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Influence of Copper Resistance Determinants on Gold Transformation by *Cupriavidus metallidurans* Strain CH34

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***Cupriavidus metallidurans* is associated with gold grains and may be involved in their formation. Gold(III) complexes influence the transcriptome of *C. metallidurans* (F. Reith et al., Proc. Natl. Acad. Sci. U. S. A. 106:17757–17762, 2009), leading to the upregulation of genes involved in the detoxification of reactive oxygen species and metal ions. In a systematic study, the involvement of these systems in gold transformation was investigated. Treatment of *C. metallidurans* cells with Au(I) complexes, which occur in this organism's natural environment, led to the upregulation of genes similar to those observed for treatment with Au(III) complexes. The two indigenous plasmids of *C. metallidurans*, which harbor several transition metal resistance determinants, were not involved in resistance to Au(I/III) complexes nor in their transformation to metallic nanoparticles. Upregulation of a *cupA-lacZ* fusion by the MerR-type regulator CupR with increasing Au(III) concentrations indicated the presence of gold ions in the cytoplasm. A hypothesis stating that the Gig system detoxifies gold complexes by the uptake and reduction of Au(III) to Au(I) or Au(0) reminiscent to detoxification of Hg(II) was disproven. ZupT and other secondary uptake systems for transition metal cations influenced Au(III) resistance but not the upregulation of the *cupA-lacZ* fusion. The two copper-exporting P-type ATPases CupA and CopF were also not essential for gold resistance. The *copABCD* determinant on chromosome 2, which encodes periplasmic proteins involved in copper resistance, was required for full gold resistance in *C. metallidurans*. In conclusion, biomineralization of gold particles via the reduction of mobile Au(I/III) complexes in *C. metallidurans* appears to primarily occur in the periplasmic space via copper-handling systems.**

Gold was previously thought to be inert and immobile under Earth surface conditions, and hence not biologically active, but recent research has documented the occurrence of a biogeochemical cycle of gold in the environment (1). Microbial weathering of gold-bearing minerals contributes to the mobilization of gold by releasing elemental gold trapped within minerals and by solubilizing gold via oxidation-promoting complexation, for instance with thiosulfate or cyanide (1, 2). Subsequent microbial destabilization of gold complexes coupled with precipitation and biomineralization can immobilize gold, completing the cycle (2). Secondary gold can occur as nanoparticulate, bacteriomorphic, sheet-like, and wire gold, as well as euhedral, hexagonal, octahedral, and triangular crystals and secondary grains (3).

In contrast to other heavy metals, gold does not form free ions in aqueous solution at surface conditions but occurs as metallic nanoparticles (0) and as aurous (I) and auric (III) complexes (4). Based on thermodynamic calculations and natural abundances of possible ligands, complexes with chloride, ammonium, thiosulfate, amines, and cyanide appear to be the most important complexes in surface solutions (see, for example, reference 1). Hence, the speciation of Au (i.e., oxidation state, complexing ligand, and stability of the aqueous complexes), and not only its concentration, determines its toxicity and consequently the genetic and biochemical responses of cells.

The betaproteobacterium *Cupriavidus metallidurans* strain CH34 contains a variety of metal resistance factors that allow it to thrive in metal-contaminated environments (5, 6). The respective metal resistance determinants are located on the two native megaplasmids pMOL28 and pMOL30 and on chromosomal DNA, mainly on chromosome 2 (6, 7). *C. metallidurans* dominated bacterial biofilms associated with secondary gold grains from three sites in Australia (8). The bacterium rapidly accumulates toxic Au(III) complexes from solution. This process is coupled to the swift formation of Au(I)-S complexes associated with the cells and the further transformation to Au(I)-C compounds and nanoparticulate Au(0). In particular, oxidative stress and copper resistances gene clusters are induced, possibly to promote cellular defense (9). The products of these gene clusters suggest gold-handling systems, involved in import of gold complexes into the cytoplasm, export of Au(I) back to the periplasm after reduction, and further chemical reduction to Au(0) in the periplasm.

Uptake of Au(III) complexes into the cytoplasm might constitute an active process to prevent a harmful action of Au(III) in the periplasm. This is reminiscent of binding of Hg(II) by MerP and uptake of Hg(II) by MerT to allow reduction to metallic Hg(0) by MerA, particularly since *mer* genes in *C. metallidurans* were also upregulated by Au(III) complexes (9). The strongest upregulation by Au(III) complexes observed (9) was that of a genomic region renamed here "*gig*" for "gold-induced genes." GigP (Rmet_4685) may be a small (9.7 kDa in the preform) putative periplasmic protein with a pI close to 9, GigA (Rmet_4684) a cytoplasmic

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31-kDa protein with a pI of 5, GigB (Rmet_4683) another 28-kDa cytoplasmic protein with a pI of 4.6, and GigT (Rmet_4682) a 17-kDa protein with three possible transmembrane spans. GigT is loosely related to DoxD, a subunit of the terminal quinol oxidase present in the plasma membrane of an archaeon (10). Just upstream in the opposite direction are genes for the extracytoplasmic function (ECF) (11) sigma factor RpoQ, followed by the gene for a putative anti-sigma factor (Rmet_4686/7). Both genes are transcribed as an operon (12). Expression of *rpoQ* was upregulated by copper and not by other metals tested. Although a Δ*rpoQ* deletion did not affect copper resistance in *C. metallidurans* (12), the *gig* products are candidates for an import system for Au(I/III) complexes.

Despite the commonly rather low concentrations of mobile gold in the environment, some *Salmonella* strains harbor homeostasis systems exclusively dedicated to gold complexes (13, 14). These resistance systems exhibit extensive similarities with those for copper. First, the P_{IB1}-type ATPase GolT transports Au(I) from the cytoplasm to the periplasm. Although *Escherichia coli* CopA is a Cu(I) and Ag(I) transporter, but not an Au(I) transporter (15), *Salmonella* CopA might also recognize Au(I) as a substrate (13). GolB is a cytoplasmic gold chaperone, probably shuffling the metal substrate to either efflux ATPase. Second, periplasmic Au(I) is exported by the GesCBA RND-driven system back to the outside. Control of *gol* gene expression is exerted by two MerR-type regulators: GolS and SctR, which is an ortholog of CueR from *E. coli* (13). *C. metallidurans* contains the *cupC/AR* gene region (see Results) in a divergon situation that was strongly upregulated by Au(III) complexes (9). The products of these genes exhibit a high similarities to the *gol* products from *Salmonella*: GolS/CupR with 45% amino acid identity and GolT/CupA with 40% amino acid identity. Thus, the products of the *cupC/AR* divergon are prime candidates for a possible Au(I) export system in *C. metallidurans*.

Due to the chemical similarities of Au(I) and Cu(I) ions, the products of gold-induced copper resistance factors may be involved in the export of Au(I) from the cytoplasm to the periplasm and in further modification of the gold complexes in this cellular compartment. Bacterial copper homeostasis has been studied in several bacteria (16, 17). Common motifs include (i) the export of surplus copper from the cytoplasm by P_{IB1}-type ATPases (TC 3.A.3) (18–21), often regulated at the level of transcription by MerR-type regulatory proteins such as CueR (22), (ii) cytoplasmic and periplasmic copper chaperones, (iii) the transport of copper ions to the outside of the cell by RND-driven (TC 2.A.6, resistance-nodulation-cell division protein family) transenvelope efflux complexes such as CusCBA (23–26) that are regulated by two-component regulatory systems (27), and (iv) oxidation of Cu(I) to Cu(II) in the periplasm. Unfortunately, there is some confusion with names since "CopA" designates a copper-exporting P_{IB1}-type ATPase from *E. coli* but also a periplasmic copper oxidase in other bacteria, including *C. metallidurans*. This bacterium contains a chromosomal *cupC/AR* determinant and a huge copper resistance cluster on plasmid pMOL30 (5, 28), which both might be involved in periplasmic modification of gold complexes.

