. metallidurans* is able to counteract the resulting synergistic toxicity of cytoplasmic Cu(I) and Au(I) by CopA- and O_2-dependent oxidation to Cu(II) and Au(III). This reaction removes the toxic Au(I) intermediate and allows reduction Au(III) to stable metallic Au nanoparticles (golden hexagon) as final product with a low toxicity burden.

as Au(I)-oxidase when fully metallated in the presence of Cu (Fig. 2). CopA interfered with HEPES-mediated production of nanoparticulate Au (Fig. 3–5) leading to production of stable nanoparticles. Only fully metallated CopA produced ''perfect'' large and smooth nanoparticles while particles produced by under-metallated CopA were rough and smaller (Fig. 3B and D, compare also ESI,† Fig. S4D and E). Although CopA failed to liberate sufficient Au from Au(I)-thiosulfate to produce Au nanoparticles in HEPES buffer *in vitro*, CopA was obviously able to prevent toxic effects of Au(I) *in vivo*, *e.g.*, by inhibition of CupA after uptake into the cytoplasm, binding to thiol groups in the periplasm or negative interference with the formation of periplasmic Au nanoparticles serving as Au sink.

It is known since medieval times that Au(III)-complexes are reduced to colloidal nanoparticulate Au, the fabled '*aurum potabile*', upon contact with the reducing milieu of cells or their extracts.[46] This reduction process produces highly toxic Au(I)-intermediates. Gold(III)-complexes are also rapidly reduced to Au nanoparticles *in vitro* with water as electron donor if HEPES as buffer substance that contains a sulfonic acid group removes the protecting chloride groups. Another sulfonic acid-containing buffer substance, MOPS (3-morpholinopropane-1-sulfonic acid, functioned in a similar manner, but not Tris (data not shown). However, although presence of the resulting Au nanoparticles could be clearly demonstrated using UV-VIS spectroscopy, they were not stable enough to be seen by spICP-MS and helium ion microscopy.

In contrast, the presence of fully metallated CopA, which functions as Au(I)-oxidase, allowed the formation of stable Au nanoparticles from Au(III). Gold(I) the first product of the Au(III) reduction process by *C. metallidurans*,[9] appears in the cytoplasm because it binds to the cytoplasmic Cu chaperone CupC,[23] the cytoplasmic regulator CupR[10,13] and inhibits Cu efflux by the P-type ATPase CupA,[17] which results in synergistic Cu/Au toxicity. The chromosomal *copABCD* determinant is up-regulated by pre-treatment with Cu and Au,[9] CopA exported into the periplasm, possibly under-metallated as its ortholog CueO,[41] so that presence of Cu is also required for full catalytic activity of CopA. In the periplasm, CopA oxidises Cu(I) and Au(I) to Cu(II) and Au(III), respectively. Cu(II) is a worse substrate for uptake than Cu(I)[33,34] so that oxidation of Cu(I) to Cu(II) leads to decreased Cu accumulation in Cop-containing cells (Table 1). In the absence of Cu, CopA seems to lose its Cu cofactor rapidly, leading to inactivation of its enzymatic activity. This would prevent catalysis of Fenton reaction by an idle, fully Cu-loaded oxidase in the periplasm to protect the respiratory chain and periplasmic components.[47,48]

Oxidation of Au(I) back to Au(III) by CopA not only prevents uptake of Au(I) and/or formation of stable Au(I)–S adducts,[9] it also allows formation of stable Au nanoparticles by removal of Au(I). The reduction pathway of Au(III) *via* Au(I) to Au(0) may not lead to stable Au nanoparticles while direct reduction of Au(III) to Au(0) does, because, similar to high concentrations of Cu ions, Au(I)-intermediates could de-stabilize these particles by binding to their surface or integrating into the Au(0) crystal lattice.

