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2 **Impact of a model soil microorganism and of its secretome on the**
3 **fate of silver nanoparticles**

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21

22 **Abstract**

23 Sulfidation is a key process for silver nanoparticles released from consumer products in the
24 environment. This study focuses on the impact of a model soil microorganism, *Bacillus subtilis*,
25 on the fate of pristine and already sulfidized Ag-NPs. The nanoparticles were incubated with
26 the initial growth medium, isolated secretome and living bacteria, and characterized for their
27 size and morphology, agglomeration state, structure and Ag speciation. No Ag internalization
28 or sorption on the cell wall was detected. A partial sulfidation, leading to an Ag-Ag₂S core-
29 shell structure, was observed in the presence of the secretome, and the rate limiting step of the
30 reaction was the oxidation of Ag⁰, and it was favored near the crystal dislocations. The
31 sulfidation was complete in the presence of the living bacteria and followed an indirect
32 pathway. Both crystalline Ag₂S and amorphous Ag₂S and/or Ag-thiol were identified. At the
33 opposite, the bacteria had no impact on Ag₂S. These results suggest that microorganisms
34 participate to the sulfidation of Ag-NPs in aerobic systems such as unsaturated soils, and thus
35 affect the bioavailability of Ag. It is important to take these transformations into account during
36 exposure experiments, since they drastically change the exposure conditions. Finally, the
37 secretome of *B. subtilis* might be used for the green synthesis of Ag-Ag₂S core-shell
38 nanoparticles.

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44 **Introduction**

45 Because of their broad-spectrum antimicrobial properties, silver nanoparticles (Ag-NPs)
46 are increasingly produced and used in consumer products (cosmetics and personal care
47 products, food and food packaging, textile, electronics) and also in medicine and agriculture.¹
48 This increased use has raised some concerns, both from a toxicological point of view for
49 consumers during the use of these products, and from an ecotoxicological point of view since
50 Ag-NPs and their secondary products are expected to end up in environmental compartments,
51 especially in soils, at the end of their life cycle.²⁻³ Indeed, Ag-NPs released in wastewater
52 accumulate in sewage sludge during wastewater treatment.⁴ In Europe, 40 to 50% of this
53 biosolid is applied on agricultural soils as amendment.⁵

54 Since the last decade, the ecotoxicity of Ag-NPs has been studied at various levels of
55 complexity, from single organisms in growth media to complex systems such as soils and
56 natural waters⁶⁻⁷. The impact on bacteria has been the focus of many studies since Ag-NPs are
57 primarily used as biocides. This property results from several effect of Ag-NPs on bacteria
58 including the generation of reactive oxygen species (ROS), the disruption of the membrane
59 integrity, the interaction of Ag ions with proteins and disruption of their regular function, and
60 the interference with DNA replication, causing DNA damage.⁸⁻⁹ These effects are supposed to
61 result mainly from the release of Ag ions by oxidative dissolution of the Ag-NPs. As a
62 consequence, they are influenced by the physicochemical characteristics of the nanoparticles
63 including the size, the shape and the nature of the coating.¹⁰ The agglomeration state is another
64 factor influencing the toxicity of Ag-NPs. The formation of agglomerates tends to decrease
65 biocidal activity, possibly due to a decreased dissolution. Finally, chemical transformations
66 likely modify the toxicity of Ag-NPs.¹¹ Sulfidation is a key process taking place in sewage

67 systems¹² and wastewater treatment plants.⁴ This transformation decreases Ag-NPs toxicity,
68 thanks to the low solubility of Ag₂S.¹³⁻¹⁵ Sulfidation might also occur in growth media during
69 *in vitro* exposure experiments, due to the composition of the growth medium itself, and to the
70 release of thiol-containing molecules by the living organisms. To our knowledge, the impact of
71 microorganisms' activity on the speciation of Ag-NPs in growth media has never been studied.
72 Thus, it is important to assess the extent of these transformations, and to identify the agents
73 responsible for these changes (the growth medium itself vs. the bacterial activity). Bacteria may
74 also influence the chemical transformations of Ag-NPs in natural systems such as soils,
75 especially in the rhizosphere. Studies on simplified systems can help to decipher the role of the
76 various soil components in the NPs transformations.

77 The objective of this study was to characterize the physicochemical changes of Ag-NPs
78 induced by a classical growth medium for bacteria (LB), by the molecules secreted by the
79 bacteria, and by a living bacterial culture. The chosen strain is *Bacillus subtilis*, a model soil
80 bacterium ubiquitous in the environment¹⁶. In soils, it plays a role in rhizospheric processes as
81 a symbiotic organism for plants¹⁷. This Gram-positive bacterium secretes large amounts of
82 proteins, peptides and amino acids.¹⁸⁻¹⁹ It is actually used by the industry to produce
83 biosurfactant, antifungal compounds, protease and poly- γ -glutamate.¹⁹ Exposure conditions
84 were chosen as close as possible to environmentally relevant conditions: Pristine and aged (i.e.,
85 sulfidized) Ag-NPs (referred to as Ag₂S-NPs), as well as Ag lactate as a control for ionic Ag
86 were used, at a dose of 1 mg Ag L⁻¹. These various Ag forms were incubated in the initial
87 growth medium, in the secretome without bacteria (supernatant after culture), and in the
88 bacterial culture during the stationary phase. Although most exposure experiments are
89 conducted during the exponential phase (e.g.,²⁰⁻²¹). The stationary phase, during which the
90 medium is depleted in nutrients and when biopolymers and other exudates are present, is closer
91 to the physiological state of bacteria in the environment.²²⁻²³ A combination of analytical and

92 physical techniques were used, including dynamic light scattering (DLS), transmission electron
93 microscopy coupled with energy dispersive X-ray spectrometry (TEM-EDX), nano X-ray
94 fluorescence, bulk and nano X-ray absorption spectroscopy, and inductively coupled plasma –
95 atomic emission spectrometry (ICP-AES).

