

A new extracellular von Willebrand A domain-containing protein is involved in silver uptake in *Microcystis aeruginosa* exposed to silver nanoparticles

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Abstract Silver nanoparticles (AgNPs) can be toxic for cyanobacteria when present at low nanomolar concentrations, but the molecular mechanisms whereby AgNPs (or free Ag⁺ released from AgNPs) interact with these prokaryotic algal cells remain elusive. Here, we studied Ag uptake mechanisms in the prokaryotic cyanobacterium *Microcystis aeruginosa* exposed to AgNPs by measuring growth inhibition in the absence or presence of high-affinity Ag-binding ligands and by genetic transformation of *E. coli* with a protein predicted to be involved in Ag uptake. We discovered a new von Willebrand A (vWA) domain-containing protein in *M. aeruginosa* that mediates Ag uptake from AgNPs when expressed in *E. coli*. This new Ag transport protein, which is absent in eukaryotic algae, is a potential candidate explaining the higher AgNPs toxicity in cyanobacteria such as *M. aeruginosa* than that in eukaryotic algae. The present study provides new insights on Ag uptake mechanisms in the prokaryotic algae *M. aeruginosa*.

Keywords *Microcystis aeruginosa* · Silver nanoparticle·vWA domain-containing protein · Genetic transformation

Introduction

Among all metallic nanoparticles, silver nanoparticles (AgNPs) are the most widely commercialized currently accounting for 55.4 % of the total nanomaterial-based consumer products (Xiu et al. 2012). AgNPs are incorporated in various products such as in electronic devices, biosensors, catalytic applications, antimicrobials, and household products (Colvin 2003; Franke 2007; Park et al. 2010; Roco 2005). AgNPs are increasingly released in the environments. AgNPs (intact nanoparticles or free Ag⁺ released) enter sewage as well as terrestrial and aquatic ecosystems (Blaser et al. 2008; Das et al. 2014; Gottschalk et al. 2009). Approximately 8.8 t of AgNPs are released into the wastewater systems in the UK annually (Whiteley et al. 2013). As high as 130 t of Ag per year could be discharged in European rivers, and approximately 15 % of them came from AgNPs products (Blaser et al. 2008). The predicted environmental concentrations for AgNPs in different environmental ecosystems are in the range of nanograms per liter to milligrams per kilogram (Fabrega et al. 2011) and could increase by approximately 1.7 times between 2014 and 2020 (Massarsky et al. 2014). This potential increase in AgNPs release in aquatic ecosystems will increasingly pose a toxicity risk to aquatic organisms.

A number of studies have shown that nanomolar to subnanomolar concentrations of AgNPs and free Ag⁺ can be toxic for growth of bacteria and growth/photosynthesis of phytoplankton (Fabrega et al. 2011; Huang et al. 2016; Miao et al. 2009; Navarro et al. 2008; Wijnhoven et al. 2009). In eukaryotic phytoplankton species, AgNPs toxicity is often mediated by the free Ag⁺ ion released from the AgNPs since

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addition of high-affinity Ag-binding ligand totally prevents short-term Ag toxicity effects (He et al. 2012; Leclerc and Wilkinson 2014; Li et al. 2010; Miao et al. 2009; Navarro et al. 2008). However, Miao et al. (2010) suggested that direct uptake of AgNPs could occur via phagocytosis in a mixotrophic freshwater phytoplankton species (*Ochromonas danica*). In bacteria, whether AgNPs or the free Ag⁺ species can be bioavailable remains controversial, as some studies have suggested that AgNPs can be directly taken up by the cell while others have concluded the contrary (Behra et al. 2013). Cyanobacteria have been shown to be more sensitive to AgNPs than eukaryotic algae (Park et al. 2010), and our previous proteomic study has shown that the expression of an uncharacterized von Willebrand A domain-containing protein (vWA protein) potentially involved in metal transport was down-regulated in response to a 96-h AgNPs exposure (unpublished data). More knowledge on the uptake mechanisms of free Ag⁺ or AgNPs in prokaryotic phytoplankton is needed to better understand interspecific differences in Ag uptake and toxicity as well as for genetic engineering of resistant phytoplankton strains for the bioremediation of Ag-polluted sites.

