1   **Effects of sub-lethal doses of silver nanoparticles on *Bacillus subtilis* planktonic and**

2   **sessile cells.**

3   **Running title:** Effects of Ag-NPs on *B. subtilis*

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22

Abstract

24 **Aims:** Due to their antimicrobial activity, silver nanoparticles (Ag-NPs) are being

25 increasingly used in a number of industrial products. The accumulation of Ag-NPs in soil

26 might affect plant growth-promoting rhizobacteria and, in turn, plants. We describe the

27 effects of Ag-NPs on the soil bacteria *Azotobacter vinelandii* and *Bacillus subtilis*.

28 **Methods and Results:** In growth inhibition studies, *A. vinelandii* showed extreme sensitivity

29 to Ag-NPs, compared to *B. subtilis*. We investigated the effects of Ag-NPs at sub-inhibitory

30 concentrations, both on planktonic and sessile *B. subtilis* cells. As determined by 2,7-

31 dichlorofluorescein-diacetate assays, Ag-NPs increase the formation of reactive oxygen

32 species in planktonic cells, but not in sessile cells, suggesting the activation of scavenging

33 systems in biofilms. Consistently, proteomic analysis in *B. subtilis* Ag-NPs-treated biofilms

34 showed increased production of proteins related to quorum sensing and involved in stress

35 responses and redox sensing. Extracellular polysaccharides production and inorganic

36 phosphate solubilization were also increased, possibly as part of a coordinated response to

37 stress.

38 **Conclusions:** At low concentrations, Ag-NPs killed *A. vinelandii* and affected cellular

39 processes in planktonic and sessile *B. subtilis* cells.

40 **Significance and Impact of Study:** Re-direction of gene expression, linked to selective

41 toxicity, suggests a strong impact of Ag-NPs on soil bacterial communities.

Keywords

43 Rhizosphere; Biofilms; Stress response; Proteomics; *Bacillus*

44

Introduction

Nanoparticles (NPs) are defined as material that is at least one dimension below 100 nm (Handy *et al.* 2008). Such a small size confers NPs features different from the bulk material, *i.e.*, higher chemical reactivity, resistance and electrical conductivity and, potentially, higher biological activity (Nel *et al.* 2006).

Silver NPs (Ag-NPs) are widely used for medical and industrial applications, *e.g.*, for biological implants, air and water treatment filters, clothing, paints, cosmetics and food storage containers (Duncan 2011; Levard *et al.* 2012). The NP formulation increases the antimicrobial properties of silver, making Ag-NPs effective against a broad spectrum of bacterial and fungal species (Sotiriou and Pratsinis 2011; Guo et al. 2013), including antibiotic-resistant strains (Schacht *et al.* 2013).

The growing diffusion of Ag-NPs in commercially available products used daily (Benn and Westerhoff 2008) leads to a NP dispersal in the environment that is difficult to track and quantify. The release of Ag-NPs into the environment mainly occurs through the application of sewage sludge to agricultural land (Schlich *et al.* 2013). This procedure is still adopted in many countries (Gottschalk *et al.* 2009), although sludge may contain substantial amounts of heavy metals (Bourioug *et al.* 2015) and transfer them to soil. Despite scientific models identified soil as a major NP sink (Mueller *et al.* 2009), their actual concentrations in the environment are often unknown, and their biological activity still needs to be investigated (Whitley *et al.* 2013).

Some soil microorganisms, defined as plant growth-promoting rhizobacteria (PGPR), promote plant growth through several indirect or direct mechanisms, such as nutrient uptake, regulation of plant physiology by mimicking synthesis of plant hormones and increase of mineral and nitrogen availability in the soil (Philippot *et al.* 2013). PGPR can also increase

69 heavy metal solubility, helping plants withstand pollutants contamination (Vacheron *et al.*

70 2013).

71 Previous studies have shown that exposure to Ag-NPs leads to significant mortality in various

72 bacteria, mainly through membrane damage (Hachicho *et al.* 2014) and oxidative stress, via

73 Ag-NP-induced reactive oxygen species (ROS) (Fabrega *et al.* 2009). While antimicrobial

74 activity and efficacy of Ag-NP has been the focus of a variety of studies, aiming to use Ag-

75 NPs as an alternative to antibiotics (Rai *et al.* 2012), little information is available regarding

76 the possible effects of sub-lethal doses. To identify mechanisms activated by bacteria to face

77 Ag-NP presence in soil, we have studied the effects of Ag-NPs at concentrations up to 10 mg

78 l^{-1} on two plant growth-promoting rhizobacteria: the Gram negative nitrogen-fixing bacterium

79 *Azotobacter vinelandii*, and *Bacillus subtilis*, a Gram positive bacterium. We found that 10

80 mg l^{-1} Ag-NPs strongly inhibited *A. vinelandii* growth and induced oxidative stress response

81 and exopolysaccharide production in *B. subtilis*. Our results suggest that Ag-NPs, at a

82 concentration range locally found in the soil environment, can induce ROS production and

83 select soil microbial population. Interestingly, we also found that, in *B. subtilis*, plant growth-

84 promoting activities, in particular inorganic phosphate solubilization, were activated by sub-

85 lethal Ag-NP concentrations. Possible implications on soil microbial community are

86 discussed.