96

97 **Material and methods**

98 **Nanoparticles**

99 The silver nanoparticles and silver lactate were purchased at Sigma: PVP-coated Ag-
100 NPs (ref 758329, 5 wt% in ethylene glycol, <100 nm by TEM) and silver lactate (ref 85210,
101 CH₃CH(OH)COOAg, Molecular Weight: 196.94). Silver lactate was preferred to silver nitrate
102 to avoid the formation of AgCl in the medium. Ag₂S-NPs were obtained by sulfidation of PVP-
103 Ag-NPs as described in Levard et al.²⁴. More details and characterization are provided in
104 [Figures S1 and S2](#).

105

106 **Bacterial strain and culture media**

107 The *B. subtilis* strain used was the 3610 strain (wild type) (personal gift, Maria
108 Laaberki). The growth medium was LB: 10 g.L⁻¹ tryptone (Becton Dickinson ref: 212750), 5 g
109 L⁻¹ yeast extract (Becton Dickinson ref: 21705) and 5 g L⁻¹ NaCl (Sigma: S9888). Isolation of
110 the secretome: *Bacillus subtilis* strain 3610 was grown in Erlenmeyer flasks (250 mL)
111 containing LB medium with an initial OD₆₀₀ of 0.01 with shaking at 190 rpm at 37 °C. After
112 16h, the medium was depleted in nutrients from LB, and contained essentially the molecules
113 secreted by the bacteria (> 10 g L⁻¹ poly-γ-glutamate²⁵, 360 mg L⁻¹ exopolysaccharides, 1 g L⁻¹
114 DNA, and many proteins). Although the stationary phase was established since several hours,
115 we did not observe significant cell mortality (as evaluated by lactate dehydrogenase assay). The

116 culture was centrifuged at 8000 rpm for 15 min, and the cell-free supernatant (SN), considered
117 as the secretome, was recovered and used for incubations.

118

119 **Incubations with Ag-NPs, Ag₂S-NPs and Ag lactate**

120 Incubations with living bacteria: Cells were grown at 37°C in LB medium in 250 mL
121 Erlenmeyer flasks during 16h with an initial OD₆₀₀ of 0.01. After 16h of growth, the cells
122 culture were further incubated alone (for the control) or with 1mg L⁻¹ of Ag-NPs, or Ag₂S-NPs
123 or 1.8 mg L⁻¹ of silver lactate ((= 1mg L⁻¹ of Ag) during 5h under shaking. Viability and central
124 metabolism **were** not modified after exposure in these conditions (Eymard Vernain et al., subm).

125 Incubations in LB and SN without bacterial cells: SN and LB media were incubated without
126 (for the control) and with 1mg L⁻¹ of Ag-NPs or Ag₂S-NPs or silver lactate during 5h at 37°C
127 in 250 mL Erlenmeyer flasks filled up to 50 mL with shaking (referred to as “aerated condition”,
128 A, same as the bacterial culture), and in closed 50 mL falcon tubes (referred to as “unaerated
129 condition”, NA) at 25°C. These two conditions allowed us to test the influence of the aeration
130 on the transformations.

131

132 **Sample preparation after incubation**

133 All samples were obtained after centrifugation at 8000 rpm for 30 min. Different sample
134 preparations were used depending on the technique.

135 For TEM-EDX, samples without bacteria were deposited on a TEM copper grid (Ted Pella, ref
136 01824) and dried in inert atmosphere, and bacterial samples were fixed by high pressure
137 freezing, freeze-substitution and resin embedding ([Supporting Information](#)), prepared as 400
138 nm sections and deposited on a TEM copper grid.

139 For nanoXRF/nanoXANES analyses, 400 nm sections of embedded bacteria were deposited on
140 a grid with a Si₃N₄ window (Ted Pella, ref 21500-10). Other samples were prepared by

141 deposition of a drop of the fresh pellet on an ultralene film, drying in inert atmosphere, and
142 fixation of this film on a PEEK sample holder.

143 For bulk EXAFS analysis, pellets were mixed with 20% glycerol as cryoprotectant, placed in
144 the 5 mm diameter holes of the cryo-sample holder, plunged in liquid N₂, and introduced in
145 frozen hydrated state in the cryostat. For Ag lactate samples, Ag could not be recovered by
146 centrifugation, so the whole medium after incubation was freeze dried, diluted in BN and
147 pressed into 5 mm diameter pellets.

148

149 **TEM-EDX**

150 Sample were observed using a Tecnai OSIRIS (FEI) in STEM mode operating at 200
151 kV with a probe current close to 0.7 nA. Elemental analysis was performed using four
152 windowless energy dispersive X-ray detectors (Bruker) offering almost 1 sr of solid angle.
153 Hyperspectral EDX maps were obtained, from which elemental profiles for S and Ag were
154 extracted, and Ag/S peak intensity ratios were calculated. Diffraction patterns were recorded in
155 TEM mode using selected area diffraction (SAD) technique.

156 **NanoXRF**

157 NanoXRF and XANES analyses were performed on beamline ID16b at the European
158 Synchrotron Radiation Facility (ESRF, Grenoble, France). The optics used were a double white
159 mirror with Pt coating and a KB mirrors system which focus the beam down to 62 (vertical) ×
160 83 (horizontal) nm². Two fluorescence detectors (three-elements SDD arrays from SGX
161 Sensortech) were used. The photon flux was 5.8×10^9 ph s⁻¹ at 25.61 keV. NanoXRF maps
162 were recorded at 25.61 keV, with a step size of 50 x 50 nm, and a dwell time of 100 ms.

