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Revealing Silver Effects on Bacterial Cell Structure
by Atomic Force and Scanning Electron Microscopies

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Abstract

The use of silver ($\text{Ag}^+$) as an antimicrobial under different forms and at different scales, appears in numerous applications such as in health care, food industry, clothing, fabrics and disinfectants. Yet, there is still important gaps regarding the complete comprehension of the mechanisms of its actions on bacteria. In a previous work\(^1\), we demonstrated that, silver and copper severely damage membrane proteins involved in photosynthesis and respiration in bacteria exposed to metal excess. Here, we are presenting complementary data using AFM and SEM microscopies, that reveals (i) the drastic effects of $\text{Ag}^+$ ions on the morphology and structure of cell membrane and (ii) the formation of $\text{Ag}^+$ aggregates that adhere to the bacterial cell surface in *Rubrivivax (R.) gelatinosus*. Impacts of $\text{Ag}^+$ ions on *R. gelatinosus* are compared to those on the most commonly studied bacteria (*Escherichia (E.) coli* and *Bacillus (B.) subtilis*), while considering the effect of culture grown media on the modification of silver ions. Altogether, these results reveal other levels and subtle aspects of $\text{Ag}^+$ toxicity to be taken into account in understanding the general mechanisms of metal toxicity in bacteria.
Introduction

In the context of antibiotic crisis, where lack of efficiency is linked to both multi-resistance bacteria and increasingly reduced offer of antibiotic solutions\(^2\)\(^-\)\(^5\), silver has been considered as a very promising alternative proposal\(^4\)\(^,\)\(^6\). It is therefore, widely used in industry, agriculture and health care as an antimicrobial agent\(^5\)\(^,\)\(^7\)\(^,\)\(^8\). It can be found as pure and/or alloy with other metals, at different scale and forms. It inhibits, causes or enhances mechanisms of bacterial death, disrupts growth or reduces bacterial biofilm proliferation\(^9\)\(^,\)\(^10\). However, its use is not without danger, since excess of silver in both ions (Ag\(^+\)) and nanoparticles (Ag-NPs) forms shows toxic impact toward all types of living cells. Yet, the underlying mechanisms driving its toxicity and its impact on bacteria, are still under intense investigations\(^11\)\(^-\)\(^13\).

Unlike copper, iron or zinc for example\(^14\), silver is not an essential metal for cell growth. In bacteria, iron or zinc enter the cell trough specific import systems. Silver ions could diffuse and enter the cells through the outer membrane via nonspecific importers and poison the membrane or the cytoplasm of the bacterium\(^15\). Ag\(^+\) could also affect the membrane integrity and damage the cell structure if it accumulates outside the cell, although direct evidences are still missing. In general, bacterial cells are programmed to maintain and to resist metal effects and toxicity. They have the ability to reach metal homeostasis by using different defense systems. They can, for example, repress the import system, induce the required detoxification enzymes including metal sequestration and/or active efflux systems\(^16\)\(^,\)\(^17\). A dysfunction in the homeostasis system (import/efflux) of these metals can cause physiological disorders in both prokaryotes and eukaryotes cells\(^18\). Many studies have reported these damages\(^1\)\(^,\)\(^4\)\(^,\)\(^12\)\(^,\)\(^19\). For example, human cells (skin and lung cells) are easily exposed to silver nitrate or silver oxide by touching and breathing\(^20\); this exposure can cause breathing problems, lung and throat irritation, stomach pain, and argyria\(^21\)\(^,\)\(^22\). In the green algae *Chlamydomonas reinhardtii*, silver ions disrupt cellular metabolism and inhibit
important functions such as photosynthesis. In *Escherichia (E.) coli*, *Bacillus (B.) subtilis* or *Salmonella (S.) typhimirium* model bacteria, Ag\(^+\) inhibits the growth by targeting various metabolisms. In photosynthetic bacterium only few studies have addressed metal toxicity mechanisms and homeostasis of silver. Therefore, we used to tackle the effect of Ag\(^+\) on photosynthesis and respiration.

In a previous study, we have reported results on a molecular level regarding the metals (Cu\(^+\) and Ag\(^+\)) toxicity in *Rubrivivax (R.) gelatinosus* an environmental and gram-negative photosynthetic purple bacterium. The results revealed new findings on the targets of both Cu\(^+\) and Ag\(^+\) ions in the inner membrane. The membrane complexes affected are specifically the peripheral light harvesting complex LH2 and the cytochrome c oxidase; required for photosynthesis and respiration respectively. Moreover, our data showed that Ag\(^+\) has effects on the succinate dehydrogenase (SDH) complex in *E. coli*, but not in *B. subtilis*.