Here, we examined AgNPs and free Ag⁺ uptake mechanisms in two freshwater algae of contrasting sensitivity to AgNPs, i.e., the eukaryotic phytoplankton *Chlorella vulgaris* (less sensitive to AgNPs) and the prokaryotic cyanobacterium *Microcystis aeruginosa* (more sensitive to AgNPs). We studied AgNPs and free Ag⁺ uptake mechanisms using growth experiments with/without high-affinity Ag-binding ligands. In addition, we isolated and characterized a new external protein predicted to be involved in metal transport and studied its potential role in Ag uptake using *E. coli* as a vector for protein expression. Our results show that this new protein (absent in *C. vulgaris*) is involved in Ag transport in *M. aeruginosa*, which partly explains the higher AgNPs toxicity on the growth of *M. aeruginosa* than that of *C. vulgaris*.

Materials and methods

Algal cultures

All toxicity tests were conducted using the freshwater unicellular cyanobacterium *M. aeruginosa* (FACHB-905) and the green alga *C. vulgaris* (FACHB-24), obtained from the Institute of Hydrobiology, Chinese Academy of Sciences. The two algae were cultured in 250-mL flasks containing 50 mL of sterilized BG-11 medium (initial pH = 7.1). Illumination was provided with cool-white fluorescent light bulbs at 54 $\mu\text{mol m}^{-2} \text{s}^{-1}$ using a 12:12-h light-dark photoperiod cycle. The temperature was maintained at 22 ± 0.5 °C. The flasks were shaken three times per day. Algal cell density was monitored using a hemocytometer. Algal cells in the stationary growth phase were used for almost all experiments

(initial cell density approximately 3×10^6 cells/mL). We repeated the AgNPs toxicity experiment in the absence or presence of high-affinity Ag-binding ligands using exponentially growing algal cells (initial cell density of 1×10^5 cells/mL) in order to evaluate the effect of the algal growth phase on AgNPs toxicity.

Algal exposure to AgNPs or free Ag⁺

The citrate-coated AgNPs were purchased from Shanghai Huzheng Nano Technology Co., Ltd (AGS-WMB1000C, Shanghai, China), and the particle mean diameter was approximately 10 nm according to the manufacturer's data and our previous scanning electron microscopy results (Qian et al. 2013). AgNPs were diluted in Milli-Q water just before every experiment and then were dispersed by ultrasonic vibration before addition into algal culture medium. For the positive control, we performed the experiments with the Ag⁺ (from AgNO₃) to compare the toxicity of AgNPs and Ag⁺. For both species, we also performed 24-, 48-, 72-, and 96-h exposures to 460 nM AgNPs or 46 nM AgNO₃ in the presence or absence of a high-affinity ligand for the free Ag⁺ ion (120 nM 2,3-dimercaptopropanesulfonate or DMPS, 230 and 460 nM cysteine or Cys). Here, the medium containing silver (AgNPs or AgNO₃) with Cys or DMPS was pre-equilibrated for 30 min before inoculating the algae. Note that for this experiment, the pH of the culture medium was adjusted daily to 7.1.

RNA extraction, reverse transcription, and real-time quantitative PCR analysis of the von Willebrand A domain-containing protein

The transcription of the gene coding for the vWA domain-containing protein was analyzed by real-time quantitative PCR (qRT-PCR). To do so, 60 mL of *M. aeruginosa* culture exposed for 4, 8, 12, 24, 48, 72, and 96 h to 460 nM AgNPs was collected and centrifuged at $7000 \times g$ for 15 min (as well as the control). RNA was then extracted, reverse transcribed, and performed by qRT-PCR using an Eppendorf Mastercycler® ep realplex4 system (Wesseling-Berzdorf, Germany) following the method described in Qian et al. (2014). The specific primer of the *vWA* gene for qRT-PCR was 5'-AGGA TCAGGAAGCACACTGG-3' (forward) and 5'-TCAT CCCGATAGCTTCCAC-3' (reverse).

Functional characterization of the new vWA protein using transformed *E. coli* cells

E. coli strain BL21 (DE3) was used for protein expression to assess whether or not the vWA protein was involved in Ag uptake. The *vWA* gene in *M. aeruginosa* was amplified by PCR using the primers (forward, ggatccATGTATCA AGAACTACTGAGTA; reverse, gcggccgcGCGTAATG