With all of these candidates in mind, we investigated which of them are involved in gold biomineralization in *C. metallidurans*. Since Au(III) complexes are reduced to Au(I) complexes at the beginning of this process, differences in the transcriptome of *C. metallidurans* after treatment with Au(I) complexes compared to those with Au(III) as the central metal ion may sort the upregulated genes into groups involved in Au(III) uptake/reduction or subsequent Au(I) handling. Therefore, a gene array experiment was the first step, which compared the effect of Au(I) complexes to the published results obtained with Au(III) complexes. Second, we sought to determine whether gold complexes enter the cytoplasm. CupR from *C. metallidurans* is a MerR-type regulatory protein able to bind gold complexes (29). MerR-type regulators usually stay at their operator on the DNA and repress the respective downstream operon in the absence of their target metal but shift to an activator conformation when the target metal is bound (30). Upregulation of the *cup* determinant might therefore indicate the presence of gold in the cytoplasm of *C. metallidurans*. If there is uptake, the *gig* products may be involved in this process and P_{IB1}-type ATPases such as the GolT-ortholog CupA or its paralog CopF needed to transport gold ions back into the periplasm. Finally, the contribution of the megaplasmids pMOL28 and pMOL30 and that of the chromosomal *cop* determinant to gold resistance and biomineralization in *C. metallidurans* was evaluated.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used for experiments were *C. metallidurans* CH34 wild type, its plasmid-free derivative strain AE104 (7), and further derivatives of these strains. Tris-buffered mineral salts medium (7) containing 2 g of sodium gluconate (TMM)/liter was used to cultivate these strains aerobically with shaking at 30°C. Solid TMM contained 20 g of agar/liter. Analytical-grade salts of copper chloride ($CuCl_2$), silver nitrate ($AgNO_3$), sodium tetrachloroaurate(III) ($NaAuCl_4$), sodium gold(I) thiosulfate hydrate [$Na_3Au(S_2O_3)_2{\cdot}2H_2O$], potassium dicyanoaurate(I) [$KAu(I)CN_2$], and sodium aurothiomalate(I) ($C_4H_4AuNaO_4S$ [Sigma-Aldrich, USA, or Alfa Aesar, Germany]) were used to prepare 0.1 or 1 M stock solutions. To establish the initial composition of aqueous gold species in TMM was important because the gold speciation influences cellular responses, and it cannot be assumed that the addition of a solid gold compound, e.g., sodium tetrachloroaurate(III), to TMM will result in the formation of Au(III)-chloride complexes in TMM. Hence, a chemical model for the speciation of sodium tetrachloroaurate(III) hydrate, sodium gold(I) thiosulfate hydrate, and potassium dicyanoaurate(I) in TMM was calculated. Calculations were conducted using Geochemist's Workbench (GWB [31]). Thermodynamic properties were taken from the Lawrence Livermore National Laboratory database (version R9), and the properties for gold complexes were from Usher et al. (32); the thermodynamic properties for sodium aurothiomalate(I) were not available. Using the REACT routine in GWB, titration curves for the addition of up to 50 μM sodium tetrachloroaurate(III) hydrate, up to 500 μM sodium gold(I) thiosulfate hydrate, and 5,000 μM potassium dicyanoaurate(I), to TMM under thermodynamic equilibrium conditions were calculated. The results showed that in TMM amended with sodium tetrachloroaurate(III), >99.9% of gold exists as a Au(III)-tetraammonium complex. In TMM titrated with gold(I) sodium thiosulfate, >99.9% of gold exists as a Au(I)-thiosulfate complex, and in TMM amended with $Au(CN)_2^-$, >99% exists as a dicyanoaurate(I) complex (see Fig. S1 in the supplemental material).

Genetic techniques. Standard molecular genetic techniques were used (33, 34). For conjugal gene transfer, overnight cultures of donor strain *E. coli* S17/1 (35) and of the *C. metallidurans* recipient strains grown at 30°C in Tris-buffered medium were mixed (1:1) and plated onto nutrient broth agar. After 2 days, the bacteria were suspended in TMM, diluted, and plated onto selective media as previously described (33). All of the primer pairs used are listed in Table S1 in the supplemental material. Plasmid pECD1003 (36) was used to construct deletion mutants. It is a derivate of plasmid pECD889 (12) and therefore a derivative of plasmid pCM184 (37). These plasmids harbor a kanamycin resistance cassette flanked by

loxP recognition sites. Plasmid pECD1003 also carries an exchange of 5 bp at each *loxP* site. Using these mutant *lox* sequences, multiple gene deletions within the same genome are possible without interference and secondary recombination events (38, 39).

Deletion mutants. Fragments 300 bp upstream and downstream of the target gene were amplified by PCR and cloned into vector pGEM-T Easy (Promega, Madison, WI), further cloned into plasmid pECD1003, and sequenced. The resulting plasmids were used in a double-crossover recombination in *C. metallidurans* strains to replace the respective target gene by the kanamycin resistance cassette, which was subsequently also deleted by transient introduction of the *cre* expression plasmid pCM157 (37). Cre recombinase is a site-specific recombinase from the phage P1 that catalyzes the *in vivo* excision of the kanamycin resistance cassette at the *loxP* recognition sites. The correct deletions of the respective transporter genes were verified by Southern DNA-DNA hybridization. For the construction of multiple deletion strains, these steps were repeated. The resulting mutants carried a small open reading frame (ORF) instead of the wild-type gene to prevent polar effects. For gene disruption experiments, only the first step was performed.

MIC. The MIC was determined in triplicate as the lowest concentration inhibiting bacterial growth on solid TMM. A preculture was first incubated at 30°C and 250 rpm for 30 h, diluted 1:20 in fresh medium, and incubated for 24 h at 30°C and 250 rpm. This 24-h culture was diluted 1:100 in fresh medium and used for streaking onto plates containing different concentration of the respective metal salts. The plates were incubated at 30°C for 4 to 5 days, and cell growth was monitored.

Dose-response growth curves in tubes. Growth curves for *C. metallidurans* were conducted in TMM. A preculture was incubated at 30°C and 250 rpm for 30 h, diluted 1:20 in fresh medium, and incubated for 24 h at 30°C and 250 rpm. The culture was then diluted parallel 1:10 into fresh medium containing increasing metal concentrations. The cells were cultivated for 20 h at 30°C and 250 rpm, and the optical density (OD) was determined at 600 nm. To calculate the IC_{50} value (i.e., the metal concentration that led to turbidity reduction by half) and the corresponding *b* value (i.e., the measure of the slope of the sigmoidal dose-response curve), the data were adapted to the formula $OD(c) = OD0/\{1 + \exp[(c - IC_{50}]/b)\}$, which is a simplified version of a Hill-type equation as introduced by Pace and Scholtz (40) as published previously (41). The $OD(c)$ is the turbidity at a given metal concentration, OD0 is the turbidity with no added metal, and *c* is the metal concentration.

Dose-response growth curves in 96-well plates. Dose-response growth curves in 96-well plates were also determined in TMM. A preculture was incubated at 30°C and 250 rpm up to the early stationary phase, diluted 1:20 in fresh medium, 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 (Greiner). Cells were cultivated for 20 h at 30°C and 1,300 rpm in a neoLab Shaker DTS-2 (neoLab, Heidelberg, Germany), and the OD was determined at 500 or 600 nm as indicated in a Tecan Infinite 200 Pro Reader (Tecan, Männersdorf, Switzerland).

β-Galactosidase assay and *lacZ* reporter constructions. *C. metallidurans* cells with a *lacZ* reporter gene fusion were cultivated as a preculture in TMM containing 1.5 mg of kanamycin/liter at 30°C and 250 rpm for 30 h, diluted into fresh medium, and incubated at 30°C. At a cell density of 60 to 70 Klett units, metal salts were added in different concentrations, and the cells were incubated with shaking for a further 3 h. The specific β-galactosidase activity was determined in permeabilized cells as previously published, with 1 U defined as the activity forming 1 nmol of *o*-nitrophenol min^{-1} at 30°C (42). The *lacZ* reporter gene was inserted in several target genes to construct reporter operon fusions. This was done by single-crossover recombination in *C. metallidurans* strains. PCR products (300 to 400 bp) of the 3′-end regions of the genes *gigA*, *gigT*, *cupR*, *cupA*, and *cupC* were amplified from the total DNA of strain CH34, and the resulting fragments were cloned into plasmid pECD794 (pLO2-*lacZ*) (36). The respective operon fusion cassettes were inserted into the ORF of the target gene by conjugation and single-crossover recombination.

RNA isolation. *C. metallidurans* CH34 cells were cultivated as described above. At a cell turbidity of 100 Klett units, the different metal salts were added. After a 10- or 30-min incubation at 30°C, the cells were rapidly harvested at 20°C and stored at −80°C. Total RNA was isolated with an RNeasy minikit (Qiagen, Hilden, Germany) according to the manufacturers' instructions. A second DNase treatment with a precipitation step was performed. To exclude experimental artifacts resulting from DNA contamination, only RNA was used that did not generate products in a PCR with chromosomal primers. RNA concentration was determined photometrically, and RNA quality was checked on formamide gels (34) and measured as the RNA integrity number (RIN) on an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany).