163

164 **X-ray absorption spectroscopy**

165 Ag speciation was studied by Ag K-edge (25.5 keV) bulk EXAFS and XANES
166 spectroscopy on FAME (BM30B) beamline at the ESRF and Samba beamline at SOLEIL
167 synchrotron (Paris, France) at 15-20°K using a liquid He cryostat, by Ag L_{III}-edge (3.35 keV)
168 bulk XANES spectroscopy on ID21 beamline at the ESRF at 80°K using a liquid N₂ cryostat,
169 and by Ag K-edge nanoXANES spectroscopy at room temperature on ID16b beamline at the
170 ESRF. Experimental details have been described previously for Ag K-edge bulk EXAFS on
171 FAME²⁶ and Ag L_{III}-edge XANES on ID21²⁷. For FAME and Samba data, a Si(220)
172 monochromator, a 30- and 35-element, respectively, Ge detector were used. For Ag K-edge
173 NanoXANES spectroscopy on ID16b, a Si(111) double crystal monochromator was used and
174 spectra were recorded using the full XAS mapping mode.²⁸ S speciation was also studied by S
175 K-edge XANES spectroscopy on ID21 (details in supporting information).

176 XANES and EXAFS data treatment was done with ATHENA from the Demeter software
177 package.²⁹ After normalization, spectra were analyzed by linear combination fitting using
178 libraries of Ag K- and L-edge and S K-edge references spectra detailed in Supporting
179 Information.

180

181 **ICP-AES**

182 The intracellular silver was determined by ICP-AES after cell lysis (details in
183 Supporting Information).

184

185 **Dynamic light scattering (DLS)**

186 Nanoparticles at 1 mg L⁻¹ were incubated in ultrapure water (referred to as H₂O), LB
187 medium or supernatant during 5 h in NA conditions before the DLS (dynamic light scattering)

188 measurement. The DLS apparatus was a DynaPro NanoStar (Wyatt Technology). Each
189 measurement was performed at least on biological triplicates, and with four technical replicates.
190 For each DLS analysis, between 2 and 5 hydrodynamic diameters were obtained. The results of
191 the various measurements were plotted on the same figure, with no averaging. The absence of
192 detectable particle in H₂O, LB and SN media was validated.

193 **Statistical tests**

194 Statistical tests (Tukey post hoc after ANOVA) were done on the EDX data (S/Ag peak heights)
195 using the software SPSS.

196

197 **Results**

198 **Distribution of silver in presence of *Bacillus subtilis***

199 Bacteria exposed to Ag-NPs, Ag₂S-NPs and Ag lactate were studied by nanoXRF in
200 order to evaluate the possible internalization of Ag, or binding to the cell wall. [Figure 1](#) shows
201 the nanoXRF maps obtained on 400 nm sections of bacteria. Cells appear on the Os maps,
202 thanks to OsO₄ staining (Os in [Figure 1](#), details on freeze-substitution in [Supporting](#)
203 [information](#)). For *B. subtilis* after exposure to Ag-NPs, Ag-rich spots of a few tens of nm, not
204 in contact with bacteria, were observed. No concentrated or diffuse Ag was detected in the
205 cytoplasm or cell wall of the bacteria. The detection limit for Ag in our experimental conditions
206 is estimated to 27 ± 7 mg Ag L⁻¹ (Supporting information). For *B. subtilis* after exposure to
207 Ag₂S-NPs, aggregates of several micrometers of Ag₂S-NPs were observed in the medium.
208 Beside this effect, there was no correlation between Ag and Os localization, suggesting no
209 internalization of Ag. This finding was confirmed by analyzing bacteria far from the Ag₂S-NPs

210 aggregate, which shows the absence of silver (fourth row in [Figure 1](#)). Finally, for bacteria after
211 exposure to Ag lactate, no Ag was detected inside and outside bacteria.

212 The absence of accumulation of silver in the bacteria for all conditions was confirmed
213 by ICP-AES after cell lysis. Indeed, for all conditions, silver concentration in the samples after
214 digestion was below the quantification limit of $3 \mu\text{g L}^{-1}$, corresponding to $4.5 \cdot 10^{-2} \mu\text{g Ag per}$
215 15 mL tube . This amount corresponds to 0.018% of the initial Ag (1 mg L^{-1} in 250 mL of
216 bacterial culture), to 4.5 attograms Ag per cell (considering about 10^{10} cells in 250 mL), and to
217 an intracellular concentration of 8.6 mg L^{-1} . Bondarenko et al.²¹ determined an intracellular
218 concentration of 0.7 mg L^{-1} at the EC_{50} of *B. subtilis* exposed to Ag-NPs. The absence of toxicity
219 observed in the present study suggests that the intracellular Ag concentration is below this
220 value. A more sensitive method would be necessary to confirm this point. To sum up, for the
221 three conditions, no internalization or association with the cell wall was observed, which is
222 consistent with the absence of effects on the bacterial growth in our experimental conditions
223 (Eymard Vernain et al., *subm.*).

224 After evaluating the possible internalization of Ag, we investigated the effects of *Bacillus*
225 *subtilis* on the physicochemical state of the nanoparticles, and possible chemical
226 transformations, by characterizing the Ag-NPs after incubation with the bacteria, and with the
227 growth medium (LB) alone and the secretome (SN) alone.

228 **Size and morphology of the NPs**

229 The nominal size and morphological characteristics of Ag-NPs and Ag₂S-NPs were observed
230 by transmission electron microscopy (TEM), after incubation for 5 h in H₂O, LB and SN (in
231 NA conditions) and cell culture. [Figure S3](#) shows representative images for these conditions,
232 and compares the size distribution for Ag-NPs. A decrease in nominal size was observed
233 between H₂O (majority of the nanoparticles between 40 and 80 nm) and LB or SN (majority of

234 NPs between 10 and 60 nm)(Figure S4). This observation suggests some partial dissolution of
235 the Ag-NPs in LB and SN. In addition, TEM observation showed that the original and partly
236 dissolved Ag-NPs were multi-twinned crystals (Figure S3 A-C). For the nanoparticles
237 incubated with *B. subtilis*, there was no significant change in diameter compared to H₂O, but a
238 dramatic change in morphology. Particles were actually aggregates of small (5 to 20 nm in
239 diameter) single crystal nanoparticles (Figure S3).