Materials and Methods

Bacterial strains and growth conditions

89 *Bacillus subtilis* wild type strain Cu1065 and *Azotobacter vinelandii* wild type strain UW136

90 were maintained at -80°C in suspensions containing 20% glycerol. *B. subtilis* was grown

91 aerobically in Tryptic Soy Broth (TSB) medium for 12 h at 30° C. *A.vinelandii* was grown in

92 Burk's medium supplemented with 1% sucrose and 15 mmol l^{-1} ammonium acetate for 30 h

93 at 30°C. Silver nanoparticles (Ag-NPs; 10 nm OECD PVP BioPure Silver Nanoparticles,

94 nanoComposix, USA) were stored at 4°C as 1 mg/ml suspension in water, and were added to

95 liquid medium, or uniformly distributed on the agar surface, immediately prior to the start of

96 the experiments. According to the supplier, purchased Ag-NPs have a diameter of 8.3 ± 1.5

97 nm, hydrodynamic diameter smaller than 20 nm and negative zeta potential (-19 mV).

98 **Effects of Ag-NPs on planktonic growth**

99 *B. subtilis* and *A. vinelandii* growth in the presence of Ag-NPs at various concentrations (0,

100 0.01, 0.1, 1, 10, and 100 mg l^{-1}) was monitored, registering the optical density (OD) at 600

101 nm every 45 min with a microtiter reader (Biotek-Power Wave XS2). The results were

102 confirmed plating cell suspensions from stationary phase serially diluted on agarized media,

103 incubated at 30°C (overnight for *B. subtilis*, 36 h for *A. vinelandii*) and the colony forming

104 units (CFU) were enumerated using the drop-plate method (Herigstad *et al.* 2001).

105 Experiments were conducted in triplicate. Growth curves were used to calculate the

106 generation time for each condition.

107 **Transmission electron microscopy (TEM) study**

108 Samples for TEM analysis were collected from liquid cultures both in exponential and

109 stationary phases, respectively after 3 h and 8 h of growth in contact with 0 and 10 mg l^{-1} of

110 NPs. Cells were centrifuged (30 min, 7000 *g*) and fixed in an equal volume of 2.5%

111 glutaraldehyde in cacodylate buffer (pH 7.4) at 4°C overnight. The samples were then rinsed

112 with 0.1 mol l^{-1} cacodylate buffer followed by postfixation in cacodylate buffer supplemented

113 with 1% (wt/vol) osmium tetroxide. Fixed cell suspensions were washed with cacodylate

114 buffer, dehydrated in an ethanol gradient (once for 15 min in 25%, 50%; once for 1 h in 70%;

115 once for 15 min in 90%, 95% and two times for 15 min in 100%) and then in propylene oxide

116 for 20 min. The samples were infiltrated and finally embedded in Epon Araldite at 60°C for

117 24 h. The polymerized samples were sectioned into ultra-thin slices (80 nm in thickness) and

118   placed on collodion-coated copper grid (400 meshes). The slices were examined by TEM

119   with Leo912ab (Zeiss, Jena, Germany) at 80 KV.

120   Ten images with a reduced enlargement of both the control and the treated samples were

121   analyzed after exposure to uranyl acetate (10 min) and to lead citrate (5 min) to count live

122   and dead cells, considering cells with no significant morphological alterations as live cells.

123   TEM analysis was also used to verify the absence of aggregated NPs in the conditions used.

124   **Biofilm formation**

125   Colony biofilms of *B. subtilis* were prepared following the method reported (Anderl *et al.*

126   2000). Briefly, 10 µl of cell suspension containing 1.5 x 10^6 cells were used to inoculate

127   sterile black polycarbonate filter membranes (0.22 mm pore size, Whatman, UK) that were

128   placed on TSA plates, at 30°C, either in the absence or in the presence of Ag-NPs (1 or 10 mg

129   l^{-1}). Ag-NPs were poured on agar plates and let adsorb. The membranes were transferred

130   every 48 h to fresh media, and grown for 8 days in total.

131   **Colony biofilm quantification with Bradford assay and ATP assay**

132   Total protein amount and average ATP consumption were determined to assess relative

133   amounts of biomass and metabolic activity in colony biofilms.

134   For protein determination, a membrane was collected every 24 h and resuspended in a 10-ml

135   tube with 2 ml of sterile phosphate buffered saline (PBS, 10 mmol l^{-1} phosphate buffer, 0.3

136   mol l^{-1} NaCl, pH 7.4). Cells were broken by 5 cycles of 30 s sonication with 30 s intervals;

137   cell lysates were centrifuged 15 min at 4°C at 19000 *g* and supernatant was collected. The

138   protein amount was quantified with Bradford assay (Bradford 1976), using bovine serum

139   albumin as a standard. Experiments were performed in triplicate.

140   Bacterial metabolic activity in colony biofilm was assessed using the biomass detection kit

141   (Promicol). The experiments were performed according to the manufacturer's protocol using

142   the FB 14 Vega bioluminometer (Berthold Detection Systems). Relative light units per

143   second (RLU s^{-1}) values were converted to ATP concentrations (nmol ml^{-1}) using the standard

144   provided. Colony biofilm was resuspended in 100 mmol l^{-1} Tris pH 7.75, vortexed and

145   sonicated for 30 s (Kobayashi *et al.* 2009). A calibration curve was generated measuring RLU

146   s^{-1} in *B. subtilis* planktonic cells. Tests were performed in triplicate.