CTCCTTGAGAAC) and cloned into T vector (TaKaRa, China) after purification. This gene was then subcloned into a bacterial expression vector (pET-28a, Novagen company, Darmstadt, Germany) and transformed into *E. coli* BL21 (DE3) (purchased from TransGen, Beijing, China; code number CB105-02). The potential of the new vWA protein to enhance Ag and Cu transport was evaluated in the *E. coli* strains expressing or not the vWA protein and treated or not with IPTG. *E. coli* cells were grown in the LB liquid culture medium for 4 h in the absence or presence of 460 nM AgNPs and 460 nM CuSO₄. After the exposure, an aliquot of *E. coli* culture was diluted 10⁶-fold in fresh LB liquid culture medium and 100 µL of diluted culture was put on an LB solid culture medium within a petri dish, which was incubated for 24–36 h at 37 °C. After the incubation, bacterial colonies uniformly distributed on the surface of the solid medium were counted for each treatment. Another aliquot of metal-exposed bacterial cells were centrifuged at 2100×g, and the pellet was rinsed three times with 10 mL of 460 nM Cys to remove Ag adsorbed at the cell surface. After the last rinsing step, the pellet was resuspended in fresh LB liquid culture and bacterial cell density was inferred from the OD₆₀₀ using a calibration curve of OD₆₀₀ as a function of cell density. The rinsed bacterial pellets were finally digested in concentrated nitric acid at 200 °C for 30 min. The clear digested bacterial samples were then diluted in Milli-Q water (final HNO₃ concentration of 13 % v/v). Silver and copper cell content was analyzed by inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7500a, USA).

Analysis of protein interactions using yeast two-hybrid experiments

A Matchmaker™ Gold Yeast Two-Hybrid System Kit (Clontech, USA) was used for yeast two-hybrid experiments aiming to identify the proteins interacting with the vWA domain-containing proteins we isolated in *M. aeruginosa* (MAE_10560) and to better understand its cellular function. To do so, we first cloned the complementary DNA (cDNA) of MAE_10560 in the binding domain (BD) plasmid vector. Second, the cDNA of three other hypothetical proteins (MAE_10570, MAE_10550, MAE_32880), which all share structural similarities with MAE_10560 based on a search in the STRING database, was cloned in the activation domain (AD) plasmid vector. Third, BD and AD plasmid vectors with a reporter gene were co-transformed into a mutant yeast strain of AH109.

Data analysis

Experiments were repeated three times with new algal batches, each time with four replicates. The results are expressed as the mean ± standard error of the mean (SEM).

Significant differences among means were evaluated with Student's *t* test and ANOVA, which were performed with the StatView 5.0 program. Statistically significant differences were reported when the *p* value (*p*) was less than 0.05.

Results

Silver speciation calculations at equilibrium with Cys

As performed by Navarro et al. (2008), we computed initial Ag speciation at equilibrium in the presence of different Cys concentrations in the BG-11 medium using the stability constants listed in Table 1. According to MINEQL simulations, regarding the AgNO₃ exposure, more than 99 % of total Ag (46 nM AgNO₃) in the BG-11 medium is complex to Cys if 230 or 460 nM Cys is added to the exposure medium. Therefore, at an initial concentration of 460 nM, even assuming that 10 % of AgNPs dissolved over the long-term exposure, our calculations suggest that addition of 230 or 460 nM Cys could bind most of the free Ag⁺ ion released from the AgNPs. However, assuming that all AgNPs (460 nM) could dissolve in free Ag⁺ in the presence of 230 nM Cys, then there would be around 50 % of total Ag bound to HCys, and the remaining Ag species would be the free Ag⁺ and AgCl complex. If 460 nM Cys is added and all AgNPs are assumed to dissolve (460 nM Ag⁺), then 96.8 % of Ag would be predicted to be bound to HCys and only around 3.2 % (14 nM) of Ag would remain as free Ag⁺ and AgCl. Note that calculations of initial Ag speciation at equilibrium with DMPS could not be done since no Ag-binding constants were found in the literature. However, DMPS is a chelating agent and should form very strong complexes with divalent and monovalent metals (likely more stable than those form with Cys).

Table 1 Stability constant of cysteine (Cys) complexes at I=0 and T=25 °C

Reaction	Log K	Reference
$\text{Ag}^+ + \text{Cys}^{2-} + \text{H}^+ \leftrightarrow \text{AgCysH}$	22.7	Adams and Kramer 1999
$\text{Ag}^+ + 2\text{Cys}^{2-} + 2\text{H}^+ \leftrightarrow \text{Ag}(\text{CysH})_2^-$	36.7	Adams and Kramer 1999
$\text{Ag}^+ + \text{H}_2\text{O} \leftrightarrow \text{AgOH} + \text{H}^+$	-12	Martell et al. 2001
$\text{Ag}^+ + 2\text{H}_2\text{O} \leftrightarrow \text{Ag}(\text{OH})_2^- + 2\text{H}^+$	-24	Martell et al. 2001
$\text{Cys}^{2-} + \text{H}^+ \leftrightarrow \text{CysH}^-$	10.8	Martell et al. 2001
$\text{Cys}^{2-} + 2\text{H}^+ \leftrightarrow \text{CysH}_2$	19.1	Martell et al. 2001
$\text{Cys}^{2-} + 3\text{H}^+ \leftrightarrow \text{CysH}_3^+$	20.8	Martell et al. 2001