In this study, we carried out an investigation, using Scanning Electron Microscope (SEM) and Atomic Force Microscope (AFM), to provide data on the morphological and structural changes that occur upon exposure of bacteria to silver ions due to metals influence. The aim of these investigations, in this context, was to elucidate the influence of silver Ag\(^+\) ions outside the bacterial cells and essentially to reveal Ag\(^+\) interaction with the cell membrane surface. The results showed that high resolution images could discern and characterize the detailed changes that occurred on the bacterial cell membrane after treatment with AgNO\(_3\). To our knowledge, this presents the direct evidence of Ag\(^+\) silver ions morphological damages to *R. gelatinosus* cell membrane. Our data analyses showed drastic changes that increased with the increasing of incubation time, while untreated samples remain unaffected. Ag\(^+\) exposure leads to i- the formation of irregular deep grooves vesicles on the cell surface, ii- to cell membrane shrinking and iii- to structure breaking in the extreme case of extended exposure. A phenomena of silver particles accumulation on the membrane surface has also been
observed using SEM, raising the questions of the media effect on silver precipitation, the influence of cell membrane surface and eventually suggest a hypothetical scenario of cell defense.
**Results and discussion**

*Consequences of Ag⁺ exposure on cell surface and morphology revealed by SEM and AFM microscopy*

To assess the toxicity effect of Ag⁺ on cell’s morphology and structure, we imaged *R. gelatinosus* cells that were exposed to 1 mM of AgNO₃ and deposited using dip coating on substrate for both SEM (on freshly cleaved graphite HOPG) and AFM (on freshly cleaved Mica). We have prepared same samples, under same conditions, for both untreated and treated cultures after 1 and 24 hours (h) incubation with AgNO₃. Results showed that untreated cells of *R. gelatinosus* have rod-shape and a relatively smooth surface with no ruptures or swellings (Fig. S1)). The images, at the same scales, showed also that the morphology and physical appearance of the untreated bacteria were not dependant on the imaging tool (AFM vs SEM) or the nature of the substrate (HOPG vs Mica). Bacteria were sometimes grouped in clusters, but most often isolated cells were easily detected, enabling individual and non-perturbed study of their morphology. No modification due to the deposition on the substrate and no physical stress or surface deformation were observed. No ageing effect or alteration during the observation time window was observed. Furthermore, there was no effect of different deposition procedure, nor drying technics, nor ambient air or high vacuum. Membranes cells were continuous, and homogeneous. We did not observe any charge effect on the bacteria in SEM images and there was no observable effect of electron beam tension. For the AFM imaging in non-contact mode, we did not saw displacement of cells, and the tip interaction with the membrane caused no damage. Thus, bacteria were completely stable (shape and position) during the observation time window.

Interestingly, in the samples that were exposed to 1 mM of AgNO₃ for one hour, SEM images showed accumulation of metals around some bacteria (Fig. 1). With a bigger scale we
observed bacteria that appeared very bright (statistically one fourth, at that concentration of cells and ions) (Fig. 1A). Higher magnification showed an impressive accumulation of metallic aggregates on the cell surface (Fig. 1B and Fig. S2). The size distribution of these aggregates on the membrane was compared with the distribution size of the ones that can be observed also around the cells (Fig. 1C). It seems, that during the first hour, the first stage was silver precipitation to form these aggregates. The superposition of several normal distributions of the aggregates size distribution suggested several, may be competitive, stochastic nucleation processes\textsuperscript{29,30}. However, we observed a difference between distribution on and around the cell’s membrane. Some of the aggregates around the cells (2\textsuperscript{nd} and 3\textsuperscript{rd} pics of density profile) can be attributed directly to detached particles from the membranes. These data raised questions related to the interaction of bacteria with silver ions in the medium. First, since not all bacteria displayed these particles on their surface, this may suggest a difference in the surface component (exopolysaccharides/ capsule) of our cells, and/or a difference in the growth stage (dividing versus static or dying cells). Second, we might consider that these particles arose from interaction of silver with components in the growth media. Nevertheless, we can also speculate at that point, that precipitation of silver ions was mediated by cells eventually as a first stage of a defence mechanism, since insoluble particles could be more difficult to penetrate into the cell with increasing size of the particles. This was suggested in \textit{Pseudomonas aeruginosa} in which extracellular synthesis of nanoparticles was also observed after exposure to cadmium or selenium\textsuperscript{31–33}. This mechanism was suggested to contribute to metal resistance in \textit{Pseudomonas}.  