Level of oxidative stress on planktonic and sessile cells

148   The level of oxidative stress in planktonic and sessile cells of *B. subtilis* was determined

149   using the 2,7-dichlorofluorescein-diacetate (H$_2$DCFDA) assay (Jakubowski *et al.* 2000).

150   *B. subtilis* planktonic cells grown at 30°C for 12 h in TSB, with either 0, 1, or 10 mg l^{-1} of

151   Ag-NPs, were washed with PBS and resuspended in 50 mmol l^{-1} PBS, while, for the colony-

152   biofilm, one membrane biofilm was collected for 8 days, scraped and homogeneously

153   resuspended in 2 ml of PBS 50 mmol l^{-1}.

154   Seven hundred and fifty μl of cell suspension was incubated with 10 μmol l^{-1} H$_2$DCFDA at

155   30°C for 30 min, vortexed and centrifuged. The supernatant was collected to measure

156   fluorescence relative to the extracellular reactive oxygen species (ROS) presence. To evaluate

157   intracellular ROS concentrations in either planktonic or biofilm cultures, cells were washed

158   three times and broken with 5 cycles of 30 s sonication with 30 s intervals. The fluorescence

159   of the supernatant collected before (outer oxidative stress) and after cell sonication (inner

160   oxidative stress) was measured using the fluorometer VICTOR TM X Multilabel Plate

161   Readers (Perkin Elmer, Italy), excitation 490 nm and emission 519 nm. The emission values

162   were normalized against the protein concentration, obtained from the remaining 750 μl of cell

163   suspension with the Bradford assay. Experiments were conducted in triplicate.

Extraction and characterization of the extracellular polymeric substances (EPS)

165   EPS extraction and characterization was conducted as described by Villa and collaborators

166   (2012) on five-days old biofilm biomass, grown in contact with 0 and 10 mg l^{-1} Ag-NPs. The

167   cetyltrimethylammonium bromide (CTAB)-DNA method described by Corinaldesi and

168 collaborators (2005) was used to quntify extracellular DNA (eDNA). The Bradford method

169 was applied to analyze protein concentrations, whereas the optimized microplate phenol-

170 sulfuric acid assay was applied for carbohydrate determination (Masuko *et al.* 2005) using

171 glucose as standard. The results obtained were normalized by the cellular protein

172 concentration. Experiments were performed in triplicate.

173 Proteomic analysis

174 Protein extracts were obtained by lysing, homogenizing and sonicating the whole colony

175 biofilm (ten 5-days old biofilms for each condition), grown either in the presence or in the

176 absence of 10 mg l^{-1} Ag-NPs, in lysis buffer (10 mmol l^{-1} Tris-HCl pH 7.5, 100 mmol l^{-1}

177 NaCl) with protease inhibitor. Protein extracts were precipitated adding a cold mix of ethanol,

178 methanol and acetone (ratio 2:1:1, v/v), and redissolved in 6 mol l^{-1} urea, 100 mmol l^{-1}

179 triethylammonium bicarbonate buffer pH 8.5. After reduction with 10 mmol l^{-1} dithiothreitol

180 and alkylation with 20 mmol l^{-1} iodoacetamide, equal amounts of protein samples were

181 digested 50:1 (w/w) with sequence grade trypsin (Promega, USA) at 37 °C overnight. In-

182 solution dimethyl labeling on peptides was performed as described by Boersema and

183 collaborators (2009) with sodium cyanoborohydride ($NaBH_3CN$), formaldehyde (CH_2O, light

184 labeling) and deuterated formaldehyde (CD_2O, heavy labeling). In Experiment A, tryptic

185 peptides deriving from control and Ag-NP treated biofilm were reacted with light and heavy

186 formaldehyde, respectively. A second experiment (Experiment B) was also performed,

187 inverting isotope labelling. After mixing equal quantities of labeled tryptic peptides, the

188 samples were loaded on 18 cm Immobiline DryStrip gels (GE Healthcare, Sweden), pH 3-10,

189 for peptide separation. Isoelectric focused strips were cut in 18 pieces and extracted peptides

190 were analyzed by liquid chromatography-electrospray ionization-tandem mass spectrometry

191 (LC-ESI-MS/MS) on an Ultimate 3000 Micro HPLC apparatus (Dionex, USA) equipped with

192 a FLM-3000-Flow manager module directly coupled to a LTQ Orbitrap XL hybrid FT mass

193 spectrometer (Thermo Fisher Scientific, USA). Reverse-phase chromatography was

194 performed on a Jupiter C18, 5 μm, 150 x 1.0 mm column (Phenomenex, USA) and a 95 min

195 run (gradient 1.6 to 44 % acetonitrile in water with 0.1% formic acid over 60 min) at a flow

196 rate of 80 μL min^{-1}. Mass spectra were collected in FT-IT data dependent scan mode (MS

197 scan at 60000 of resolution in the Orbitrap and MS/MS scan on the three most intense peaks

198 in the linear ion trap, mass range 300-2000 Da). Selected peptide charge states were isolated

199 with a width of m z^{-1} 6-10 and activated for 30 ms using 35% normalized collision energy

200 and an activation q of 0.25. Protein identification and quantification was obtained with the

201 embedded ion accounting algorithm (Sequest HT) of the software Proteome Discoverer

202 (version 1.4, Thermo) after searching a UniProtKB/Swiss-Prot Protein Knowledgebase

203 (release 2013_08 of 24-Jul-13 containing 540732 sequence entries; taxonomical restrictions:

204 *Bacillus subtilis*, 4188 sequence entries). The search parameters were 10 ppm tolerance for

205 precursor ions and 0.8 Da for product ions, 2 missed cleavages, carbamydomethylation of

206 cysteine as fixed modification, oxidation of methionine as variable modification, light and

207 heavy dimethylation of peptide N-termini and lysine residues as fixed modification on two

208 different search nodes, and Percolator calculated false discovery rate under 5%. Relative

209 peptide abundance was calculated from extracted ion chromatograms of the different isotopic

210 variants with 1.5 fold change threshold value for up/down regulation.