C. metallidurans had to evolve this complicated Au resistance mechanism because its Cu-exporting P-type ATPase CupA was unable to export Au(I) similar to GolT from *Salmonella*[15] but was inhibited instead.[17] Nevertheless, this mechanism was necessary to survive in auriferous soils as standard *C. metallidurans* metal desert ecosystems[49] additionally containing Au-complexes (Fig. 6). Overall, CopA permits *C. metallidurans* and other CopA-positive bacteria to survive in highly Au-polluted environments, hence assigning *C. metallidurans* a key function in environmental Au cycling.[39]

Experimental

Microbial and molecular techniques

Bacterial strains and growth conditions. Strains used for experiments were the plasmid-free *C. metallidurans* strain AE104[50] and the Δ*copABCD* deletion mutant of strain AE104.[10] Tris-buffered mineral salts medium[50] containing 2 g L^{-1} sodium gluconate (TMM) was used to cultivate these strains shaken aerobically at 30 °C. Solid Tris-buffered media contained 20 g L^{-1} agar. Analytical grade heavy metal chlorides were used to prepare 0.5 or 1 M stock solutions, which were sterilized by filtration. Standard molecular genetic techniques were used.[51,52] All primer pairs, plasmids and bacterial strains are listed in Table S2 (ESI†).

Dose–response growth curves in 96 well plates. Growth curves for *C. metallidurans* were established in TMM. A pre-culture was incubated at 30 °C, 250 rpm up to early stationary phase, then diluted 1 : 20 in fresh medium without or with distinct metal concentration and incubated for 24 h at 30 °C and 250 rpm. Overnight cultures were used to inoculate parallel cultures with increasing metal concentrations in 96 well plates. Cells were cultivated for 20 h at 30 °C and 1300 rpm in a neoLab Shaker DTS-2 (neoLab, Heidelberg, Germany) and the optical density was determined at 500 nm or 600 nm as indicated in a TECAN infinite 200 PRO reader (TECAN, Männersdorf, Switzerland).

Purification of CopA. CopA without TAT-leader sequence was heterologous expressed in *E. coli* Rosetta (without pLysSRARE) with pG-Tf2 as a C-terminal Strep-TagII fusion protein using the vector pECD1569. The host cells were cultivated for 5 h in TMM (pH 7) with 2 g L^{-1} % glycerol and 3 g L^{-1} casamino acid anaerobically at 37 °C until a turbidity of 0.1 at 600 nm was reached. Expression of *copA* was induced by 100 μg AHT (anhydrotetracycline) per litre and incubation was continued anaerobic for 20 h at 30 °C. Cells were harvested by centrifugation (30 min, 7650 × *g*, 4 °C), suspended in 1 mL of buffer P (100 mM Tris–HCl, pH 8, 0.5 M sucrose) and incubated for 30 min on ice with 1 mM protease inhibitor PMSF (phenylmethylsulfonylfluoride) and DNase I (10 mg L^{-1}). The spheroplasts were separated from periplasm by centrifugation (30 min, 7650 × *g*, 4 °C). The pellet was suspended in buffer (100 mM Tris–HCl, pH 7, 150 mM NaCl) and the spheroplasts were ultra-sonicated. For purification of CopA, the cell debris and unbroken cells were removed by centrifugation (15 min, 23 400 × *g*, 4 °C). CopA was purified anaerobic using a Strep-tactin affinity chromatography column

according to the manufacturer's protocol (IBA GmbH, Göttingen, Germany). Fractions containing the CopA protein, monitored by SDS (sodium dodecylsulfate) polyacrylamide gel electrophoresis, were pooled and concentrated with Vivaspin concentrator columns (Sartorius AG, Göttingen, Germany). Purified CopA was analysed by mass spectrometry. The protein concentration was determined by Bradford (1976) assay,[53] using bovine serumalbumin as a standard or by using the NanoDrop®ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, United States).