Effect of high-affinity Ag-binding ligands on AgNP toxicity

Addition of 460 nM AgNPs or 46 nM AgNO₃ without DMPS or Cys did not affect *C. vulgaris* growth over the 120-h exposure (Fig. 1a). However, exposure of *M. aeruginosa* to 460 nM AgNPs and 46 nM AgNO₃ without DMPS or Cys decreased the cell yield of *M. aeruginosa* up to around 50 and 40 % after 72–120 h, respectively (Fig. 1a). The addition of 120 nM DMPS or 230 and 460 nM Cys in the absence of AgNPs or AgNO₃ did not significantly affect *M. aeruginosa* growth over the 96-h period (Fig. 1 b, c). Complexation of free Ag⁺ released by AgNPs using DMPS did not affect AgNPs toxicity to *M. aeruginosa* after 24 h of growth (Fig. 1b). As time increases, DMPS could not totally relieve the toxicity of AgNPs to *M. aeruginosa* (Fig. 1b). By contrast, addition of 120 nM DMPS abolishes the toxicity of 46 nM Ag⁺ in *M. aeruginosa* (Fig. 1b). Similar trends were observed for the experiments involving addition of Cys in the culture (Fig. 1c). Subsequent experiments with *M. aeruginosa* using an initial cell density of 1 × 10⁵ cells/mL rather than 3 × 10⁶ cells/mL also demonstrated that addition of 230 nM Cys could not completely relieve AgNPs toxicity over 48 h of exposure, whereas the same Cys addition

abolished the toxicity of 46-nM free Ag⁺ (Fig. 1d). However, after 24 h of exposure of exponentially growing *M. aeruginosa* cells to 460 nM AgNPs with or without Cys, no significant toxicity occurred on cell yield (Fig. 1d).

The predicted function of the vWA protein and its interaction with related proteins by yeast two-hybrid experiments

We obtained the whole amino acid sequence and gene sequence from NCBI of the vWa protein (MAE_10560). The vWA protein included 252 amino acid residues. From the blast results, we found a binding motif, i.e., a metal ion-dependent adhesion site (MIDAS), in the vWA protein (Fig. 2a). The cellular location (inside the cell, transmembrane, or outside the cell) of the vWA protein was predicted using the software TMHMM 2.0 (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>). No transmembrane helice was detected in the whole protein. Rather, the model predicts that the protein is located outside the cell (Fig. 2b). However, we could not identify the proteins with which our target protein interacts using yeast two-hybrid assays since no positive yeast clone was found in defective medium indicating that the target protein MAE_10560 could not interact with predicted proteins. We also analyzed the