211 **Bioinformatic analysis**

212 Modulated proteins identified by proteomic analysis were further analyzed by the Protein

213 Analysis Through Evolutionary Relationships Classification System (PANTHER, version 9.0,

214 http://www.pantherdb.org) (Mi *et al.* 2013) to highlight the most relevant Gene Ontology

215 (GO) terms and the enriched functional-related protein groups. By the PANTHER Statistical

216 overrepresentation tool, the over- and under- representation of any protein class was assayed

217 using the binomial test (Cho and Campbell 2000) with Bonferroni correction for multiple

218   comparisons, comparing the protein list to the whole *B. subtilis* proteome. The most

219   significant categories were identified by calculating the related significance (*p*-value).

220   ***In-vitro* Plant Growth-Promoting Rhizobacteria (PGPR) and motility assays**

221   PGPR assays were performed inoculating planktonic cells either in direct contact with Ag-

222   NPs or just pre-exposed to Ag-NPs. In the first case, media used for PGPR assays were

223   inoculated with 100 µl of culture of *B. subtilis* at 0.3 as OD (600 nm) either in the absence or

224   in the presence of 10 mg l^{-1} Ag-NPs. In the case of pre-exposition to Ag-NPs, 100 µl of *B.*

225   *subtilis* grown in the absence or in the presence of 10 mg l^{-1} Ag-NPs for 24 h at 30°C, washed

226   in PBS and resuspended to obtain 0.3 as OD (600 nm), were used as inoculum for the PGPR

227   assays.

228   Indole-3-acetic acid (IAA) production was detected as described by Brick and collaborators

229   (1991). Bacterial cultures were grown for 72 h in TSB supplemented with tryptophan (500

230   mg ml^{-1}). After centrifugation, the supernatant (2 ml) was mixed with 40 µl of

231   orthophosphoric acid and 4 ml of the Salkowski reagent (35% of perchloric acid, 1 ml 0.5

232   mol l^{-1} FeCl$_3$ solution). After incubation for 25 min, the OD (530 nm) was taken.

233   Concentration of IAA produced by cultures was measured using a calibration curve of IAA in

234   the range of 10-100 mg ml^{-1}.

235   To verify the capacity to solubilize inorganic phosphate, the colorimetric method described

236   by Ahmad and collaborators (2008) was used. After 72 h of growth at 30°C, the OD (600 nm)

237   of centrifuged bacterial cultures was measured. Values obtained from inoculated medium

238   were subtracted from the control.

239   Production of siderophores was studied by cultivation of the isolates on chrome azurol sulfate

240   (CAS) agar plate, prepared as described by Schwyn and Neilands (1987). After solidification,

241   TSA plates were cut into halves and one half was replaced by CAS agar. The halves

242   containing TSA were inoculated and the plates were incubated at 30°C for a week. The

243   chromatic change of CAS agar was evaluated to state the siderophores production.

244   Nitrogen fixation was evaluated inoculating the medium described by Tarrand and

245   collaborators (1978). After 72 h of growth at 30°C, 100 µl of grown bacteria were inoculated

246   again in new medium and let grow at 30°C for a week. The OD (600 nm) was measured.

247   Each assay was conducted with ten replicates for control and ten replicates for treated cells.

248   Swarming and swimming motility were determined as previously described by Villa and

249   collaborators (2012) in TSB medium added either with 0.3% (wt/vol) agar (for swimming

250   motility) or with 0.7% (wt/vol) agar (for swarming motility). Plates were allowed to dry for 2

251   h and were inoculated with 10 µl of a 24 h-old culture of *B. subtilis*, incubated with either 0

252   or 10 mg l^{-1} Ag-NPs, washed with PBS, resuspended to obtain 0.3 as OD (600 nm), added to

253   the top of the agar and incubated at 30°C for 48 h. Results were expressed as the diameter

254   (cm) of the area of observed motility.

255   **Statistical analysis**

256   A t-test or analysis of variance (ANOVA) via Graphpad Software (San Diego California

257   USA) was applied to statistically evaluate any significant differences among the samples.

258   Tukey's honestly significant different test (HSD) was used for pairwise comparison to

259   determine the significance of the data. Statistically significant results were depicted by *p*-

260   values 0.05.

261   # Results

262   **Effect of Ag-NPs on planktonic growth of rhizobacteria *A. vinelandii* and *B. subtilis***

263   In order to evaluate Ag-NP effects on two important representatives of rhizobacteria, namely

264   *A. vinelandii* and *B. subtilis*, we performed growth inhibition tests in liquid media. Ag-NP

265   concentrations chosen ranged from 0.1 mg l^{-1}, *i.e.*, a concentration close to the proposed "no

266   effect" concentration in soil (0.05 mg/kg; Schlich *et al.* 2013) to 100 mg l^{-1}. As shown in Fig.