For the co-factor reconstruction 10 μM of the purified CopA protein were incubated with 100 μM $CuCl_2$ for 2 h at 4 °C. To remove unbound Cu the solution was loaded on a PD10 column (Biorad, München, Germany) and the eluted protein was up concentrated with a 30 kDa VivaSpin column Sartorius AG, Göttingen, Germany) at low rpm.

Analytical methods

The formation of nanoparticulate Au was measured in a spectroscopic (extinction/absorbance spectra) assay in combination with single particle inductively coupled plasma mass spectrometry (spICP-MS) and helium ion microscopy (HeIM). Commercially available nanoparticles (5/50 nm Citrate BioPure Gold DAG2151/MGM2244, nanoComposix, San Diego, United States) with concentrations from 0.1 to 1 g L^{-1} served as controls. Controls exhibited an increase in absorption maxima with increasing Au concentration, as well as a shift of the maximum at 520 nm to 580 nm.[54] Particles were clearly visible in the HeIM and spICP-MS (ESI,† Fig. S3 and S5). The spectra were measured in a TECAN infinite m200 PRO reader (TECAN, Männersdorf, Switzerland) from 400 nm to 850 nm. For samples 1 mM $CuCl_2$, $Na_3Au(I)(S_2O_3)_2$ and/or $NaAu(III)Cl_4$ were diluted to final concentrations of 50 μM, respectively, with or without 0.85 nM CopA in 50 mM HEPES buffer, pH 7 or 50 mM MOPS buffer, pH 7. As negative control the assays were performed with $CopA_{WM}$, which was inactivated by heating to 95 °C for 10 min, or with 2.5 mM DTT or GSH.

Oxidase activity of CopA. To investigate the oxidase activity of CopA by O_2 consumption 0.1 g L^{-1} Oxygen Nanoprobes (Pyro Science GmbH, Aachen, Germany) with excitation maxima at 620 nm were used. The oxygen nanoprobes were anaerobically diluted into distilled water. Saturation of the nanoprobes with molecular oxygen from air was conducted before every experiment for at least 3 h. In the assay 0.1 g L^{-1} oxygen-saturated oxygen nanoprobes were incubated in 50 mM HEPES buffer (pH 7) with 0.85 nM $CopA_{WM}$, 50 μM $Cu(II)Cl_2$, $Na_3Au(I)(S_2O_3)_2$, $Na_2S_2O_3$ and/or $NaAu(III)Cl_4$. Oxygen consumption was measured for 30 min at 23 °C with an excitation wavelength of 620 nm. Emission was recorded at 750–800 nm in a 384-well plate with TECAN infinite m200 PRO reader (TECAN, Männersdorf, Switzerland). As controls served: (i) heat-inactivated CopA; (ii) only one compound; (iii) only two compounds; (iv) addition of 2.5 mM dithiothreitol or (v) reduced glutathione.

ICP-MS analysis. To determine the metal content of cells, cells were cultivated up to 100 Klett units in TMM and the different metal salts were added. When the cells reached the mid of their exponential phase of growth at 150 Klett units, 10 mL cell suspension were centrifuged for 30 min at 4500 × *g*. Cells washed twice with 50 mM Tris–HCl buffer containing 10 mM EDTA (pH 7). The supernatant was discarded and the residual liquid carefully removed at each step. The pellet was suspended in concentrated *aqua regia* (analytical grade 30% hydrochloric acid and 67% nitric acid in a 3 : 1 ratio; Normatom/PROLABO) and digested at 70 °C for 2 h. Samples were diluted to a final concentration of 5% hydrochloric acid and 2% nitric acid. Indium was added as internal standard at a final concentration of 10 ppb. Elemental analysis was performed *via* inductively coupled plasma mass spectrometry (ICP-MS) using ESI-sampler SC-2 (Elemental Scientific, Inc. Omaha) and an X-Series II ICP-MS instrument (Thermo Fisher Scientific, Bremen) operating with a collision cell and flow rates of 5 mL min^{-1} of He/H_2 (93%/7%; ref. 55), with an Ar carrier flow rate of 0.76 L min^{-1} and an Ar make-up flow rate at 15 L min^{-1}. An external calibration curve was recorded with ICP-multi-element standard solution XVI (Merck) in 5 vol% HCl. The samples were introduced *via* a peristaltic pump and analysed for their metal content. For blank measurement and quality/quantity thresholds, calculations based on DIN32645 TMM were used. The results were transformed from parts-per-million (ppm), parts-per-billion (ppb) or parts-per-trillion (ppt) *via* molar units into atoms per sample and divided by the number of cells per sample, which had been determined before as colony-forming units. Similarly, HNO_3 (trace metal grade, Normatom/PROLABO) was added to the CopA samples and the measurement was performed as described above.