Furthermore, the AFM images of untreated or exposed bacteria to AgNO\textsubscript{3} for one hour, confirmed the presence of silver aggregates on the cell’s membrane (Fig. 2). The size distribution corresponds to the one observed with the SEM. Aggregates on the cells were not displaced by the scanning and showed important adhesion to the cell surface. Yet, we cannot
exclude that some aggregates grown on cell surface can be detached during washing and
deposition on substrate processes. No membrane deformation, breaking or change in
hardness-softness were observed at that point. We imaged also, using AFM, bacteria cells
after extended incubation time (24 h) to AgNO₃. Figure 3 shows the comparison between
untreated cells, and AgNO₃ exposed bacteria after 24 h incubation. The figure shows, again
topography and peak force error images, but also longitudinal and transverse cross section
profiles. These images indicated that silver stress caused the cell surface to become rough in
*R. gelatinosus*. Cells start to lose their clear-cut rod-shaped morphologies with the increasing
of incubation time. We could not observe metallic aggregates on the membranes. The edges
of the cells seem to be petrified as shown on the peak and Force Error and we can see
numerous large patches.

Cross section curves showed clearly an increase of the rugosity, leading to cell’s
membrane breaks transversally to the bacteria length axis. However, to establish a better
quantitative analysis of the morphology, we focused our study on the distribution of heights,
skewness and kurtosis moment who are known to be fundamental for describing surface
asymmetry and flatness features. We measured for that the following parameters, along
(longitudinal) and across (transverse) bacteria length axis, taking into account only the cell,
without substrate variation possible modification:

- Arithmetic average height ($\bar{z}$) gives general description of height variation:

$$\bar{z}(N) = \frac{1}{N} \sum_{i=1}^{N} z(x, y)$$

- RMS roughness ($R_q$) represents the standard deviation of surface heights:
\[ R_q(N) = \sqrt{\frac{1}{N} \sum_{i=1}^{N} [z(x,y) - \bar{z}(N)]^2} \]

- Average total roughness \( (R_{tm}) \), the sum of mean of maxima profile for peak height \( (R_{pm}) \) and for valleys depths \( (R_{vm}) \), calculated over the surface \( R_{tm} = R_{pm} + R_{vm} \) with

\[ R_{pm} = \frac{1}{N} \sum_{i=1}^{N} \max(z_j - \bar{z}) ; 1 < j < M \text{ and } R_{vm} = \frac{1}{N} \sum_{i=1}^{N} |\min(z_j - \bar{z})| ; 1 < j < M \]

- Skewness \( (Sk) \), the 3\(^{rd}\) moment of profile amplitude probability density function, measures the profile symmetry about mean line.

\[ Sk = \frac{1}{NR_q^3} \sum_{i=1}^{N} [z_i - \bar{z}]^3. \]

If the height distribution is symmetrical \( Sk \) is zero. It is positive if the surface has more peaks than valleys and negative if the valleys are predominant.

- Kurtosis \( (Ku) \), the 4\(^{th}\) moment of profile amplitude probability function, measure surface sharpness

\[ Ku = \frac{1}{NR_q^4} \sum_{i=1}^{N} [z_i - \bar{z}]^4. \]

When \( Ku \), is equal to 3, this indicates a gaussian amplitude distribution, smaller than 3 means that the surface is flattening and higher than 3, that the surface has a lot of peaks.

Table 1, summarize these morphological parameters for the control and the exposed bacteria to silver ions. Errors on the values are estimated around 10\% for average height and rugosities, and around 20\% for 3\(^{rd}\) and 4\(^{th}\) moments.

The most important change was an important increasing of surface roughness, along bacteria length axis, till the integrity of cell’s membranes was not ensured anymore. It was difficult to localise silver aggregates under these extended exposure condition. Cells with
longer incubation time (> 24 h) showed greater damages (Fig. 4) and the broken cells were
difficult to locate by the microscope. Interestingly, for those we were able to locate, they were
so fragile that they were broken in parts after being deposited (during drying process) on the
surface of the substrate or during the tip scanning.