267   1b, Ag-NPs inhibited *A. vinelandii* growth, albeit partially, already at concentrations as low as

268   0.1 mg l^{-1}. Low OD values are caused by the low oxygen concentration in the medium;

269   however, similar sensitivity has been observed also in *A. vinelandii* cultures grown with

270   vigorous shaking. In contrast, *B. subtilis*, growth rate was only affected at 100 mg l^{-1} Ag-NPs,

271   with consistent decrease in biomass accumulation (Fig. 1a). Determination of generation

272   times during growth phase confirmed that, unlike *B. subtilis* (Fig. 1a), *A. vinelandii* growth

273   rate was already affected at the lowest concentration tested (Fig. 1b). These results were also

274   confirmed by viable counts on aliquots of stationary phase cultures treated with various Ag-

275   NP concentrations, showing reduction in colony forming units (CFU) consistent with

276   reduction in OD (600 nm) (data not shown). The results of this experiment would suggest

277   that, even at concentrations as low as 0.1 mg l^{-1}, Ag-NPs might affect the composition of soil

278   bacterial community by selective bacterial growth inhibition. We investigated whether 10 mg

279   ml^{-1} Ag-NPs, a sub-inhibitory concentration in *B. subtilis*, might trigger specific cellular

280   responses in this bacterium.

281   **Study of the interaction between Ag-NPs and *B. subtilis* by TEM observations**

282   Interaction of Ag-NPs with *B. subtilis* cells was monitored by direct TEM observations,

283   which showed that no Ag-NP aggregates were present in the media used for bacterial growth.

284   Planktonic cultures, grown either in the absence or in the presence of 10 mg l^{-1} Ag-NPs, were

285   observed to determine specific localization of Ag-NPs, and possible effects on cell

286   morphology. During exponential phase (Fig. 2a-c), Ag-NPs appear to gather preferentially as

287   aggregates around specific cells, with a non-homogenous distribution (Fig. 2b). Ag-NPs were

288   also visible inside microbial cells, as single or aggregated Ag-NPs (Fig. 2c). Phase contrast

289   images revealed that the cell walls of bacteria with internalized Ag-NPs showed no

290   interruption, and the cells were not affected morphologically (data not shown).

291   During the stationary phase (Fig. 2d-f), for both control and treated samples, the cell wall was

292   no longer stretched, resulting in a rougher surface. As highlighted in Fig. 2d-f, both in control

293   and treated samples, some dead or dying cells were present. Interestingly, in the treated

294   samples, the Ag-NPs gather preferentially within dead cells or on what remains of the cell

295   wall (Fig. 2e and 2f). This would suggest that Ag-NPs might be more toxic to *B. subtilis*

296   cultures during stationary phase. To verify this, intact versus lysed *B. subtilis* cells were

297   counted in TEM pictures on a total of six thousand cells, both for control and treated (10 mg

298   l^{-1} Ag-NPs) samples during stationary phase. No statistically significant differences were

299   observed (control: 2.83 ± 0.02 % dead /live cells; treated: 4.00 ± 0.01 % dead /live cells),

300   confirming that, at 10 mg l^{-1}, Ag-NPs does not affect *B. subtilis* viability.

301   **Effect of Ag-NPs on sessile growth of *B. subtilis***

302   Ag-NPs accumulating in soil are likely to interact with *B. subtilis* growing as a biofilm, rather

303   than in planktonic cells. For this reason, we tested inhibition of colony biofilm by Ag-NPs.

304   This condition mimics growth in soil, in which bacteria are attached to a solid surface and

305   where water availability is influenced by the solute potentials (Chang *et al.* 2003). *B. subtilis*

306   colony biofilm showed rapid growth, reaching maturity in 4 days. At later times, the colony

307   biofilm seemed to undergo a phase of dispersion, as suggested by a reduction in total proteins

308   (Fig. 3). Although the presence of 10 mg l^{-1} of Ag-NPs did not hinder biofilm biomass as

309   determined both by total protein determination (Fig. 3) and ATP consumption levels (Fig.

310   S4), it appeared to slow down growth rate, in particular at days 2 and 3, corresponding to the

311   exponential phase of biofilm growth. In this growth phase, the lower ATP concentration of

312   Ag-NPs treated biofilm with respect to the control, suggested a more extended lag phase in

313   the presence of Ag-NPs. In contrast, the presence of 1 mg l^{-1} of Ag-NPs seemed to enhance

314   biofilm growth by day 4.

315   **Level of oxidative stress in planktonic cells and biofilm of *B. subtilis***

316 Results of the biofilm growth inhibition experiments highlight a phase of adaptation to Ag-

317 NPs of biofilms that is not visible in planktonic cells. Since inhibition of bacterial growth by

318 Ag-NP might be associated to induction of oxidative stress, we measured Ag-NP-induced

319 ROS production both in planktonic (Fig. 4) and biofilm (Fig. 5) cells. Due to the complex

320 structure of the biofilm, ROS production was determined both intracellularly and in the

321 biofilm matrix. In planktonic cells, collected during stationary phase, 10 mg l^{-1} Ag-NP

322 increased intracellular ROS concentrations by 3-fold compared to the untreated control (Fig.