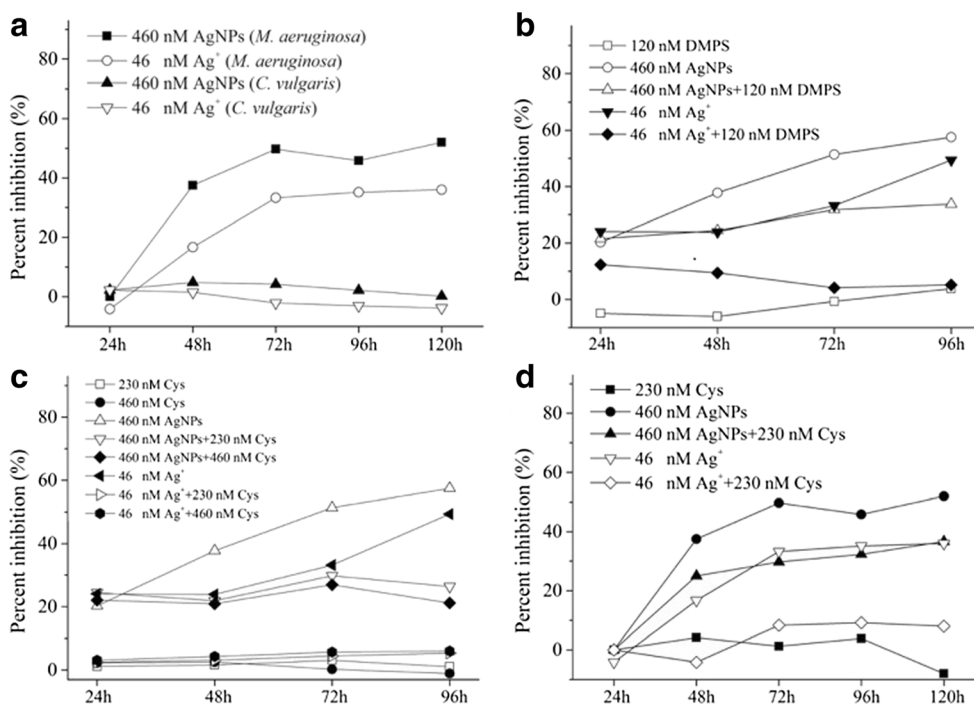


Fig. 1 Effects of high-affinity Ag-binding ligand (cysteine and DMPS) on the toxicity of AgNPs and AgNO₃ to *M. aeruginosa* and *C. vulgaris* over a 96-h exposure. **a** The effects of 460 nM AgNPs or 46 nM AgNO₃ on the growth of *M. aeruginosa* and *C. vulgaris* (high initial cell density). **b** The effects of DMPS (120 nM) on the toxicity of 460 nM AgNPs or 46 nM AgNO₃ on *M. aeruginosa* cell yield over time (high initial cell

density). **c** The effects of Cys (230 or 460 nM) on the toxicity of 460 nM AgNPs or 46 nM AgNO₃ on *M. aeruginosa* cell yield over time (high initial cell density). **d** The effects of Cys (230 nM) on the toxicity of 460 nM AgNPs or 46 nM AgNO₃ to *M. aeruginosa* cell yield over time (low initial cell density)