Morphological alterations caused by silver ions, comparison with other bacteria

There are various studies which have reported the effect of silver on other bacteria
such as E. coli and B. subtilis\textsuperscript{35,36}. It was then important to extend our study to these two
species as controls and comparison to our results on the photosynthetic model bacterium R.
gelatinosus. In these experiments, Ag\textsuperscript{+} stress was applied to E. coli and B. subtilis cells grown
in LB medium since it was difficult to grow them in Malate. Experiments were achieved with
the same concentration of Ag\textsuperscript{+} ions (1 mM), and on cultures with same optical density.
Samples were collected after incubation time and they were prepared to be imaged by SEM
and AFM, using same protocol for deposition and same substrate as above for R. gelatinosus.
For E. coli, the results showed that cells in the control sample had smooth and soft surface,
with intact rod-shaped cells (Fig. S3). The AFM images (Fig. 5) confirmed the SEM results
on untreated E. coli as cells appeared regular and smooth. In contrast, exposure to silver ions,
affected the surface topology. Indeed, cells exhibited more irregular membrane surface and
some grooves appeared on the top even after just one hour of exposure (Fig. 5). The grooves
increased with increasing incubation time up to 24 h and very likely Ag\textsuperscript{+} stress could promote
cell wall breakdown and cytoplasm release after extended exposure (24 h in Fig. 5). Similar
AFM experiments on E. coli exposed to Ag\textsuperscript{+} ions were previously reported\textsuperscript{37} and showed that
vesicles appeared on the bacterium surface and became larger with the increase of the
incubation time. Nevertheless, contrary to R. gelatinosus samples, no accumulation of
metallic aggregates on E. coli cells was observed. In the case of B. subtilis, we observed
electron beam charge effect, and brighter aspect in the case of SEM imaging. In addition, silver exposed *B. subtilis* cells were most often aggregating in clusters, while the control sample cells were evenly individually distributed on the surface of the substrate (Fig. S4). For AFM imaging, we could observe a slight displacement of the cells under tip scanning, suggesting a weaker adhesion to the Mica substrate than previous cases. The cells aggregation effect on the other hand, might relate to the interactions between cells and Ag\(^+\) that accumulate on the surface of the cells; in addition to the interaction with the substrate used. This phenomenon was reported with *E. coli* cells exposed to carbon dots stress for instance\(^{38}\).

The imaging of *B. subtilis* exposed to silver ions showed that Ag\(^+\) had less effect comparing to *R. gelatinosus* and *E. coli* during the first hour of incubation (Fig. 6). Cell morphology differs slightly from control one. However, we could observe a drastic effect after 24 h exposition to silver. Apparent smaller size and irregular shape were the main morphological transformations, as reported for *E. coli* cells exposed to silica nanoparticles\(^{39}\).

Collectively, these results showed that silver has a strong impact on bacteria. It changes the structure of the cell surface which could be due to the accumulation of ions on the surface or/and deterioration of specific components of the cell wall. The impact of silver on bacteria differed from one type to another. The most drastic effect was observed with *R. gelatinosus*. This could originate from various factors. The thickness and structure of the cell wall for instance, is considered to be an important factor of metal stress resistance\(^{40}\). Another difference could arise from culture media composition: the speciation and bioavailability of metals in malate vs LB medium.

**Effect of culture media (malate medium versus LB medium) on Ag\(^+\) toxicity in *R. gelatinosus***
Studies have reported that bacterial growth media could impact on ions bioavailability and therefore on cell’s response and shape under stress condition [41-43]. We can suspect that, since culture media contains different chemical compounds as phosphates and chlorates, silver precipitation may occur distinctly depending on the medium composition. Growth of *E. coli* and *B. subtilis* in malate medium is very limited and did not allowed analyses of cells in this culture medium. In contrast, *R. gelatinosus* has the ability to grow on both malate and LB, although growth is slower in LB medium. To assess the effects of culture medium content on Ag⁺ toxicity, we first monitor the effect of Ag⁺ in LB, in comparison with malate medium, on the photosynthetic complexes of *R. gelatinosus*. In this experiment, *R. gelatinosus* cells were grown in malate or in LB medium. AgNO₃ was then added, and spectra were recorded after different incubation times. In agreement with our previous data [1], the results showed that for cells grown in malate medium, the light harvesting LH2 bacteriochlorophyll Bch800 decreased with the increasing incubation time. After 10 h of incubation, the Bch800 bacteriochlorophyll was completely lost (Fig. 7A). On the other hand, and in contrast to malate, for cells grown in LB, Ag⁺ took longer time to show the same impact on LH2. After 10 h of incubation, Bch800 decreased only slightly and was lost only after 24 h post incubation (Fig. 7B). These results indicate that silver in LB medium still affect LH2 but its impact and interaction with the membrane complexes, could be slowed down by Ag⁺ ligands in the LB medium.