Design of *C. metallidurans* microarrays. The genome sequence of *C. metallidurans* CH34 (NCBI reference sequence NC_007973.1/NC_007974.1/NC_007971.1/NC_007972.1; November 2008) was used by ImaGenes GmbH (Berlin, Germany) to design an Agilent custom microarray for all predicted ORFs and intergenic regions. At first a 2×244K array with seven different oligomers (50- to 60-mers) per target was produced on the basis of bioinformatic predictions from the sequence (6,426 ORFs; 3,465 intergenic regions) using the ImaGenes preselection strategy. A pool of five RNAs from differently treated cells (300 μM $ZnCl_2$, 50 μM $NaAuCl_4$, 0.5 mM EDTA, 1 mM H_2O_2, untreated) were hybridized to one 244K array (SurePrint Inkjet Technology; Agilent Technologies) to cover all expressed genes, and genomic DNA was hybridized to cover nonexpressed genes to a second 244K array. A total of 50,622 oligomers from the RNA hybridization and 55,188 oligomers from the DNA hybridization gave positive results and were used to extract the most informative oligomers with ImaGenes probe selection algorithm for a 15K array covering the whole genome of *C. metallidurans* with one to two oligomers per target ORF or intergenic region.

Microarrays of *C. metallidurans*. *C. metallidurans* strain CH34 wild-type was treated as follows: (i) 10 min without metals, (ii) 10 min with 500 μM $C_4H_4AuNaO_4S$ [Au(I)-thiomalate], (iii) 10 min with 500 μM $Na_3Au(S_2O_3)_2$ [Au(III)-thiosulfate], (iv) 10 min with 50 μM $KAu(CN)_2$ or (v) KCN, and (vi) 30 min with 5 mM $KAu(CN)_2$ or (vii) KCN. Each set of conditions was performed in triplicates, including three independent bacterial cultures. Additional controls were treated 10 min with 50 μM $NaAuCl_4$ and 30 min with 500 μM $KAu(CN)_2$ or KCN. Isolated and quality-checked RNA were provided to ImaGenes GmbH or IMGM Laboratories GmbH (Martinsried, Germany) for hybridization with an *C. metallidurans* Agilent Custom GE microarray (8×15K) with a one-color (Cy3)-based protocol. Signals were detected using an Agilent DNA microarray scanner. The software tool Feature Extraction 10.7.3.1 was used for raw data extraction.

In the algorithm used, the mean intensity of the pixels of the surrounding area was first subtracted from the mean density of the pixels of the spots to give the signal strength. Its deviation was half of the sum of both intensity deviations. The distance (*D*) value was the distance between spot and background pixel intensities divided by the sum of the deviations. *D* values were a more useful than the Student *t* test values because nontouching deviation bars of two values ($D > 1$) at three repeats always mean at least a significant (>95%) difference. Signals were further processed if $D > 1$. Following that, the mean values of the three biological repeats were calculated, along with their deviation and the smallest *D* value (D_{min}). Third, the values from different spots, positions, or oligonucleotides assigned to the same gene were taken to calculate a gene-specific mean value and minimum D value. Finally, the respective spot signals coming from various growth conditions were compared (Q values; $Q' = Q$ if $Q > 1$; otherwise, $Q' = 1/Q$).

Transmission electron microscopy. A preculture was incubated at 30°C and 250 rpm for 30 h, diluted 1:20 in fresh medium, and incubated for 24 h at 30°C and 250 rpm. Cells were harvested by centrifugation at 5,000 rpm for 10 min and suspended in TMM containing 50 μM

$NaAuCl_4$. After incubation for 72 h at 30°C on a shaking incubator at 100 rpm, the cells were fixed directly with 3% glutaraldehyde (Sigma, Taufkirchen, Germany) in 0.1 M sodium cacodylate buffer (SCB) for 3 h, centrifuged at 5,000 rpm for 5 min, and taken up in 4% agar-SCB, followed by one wash step with SCB overnight at 4°C and three wash steps with the same buffer for 5 min. After fixation with osmium tetroxide for 1 h, the samples were dehydrated in a series of ethanol (10, 30, and 50%). The cells were then treated with 1% uranylacetate–70% ethanol for 1 h and further dehydrated with a series of 70, 90, and 100% ethanol. Thereafter, the samples were infiltrated with epoxy resin according to the method of Spurr (43) and polymerized at 70°C. The ultrathin sections (80 nm) were observed with an EM900 transmission electron microscope (Carl Zeiss SMT, Oberkochen, Germany) operating at 80 kV. The images were recorded using a Variospeed SSCCD camera SM-1k-120 (TRS, Moorenweis, Germany).

Microarray data accession number. The microarray data were deposited in the GEO database at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE42199.

RESULTS

Influence of Au(I) complexes on gene regulation in *C. metallidurans*. Previous research had demonstrated that Au(III) complexes were taken up by *C. metallidurans* cells and reduced to Au(I) in a process leading to the upregulation of the genes involved, e.g., in mercury resistance, copper homeostasis, and oxidative stress response (9). Since *C. metallidurans* also faces Au(I) complexes in the environment (1), the effect of $Au(I)(CN)_2^-$, Au(I)-thiosulfate, and Au(I)-thiomalate on the global transcriptome of *C. metallidurans* was investigated. Moreover, this might also identify genes specifically involved in handling of Au(I) complexes (see Table S2 in the supplemental material) in contrast to those mediating accumulation of Au(III) complexes and/or the reduction of Au(III) to Au(I).

Compared to the effect of 50 μM KCN, 359 genes and the transcription of 223 intergenic regions were upregulated by 50 μM $KAu(I)CN_2$, bioavailable as $Au(I)(CN)_2^-$, according to calculations (see Fig. S1 in the supplemental material), whereas 182 genes and 85 intergenic regions were downregulated (data not shown). To assess the most important effects, only >10-fold up- or downregulation of genes was considered (see Table S2 in the supplemental material). With the exception of an intergenic region adjacent to the *lpdA* gene for a FAD-dependent dihydrolipoamide dehydrogenase, the gene most strongly upregulated (102-fold) was *cupR* for a MerR-type regulator of the Cup system. The adjacent genes in a divergon situation—(i) *cupC* for a possible cytoplasmic copper chaperone on the other DNA strand than *cupR* and (ii) upstream of *cupR* the gene *cupA* for a P_{IB1}-type copper ATPase—were also strongly upregulated. These high ratios, however, were also due to a very low expression signals coming from the *cupC/AR* genes in the cyanide-treated control cells (data not shown). Nevertheless, nine genes and six intergenic regions downstream of *cupAR* in the same, uninterrupted direction of transcription were also upregulated. Since the functions of their gene products are not connected to copper metabolism (e.g., a putative ABC-importer for branched amino acids), upregulation might have been a result of a readthrough following the transcriptional activity of the *cupAR* operon.

Other gene regions upregulated in the $Au(CN)_2^-/CN^-$ comparison fell into one of five categories: (i) detoxification of H_2O_2 (alkyl hydroperoxide reductase and catalase), (ii) mercury resistance determinants on chromosome 1 and on both megaplasmids pMOL28 and pMOL30, (iii) multiple drug resistance (the inner membrane exporter MatE and an HAE-RND-driven efflux system), (iv) genes in the vicinity of those encoding CzcJ-like metal response proteins (MmtQ and MmsQ), and (v) possible targets of a toxic action (LpdA). All of these genes were not downregulated in the presence of KCN compared to untreated cells (data not shown).

The genes most strongly downregulated were *appC* and *appD* for a cytochrome *bd* ubiquinol oxidase and genes in a putative operon with them (>67-fold), followed by genes for an isoenzyme of this type (CydAB, >25-fold), and other genes for enzymes of the central metabolism (see Table S2 in the supplemental material). These genes were downregulated in the $Au(CN)_2^-/CN^-$ comparison; however, they were not changed in the comparison of $Au(CN)_2^-$ to nontreated cells but strongly upregulated in the CN^-/untreated comparison (data not shown). Therefore, incubation in the presence of cyanide led to a strong upregulation of genes for cytochrome *bd*-containing ubiquinol oxidases and for some other genes of the central metabolism. The fact that this cyanide effect was prevented in the presence of Au(I) indicated that $Au(CN)_2^-$ was not able to poison cytochromes by the transfer of cyanide to the central iron atom.