323 4). The effect of 1 mg l^{-1} Ag-NPs was also tested, and, surprisingly, determined a reduction in

324 intracellular ROS levels, possibly suggesting that, at low concentrations, Ag-NPs might

325 induce an adaptive response to oxidative stress, leading to a reduction of detectable ROS.

326 A different picture emerged from experiments on biofilm cells: indeed, ROS levels were

327 lower or similar in Ag-NP-treated samples in comparison to the control throughout biofilm

328 growth (Fig. 5). High levels of ROS were detected in the extracellular matrix, regardless of

329 the presence of Ag-NPs (Fig. 5a). In contrast, intracellular ROS formation in biofilm cells

330 was lower than those measured in planktonic cells (Figs. 4 and 5b) being undetectable on

331 days 3-4, *i.e.*, during the late exponential/stationary phase of biofilm formation, while

332 reaching a peak on day 8 (Fig. 5b). In biofilm cells, exposure to Ag-NPs reduced intracellular

333 ROS concentrations, with the only exception of day 1 for the higher Ag-NP concentration

334 tested (10 mg l^{-1}).

335 To gather additional information on their effects on *B. subtilis* biofilm, we characterized the

336 composition of the biofilm matrix in the presence and in the absence of Ag-NPs. In particular,

337 we quantified the amounts of proteins, EPS and eDNA. Exposure to either 1 or 10 mg l^{-1} of

338 Ag-NPs did not affect protein or eDNA amounts, while significantly stimulating EPS

339 production in the biofilm matrix (ca. 2.5-fold; Fig. 6).

340 **Quantitative proteomics and bioinformatic data-mining**

341 In order to further evaluate the impact of Ag-NPs on *B. subtilis*, we determined the total

342 protein composition from whole colony biofilm grown in the presence or absence of 10 mg l^{-1}

343 Ag-NPs by proteomic analysis. Biomass was collected during stationary phase. The data

344 revealed a total of 19 proteins differentially expressed at significant levels in the Ag-NP

345 treated samples compared to the control (Table 1, Table S1 and Table S2). No down-regulated

346 protein in Ag-NP treated biofilm were detected.

347 Data were further analyzed by the Statistical overrepresentation test of the software

348 PANTHER to highlight the most relevant GO term group annotation associated with our

349 proteomic dataset. This analysis showed a statistically significant higher expression of

350 proteins with oxidoreductase activity (*p*-value = 0.0487) (Table S3).

351 As shown in Table 1, Ag-NPs appeared to positively affect production of proteins either

352 belonging to stress responses or able to sense the cell's redox potential. Indeed, two proteins

353 directly involved in the response to oxidative stress (Alkyl hydroperoxide reductase subunit C

354 and FeS cluster assembly protein SufD) and two proteins able to sense redox conditions

355 (Thioredoxin A and the iron-sulfur cluster protein YutI) were more expressed in the presence

356 of Ag-NPs. In addition, exposure to Ag-NPs also induced other stress response-related

357 proteins, namely, oxalate decarboxylase (OxdC), involved in protection against low-pH stress

358 (MacLellan *et al.* 2009), Tig (trigger factor), a chaperone protein activated in response to

359 heat-shock (Reyes and Yoshikawa 2002), and the cell-wall associated protease WprA,

360 induced by phosphate starvation and necessary for the secretion of the peroxidase YwbN

361 (Monteferrante *et al.* 2013). Our results suggest Ag-NP induction of some quorum-sensing

362 related genes, as indicated by increased production of SrfAB, DegU, OppF and CotE

363 proteins. DegU is able to induce competence in *B. subtilis* through positive regulation of

364 *comK* (D'Souza *et al.* 1994; Kobayashi 2007); *oppF* is part of *oppABCDF* operon, encoding

365 Opp, an oligopeptide permease (Lazazzera 2001), which allows uptake of quorum-sensing

366   related peptides. Interestingly, the *srfAB* gene, encoding a subunit of surfactin synthase, also

367   contains the competence stimulating peptide ComS (Zafra *et al.* 2012), another quorum

368   sensing signal. Finally, another Ag-NP-induced protein, CotE, is produced during sporulation,

369   which is subject to a complex regulation in *B. subtilis* that also requires high cell density and

370   production of quorum sensing signals (Hilbert and Piggot 2004).

371   **Plant growth-promoting activity and motility**

372   *B. subtilis* is considered an important plant growth-promoting rhizobacterium (PGPR)

373   (Saharan and Nehra 2011). Since Ag-NPs in soil might affect plant growth through

374   modulation of PGPR composition and metabolic activities, their effects on PGP activities in

375   *B. subtilis* (Barriuso *et al.* 2008) were evaluated, either pre-exposed to or grown in presence

376   of Ag-NPs (10 mg l^{-1}) (Fig. 7). Although bacteria in the rhizosphere are thought to be mostly

377   present as a biofilm, no reliable assays are currently available to test PGPR activities on

378   sessile cells. Thus, we tested the effects of Ag-NPs on *B. subtilis* planktonic cells. Among the

379   different PGP activities, we examined nitrogen fixation and phosphate solubilization, as they

380   increase bioavailability of nitrogen and phosphate in soil, essential for plant growth

381   (Bhattacharyya and Jha 2012). We also determined production of IAA, an auxin

382   phytohormone that regulates plant development and stimulates nitrogen, phosphorous and

383   potassium uptake by plants (Etesami *et al.* 2009); finally, we measured production of

384   siderophores, high-affinity iron chelating compounds used to solubilize mineral iron and

385   promote its bioavailability (Saharan and Nehra 2011). The *B. subtilis* showed no nitrogen

386   fixation activity in the conditions tested, while comparable levels of IAA and siderophore

387   production were measured either in the presence or in the absence of Ag-NPs. In contrast,

388   treatment with 10 mg l^{-1} of Ag-NPs increased the ability of *B. subtilis* to solubilize inorganic

389   phosphate (OD 600 nm control: 0.754 ± 0.139; treated: 1.882 ± 0.145).