**Effect of culture media (malate medium versus LB medium) on the interaction of *R. gelatinosus* with silver ions.**

Given that accumulation of silver aggregates was observed for *R. gelatinosus* in Malate medium, but not in *E. coli* or *B. subtilis* in LB, we asked whether these aggregates could also form when *R. gelatinosus* was grown in LB medium. To answer this question,
bacteria were grown as previously in Malate or LB medium. SEM imaging was used to
discriminate any effects between untreated or AgNO$_3$ exposed cells in LB or Malate medium.
The images revealed several differences. First for unknown reason, *R. gelatinosus* cells grown
in LB were longer than those grown in Malate medium (Fig. S5). Second, the comparison
between exposed bacteria to silver showed that unlike malate case, bacteria grown in LB did
not exhibit any silver accumulation on their surface. Bright halos were observed around the
cells; likely, due to trace of the medium, but we did not observe any metallic aggregates on
the bacterial surface (Fig. 8) in the LB medium. Together with the effects of Ag$^+$ in LB
medium on the photosynthetic LH2 complexes, the SEM results showed and confirmed that
the susceptibility of bacterial cells to silver ions and very likely to AgNPs is dependent on the
culture medium content. This is in agreements with previous studies showing that the thiol-
containing compounds and some amino acids of LB medium could sequester Ag$^+$ and other
cations, protecting thus bacteria from Ag$^+$ toxicity$^{44,45}$. Indeed, while Malate medium includes
high concentration of phosphate (K$_2$HPO$_4$), LB on the other hand contains NaCl and reduced
amount of phosphate. Both react with AgNO$_3$ and can lead to precipitate Ag$^0$ as Ag$_3$PO$_4$ and
AgCl$^{46}$, respectively. Accordingly, we suggest that in Malate medium, Ag$_3$PO$_4$ precipitate
forms aggregates in the medium and adhere to some cells, while in LB most Ag$^+$ could be
sequestered by ligands such as thiols preventing thus formation of AgCl aggregates.

Formation of Ag$^+$ aggregates in Malate/LB or citrate.

It was difficult, at that point to identify precisely the composition of this aggregates,
the focusing of the electron beam for EDX analysis for instance, induced contamination and
charge effect on the sample and the signal even accumulated was difficult to separate from the
carbon (HOPG) main signal. Furthermore, we suggest that these dots/aggregates may
correspond to the forming of Ag$_3$PO$_4$ particles due to the reaction between AgNO$_3$ and
K$_2$HPO$_4^{47,48}$. The latter considered to be a necessary component for Malate medium formulation, beside other chemical elements, at the reactant’s concentration range of phosphate derivatives and malate molecule in malate medium. Indeed, these components are the more concentrated in the malate medium, and their reaction with AgNO$_3$ is thus more probable. The reaction between silver nitrate and phosphate is: $2$K$_2$HPO$_4$ + $3$AgNO$_3$ → Ag$_3$PO$_4$ + $3$KNO$_3$ + KH$_2$PO$_4$. The chlorate can also lead to AgCl precipitates$^{49}$, but since there are present in both media, and before further and more detailed study, we decided to drive them away at that point of comparison. Phosphates as far as they are concerned are at very low concentration in LB formulation. As shown in figure 1, the size distribution density profiles show different superposed size distribution, suggesting that the nucleation stochastic process leading to the first distribution (red line) around the cells is different from the double centred distribution (blue line) on the membranes. We hypothetically attributed the first process, to silver precipitation due to chemical compounds present into malate medium, distinguished from other process involving bacteria response to the metallic stress concerning as showed in previous work$^1$ membranes proteins.

To demonstrate rapidly this assumption, we carried the following experiment. 6 mM of AgNO$_3$ was directly added to the media, malate and LB, under same conditions. The concentration was increased to enhance the suspected effects. We analysed then the optical properties, using spectrophotometry, of the compounds to detect possible formation of silver nanoparticles throw their well-known plasmonic response$^{50-52}$. (Fig. 9) shows the optical absorption for AgNO$_3$ added to culture media (Malate vs LB) and for comparison with citrate that is commonly used to reduce AgNO$_3$ to produce Ag nanoparticles. Usually in citrate assisted production of silver nanoparticles, borates are used instead of phosphates$^{53}$. The spectrophotometric curve showed clearly in all cases the formation of silver nanoparticles with a higher efficiency in the case of Malate compare to LB. The nanoparticles were not very
stable and underwent probably fragmentation process and dilution into the solution. We can see that in case of Malate and LB, nanoparticles underwent fragmentation and/or dilution, while for citrate they underwent also growing mechanisms.