In contrast to the strong transcriptomic response mediated by $Au(CN)_2^-$, only a few genes changed their expression significantly when *C. metallidurans* cells were treated with Au(I)-thiomalate and even fewer when they were incubated in the presence of Au(I)-thiosulfate (see Table S2 in the supplemental material). Au(I)-thiomalate led to a 2-fold upregulation of the genes for an HME-RND-driven efflux system related to CusCBA/F in *E. coli* (24) and a gene of unknown function in the vicinity of *cus* (but not in a putative divergon with *cus*) and to a 2- to 3-fold downregulation of the genes for enzymes required for sulfate reduction and sulfur metabolism. Obviously, *C. metallidurans* was able to use thiomalate as a convenient sulfur source. No gene was upregulated by Au(I)-thiosulfate, and four were downregulated, which encoded TonB-dependent outer membrane receptor proteins.

In summary, $Au(I)(CN)_2^-$ and Au(I)-thiomalate led to the upregulation of genes that were also upregulated by Au(III) complexes (9). The intensity of the response was highest in Au(III) complexes and $Au(CN)_2^-$, but the CN^- itself also caused a transcriptome response due to its toxicity, obscuring the effect of the Au(I) ions. Thus, Au(III) complexes remain the most suitable mobile form of gold for studies of the interaction of gold compounds on *C. metallidurans* cells, and the products of Au(III)-induced genes are ideal candidates for gold transporters and modifying systems.

Influence of megaplasmids pMOL30 and pMOL28 on gold resistance and biomineralization. *C. metallidurans* relies with its heavy metal resistance on its two megaplasmids, pMOL28 and pMOL30 (7), but only a few gene regions on these plasmids were upregulated by Au(III) complexes (9) or $Au(CN)_2^-$ (see Table S2 in the supplemental material), mainly the *cop* cluster on pMOL30 and the two *mer* determinants on either plasmid. When the resistance of *C. metallidurans* wild type and its plasmid-free derivative strain AE104 to Au(III) complexes and to Au(I)-thiosulfate was compared, however, there were no significant differences between both strains (Table 1). The resistance to Ag(I) was also similar, but strain CH34 was slightly more resistant to Cu(II) than was strain AE104.

This result was confirmed in liquid culture (Table 2). Wild-

TABLE 1 Resistance of derivatives of *C. metallidurans* wild type and its plasmid-free derivative strain AE104 to group 11 metals[a]

Bacterial strain	MIC (μM)			
	$NaAuCl_4$	Au(I)-thiosulfate	$CuCl_2$	$AgNO_3$
AE104	1	0.5	1,750	0.3
AE104Δ*cupC/AR*	1	0.5	**200**	0.3
AE104Δ*gigPABT*	1	0.5	1,750	0.3
AE104Δ*rpoQ*	1	0.5	1,750	0.3
AE104Δ*rpoQ-rsqA*	1	0.5	1,750	0.3
CH34	0.9	0.5	2,000	0.3
CH34Δ*cupC/AR*	0.9	0.5	**100**	0.2
CH34Δ*copF*	0.9	0.5	2,000	0.3
CH34Δ*cupC/AR*Δ*copF*	0.9	0.5	**50**	0.2

[a] Cells were precultivated for 30 h in TMM at 30°C with shaking, diluted 1:20 into fresh TMM, incubated again for 24 h at 30°C with shaking, diluted 1:100 into fresh TMM, plated onto TMM agar plates containing increasing metal concentrations, and incubated in the dark at 30°C. Growth was recorded as the ability to form colonies after 4 days. The three biological reproductions gave identical MICs. Thus, the deviation would be "±0" for each value and was omitted. Strongly decreased MIC levels are indicated in boldface. Au(I)-thiosulfate, $Na_3Au(S_2O_3)_2$.

type strain CH34 was slightly less resistant to Au(III) complexes than its megaplasmid-free derivative strain AE104, but the difference was not significant. However, CH34 was significantly (6-fold) less resistant to Au(I)-thiosulfate than was strain AE104. Therefore, the two megaplasmids did not mediate gold resistance, but they might increase the gold sensitivity of *C. metallidurans*.

Interestingly, the IC_{50} values for gold compounds, which define 50% growth inhibition in a dose-response experiment in a 20-h liquid culture, were much higher than the MIC data for growth in solid media after 4 days, e.g., for strain AE104 the MIC of $NaAu(III)Cl_4$ was 1 μM and the IC_{50} was 50 μM, and for Au(I)-thiosulfate the MIC was 0.5 μM but the IC_{50} was 130 μM (Tables 1 and 2). In contrast, these values were very similar for silver and copper compounds. For strain AE104, e.g., the MIC of silver nitrate was 0.3 μM and the IC_{50} was 0.65 μM, while for Cu(II) chloride the MIC was 1.74 mM and the IC_{50} was 1.17 mM. This indicates a difference in the ability of *C. metallidurans* to deal with gold toxicity depending on the growth conditions. Although a large number of planktonic cells, as present in liquid culture, are able to deal with toxic gold complexes, individual cells struggle to grow on solid Au-containing media.

In contrast to the untreated *C. metallidurans* cells, cells of strain CH34(pMOL28, pMOL30), AE104, AE126(pMOL28), or AE128(pMOL30) incubated in the presence of 50 μM Au(III) complexes displayed a fibrillary structure of the cytoplasm and single electron-dense metallic gold nanoparticles (strains CH34, AE104, and AE126 and the negative control in the case of strain CH34 [Fig. 1], AE128, and other negative controls [not shown]). This indicated that the plasmids pMOL28 and pMOL30 are not essential for the biomineralization of metallic gold particles.

Expression of the chromosomal *cup*, *cop*, and *gig* genes by group 11 metal cations and complexes. If the two megaplasmids pMOL28 and pMOL30 with the huge copper resistance determinant *cop* on pMOL30 and the two additional *mer* determinants on either plasmid are not required for gold resistance or transformation, the chromosomal copper resistance determinants and the gold-induced *gig* genes were the primary candidates that might encode a gold uptake (Gig) and efflux (Cup) system, respectively. Thus, the expression of the *gol*-homologous *cup* and the *gig* determinant was studied using *lacZ* reporter gene fusions, and this experiment also indicated the arrival of gold ions in the cytoplasm of *C. metallidurans*.

The genes *gigA* and *gigT* were most strongly upregulated when the cells were treated with Au(III) complexes (Table 3) but also to some extent by Cu(II), Ag(I), and Au(I)-thiosulfate. The genes were not influenced by Ni(II) treatment, which served as a control. Au(III) was indeed the best inducer for these genes at a concentration between 40 and 50 μM. Expression of the gene for the ECF sigma factor RpoQ, which is located adjacent to the *gig* genes on the other DNA strand in a divergon situation, was not influenced by one of the metals tested.

Au(III) complexes were also the best inducer for the three *cup* genes (Table 3). They were also upregulated after treatment with copper and silver, and—except for *cupR*—after treatment with Au(I)-thiosulfate but not by Ni(II). If the expression levels of the *gig* and *cup* genes were compared, the specific activity of *gig-lacZ* reporter gene fusion was very low in cells not treated with any metal, ~4 U mg (dry mass)$^{-1}$, and went up to a maximum specific activity of 646 ± 81 U mg^{-1} [*gigA-lacZ*, 50

TABLE 2 Resistance to group 11 metals in liquid culture of various derivatives of *C. metallidurans* wild type and its plasmid-free derivative strain AE104[a]