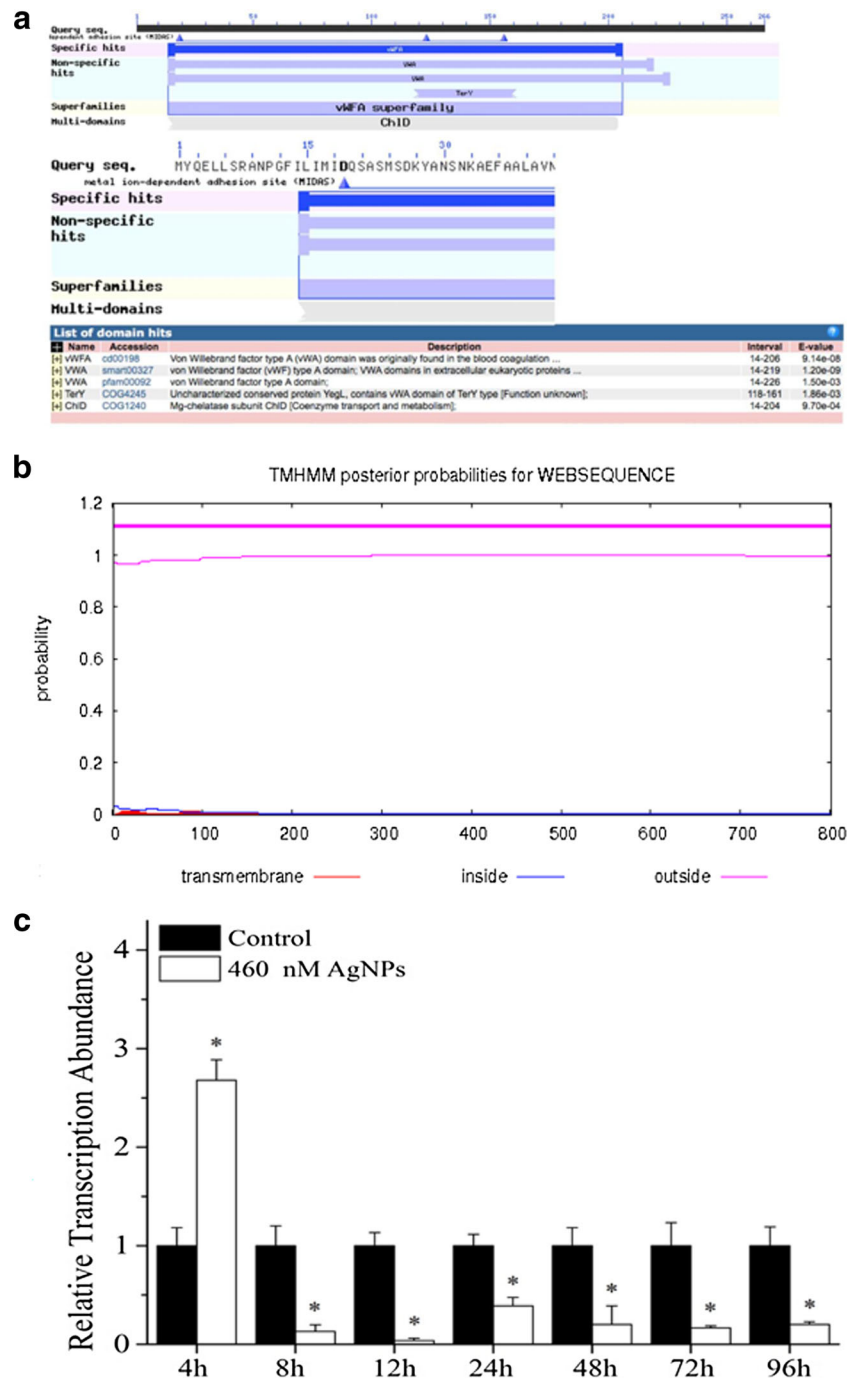


Fig. 2 The function and location of vWA protein. **a** The vWA domain sequence of the vWA protein using the BLAST algorithm of the NCBI. **b** The cellular location (inside the cell, transmembrane, or outside the cell) of the vWA isolated in *M. aeruginosa* predicted by using the web-based software TMHMM 2.0. **c** Transcription of the gene coding for the vWA

domain-containing protein in *M. aeruginosa* grown for 4 to 96 h in the presence or absence (control) of 460 nM AgNPs. Asterisks (*) represent statistically significant differences when compared with the control at the $p < 0.05$ level

transcription of the vWA gene in *M. aeruginosa* after AgNPs treatment and found that it was up-regulated to approximately 2.8-fold of the control after 4 h of exposure to 460 nM AgNPs, but down-regulated to less than 50 % of the control after an 8- to 96-h exposure (Fig. 2c).

The function of vWA protein overexpression in *E. coli* by genetic transformation

To identify the function of the new vWA protein in *M. aeruginosa*, we cloned the whole vWA gene with

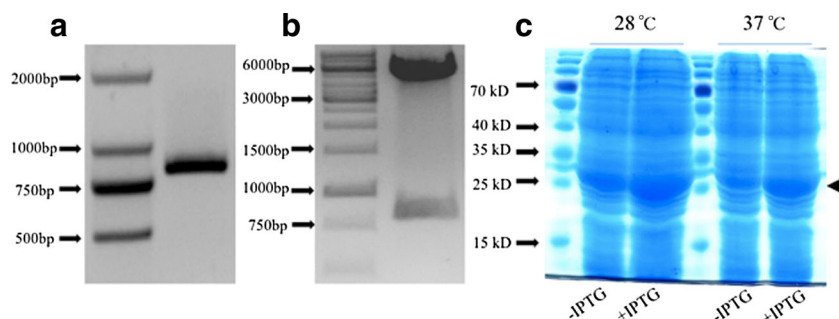


Fig. 3 The vWA protein was overexpressed in *E. coli*. **a** Fragment size of the vWA gene amplified by PCR (798 bp). **b** Size of the vWA gene when subcloned between the genes *NotI* and *Bam*HI of pET-28a. **c** Detection of

the vWA protein by Coomassie brilliant blue staining in SDS-PAGE for samples incubated or not with IPTG at 28 or 37 °C

approximately 800 bp (Fig. 3a), linked with pET-28a to be transformed into the BL21(DE3) *E. coli* (Fig. 3b). The BL21(DE3) *E. coli* containing the pET-28a-vWA vector was cultured in the LB medium until reaching an OD₆₀₀ of 0.6–0.8. Then, IPTG (final concentration of 0.1 mM) was added to induce the expression of the vWA protein under 28 and 37 °C. After 6 h of induction, the total protein was extracted and loaded into the SDS-PAGE. The predicted molecular weight of the target protein (30 kDa) was confirmed by Coomassie brilliant blue (CBB) staining (Fig. 3c).