More details analysis, trying to distinguish between several chemical compounds (phosphates, malic acid, carboxylates, chlorates, …) in broth composition and also light effect, is actually carried in our group. Furthermore, these preliminary results are sufficient to establish a link between observed aggregates and silver precipitation process due to medium solution. Considering Mie theory for the plasmonic response of silver nanoparticles, we can establish that the response around 440 nm corresponds to an average size distribution around 20 nm, in agreement with the distribution size observed around the cells but not the one on the membranes. The second distribution seems induced by species on the membranes, and hypothetically considered as a part of a defence mechanism.

Conclusions and perspectives

Metals antimicrobial activity has been investigated in different model bacteria. Metals exert their antimicrobial activity by affecting various components of the cell, including very likely, the cell envelope integrity. In this context, we present here, the morphological changes triggered by Ag+ ions on the structure of R. gelatinosus, E. coli and B. subtilis cells, using AFM and SEM microscopies. It is obvious from this study and others, that AFM and SEM can provide a useful information regarding the antimicrobial mechanisms of metal ions. The results helped us to build a 2 steps scenario: a first step (occurring in the 1st hour of exposure) where silver ions precipitate around and on the bacterial membrane following apparently different stochastic nucleation and growth mechanism. A second step (extended exposure) where silver ions and/or small particles impact and penetrate cell’s membrane inducing huge
transformation of bacterial membranes morphology and mechanical properties leading to cell
cell wall breaking. However, several open questions remain concerning the localisation of silver
fixation points, defense mechanism implying diffusion and nucleation of silver ions on the
membranes and optimal particles size for membranes penetration. Interaction with
synthesized nanoparticles with controlled size and shapes is under investigation to clarify that
point.

Comparison between bacteria and media enable to clarify specific behaviour of some
species. While *R. gelatinosus* cells showed high susceptibility to Ag⁺ after short exposure, in
*B. subtilis*, the impact of Ag⁺ took longer. Only cells incubated for 24 h with AgNO₃ appeared
in smaller size and irregular shape. This might be explained by the cell wall structure of *B.
subtilis* and very likely of other gram-positive bacteria. The thicker membrane built of layers
of peptidoglycan in gram-positive species, could improve the cell ability to resist external
environmental stress including metals stress⁴⁰. On the other hand, our results showed also
clearly the effect of culture media on those processes. A better understanding of the specific
role of the different chemical components present in the media should help addressing this
issue. It was found that silver reacts differently in different media used for cells growth. It has
more toxic impact on cells in Malate than in LB medium, at least for *R. gelatinosus*, due, as we
suggest, to the presence of phosphate in Malate medium. The reaction between AgNO₃ and
K₂HPO₄ lead to the precipitation of Ag₃PO₄ that appeared as whitish particles adhering to the
cells surface. These dotes were detected by the SEM due to the backscattered light of
identified Ag atoms on the sample surface. It is tempting to suggest that the accumulation of
Ag₃PO₄ particles on cell surface could enhance or accelerate the toxic effect of silver. Indeed,
because of their direct attachment to the membrane they could potentially release Ag⁺ ions
inside the cells. Nevertheless, we should also consider the opposite hypothesis. Indeed,
reduction of soluble Ag⁺ to colloidal or nanoparticles in Malate medium could protect cells if
insoluble particles are too large to enter into the cells as reported in *Pseudomonas aeruginosa*\textsuperscript{55,56}. The composition of the Ag precipitates is not yet known. It could correspond to *Ag\textsubscript{3}PO\textsubscript{4}*; nevertheless, there is also a strong reaction between *AgNO\textsubscript{3}* and Malic acid (main ingredient for Malate medium formula). More investigations are required to determine the nature of these precipitates in Malate medium.

### Materials and Methods

**Bacterial strains and growth** - *R. gelatinosus* (wild type S1 strain)\textsuperscript{1} was grown at 30°C, in the dark micro-aerobically (low oxygenation: 50 ml flasks containing 50 ml medium) or in light by photosynthesis (filled tubes with residual oxygen in the medium) in malate or LB growth medium.

**Spectrophotometric measurements** - Absorption spectroscopy was performed with an Agilent Cary 500 spectrophotometer for both bacteria measurement and the characterisation of the aggregates described on the culture media influence. For spectra on whole cells, cells were in a 60 % (wt/vol) sucrose solution.

**Atomic Force Microscopy (AFM)** - AFM images were taken under ambient air condition at room temperature, using the tapping mode of a Bruker Nanoscope AFM. Probe and cantilever used from Nanosensors type: (PPP-NCHR, tip radius of curvature < 10 nm, tip height 10-15 mm, highly doped silicon to dissipate static charge with high mechanical Q-factor for high sensitivity, nominal resonance frequency is 330 kHz and a force constant of 45 N/m). Images in this paper are taken at 50 μm for ensemble imaging or at 5 μm scale for individual bacteria imaging. Image resolution are at 512*512 Pixels. The scan speed is kept at 1 Hz. One image acquisition takes less than 10 min, ensuring a stable observation time window. Data acquired
include height (topographical), peak force Error image (mechanical), 3D and cross-section. Data analysis were carried using Gwyddion open-source software.