240 Contrary to the Ag-NPs, Ag₂S-NPs produced by sulfidation with Na₂S formed aggregates
241 between 350 nm and 10 μm, in which it was not possible to distinguish individual nanoparticles
242 and to determine their nominal size (Figure S5).

243 **Agglomeration state of Ag-NPs**

244 The study of the agglomeration state by DLS was restricted to Ag-NPs since Ag₂S-NPs
245 particles were not in a nano state (see below). Suspensions of Ag-NPs after incubation in H₂O,
246 LB or SN in NA conditions were analyzed. For H₂O and LB, the DLS measurements were
247 highly reproducible, and showed two populations: one with a diameter between 5 and 20 nm
248 and the other one with diameter of about 100 nm (Figure S6). For SN, a variety of diameters,
249 from a few nm to 1 μm, were obtained. Large diameters suggest an agglomeration due to SN,
250 but the agglomeration state was not stable. Indeed, results were not reproducible. Figure S6
251 shows the various diameters obtained on the triplicate samples, with four analyses per sample.
252 There results suggest that some molecules secreted by *Bacillus subtilis*, present in SN, induce
253 an agglomeration of the silver nanoparticles.

254 The combination of DLS and TEM results suggest a partial dissolution of the individual
255 nanoparticles in LB and SN, and their agglomeration in SN only.

256

257 **TEM-EDX analyses**

258 The Ag-NPs after incubation in the various media (H₂O, LB, SN, and bacterial culture)
259 were then studied by TEM-EDX. This technique allowed imaging the distribution of S and Ag
260 within the nanoparticles (Figure 2A). Different patterns were evidenced for Ag-NPs depending
261 on the medium. With *Bacillus subtilis*, Ag and S distribution was uniform, with a Ag/S
262 elemental ratio of 2/1, and SAED and HRTEM showed that the aggregates were composed of
263 single crystals of acanthite (Ag₂S, Figure S7). Thus, Ag-NPs were sulfidized in the presence of
264 bacteria. For Ag-NPs incubated in SN in aerated conditions, a coating of Ag₂S was observed,
265 leading to an Ag-Ag₂S core-shell structure. The Ag₂S coating had a thickness of a few nm
266 (Figure 2A and S8A), and was thicker near the dislocations of the multi-twinned Ag-NPs
267 (Figure S9). HRTEM analyses showed that the Ag₂S shell was crystalline but surrounded by
268 amorphous or organic phases (Figure S9). These observations suggest that the sulfidation
269 occurs at the surface of the NPs, preferentially near the crystal defaults, possibly driven by some
270 organic compounds. These observations suggest that the sulfidation progresses from the surface
271 to the inner part of the NPs. This coating was less developed for SN in non-aerated conditions,
272 even less for LB in aerated conditions (Figure 2A and Figure S8B), and almost absent for LB
273 in non-aerated condition (Figure 2A). For Ag₂S-NPs after incubation of the various media
274 (H₂O, SN, *B. subtilis*), a homogeneous distribution of Ag and S was observed, except in some
275 localized regions with only Ag (Figure 2B). These areas are probably due to electron beam
276 damage, since they increased in size with beam exposure. Moreover, bulk XRD analyzes of
277 Ag₂S-NPs before incubation (Figure S1) and bulk XAS analyses after incubation (see next
278 section) showed the absence of Ag⁰ in these samples.

279 In order to compare more quantitatively the extent of sulfidation between the conditions, we
280 extracted the EDX spectra of the whole NPs, and compared the height of the S peak after
281 normalization to the height of the Ag peak for each condition (Figure 2C). As expected from
282 the EDX maps, the S/Ag peak height increased in the order LB (NA) < SN (NA) < LB (A) <

283 SN (A) < *B. subtilis*. The difference was not significant for the first three, and significant ($p <$
284 0.05) for the latter two. The S/Ag peak was slightly lower for Ag-NPs + *B. subtilis* than for the
285 Ag₂S-NPs reference. Thus, SN was more efficient at sulfidizing the NPs than LB, and aeration
286 enhanced the sulfidation, but in all these conditions the sulfidation remained partial. Only the
287 living bacteria led to an almost complete transformation to Ag₂S.

288 **X-ray absorption spectroscopy**

289 The nature and proportions of Ag species in each sample were then studied by X-ray
290 absorption spectroscopy. XANES and EXAFS spectra, or XANES spectra only, were treated
291 by LCFs using model compound spectra (Figure 3, Table 1). Principal component analysis was
292 not used because the set of spectra at each edge was too small. Among the reference spectra of
293 our K-edge database, four standards including Ag-NPs, Ag₂S-NPs, amorphous Ag₂S and Ag-
294 thiol were sufficient to reproduce the spectra. Because of their similarity, the last two species
295 were merged into a single pool. Although the LB medium contains 5 g L⁻¹ NaCl, AgCl was not
296 detected in any sample. No significant change in speciation was detected for Ag-NPs after
297 incubation in LB or SN in NA conditions. By contrast, in A conditions, about 22% of sulfidation
298 was detected for LB and 14 to 23% for SN (Table 1). The nature of the secondary species, either
299 crystalline Ag₂S or amorphous Ag₂S/Ag-thiol, remains unclear for these samples because it is
300 a minor phase (the error bar for this type of analysis is estimated at $\pm 10\%$). In the presence of
301 *B. subtilis*, about 80% of Ag-NPs were sulfidized. The proportion of the secondary forms
302 slightly differ between XANES and EXAFS LCFs (64 and 45% for crystalline Ag₂S,
303 respectively, and 18 and 47% for amorphous Ag₂S/Ag and/or Ag-thiol, respectively, Table 1),
304 but it remains clear that both species were present. The fit quality was clearly weaker (R factor
305 increased by 20%) when only crystalline Ag₂S was used.