Bacterial strain	Mean IC_{50} (μM) and slope ± SEM of the sigmoidal dose-response curve							
	$NaAuCl_4$		Au(I)-thiosulfate		$CuCl_2$		$AgNO_3$	
	IC_{50}	Slope (*b*)	IC_{50}	Slope (*b*)	IC_{50}	Slope (*b*)	IC_{50}	Slope (*b*)
AE104	50.0 ± 9.4	12.7 ± 4.9	131 ± 5	70.9 ± 17.0	1,169 ± 19	61.5 ± 10.5	0.65 ± 0.08	0.13 ± 0.01
AE104Δ*cupC/AR*	56.4 ± 2.6	9.3 ± 0.3	128 ± 4	57.8 ± 2.9	154 ± 10	36.8 ± 16.1	0.70 ± 0.08	0.21 ± 0.06
AE104Δ*gigPABT*	48.2 ± 3.7	11.4 ± 1.6	128 ± 4	65.0 ± 6.3	1,146 ± 20	67.7 ± 18.8	0.67 ± 0.05	0.10 ± 0.02
AE104Δ*rpoQ*	56.8 ± 0.8	7.42 ± 1.22	129 ± 1	75.9 ± 50.5	969 ± 14	183 ± 14	0.67 ± 0.10	0.13 ± 0.03
AE104Δ*rpoQ-rsqA*	55.6 ± 4.4	9.43 ± 0.71	142 ± 8	115 ± 20	828 ± 13	180 ± 30	0.64 ± 0.02	0.12 ± 0.02
CH34	39.5 ± 5.9	12.2 ± 0.3	21.2 ± 10.3	80.8 ± 18.5	1,224 ± 40	385 ± 191	0.82 ± 0.09	0.16 ± 0.06
CH34Δ*cupC/AR*	31.5 ± 0.5	15.6 ± 0.6	33.7 ± 3.1	48.3 ± 16.2	214 ± 13	50.0 ± 9.4	0.92 ± 0.04	0.23 ± 0.04
CH34Δ*copF*	42.7 ± 4.8	11.7 ± 2.6	26.6 ± 11.9	120 ± 16	1,131 ± 21	140 ± 13	0.43 ± 0.05	0.12 ± 0.09
CH34Δ*cupC/AR*Δ*copF*	47.6 ± 0.3	10.5 ± 0.1	32.4 ± 10.9	119 ± 10	134 ± 4	76.9 ± 55.5	0.24 ± 0.05	0.12 ± 0.03

[a] Cells were precultivated for 30 h in TMM at 30°C with shaking, diluted 1:20 into fresh TMM, incubated again for 24 h at 30°C with shaking, diluted 1:10 into fresh TMM in parallel cultures containing various metal concentrations, and incubated for 20 h with shaking at 30°C (silvercultures were protected against light). The OD at 600 nm was determined and used to calculate the IC_{50} (half-maximum inhibition) and slope of the sigmoidal dose-response curve. Au(I)-thiosulfate, $Na_3Au(S_2O_3)_2$.

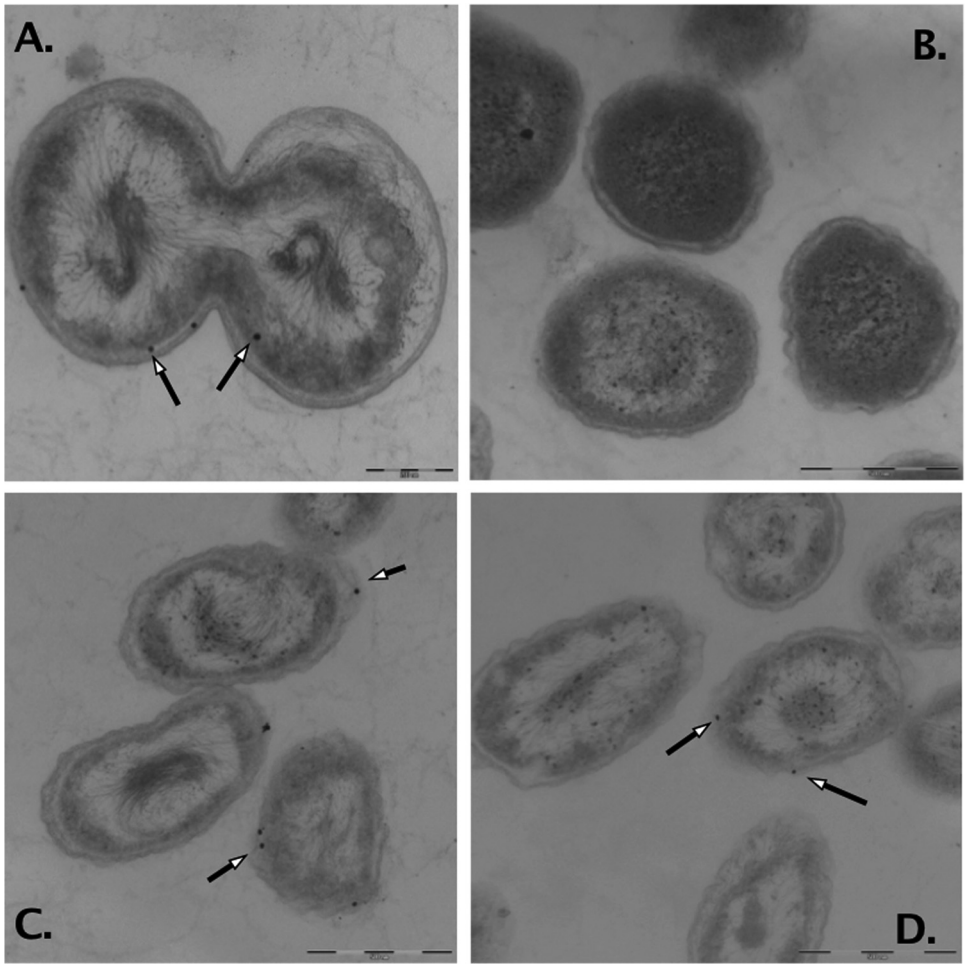

FIG 1 Formation of electron-dense particles by *C. metallidurans* strains. *C. metallidurans* strain CH34 was incubated in liquid culture with (A) or without (B) 50 μM $NaAuCl_4$, or strain AE104 (C) or AE126(pMOL28) (D) was incubated in the presence of 50 μM $NaAuCl_4$. The arrows point to electron-dense particles that are metallic gold nanoparticles as published (9). Scale bar, 0.2 μm (A) or 0.5 μM (B, C, and D).

μM NaAu(III)Cl_4, value for Table 3]. In contrast, the *cup-lacZ* fusions started from a much higher basic expression value, ~100 U mg (dry mass)$^{-1}$, so that the maximum specific activity reached was much higher than that of the *gig-lacZ* fusions, 1,640 ± 80 U mg^{-1} [*cupA-lacZ*, 50 μM NaAu(III)Cl_4, value for Table 3], although the actual induction quotient was lower (Table 3). Assuming that the specific activity is a measure for the expression level of the actual target gene product, this would indicate a strong expression level of *cup* and *gig* genes in gold-treated cells.

Since CupR is a MerR-type regulatory protein able to bind gold complexes (29), this may indicate the presence of gold ions in the

TABLE 3 Maximum upregulation of reporter gene fusions after treatment with transition metals[a]

Gene	Mean BG[b] (U mg^{-1}) ± SD	Fold upregulation ±SD (optimum inducer concn [μM])				
		$NaAuCl_4$	Au(I)- thiosulfate	$CuCl_2$	$AgNO_3$	$NiCl_2$
gigA	4.4 ± 2.27	150 ± 19 (50)	2.73 ± 0.40 (80)	34.6 ± 5.9 (500)	17.4 ± 3.2 (0.75)	1.59 ± 1.55 (200)
gigT	3.9 ± 1.90	37.3 ± 3.5 (40)	2.82 ± 0.79 (80)	22.1 ± 5.2 (500)	5.77 ± 0.85 (0.75)	1.89 ± 1.74 (300)
rpoQ	490 ± 282	1.16 ± 0.05 (10)	1.17 ± 0.11 (40)	1.28 ± 0.06 (50)	1.16 ± 0.19 (0.25)	1.36 ± 0.24 (50)
cupR	109 ± 54	13.2 ± 2.8 (50)	1.16 ± 0.15 (40)	2.79 ± 0.33 (500)	5.46 ± 1.31 (0.75)	1.05 ± 0.17 (50)
cupA	107 ± 53	10.7 ± 0.5 (50)	2.87 ± 0.08 (100)	3.19 ± 0.40 (400)	6.19 ± 0.94 (1.00)	1.71 ± 0.58 (100)
cupC	82 ± 37	19.6 ± 1.6 (50)	3.87 ± 0.83 (80)	3.48 ± 0.37 (500)	9.17 ± 1.70 (1.00)	1.37 ± 0.37 (50)

[a] Exponentially growing cells of strain AE104 with *lacZ* insertions in the respective genes were divided into parallel cultures, and the indicated metal compounds were added. Incubation was continued with shaking at 30°C (silver cultures in the dark), and the specific activity of the β-galactosidase was determined after 3 h. The fold upregulation of the optimum inducer concentration is shown plus the deviations from four independent experiments. The values are means for all 20 experiments in U mg^{-1} (dry mass).
Au(I)-thiosulfate, $Na_3Au(S_2O_3)_2$.
[b] BG, background expression level before induction.