Single particle ICP-MS (spICP-MS) elemental analysis was performed by inductively coupled plasma mass spectrometry (ICP-MS) using an ESI-sampler SC-2 (ESI Elemental Service & Instruments GmbH, Mainz, Germany) and a X-Series II ICP-MS instrument (Thermo Fisher Scientific, Bremen, Germany), equipped with a Miramist nebuliser and cyclonic spray chamber (ESI). All samples and nanoparticle solutions were prepared using analytic grade water for dilution and aspirated by a peristaltic pump directly into the plasma. Nanoparticle produces an ion plume in the plasma with a process duration of ~300–500 μs, produce a Single Particle Event (SPE) and were measured with dwell time of 5 ms in time depend scan of 400 ms, in triplicates. Standard mode was used for all measurements of Au_{197} with an Ar carrier flow rate of 0.76 L min^{-1} and an Ar make-up flow rate at 15 L min^{-1}.

Helium ion microscopy is a novel and unique method for investigating and structuring sub-micron features. Helium atoms are ionised at a W tip and accelerated onto a sample. Due to the higher mass of these helium ions with respect to electrons, which are used at scanning electron microscopy, higher resolutions can be achieved. The helium ions penetrate deeper into the surface leading to a smaller area from which secondary electrons, used to generate an image, leave the sample. An ORION Nanofab of ZEISS was operated at 28 to 30 kV acceleration voltage with a beam current of approximately 1 pA. As a substrate for the samples a silicon wafer (orientation ⟨100⟩, thickness 500 ± 25 μm) was cleaned with isopropanol and plasma treated (Electron Microscopy Sciences) at 2 × 10^{-1} mbar

with 35 mA for 45 s. 10 μL of the samples (respectively 5 μL of sample 5 nM Au) were pipetted onto the substrate, pre-dried and finally outgassed at medium vacuum using a desiccator.

Statistics. Students' *t*-test was used but in most cases the distance (*D*) value, *D*, has been used several times previously for such analyses.[10,45,56] It is a simple, more useful value than Student's *t*-test because non-intersecting deviation bars of two values ($D > 1$) for three repeats always means a statistically relevant ($\geq$95%) difference provided the deviations are within a similar range. At $n = 4$, significance is $\geq$97.5%, at $n = 5 \geq 99\%$ (significant) and at $n = 8 \geq 99.9\%$ (highly significant).

Conclusions

Here, we show on the molecular level how and why the bacterium *C. metallidurans* "makes" gold in its natural environment. In auriferous soils, most metals do not interfere with the resistance of this bacterium to toxic gold complexes and can be removed from the cell by plasmid-encoded metal efflux systems. Exception is copper: gold ions block export of toxic intracellular copper ions, leading to synergistic Au/Cu toxicity. *C. metallidurans* solves this problem by oxidation of Au(I) and Cu(I) ions in the periplasm to Au(III) and Cu(II), which prevents uptake of these ions, and also enhances formation of periplasmic gold nanoparticles from Au(III) as dead-end compound of the bacteriums' gold detoxification pathway.

Conflicts of interest

There are no conflicts to declare.

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