**Bacterial immobilization for imaging by AFM** - Acquisition of AFM images in air was much more convenient and had higher resolution than that in liquid. Our air/nitrogen dried microbial samples provided a suitable hardness for scanning without significant topographic changes and good resolution. Bacteria were immobilized on Mica substrate with gelatin. MICA substrate was used for its properties as it has no known effect on biological samples, transparent, easy to cut and to manipulate, atomically flat and inexpensive. The substrate must be coated to immobilize the living cells on it by creating electrostatic interaction between the negative charge of bacteria and the positive charge of gelatin. *Gelatin preparation (sigma G-6144)*: we used 0.5 g in 100 ml of hot distilled water. We used around 15 ml in a beaker, enough to dip mica substrate in it. We leave substrate to dry, to be used after 24 h. **Placing bacteria on gelatin coated mica**: 1 ml bacterial culture (0.5 OD at 680 nm) was centrifuged (5000 rpm) washed and rapidly resuspended in 500 µl of deionized water. 5 µl of this cell suspension was applied on the gelatin-coated mica substrate. Carefully, spread on the surface of the gelatin to avoid damaging it, and let it dry again as described in\textsuperscript{29,57}.

**Scanning Electron Microscopy (SEM)** – We imaged bacteria, which had been fixed, dehydrated and dried with a Zeiss Semfeg. It was not necessary to frozen them. We used low tension (2.0 – 3.0 kV) to avoid contamination and electron damaging of structures. Images were taken at different magnifications. The specimen did not release any volatile substance even at high vacuum. We kept same resolution as AFM to enable image comparison and control.

**Preparation of bacterial sample for SEM** – overnight PS grown cells (OD 680nm = 1) were exposed or not to AgNO\textsubscript{3}. After incubation time, samples were washed in deionized water,
and then diluted 10 times. Then, we use a clean graphite substrate (freshly cleaved) and dip coat in the cellular culture, and then let to dry\textsuperscript{30}.

**Statistical Analysis** - All experiments were performed in triplicate, unless otherwise stated, and data analysis were carried on different bacteria from different samples, showing a great reproducibility.

**Data availability** – All data generated or analysed during this study (and its supplementary files) are included in this publication article (Supplementary information). Any remaining information can be obtained from the corresponding authors upon reasonable request.

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**Author contributions**

N.K. conceived and administered the project; collected and analyzed the AFM and SEM data. R.T. contributed to perform most of the experiments, including all the AFM images; and wrote a first draft of the paper. J.D. participated in experiments related to the chemical investigation on media. S.L. and S.O. coordinate the biological part of the project, and with N.K. supervised research activities and defined the final version of the manuscript. Authorship is limited to those who have contributed substantially to the work reported. All authors have given approval to the final version of the manuscript. All authors reviewed the manuscript.
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Ethics approval and consent to participate

Not applicable.

Consent for publication

All the authors agree with the publication.

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Fig. 1 - SEM image at different scale (A) and their zoom (B) of *R. gelatinosus* bacteria, exposed to AgNO₃ for 1 h, deposited on HOPG graphite as described previously. One can observe silver (bright dots) concentration on the outer membrane. In (C) the graph shows the size distribution (histogram and density) of metallic aggregates on cells surface and around the cells.
Fig. 2 - AFM image of *R. gelatinosus* deposited as described previously. Images shows topological i.e. height (A and C) and peak force error (B and D) of untreated bacteria (1st line) and bacteria exposed to AgNO₃ for 1 h (2nd line). The profile curves correspond to relative (difference with mean height) average height across (transversal (E)) and along (longitudinal (F)) bacteria cell axis for control bacteria as profile references.
Fig. 3 – Images show topological, peak force error and profile cross section analysis. AFM image of *R. gelatinosus* bacteria untreated (A) and exposed (C) to AgNO$_3$ for 24 h.