306 For silver lactate, the sulfidation was total in LB and SN, even in NA conditions. This
307 is not surprising since Ag is already present as Ag⁺, so there is no oxidation step required before

308 sulfidation. The final form was a mixture of crystalline Ag₂S and amorphous Ag₂S/Ag-thiol. A
309 similar result was obtained for Ag lactate in the presence of *B. subtilis*. Again, fits were clearly
310 weaker (R factor doubled) if only crystalline Ag₂S was used. Concerning Ag₂S-NPs, there was
311 no significant change in speciation after incubation with *B. subtilis* and with SN in aerated
312 conditions. The other conditions were not tested.

313 The speciation of sulfur in the LB medium before culture and in the supernatant after
314 culture (in absence of silver) were studied by sulfur K-edge XANES spectroscopy (Figure S10).
315 For both media, a mixture of thiols (R-SH), alkyl sulfides (R-S-R), sulfoxides (R-S=O) and
316 sulfate were observed. The proportion of thiols was comparable in both media (26 to 32% of
317 total S).

318 **Discussion**

319 Concerning the fate of pristine Ag-NPs in the various media, the molecules secreted by
320 *B. subtilis* (SN medium) increased the agglomeration of Ag-NPs as compared to pure water,
321 whereas the molecules of the initial growth medium (LB) did not. Meanwhile, a decrease of the
322 particle nominal diameter, suggesting a partial dissolution, was observed after incubation with
323 SN and LB. Although the toxicity of Ag-NPs strongly relies on their dissolution^{21, 30-31}, in our
324 experimental conditions (1 mg L⁻¹ Ag applied during the stationary phase), no impacts in terms
325 of cell mortality or changes in the central metabolism of *Bacillus subtilis* were observed. This
326 may be related to the presence of exopolymers produced by the bacteria known to decrease
327 metals availability³²⁻³³, and to other types of molecules excreted by the bacteria. This absence
328 of toxicity is consistent with the absence of internalization of silver inside cells, as observed by
329 nanoXRF and ICP-AES coupled with cell lysis. In addition, some transformations of Ag-NPs
330 were observed, likely affecting Ag availability. Ag-NPs were partly sulfidized (about 20% of
331 total Ag), and the secondary species (either crystalline Ag₂S or amorphous Ag₂S/Ag-thiol)

332 formed a homogeneous shell on the NPs, leading to an Ag-Ag₂S core-shell structure. Based on
333 TEM-EDX results, this surface sulfidation was slightly higher in the medium containing the
334 secretome (SN) than in the growth medium (LB). The aeration of the suspension, i.e., the
335 presence of dissolved oxygen, clearly enhanced the reaction. The sulfidation of ionic silver (Ag
336 lactate) in LB and SN was tested in non-aerated conditions. In that case the reaction was
337 complete, with formation of crystalline Ag₂S and amorphous Ag₂S/Ag-thiol. These results
338 allowed us to conclude that the limiting step of the sulfidation of Ag-NPs under our conditions
339 was not the availability of reduced sulfur, but of oxygen, necessary for the oxidation of Ag⁰
340 into Ag⁺. After incubation with *Bacillus subtilis*, the sulfidation was almost complete (80%)
341 and again, both crystalline Ag₂S (acanthite) and amorphous Ag₂S/Ag-thiol were formed, based
342 on bulk XANES and EXAFS analysis. These results are in contradiction with those obtained
343 by Hsueh et al.³⁴, who identified Ag₂O in a bacterial pellet of *B. subtilis* after exposure to 100
344 mg L⁻¹ Ag-NPs. However, to our opinion these results are not valid since Ag₂S and Ag₂O
345 reference spectra shown in this article are almost identical (which is not the case, e.g.,³⁵), and
346 the first peak for the various references (Ag₂S, Ag₂O, Ag-NPs) are surprisingly at the same
347 position.

348 The changes in speciation occurred without internalization or sorption on bacterial cell
349 walls, as observed both from TEM-EDX and nanoXRF. The composite structure of the Ag₂S
350 aggregates, composed of Ag₂S nanocrystals much smaller than the original Ag-NPs (Figure
351 S3), suggest an indirect sulfidation, with release of Ag⁺ ions followed by reaction with sulfides.
352 On the contrary, a direct sulfidation was observed by Thalmann et al.³⁶ for Ag-NPs in the
353 presence of HS⁻, with the formation of hollow Ag₂S spheres attributed to the Kirkendall effect.
354 Sulfidation of Ag-NPs can be realized by dissolved inorganic sulfide species including H₂S,
355 HS⁻ and S²⁻, and can be direct or indirect depending on the S/Ag ratio.³⁷ Inorganic sulfides are
356 likely from the aerobic culture medium of a chemoheterotrophic strain like *B. subtilis*. Thus,

357 thiol-containing molecules present in the medium (as observed from S XANES spectroscopy)
358 are the most likely sources of sulfide. This pathway would involve a cleavage of the S-C bond.
359 Such cleavage has been documented in synthesis routes of nanosized Ag₂S using cysteine as S
360 donor³⁸⁻³⁹. The proposed sulfidation mechanism is schematized in Figure S11.

361 These results show that living bacteria are more efficient at sulfidizing the Ag-NPs than
362 the growth medium or secreted molecules alone. A possible explanation of this observation
363 could be a modification of the secretome under Ag-NPs exposure. Indeed, the secretome used
364 for the SN conditions is produced by the bacteria in the absence of Ag-NPs, and may differ
365 from the one produced in the presence of Ag-NPs. Based on our conclusion on the rate limiting
366 step of the sulfidation, we can hypothesize that the bacteria under Ag-NPs exposure excrete
367 more oxidizing molecules. They might also excrete more thiol-containing molecules. These
368 hypotheses could be tested by mass spectrometry proteomics experiments. Anyhow, an
369 important result of this study is the demonstration that the bacterial activity has a strong impact
370 on the fate of Ag-NPs.

371 **Methodological and Environmental implications**

372 This study shows that the bacterial activity has a major impact on the fate of Ag-NPs,
373 by enhancing their sulfidation. The results suggest that the bacterial activity favors the first step
374 of the reaction, which is the oxidation of Ag⁰. Concerning the second step, the sulfidation itself,
375 thiol-containing proteins and peptides are the most likely source of reduced S. These results
376 suggest that microorganisms may participate to the sulfidation of Ag-NPs in aerobic systems in
377 the environment, such as unsaturated soils. On the contrary, *B. subtilis* does not modify the
378 speciation of Ag₂S-NPs.