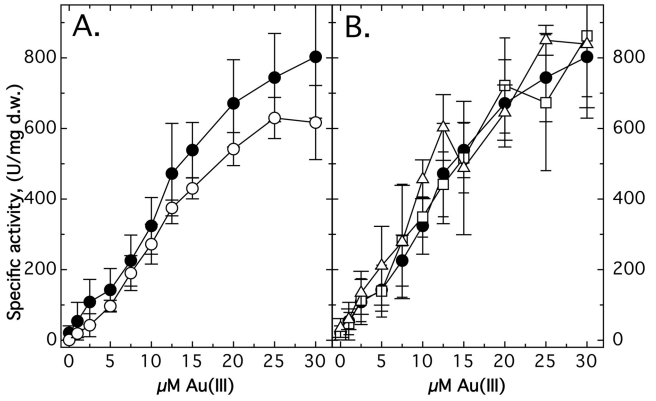

FIG 2 Regulation of a *cupA-lacZ* fusion in the presence or absence of *gig* genes or those for metal uptake systems. Exponentially growing cells of strain AE104 (●, both panels), its Δ*gig* derivative (○, panel A), its Δ*zupT* derivative (□, panel B), or the Δ7 multiple deletion strain (△, Δ*zupT* Δ*corA1* Δ*corA2* Δ*corA3* Δ*zntB* Δ*pitA* Δ*hoxN*, panel B), all with a *cupA-lacZ* fusion, were divided into parallel cultures, and various concentrations of $NaAuCl_4$ were added. Incubation was continued with shaking at 30°C, and the specific activity of the β-galactosidase (d.w., dry mass) coming from the reporter gene was determined after 3 h. At least three replicates were performed, and standard deviation bars are shown.

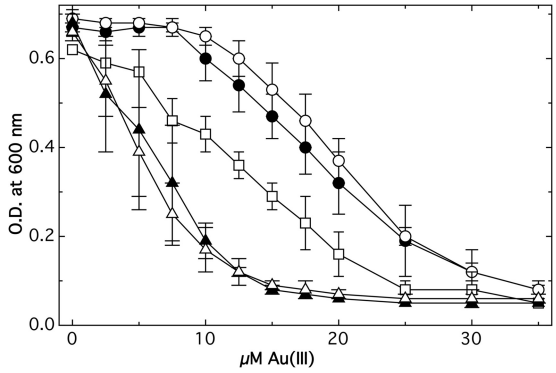

FIG 3 Gold resistance of strain AE104 and derivatives of this strain carrying deletions in metal uptake systems. Cells of strain AE104 (●) and derivatives of this strain carrying a Δ*cupC/AR* (○), a Δ*zupT* (□), the Δ7 multiple (▲), or a Δ7 Δ*cupC/AR* (△) deletion were precultivated for 30 h in TMM at 30°C with shaking, diluted 1:20 into fresh TMM, incubated again for 24 h at 30°C with shaking, diluted 1:10 into fresh TMM in parallel cultures in 96-well plates containing various concentrations or Au(III) chloride, and incubated for 20 h with shaking at 30°C. The optical density (OD) at 600 nm was determined in a Tecan multiple plate reader. Six independent experiments were performed, and standard deviation bars are shown.

cytoplasm of *C. metallidurans*. Therefore, an import route for gold complexes should exist, perhaps mediated by the Gig system.

Influence of the Gig system and secondary uptake systems for transition metals on gold transformations. When the four *gig* genes were deleted from chromosome 2 of strain AE104, resistance of the Δ*gig* mutant to gold, copper, and silver on solid TMM and in liquid culture was not different from that of the parent strain AE104 (Tables 1 and 2). Derivative strains with deletions in the *rpoQ* gene or the *rpoQ* gene plus the gene downstream of *rpoQ* (Rmet_4687), which might encode the RpoQ-specific anti-sigma factor (RsqA), were also identical to strain AE104 in the MICs and IC_{50}s of gold, silver, and copper (Tables 1, 2). Thus, the *gig* products alone were not essential for gold, silver, or copper resistance in *C. metallidurans* strain AE104.

If the *gig* products are important for import of Au(III), the Δ*gig* deletion might not lead to a decrease in resistance. Using the CupR-dependent regulation of a *cupA-lacZ* gene (29) as a cytoplasmic gold sensor, the gold-specific upregulation of a *cupA-lacZ* fusion in the *gig* deletion strain and strain AE104 was compared (Fig. 2). There was a slightly stronger upregulation caused by Au(III) complexes in strain AE104 compared to its Δ*gig* mutant, but the Gig system was obviously not essential to provide Au(III) to the CupR regulator. The two data points above 30 μM Au(III) in Fig. 2A were different with a probability greater than 90% but just below 95%, as indicated by Student *t* test analysis, so the difference was not significant.

C. metallidurans relies for its transition metal uptake on a battery of redundant and unspecific secondary metal uptake systems, namely, ZupT, PitA, CorA1, CorA2, CorA3, ZntB, and HoxN, which are transition metal transport systems with broad substrate specificity (44). The effect of deletion in *zupT* or all seven genes in the Δ7 mutant strain of parent strain AE104 was investigated in dose-response curves, determined in 96-well plates (Fig. 3), which led to a slight decrease in the overall IC_{50} values (Fig. 3). Interestingly, the deletion of *zupT* resulted in a clear decrease in Au(III) resistance, and the deletion of all seven systems resulted in an even lower gold resistance level (Fig. 3).

Deletion of an import system for toxic gold complexes should lead to an increase in resistance. If not, the mutation may have caused upregulation of other import systems as compensation, leading subsequently to an increase in the cytoplasmic gold content of *C. metallidurans*. This was again tested using the CupR-dependent expression of a *cupA-lacZ* reporter gene fusion. Expression of the β-galactosidase reporter gene at increasing Au(III) concentrations was similar in the Δ*zupT* strain, the Δ7 strain, and the parent strain AE104 (Fig. 2B). Thus, decreased gold resistance in the Δ*zupT* and the Δ7 strains seemed not to be the result of increased cytoplasmic gold accumulation in these cells but may be the result of a disturbed overall transition metal homeostasis.

So, no uptake system for gold complexes could be clearly identified. This may suggest that not many gold ions are being transported into the cytoplasm, in which case *C. metallidurans* may not need a cytoplasmic Au(I) efflux systems to deal with gold toxicity.

Influence of *cup* genes on resistance to gold complexes. Next, the contribution of the Cup system, which is related to the Gol system of *Salmonella* (14), to gold resistance was analyzed. Derivatives of strain AE104 with a Δ*cupC/AR* deletion were 8- to 9-fold less resistant to Cu(II)-chloride on solid growth medium (Table 1) and in liquid culture (Table 2), indicating the importance of CupA for copper homeostasis in strain AE104. On the other hand, there was no effect of either deletion on silver or on gold resistance under conditions tested. Deletion of *cupC/AR* in the multiple deletion strain Δ7 did also not influence Au(III) resistance (Fig. 3). Gold resistance might even increase slightly when the *cup* genes were deleted in parent strain AE104 (Fig. 3).

The *cupC/AR* region was also deleted in the wild-type strain CH34. Again, this led to a 6-fold (liquid culture) or 10-fold (solid medium) decrease in copper resistance and also to a small decrease in silver resistance on solid growth medium (Tables 1 and 2). Deletion of the gene for the CupA paralog CopF, encoded by mega-plasmid pMOL30, did not affect copper resistance but did

TABLE 4 Resistance of derivatives of *C. metallidurans* wild type and its plasmid-free derivative strain AE104 to group 11 metals[a]

Bacterial strain	MIC (μM)			
	$NaAuCl_4$	Au(I)-thiosulfate	$CuCl_2$	$AgNO_3$
AE104	0.8	0.4	1,810 ± 60	0.2
AE104Δ*copABCD*	0.7	0.4	**1,125**	0.2
CH34	0.8	0.4	2,000 ± 60	0.2
CH34Δ*copABCD*	0.7	0.4	1,875	0.2

[a] Cells were precultivated for 30 h in TMM at 30°C with shaking, diluted 1:20 into fresh TMM, incubated again for 24 h at 30°C with shaking, diluted 1:100 into fresh TMM, plated onto TMM agar plates containing increasing metal concentrations, and incubated in the dark at 30°C. Growth was recorded as the ability to form colonies after 4 days. The three biological reproductions mostly gave identical MICs. Thus, the deviation would be "±0" for each value and was omitted. Strongly decreased MIC levels are indicated in boldface. Au(I)-thiosulfate, $Na_3Au(S_2O_3)_2$. The mean ± the standard error of the mean is indicated where applicable.

affect silver resistance, albeit only in liquid culture. The Δ*cupC/AR* Δ*copF* double mutant of strain CH34 was the most copper- and silver-sensitive strain tested (Tables 1 and 2) but, again, resistance to Au(III) complexes or Au(I)-thiosulfate was unaltered.