390   In order to carry out their beneficial activity on plants, bacteria must be able to colonize plant

391 roots effectively (Achouak *et al.* 2004). Two different mechanisms of flagellar motilities can

392 be involved in this process. Swimming is an individual motility (Kearn and Whittington

393 1991), necessary for the adhesion phase; whereas, swarming is the coordinated motility of a

394 whole colony, and can be affected by signal molecules (Verstraeten *et al.* 2008). We tested

395 Ag-NPs for possible effects on cell motility: exposure to 10 mg l^{-1} Ag-NPs failed to affect

396 either swimming (control: 1.42 ± 0.13 cm; treated: 1.50 ± 0.21 cm) or swarming motility

397 (control: 1.56 ± 0.05 cm; treated: 1.58 ± 0.08 cm).

Discussion

399 Due to the constant increase in their utilization in a variety of industrial products, the possible

400 accumulation of Ag-NPs in soil raises concerns, also since the extents of their biological

401 effects, especially at low concentrations, have not been clearly determined yet. It has been

402 proposed that 0.05 mg kg^{-1} of soil might represent a "no-effect concentration" for Ag-NPs

403 (Schlich *et al.* 2013). In this work, we have shown that Ag-NPs, already at 0.1 mg l^{-1}, i.e., at a

404 concentration close to the proposed "no effect concentration", can affect growth of *A.*

405 *vinelandii*, an important rhizosphere bacterium, reducing both its growth rate and the amount

406 of culture biomass. In contrast, growth of the Gram positive rhizosphere bacterium *B. subtilis*

407 was only affected at 10 mg l^{-1}. Such discrepancy seems to depend on an increased sensitivity

408 of *A. vinelandii*, rather than of Gram negative bacteria, as *Escherichia coli* showed a similar

409 response to Ag-NPs as *B. subtilis* (data not shown). Our observation suggests that, already at

410 concentrations thought to be devoid of biological activity, Ag-NPs could impact the

411 composition of rhizosphere microbial community by affecting growth of specific bacteria.

412 Despite being ca. 200-fold higher than the proposed "no-effect concentration" in soil,

413 exposure of soil bacteria to Ag-NPs at 10 mg l^{-1} or more can occur locally, in particular in

414 instances of utilization of sewage sludge, rich in Ag-NPs, as manure on agricultural soil, a

415 procedure still widely used in many European countries (Schlich et al., 2013). Our results

416   suggest that, at this concentration, Ag-NPs can enter *B. subtilis* cells grown in liquid cultures

417   and accumulate in their cytoplasm, triggering ROS formation. However, a more complex

418   picture emerges from exposure to Ag-NPs of *B. subtilis* colony biofilms, a condition more

419   likely to resemble bacterial growth and physiology in the soil environment. Despite showing

420   some reduction in initial growth rate, fully overcome in the later stages of biofilm

421   development, 10 mg l^{-1} Ag-NPs failed to trigger ROS formation, either in the biofilm matrix

422   or inside the biofilm cells. Intracellular ROS levels were actually decreased upon exposure to

423   Ag-NPs. However, exposure to 10 mg l^{-1} Ag-NPs strongly induced polysaccharide production

424   in the biofilm matrix, suggesting that the ATP consumption required by this process might be

425   responsible for reduced growth rate in the presence of Ag-NPs in the earlier stages of biofilm

426   formation.

427   Higher polysaccharide production is often induced as part of a response to environmental

428   stresses (Sutherland 2011). Polysaccharide overproduction in the EPS matrix might be

429   involved in Ag-NP absorption, thus preventing them from entering bacterial cells, and

430   limiting ROS formation and diffusion, consistent with previous observations (Peulen *et al.*

431   2011).

432   In addition to the buffering effect of the polysaccharide matrix, reduction in ROS levels in

433   biofilm cells might suggest that, at the concentrations tested, Ag-NPs might trigger an

434   adaptive response to oxidation stress. To verify this hypothesis, we carried out a proteomic

435   analysis in *B. subtilis* biofilm either in the presence or in the absence of 10 mg/l Ag-NPs. The

436   high amount of polysaccharides in the EPS, resulting in 50% and 75% of the matrix weight in

437   the control and Ag-NP-treated biofilms respectively, made extraction of proteins for

438   proteomic analysis very challenging (Bodzon-Kulakowska *et al.* 2007). Although this

439   resulted in relatively low scores for some proteins, our proteomic analysis allowed us to

440   identify cellular processes induced in response to Ag-NP treatment of *B. subtilis* biofilm,

441 namely, stress responses and quorum sensing. Indeed, we could detect higher expression of

442 the subunit C of alkyl hydroperoxide reductase, an important enzyme in oxidative stress

443 response (Antelmann et al., 1996). Another protein induced in response to Ag-NPs was SufD,

444 part of a FeS cluster assembly which, in *E. coli,* is sensitive to disruption by ROS or by iron

445 limitation (Layer *et al.* 2007). Thioredoxin (TrxA), another enzyme linked to oxidative stress,

446 was also induced by exposure to Ag-NPs. Since many Gram positive bacteria do not generate

447 glutathione, which is the dominant low molecular thiol in most Eukaryota and many Gram-

448 negative bacteria (Newton *et al.* 2009), thioredoxins are essential to *B. subtilis* for cellular

449 thiol/disulfide balance and survival under oxidative stress (Lu and Holmgren 2013). Thus,

450 results of proteomic analysis suggested that treatment with Ag-NPs leads to a higher

451 expression of proteins involved in oxidative stress response, which would in turn lead to

452 more efficient detoxification and removal of ROS, as observed.