379 This study has some implications for nano(eco)toxicology studies. In most studies, the
380 NPs are characterized before the exposure experiment. Our study evidenced various extents of

381 chemical transformations of Ag-NPs after 5 hours of incubation, depending on the media. These
382 changes clearly modify the exposure conditions. Thus, it is very important to monitor the
383 speciation of NPs during exposure experiments. This type of information would clearly help
384 the interpretation, and the inter comparison between studies. This point is valid for silver, and
385 other NPs prone to transformations and/or redox reactions such as ZnO, CuO, CeO₂ for
386 example.

387 In this study, Ag₂S-NPs produced by reacting Ag-NPs and Na₂S, was used as a proxy
388 of aged Ag-NPs. These Ag₂S-NPs formed large aggregates (several tens of micrometers) of
389 crystalline Ag₂S (acanthite), which were not altered in the presence of *Bacillus subtilis* or with
390 their secreted molecules. This form clearly differed from the secondary products obtained after
391 incubation of Ag-NPs with bacteria, which included both crystalline and amorphous Ag₂S
392 and/or Ag-thiol, with particle size still in the nanometer range. In soils amended with sewage
393 sludge, a mixture of crystalline and amorphous Ag₂S and/or Ag-thiol, present as micro and
394 nanosized particles and as diffuse concentrations, was observed²⁶. Thus, Ag₂S produced by
395 reacting Ag-NPs and Na₂S may not be the most relevant material for toxicity studies since it is
396 highly aggregated and fully crystalline. Sulfidation protocols involving thiol-containing
397 molecules might provide more representative aged Ag-NPs.

398 The development of safer-by-design nanoparticles is a growing research topic. In the
399 case of Ag-NPs, a decrease of the release of Ag from products (which can reach large amounts,
400 for example for textiles⁴⁰⁻⁴¹) while keeping the antibacterial properties, should be targeted.
401 Partly sulfidized Ag-NPs, with an Ag-Ag₂S core-shell structure, such as those produced in this
402 study, might be a material to test in this perspective. The secretome of *Bacillus subtilis* may
403 have some potential for the green synthesis of these nanocomposites.

404

405 **Supporting Information**

406 Materials and Methods: Nanoparticles characterization, high pressure freezing and freeze-
407 substitution, Ag reference compounds for XAS analyses, nanoXRF detection limit, ICP-AES
408 after cell lysis. Results: DLS analyses, TEM coupled with EDX, electron diffraction and high
409 resolution, S K-edge XANES and proposed mechanism of sulfidation of Ag-NPs in the
410 presence of *B. subtilis* (Figures S3 to S11). All data are presented in a single PDF document.

411

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427

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- 538

Table 1 : Distribution of Ag species determined by linear combination fitting of XAS spectra

Samples	Molar percentage of Ag species			Sum	R factor ^a	Edge, type of data and temp. of acquisition
	Ag-NPs	Ag ₂ S-NPs	Amorphous Ag ₂ S or Ag-thiol			
<u>Ag-NPs incubated with:</u>						
<i>B. subtilis</i>	17	64	18	99	0.0003	K, XANES, 15°K
	19	45	47	111	0.1554	K, EXAFS, 15°K
LB (NA) ^b	100			100	0.0005	K, XANES, 15°K
	100			100	0.0310	K, EXAFS, 15°K
LB (A) ^c	78	22	n.t. ^d	100	0.0017	K, XANES, RT
SN (NA)	100			100	0.0003	K, XANES, 15°K
	100			100	0.0836	K, EXAFS, 15°K
SN-1 (A)	77	23	n.t.	100	0.0017	K, XANES, RT
SN-2 (A)	100			100	0.0400	K, XANES, 15°K
	90		15	105	0.0400	K, EXAFS, 15°K
SN-3 (A)	89		14	103	0.0080	L, XANES, 80°K
<u>Ag₂S-NPs incubated with:</u>						
<i>B. subtilis</i>		100		100	0.0005	L, XANES, 80°K
SN (A)	7	91	n.t.	98	0.0002	K, XANES, RT
SN-2 (A)		100		100	0.0003	L, XANES, 80°K
<u>Ag lactate incubated with:</u>						
<i>B. subtilis</i>		25	72	97	0.0030	K, XANES, 15°K
LB (NA)		42	58	100	0.0008	K, XANES, 15°K
SN (NA)		32	68	100	0.0008	K, XANES, 15°K

Fits were done in the [E₀-20; E₀+80] eV range for XANES spectra, and in the [2-12] Å⁻¹ range for EXAFS spectra. Up to three components were used, except for XANES spectra recorded at RT, for which only two-components (Ag-NPs and Ag₂S-NPs) were used. ^a Fit quality criterion provided by ATHENA (R factor = $\sum [k^2 \chi_{\text{exp}} - k^2 \chi_{\text{fit}}]^2 / \sum [k^2 \chi_{\text{exp}}]^2$ for EXAFS spectra, and $\sum [\mu_{\text{exp}} - \mu_{\text{fit}}]^2 / \sum [\mu_{\text{exp}}]^2$ for XANES spectra). ^b Non-aerated conditions, ^c Aerated conditions. ^d Not tested because of the absence of these reference spectra in the K-edge database at RT. LB: initial growth medium. SN: supernatant (three replicates analyzed for Ag-NPs, aerated conditions).