Bacterial plaques on agar were not significantly different for *E. coli* producing or not the vWA protein after 4 h of culture without AgNPs. However, a 4-h exposure to 460 nM AgNPs significantly inhibited the growth (as determined by the bacterial plaque surface on the agar plate) of the *E. coli* strain without the vWA gene by approximately 30 % relative to that of the control. Exposure to 460 nM CuSO₄ for 4 h also decreased the plaque surfaces of *E. coli* without the vWA gene by approximately 10 % relative to that of the control. After a 4-h exposure to 460 nM AgNPs or CuSO₄, the growth of *E. coli* expressing the vWA protein (induced by IPTG treatment) was more affected than that of *E. coli* without the vWA protein; addition of AgNPs or CuSO₄ inhibited *E. coli*

growth by 80 to 90 % relative to that of the control, respectively (Table 2). We also measured Ag or Cu cell quotas in *E. coli* exposed to AgNPs and found that *E. coli* overexpressing the vWA protein due to the IPTG treatment took up 1.2 and 5.6 times more Ag or Cu than the strain weakly expressing (with no IPTG treatment) the same vWA protein. Moreover, Ag cell quotas in the *E. coli* strain expressing the vWA protein (with or without IPTG treatment) were 2.3- to 2.8-fold higher than that in the *E. coli* strain not expressing the vWA protein (with or without IPTG treatment), whereas Cu cell contents were 3.1- to 17.3-fold higher in *E. coli* expressing the vWA protein.

Discussion

Bioavailability of ionic silver inferred from AgNO₃ toxicity experiments in the presence or not of high-affinity Ag ligands

Addition of Cys or DMPS so that the free initial Ag⁺ concentration is a negligible proportion of total initial added Ag abolished Ag toxicity in *M. aeruginosa* exposed to 46 nM AgNO₃ demonstrating that Cys-Ag or DMPS-Ag complexes

Table 2 Growth of *E. coli* and intracellular Ag or Cu cell quotas after a 4-h exposure without (control) or with addition of 460 nM AgNPs or 460 nM CuSO₄

		Control group	Colony count (4 h, AgNP treatment)	Colony count (4 h, CuSO ₄ treatment)	Ag content intracellular (ng/10 ⁹ cells)	Cu content intracellular (ng/10 ⁹ cells)
BL-21	IPTG(+)	93 ± 4	68 ± 5	83 ± 4	0.23 ± 0.01	0.47 ± 0.07
	IPTG(-)	84 ± 3	59 ± 5	76 ± 4	0.21 ± 0.04	0.43 ± 0.04
BL-21 (vWA)	IPTG(+)	86 ± 2	7 ± 2a	17 ± 4a	0.56 ± 0.06a	7.81 ± 2.30a
	IPTG(-)	77 ± 5	38 ± 2b	53 ± 4b	0.46 ± 0.06b	1.40 ± 0.07b

E. coli BL-21 expressing or not the vWA protein were used for the experiment. The IPTG treatment, IPTG(+), up-regulated the expression of the vWA protein. Different lowercase letters represent statistically significant differences at $p < 0.05$ from one-way ANOVA

Table 3 Silver cell quotas in *M. aeruginosa* and *C. vulgaris* grown for 96 h in the absence (control) or presence of 460 nM AgNPs

	Control group (ng Ag/10 ⁶ cells)	AgNPs treated group (ng Ag/10 ⁶ cells)
<i>M. aeruginosa</i>	0.42 ± 0.01	3682.38 ± 121.64
<i>C. vulgaris</i>	0.43 ± 0.01	4.17 ± 1.17

are not toxic (and therefore the complexes are not bioavailable or at least much less bioavailable than the free Ag⁺ ion) for *M. aeruginosa* (Fig. 1b–d). Note that Cys may oxidize in the presence of oxygen or other oxidizing agents (e.g., ROS produced by algae) over time even though strong Ag complexes with GSH have been shown to oxidize very slowly in oxic aqueous solution (Hsu-Kim 2007). Interestingly, the absence of any Ag toxic effects over the 96-h exposure to 43 nM AgNO₃ and 230 nM Cys (Fig. 1) also suggests that the oxidation of the Ag-Cys complex is minor and not significant over the time frame of our experiment. The results of our dissolved Ag toxicity experiments (treatments with AgNO₃ added) are in line with those of previous studies in other phytoplankton species. Indeed, several reports have shown that addition of Cys, a high-affinity Ag-binding ligand such as DMPS, could abolish short-term toxicity of Ag (I) to eukaryotic phytoplankton species (Navarro et al. 2008; He et al. 2012; Gil-Allué et al. 2015).