453 In addition to proteins involved in stress responses, exposure to Ag-NPs stimulate production

454 of competence-related peptides and to induce quorum sensing mechanisms. Indeed, we

455 observed a higher expression of DegU, a transcription regulator involved in the production of

456 the ComK, a quorum sensing depending regulator (Mhatre et al., 2014). We also observed a

457 higher expression of the quorum sensing-dependent molecule surfactin coded by the *srfAB*

458 gene. In addition, a fragment of the *srfAB* gene encodes for ComS, a quorum sensing peptide

459 able to enhance competence (Morikawa 2006). Surfactin triggers matrix production (Lopez *et*

460 *al.* 2009), in line with the observed higher polysaccharide production in Ag-NP treated

461 biofilm.). It is tempting to speculate that Ag-NPs might also trigger induction of quorum

462 sensing, thus affecting gene expression at large in *B. subtilis* biofilms.

463 Exposure of *B. subtilis* to Ag-NPs positively affects polysaccharide production, which, by

464 promoting effective colonization of plant roots, plays an important role in the PGP activity by

465 this bacterium (Chen *et al.* 2013). We also found that inorganic phosphate solubilization,

466 which results in increased phosphorous availability in the rhizosphere was stimulated by Ag-

467 NPs. Although PGP activities were determined on planktonic cultures, due to lack of reliable

468 assays on biofilm cells, our results seem to suggest that sub-lethal doses of Ag-NPs might

469 exert a positive effect on PGP activity by *B. subtilis*. In conclusion, using *B. subtilis* as a

470 model for rhizosphere organisms, we were able to show that Ag-NPs at sub-inhibitory

471 concentrations affects pivotal cellular processes such as stress responses, quorum sensing and

472 PGP activities. It is conceivable that similar effects might take place on other soil bacteria: re-

473 direction of cellular processes and of gene expression, linked to selective toxicity on some

474 bacterial species, such as *A. vinelandii*, suggest a strong impact of Ag-NPs on soil bacterial

475 communities.

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636 **Table 1**

Accession	Description	Gene name [gene ID]	Function	Ag-NPs/ Ctrl
stress response				
O32165	FeS cluster assembly protein SufD	*sufD* [938871]	repair under oxidative stress	10.52
O32119	Putative nitrogen fixation proteins	*yutI* [936658]	iron-sulfur cluster assembly	3.38
O34714	Oxalate decarboxylase OxdC	*oxdC* [938620]	acidic stress response	1.65
P14949	Thioredoxin	*trxA* [938187]	cell redox homeostasis	4.72
P54423	Cell wall-associated protease	*wprA* [936350]	proteoglycan peptide bridges in stationary phase	3.37
P80239	Alkyl hydroperoxide reductase subunit C	*ahpC* [938147]	oxidative stress response	1.86
P80698	Trigger factor	*tig* [936610]	chaperone in heat-shock response	2.39
primary metabolism				
O31669	Acireductone dioxygenase	*mtnD* [939322]	aminoacid biosynthesis	2.04
P21881	Pyruvate dehydrogenase E1 component subunit alpha	*pdhA* [936005]	pyruvate metabolism	4.93
P34956	Quinol oxidase subunit 1	*qoxB* [937303]	ATP synthesis	9.00
P37808	ATP synthase subunit alpha	*atpA* [936995]	ATP synthesis	2.06
P39062	Acetyl-coenzyme A synthetase	*acsA* [937324]	acetate utilization	13.32
P12425	Glutamine synthetase	*glnA* [940020]	glutamine synthetase	3.82
transcription and translation				
P12877	50S ribosomal protein L5	*rplE* [936981]	tRNA binding	6.40
P17889	Translation initiation factor IF-2	*infB* [936930]	protein synthesis	6.62
quorum sensing				
P13800	Transcriptional regulatory protein DegU	*degU* [936751]	recruitment of ComK	5.05
P24137	Oligopeptide transport ATP-binding protein OppF	*oppF* [936410]	transmembrane transport	2.15
P14016	Spore coat protein E	*cotE* [939508]	sporulation	4.18
Q04747	Surfactin synthase subunit 2	*srfAB* [938303]	surfactin biosynthesis	3.16

637

638 **Table 1** Differentially expressed proteins identified by LC-ESI-MS/MS. The following

639 parameters are listed: alphanumeric unique protein sequence identifier (Accession) provided

640 by UniProtKB/Swiss-Prot protein Knowledgebase, protein name (Description), Gene name

641 and numeric unique gene sequence identifier (Gene ID) provided by NCBI, Function and

642 mean of the ratio of the heavy and light quantification channels (Ag-NPs/Ctrl)