542

543

Figure captions

544

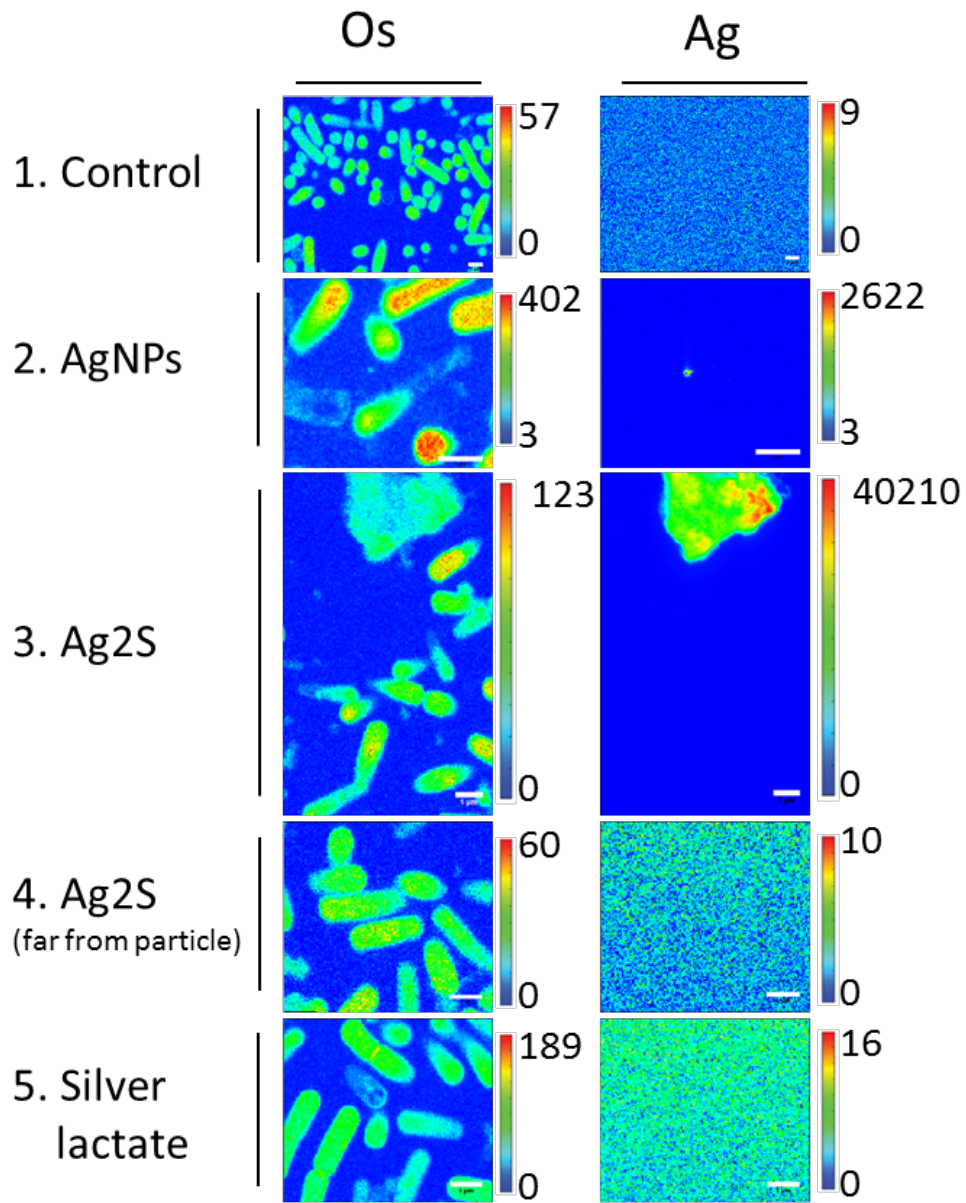
545 Figure 1: Nano-XRF analysis of *Bacillus subtilis* after incubation with Ag-NPs, Ag₂S-NPs, and
546 Ag lactate. The bacteria are imaged using the Os signal. Max number of counts for Os and Ag
547 are given for each map. Scale bar: 1 μ m. step size: 0.05*0.05 μ m, dwell time: 500ms

548

549 Figure 2: Ag (red), S (green) and Ag + S elemental maps obtained by TEM-EDX for the Ag-
550 NPs after incubation in H₂O, LB, SN, and with bacteria in aerated (A) or non-aerated (NA)
551 condition (Fig. 2A) and for Ag₂S-NPs after 5h incubation in H₂O, SN and with bacteria (Fig.
552 2B). Scale bar: 50 nm. (C) Comparison of the S/Ag peak height of the EDX spectra for
553 individual NPs, extracted from the maps. Significant differences according to the Tukey test
554 ($p < 0.05$) are indicated by letters.