Bioavailability of AgNPs inferred from toxicity experiments in the presence or not of high-affinity Ag ligands

Our results also show that the toxicity of AgNPs could not be totally prevented by the addition of Cys (Fig. 1c, d) or DMPS (Fig. 1b) over the 96-h exposure. This is the first demonstration that AgNPs remain toxic in a long-term growth experiment in the presence of high-affinity Ag-binding ligands, to our knowledge, for a non-mixotrophic phytoplankton species. Indeed, the current paradigm in eukaryotic phytoplankton states that AgNPs toxicity is mediated mostly (if not entirely) by the free Ag⁺ ion released from the AgNPs in all eukaryotic (and non-mixotrophic) species examined (Leclerc and Wilkinson 2014; Li et al. 2010; Miao et al. 2009; Navarro et al. 2008). Only the study of Miao et al. (2010) has suggested that direct uptake of AgNPs could occur perhaps via phagocytosis in a mixotrophic freshwater eukaryotic phytoplankton species (*Ochromonas danica*). Several hypotheses could explain the apparent toxic effect mediated by AgNPs (in the presence of Cys or DMPS) in *M. aeruginosa*. First, ROS released by *M. aeruginosa* could oxidize AgNPs. In those conditions, if the concentration of free Ag⁺ released from AgNPs becomes higher than that of reduced cysteine, then Ag might not remain

heavily complex by cysteine over the long-term exposure and free Ag⁺ released from AgNPs could contribute to Ag uptake and toxicity even in the presence of Cys or DMPS. Several ROS can indeed be produced by eukaryotic phytoplankton algae, and these ROS can rapidly oxidize AgNPs and enhance Ag⁺ release (Sigg and Lindauer 2015). Second, relatively small AgNPs (<20 nm in diameter) (Oukarroum et al. 2012) could enter the porous cell wall of *C. vulgaris* or might directly inhibit cell surface enzymes as shown under short-term (2-h) AgNPs exposure in periphyton in the study of Gil-Allué et al. (2015). Third, direct uptake of AgNPs could occur in *M. aeruginosa* as proposed in bacteria (Behra et al. 2013). We cannot exclude the possibility that AgNPs directly interact with *M. aeruginosa* and exert their toxicity even though the effect of Cys or DMPS on AgNPs toxicity was similar for algae in the stationary growth phase (Fig. 1b, c) and in the exponential growth phase (Fig. 1d). Therefore, if any direct AgNPs toxicity effects occurred, those would not depend on the physiological state of the cells. Further studies are needed to confirm/infirm the three above hypotheses and unravel the precise mechanisms at play here explaining the significant apparent toxicity of AgNPs in the presence of Cys and DMPS. Irrespective of the precise mechanisms explaining AgNPs toxicity in the presence of cysteine, our results clearly demonstrate, for the first time in a cyanobacterium, that even in the presence of high-affinity Ag-binding ligand in the high nanomolar concentration range, AgNPs can remain toxic on the time scale of a few days.

Metal transport function of the new vWA protein in *M. aeruginosa*

The new vWA protein isolated in *M. aeruginosa* is predicted to be located outside the cell and can enhance Ag and Cu(II) transport when expressed in *E. coli*. The absence of interacting proteins detected by the yeast two-hybrid assay indicates either that the vWA is phosphorylated before interacting with other proteins of the cell membrane or that the target protein has a new cellular function. Many proteins with vWA domains bind metal ions via MIDAS and are involved in membrane transport of cations (Fu et al. 2001; Whittaker and Hynes 2002). Proteins with vWA domains are also known to enhance Ca transport via voltage gate calcium channel in *Xenopus* oocytes perhaps by stimulating the synthesis of Ca channels (Davies et al. 2007). Even though the precise molecular mechanisms allowing Ag transport in *M. aeruginosa* via the vWA protein remain unclear, the results obtained in the present study indicate that the vWA protein is involved in Ag and Cu transport suggesting that the protein is associated to a putative cation transporter. We also found that *M. aeruginosa* took up much more Ag than *C. vulgaris* after 96 h of exposure (Table 3). However, down-regulation of vWA gene transcription in *M. aeruginosa* after 8 h of AgNPs

treatment could help decrease Ag uptake and toxicity over a long-term exposure. The new vWA protein expressed in prokaryotic organisms, such as *M. aeruginosa*, could explain partly the higher sensitivity to free Ag⁺ and AgNPs in this prokaryotic species than in the eukaryotic alga *C. vulgaris* (as measured in Fig. 1a).

The present study brings new insights into the uptake mechanisms of Ag in *M. aeruginosa* exposed to AgNPs and free Ag⁺. Genetic transformation experiments using *E. coli* as well as measurements of Ag uptake in this bacterium suggest that an Ag transport protein, vWA, which is exclusively present in *M. aeruginosa*, explains partly the higher sensitivity of this species compared with that of *C. vulgaris*. Future genetic transformation of microorganisms with this vWA protein could help improve bioremediation of metal-polluted sites.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any experiments involving human participants or animals performed by any of the authors. Informed consent was obtained from all individual participants included in the study.

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