Thus, the two GolT-related P_{IB1}-type ATPases CupA or CopF were not required for gold resistance in *C. metallidurans*. If *C. metallidurans* is indeed resistant to gold, (i) the resistance mechanism is different from that of *Salmonella*, (ii) other systems were able to substitute for the missing CupA and CopF efflux pumps, or (iii) the primary target of gold toxicity in *C. metallidurans* could be in the periplasm rather than in the cytoplasm. The latter assumption would explain the decreased gold resistance of the Δ7 strain despite lacking evidence for increased cytoplasmic accumulation of the noble metal (Fig. 2B and 3). Therefore, the influence of the *copABCD* gene region on gold resistance was tested.

Influence of *cop* genes on resistance to gold. The *copABCD* determinant located on chromosome 2 contains the genes (i) for CopA, a PcoA-like periplasmic copper oxidase (not to be confused with the CopA P-type ATPase of *E. coli*), (ii) CopB, a PcoB-like protein that should be attached to the inner face of the outer membrane, (iii) CopC, a PcoC-like putative periplasmic copper chaperone, (iv) CopD, a PcoD-like copper importer that might provide copper to the copper-dependent copper oxidase CopA, and (v) the two-component regulatory system CopRS. Paralogs of all of these proteins are also encoded by the huge second *cop* determinant on megaplasmid pMOL30, which contains, however, plenty of additional genes that might be involved in copper detoxification.

The *copABCD* determinant was deleted from chromosome 2 of strain CH34 and its plasmid-free derivative AE104. This led to a significant decrease in copper resistance in strain AE104 but not so much in CH34, which still contains the second *cop* determinant on plasmid pMOL30, and this *cop* probably substituted for the missing *cop* determinant on chromosome 2 (Table 4 and Fig. 4). The apparent higher copper resistance in AE104 compared to strain CH34 was not significant (Fig. 4C and D and Table 4). Thus, *copABCD* on chromosome 2 was important for copper resistance in strain AE104.

The gold resistance of the parent strains CH34 and AE104 in liquid culture was similar (Fig. 4A and B and Table 5). The deletion of *copABCD* decreased gold resistance in strain CH34 more than that of strain AE104. A similar decrease in gold resistance could also be observed with strain CH34 and Au(I)-thiosulfate in liquid culture, but the effect on resistance was much weaker compared to Au(III) complexes (data not shown). On solid growth media, the MICs of the Δ*copABCD* mutant strains was only slightly smaller than that of their respective parent (Table 4).

Reduction of Au(III) to Au(I). Since *mer* determinants encoding resistance to Hg(II) were also upregulated in gold-treated cells it was tested if the NADPH-dependent Hg(II) reductase MerA might be involved in Au(III) reduction and gold resistance. *C. metallidurans* contains two fully functional *mer* determinants on either plasmid, pMOL28 and pMOL30; however, the plasmids did not contribute to gold resistance as outlined above. A *mer* operon on the bacterial chromosome is interrupted in the *merA* gene (Rmet_2315) and thus inactive. The ORF Rmet_5011 might encode another mercuric ion reductase, MerA4, which was 33% identical in its amino acid sequence to the plasmid-encoded MerA proteins. This *merA4* gene was interrupted in *C. metallidurans* strains CH34 and AE104 but the gold resistance of the mutants was similar to that of its wild-type strains (Table 5).

Likewise, arsenate reductases could be involved in Au(III) reduction. *C. metallidurans* contains on chromosome 1 the *arsC1* (Rmet_0329) and *arsC2* (Rmet_0331) genes, which are part of a putative *arsMRIC2BC1HP* operon. A third *arsC*-like gene has no connection to genes involved in arsenate resistance and is proba-

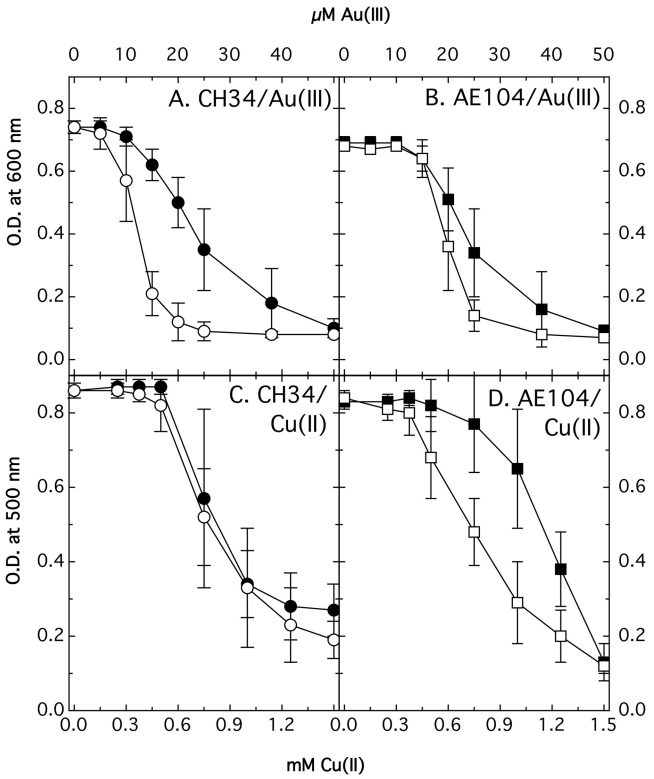

FIG 4 Gold and copper resistance of *C. metallidurans* Δ*cop* mutant strains. Cells of strain CH34 wild type (A and B, circles) or AE104 (C and D, squares) carrying a Δ*cop* gene deletion (open symbols) or not (solid symbols) were precultivated for 30 h in TMM at 30°C with shaking until the early stationary phase of growth was reached, diluted 1:20 into fresh TMM, incubated again for 24 h at 30°C with shaking, diluted 1:10 into fresh TMM in parallel cultures in 96-well plates containing various concentrations of $NaAuCl_4$ (A and B) or Cu(II) chloride (C and D) and incubated for 20 h with shaking at 30°C. The optical density (OD) at 600 nm (gold) or 500 nm (copper) was determined in a Tecan multiple plate reader.

TABLE 5 Resistance to $NaAuCl_4$ in liquid culture of various derivatives of *C. metallidurans* wild type and its plasmid-free derivative strain AE104[a]

	Mean ± SEM			
	CH34		AE104	
Bacterial strain	IC_{50} (μM)	Slope (*b*)	IC_{50} (μM)	Slope (*b*)
Parent strain	22.8 ± 1.14	3.85 ± 0.58	23.9 ± 2.31	2.65 ± 0.66
Δ*copABCD* mutant	12.9 ± 0.01	2.22 ± 0	20.7 ± 0.72	3.28 ± 0.11
Δ*arsC1* mutant	19.7 ± 1.89	4.37 ± 0.91	26.5 ± 2.40	4.16 ± 0.55
Δ*arsC2* mutant	20.8 ± 0.70	4.24 ± 0.45	23.5 ± 1.28	3.97 ± 0.39
Δ*merA4* mutant	20.8 ± 1.46	4.16 ± 0.54	25.7 ± 2.08	4.01 ± 0.58

[a] Cells of strain CH34 wild type or AE104 carrying the indicated gene interruptions were precultivated for 30 h in TMM at 30°C with shaking until the early stationary phase of growth was reached, diluted 1:20 into fresh TMM, incubated again for 24 h at 30°C with shaking, diluted 1:10 into fresh TMM in parallel cultures in 96-well plates containing various concentrations of $NaAuCl_4$, and incubated for 20 h with shaking at 30°C. The optical density at 600 nm was determined in a Tecan multiple plate reader and used to calculate the IC_{50} (half-maximum inhibition), as well as the slope of the sigmoidal dose-response curve (*b*). Please note the different method used in this experiment (small volumes in 96-well plates) versus that used to obtain the data presented in Table 2 (Erlenmeyer flasks), which led to a different IC_{50} and *b* values in all strains.

bly not an arsenate reductase. Thus, *arsC1* and *arsC2* were interrupted, but the gold resistance of the respective *C. metallidurans* CH34 and AE104 mutants was unchanged. MerA- and ArsC-like reductases may thus not be involved in gold resistance.