Relative average heights are calculated compared to height mean value, along and across the bacteria cell’s length axis (B and D).
Fig. 4 - AFM image topology (upper group) and peak force error (lower group) of several *R. gelatinosus* bacteria exposed to AgNO$_3$ for 24 h or more, showing as examples the important damages caused to membranes and cells.
Fig. 5 - Morphological changes on *E. coli* deposited as described previously. Images shows topological height (left side) and peak force error (right side) of respectively untreated bacteria (1st line) and bacteria exposed to AgNO₃ for 1 h (2nd line) and 24 h (3rd line).
Fig. 6 - Morphological changes on *B. subtilis* bacteria deposited as described previously. Images show topological (left side) and peak force error (right side) of respectively untreated bacteria (1st line) and bacteria exposed to 1mM AgNO₃ for 1 h (2nd line) and 24 h (3rd line).
**Fig. 7** - Comparative AgNO$_3$ exposure impact on photosystem *in vivo* in *R. gelatinosus* cells grown in Malate (A) and in LB (B) media.

**Fig. 8** - SEM images (and zoom) of *R. gelatinosus* bacteria grown on malate (A) and LB (B) media, exposed during 1 h to AgNO$_3$. 
Fig. 9 - Optical spectroscopy curves for Malate, LB and citrate-phosphate solution toward nanoparticles synthesis. The dashed lines are just an eye-guide around the curve maximum.
Table 1 - Morphological parameters, obtained using AFM topological imaging, along and across bacteria cell length axis, for *R. gelatinosus*, control (CT) after 1h and 24 h. and exposed to AgNO\(_3\) (Ag) for 24 h.

|                          | Rg CT 1h | Rg CT 24 hrs. | Rg Ag 24 hrs. |
|--------------------------|----------|---------------|---------------|
| **Average height \(\bar{z}\) (nm)** |          |               |               |
| trans.                   | 55       | 60            | 90            |
| long.                    | 55       | 60            | 90            |
| **Root Mean Square Roughness \(R_q\) (nm)** |          |               |               |
| trans.                   | 8        | 9             | 20            |
| long.                    | 17       | 21            | 33            |
| **Average Total Roughness \(R_{tm}\) (nm)** |          |               |               |
| trans.                   | 40       | 43            | 89            |
| long.                    | 40       | 70            | 106           |
| **Skewness \(Sk\)**     |          |               |               |
| trans.                   | 0.05     | 0.02          | 0.34          |
| long.                    | -0.51    | -0.43         | -0.34         |
| **Kurtosis \(Ku\)**     |          |               |               |
| trans.                   | 1.8      | -0.16         | +0.18         |
| long.                    | -0.8     | -1.0          | -1.0          |
Figure 1

SEM image at different scale (A) and their zoom (B) of R. gelatinosus bacteria, exposed to AgNO3 for 1 h, deposited on HOPG graphite as described previously. One can observe silver (bright dots) concentration on the outer membrane. In (C) the graph shows the size distribution (histogram and density) of metallic aggregates on cells surface and around the cells.
Figure 2

AFM image of R. gelatinosus deposited as described previously. Images shows topological i.e. height (A and C) and peak force error (B and D) of untreated bacteria (1st line) and bacteria exposed to AgNO3 for 1 h (2nd line). The profile curves correspond to relative (difference with mean height) average height across (transversal (E)) and along (longitudinal (F)) bacteria cell axis for control bacteria as profile references.
Figure 3

Images show topological, peak force error and profile cross section analysis. AFM image of R. gelatinosus bacteria untreated (A) and exposed (C) to AgNO3 for 24 h. Relative average heights are, calculated compared to height mean value, along and across the bacteria cell’s length axis (B and D).
Figure 4

AFM image topology (upper group) and peak force error (lower group) of several *R. gelatinosus* bacteria exposed to AgNO3 for 24 h or more, showing as examples the important damages caused to membranes and cells.
Figure 5

Morphological changes on E. coli deposited as described previously. Images show topological height (left side) and peak force error (right side) of respectively untreated bacteria (1st line) and bacteria exposed to AgNO3 for 1 h (2nd line) and 24 h (3rd line).
Figure 6

Morphological changes on B. subtilis bacteria deposited as described previously. Images show topological (left side) and peak force error (right side) of respectively untreated bacteria (1st line) and bacteria exposed to 1mM AgNO3 for 1 h (2nd line) and 24 h (3rd line).
Figure 7

Comparative AgNO3 exposure impact on photosystem in vivo in R. gelatinosus cells grown in Malate (A) and in LB (B) media.
Figure 8

SEM images (and zoom) of R. gelatinosus bacteria grown on malate (A) and LB (B) media, exposed during 1 h to AgNO3.

Figure 9

Optical spectroscopy curves for Malate, LB and citrate-phosphate solution toward nanoparticles synthesis. The dashed lines are just an eye-guide around the curve maximum.

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