555

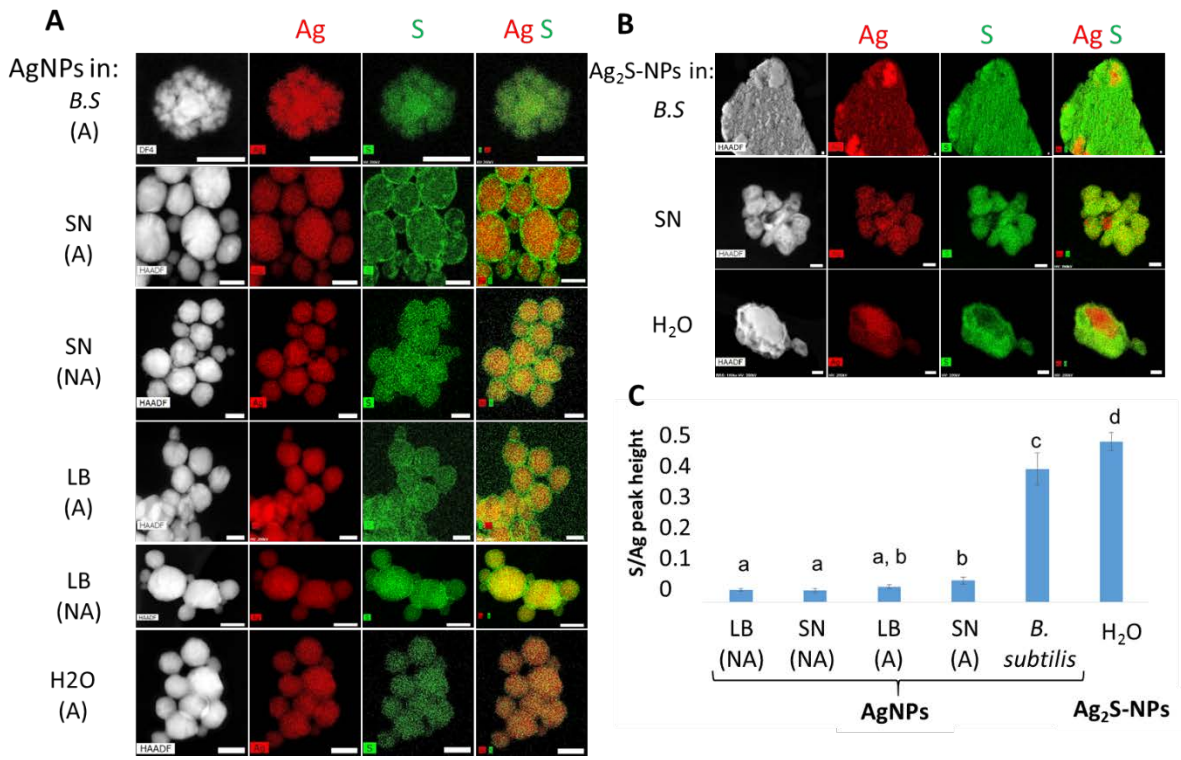
556 Figure 3: XAS spectra for Ag reference compounds and samples, recorded in various
557 conditions: A, B: Bulk Ag K-edge XANES (A) spectra and EXAFS (B) spectra recorded at
558 15°K. C: nano Ag K-edge XANES spectra recorded at room temperature. D: bulk Ag L_{III}-edge
559 XANES spectra recorded at 80°K. For the samples, the color of the spectra correspond to the
560 initial Ag form: Ag-NPs in red, Ag₂S-NPs is green, and Ag lactate in grey. Linear combination
561 fitting are shown in blue dashed lines.



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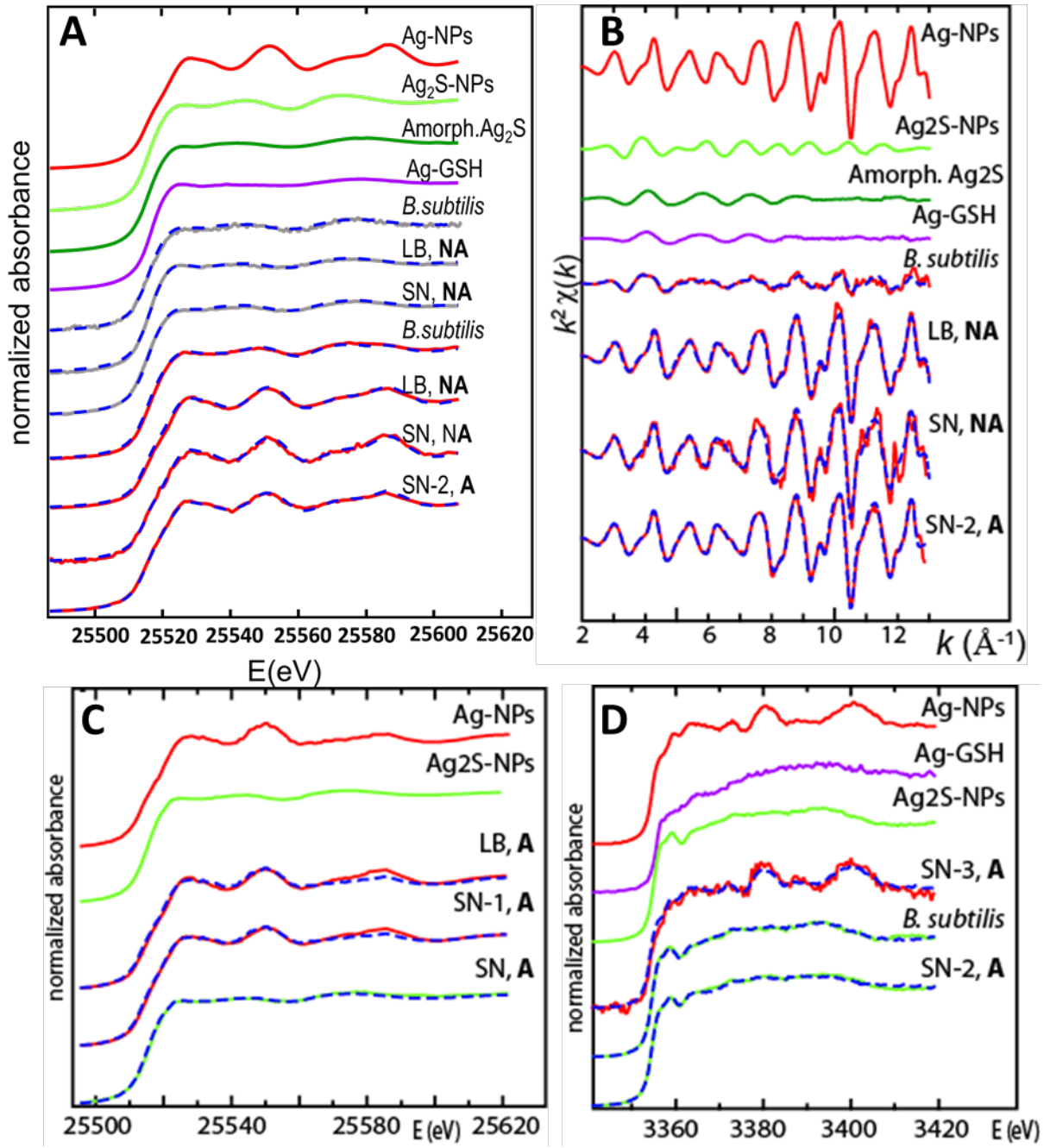
Figure 1



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Figure 2



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Figure 3

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