DISCUSSION

Gold is mobilized from primary ancient gold deposits as Au(I) and Au(III) complexes during weathering that might be enhanced by the physiological activity of microbiota (46). These gold complexes are subsequently reprecipitated and biomineralized again by other bacteria, which leads to the formation of secondary gold (1, 2, 45). The metal-resistant betaproteobacterium *C. metallidurans* dominated biofilm communities on gold grains, making an involvement of this bacterium in the gold biomineralization process highly probable (8).

When *C. metallidurans* was treated with Au(III) complexes, the complexes were rapidly (<5 min) adsorbed by the cells, sorption occurred in combination with a reduction of the Au(III) complexes to Au(I)-S complexes on the cell surfaces (9). This led to a distribution of gold all over the cell and the upregulation of genes encoding proteins involved in oxidative stress response, detoxification of Hgg(II), arsenate, and copper ions, before small metallic gold nanoparticles appeared in the periplasm (9). Since the formation of metallic gold from Au(III) complexes is a reductive process that involves an odd number of electrons per Au(III) reduced, it may very well lead to oxidative stress following the formation of radicals, explaining the observed upregulation of oxidative stress genes. Once the metallic gold nanoparticles were formed, however, reactive oxygen species may no longer be produced (46). The determinants involved in metal or metalloid resistance that are upregulated after treatment with gold complexes are controlled by MerR- or ArsR-type regulators, which usually bind "soft" metals or metalloids (30, 47, 48). Gold complexes may cause here a gratuitous induction of determinants that may not deal with the noble metal (Table 5).

Gold ions seem to arrive in the cytoplasm of *C. metallidurans*. The *cup* determinant is under the control of the MerR-type regulator CupR (29), which acts as gold-sensing regulator similar to its homologs GolS in *Salmonella* and CueR in *E. coli* (14, 15). MerR-type regulators are located on their operator as a repressor of the downstream operon and switch into an activator conformation upon metal binding. Thus, gold ions have to be present in the cytoplasm of *C. metallidurans* to stimulate expression of the *cupA-lacZ* reporter gene. The concentration of gold complexes needed to exert such a control event may be very low. The affinity of CueR for copper is in the zeptomolar range (22), and that of CupR for Au(I) might be even higher (29). Therefore, only very few gold complexes might be required in the cytoplasm for upregulation of genes by CupR.

However, the import rate of gold ions into the cytoplasm might be so slow that detoxification of cytoplasmic gold ions is not required, which is in contrast to the situation in *Salmonella* (13). In *C. metallidurans* mutant strains with multiple deletions in metal import systems, which probably synthesized an unspecific metal uptake/handling system with very low substrate specificity to deal with the resulting starvation condition, gold resistance was significantly decreased. This, however, did not lead to an increased upregulation of *cupA-lacZ* expression by gold ions, indicating that increased gold toxicity seems not to have been caused by an increased import of gold ions into the cytoplasm. Instead, the respective mutant strain may exhibit a disturbed general metal homeostasis system, which may also change the presence of periplasmic metal-handling components. In agreement with this assumption, deletion of the CupA efflux system in the Δ7 multiple deletion strain did not decrease gold resistance further on.

Therefore, there was also no real need for the export of gold ions by a P-type ATPase. *C. metallidurans* contains four P_{IB1}-type ATPases that might use Cu(I), Ag(I), or Au(I) as a substrate. Two of them are encoded in the context with genes involved in synthesis of copper-containing oxidases of the respiratory chain. These proteins might be "anabolic" exporters with a low export rate that are required to provide copper ions to these oxidases (49). The low level of copper and silver resistance of the Δ*cupA* Δ*copF* double mutant of *C. metallidurans* strain CH34 agrees with this assumption, indicating that CopF and especially CupA are the major copper efflux system for the detoxification of surplus Cu(I) from the cytoplasm. However, none of these proteins was required for gold resistance. This result agrees to recent findings from Osman et al. (50), who demonstrated that two MerR-type regulators in *Salmonella*, CueR and GolS, have a very similar affinity for copper ions. This would indicate that the Gol system in this enterobacterium actually might not be a gold resistance but rather a second copper resistance system (17).

The cytoplasmic "shunt" of Au(III) might thus not be required for transformation into gold nanoparticles, suggesting that gold ions may be reduced in the periplasm by components of the respiratory chain, reminiscent to copper (51). Treatment of *C. metallidurans* with Au(I) complexes, which had a lower bioavailability of gold ions due to the mainly covalent character of the Au(I)-C or Au(I)-S bond, resulted in a similar, albeit less pronounced, transcriptome response as Au(III). In all cases, the expression of genes involved in copper homeostasis was strongly influenced. Since the gold nanoparticles are also located in the periplasm (9), this again indicates that gold ions may be a "periplasmic problem" in *C. metallidurans*.

Even more focused, it may be a periplasmic problem in biofilm cells. With all other metal resistances tested in *C. metallidurans*,

the IC_{50}, which describes growth retardation in rather short-timed liquid culture experiments, has a similar or lower value than the MIC determined for colonies on solid growth medium (7, 36). This was also true for copper resistance (Tables 1 and 2), while for gold complexes, and to some extend for silver, the MICs were much smaller than the IC_{50}s. Au(I)-thiosulfate in particular, which caused only a minimal change in gene regulation in *C. metallidurans* cells due to the low availability of the gold ions, was even more toxic on solid growth medium than $NaAu(III)Cl_4$ (Table 1), but the situation was reversed in liquid culture (Table 2). Cultures of planktonic cells remained viable after amendment with >100 μM Au(I)-thiosulfate, whereas individual cells did not grow on plates at 100-fold lower concentrations. This suggests that in addition to genetic ability of individual cells to deal with gold complexes, biofilm formation drastically enhances the organism's ability to sustain viable populations. These populations, such as on gold grains, thus have a genetic advantage by being able remain viable while facing toxic gold complexes.

Deletion of the *copABCD* determinant from chromosome 2 led to a decrease in gold resistance, a stronger one in wild-type strain CH34 than in its plasmid-free derivative, strain AE104 (Fig. 4). The two megaplasmids of strain CH34 were not needed for gold resistance and may even decrease its level (Tables 1 and 2). Therefore, some plasmid-mediated functions may lead to increased gold sensitivity, and the products of the *copABCD* determinant might be required to compensate for this effect.

Of all the genes present on these plasmids, only *mer* genes and the huge pMOL30-located *cop* determinant were upregulated after gold treatment (9). The Mer system does not perform in the periplasm with the exception of binding of Hg(II) by MerP, but a toxic action resulting from the binding of gold ions by MerP is hardly imaginable. Moreover, the deletion of the two megaplasmids with one *mer* determinant on either plasmid decreased mercury resistance (7, 52, 53) but not gold resistance. This leaves the pMOL30-harbored *cop* determinant with more than 20 genes involved in copper transformation (28, 54), which might "mishandle" gold ions.

Compared to copper, gold is extremely rare in the environment. Although the average concentration of copper in soils is ca. 25 μg kg^{-1}, the average gold concentration is only a few nanograms per kilogram (55). In areas where biogeochemical cycling of Au and Cu has led to the formation of soil anomalies gold concentrations of up to 2 μg of Au kg^{-1} were detected, whereas these soils can contain several 100 μg of Cu per kg of soil (56). For example, at Australian sites, from which gold grains for DNA fingerprinting of associated biofilms were obtained, the ratios of Cu to Au were between 25 and 9,000 (56). Compared to gold, copper displays higher chemical solubility (under surface conditions) and environmental mobility and hence greater bioavailability (57). This suggests that organisms living in metal-rich environments are likely to encounter toxic effects from mobile copper rather than from mobile gold complexes. This is well reflected in genetic make-up of these bacteria, which harbor specific copper resistance systems used to regulate copper homeostasis (16). In contrast, toxic concentrations of gold complexes might rarely be encountered; thus, specific genetic gold resistance systems are likely to be energetically unfavorable, if other, e.g., copper, metal resistance, and homeostasis systems can be coutilized. *C. metallidurans*, which harbors a wide range of well-coordinated metal resistance factors, may have a genetic advantage of having numerous options for the coutilization of metal resistance determinants for the detoxification of gold complexes. Nevertheless, this might also lead to negative interferences between gold- and copper-handling processes.

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