Detailed Methods

A. Experiment 1: O_2 consumption and viability assay development and proof of concept

1. Maintenance and Isolation of Bacteria

Bacillus amyloliquefaciens GB03 (*Bacillus* Genetic Stock Center (BGSC) ID: 3A37) cultures were made from 1:1 (glycerol:Tryptone-Yeast (TY) extract-culture) stocks by streaking on 1% TY agar plates and incubating at 28˚C. For each experiment a single colony was inoculated into 50 mL TY + 50 mM MES media and cultured to mid-late log phase (OD_{600nm} ~0.86) at 28˚C with shaking at 200 rpm. Cells were collected by filtration by passing two separate 15 mL aliquots of culture (30 mL total) through individual 1 μm Nuclepore membranes (GE Whatman, New Jersey, USA), followed by washing with three 7.5 mL aliquots of sterile 50 mM MES. Filter membranes holding the microbes were then transferred to 12 mL of 50 mM MES in a 250 mL sterile beaker and gently swirled to liberate the cells. The cell suspension was then brought to an OD_{600nm} ~1.15 (considered cell density proportion of 1) using 50 mM MES. Prior to culturing and isolation, MES solutions were adjusted to pH 6 with 5 M NaOH, (final conc. of 25 mM NaOH), and filter sterilized. All glassware was acid washed for a minimum of 2 hours in 12.5% HCL + 12.5% HNO_3.

2. Exposure procedure

Approximately 1 h prior to completion of cell isolation, 50 µL of 50 mM MES was transferred to each well of an Oxoplate™ in order to pre-equilibrate the plate per the manufactures instructions. Metal solutions at 8 times (8x) the target concentration were prepared in advance using 50 mM pH 6 MES in a clear, sterile 96 well plate. A 10% glucose + 50 mM pH 6 MES solution was prepared and transferred to a 50 mL sterile,

plastic reagent boat, and 40 mL of *B. amyloliquefaciens* cells (OD_{600nm} ~1.15) was prepared and loaded to another reagent boat. To initiate the exposure, either 100 µL of the live cell suspension or 50 mM MES was transferred to each designated well of the Oxoplate™. 25 µL of 8x metal solutions were then added to each well, followed by 25 µL of 10% glucose + 50 mM MES. The result was an exposure media containing the targeted metal concentration (*vide infra*) and a final glucose concentration of 69 mM. The plate was then sealed with a clear, sterile PCR film, placed in the plate reader and the Oxoplate™ protocol described below was used to measure O_2 consumption over time. After 1.5 h the Oxoplate™ was removed from the reader, the PCR film was removed, and the cells were diluted 500x to a total of 100 µL in a clear, sterile 96 well plate. Then, 100 µL of 2x TY + 50 mM MES pH 6 was transferred to each well (resulting in 1000x dilution), the plate was sealed with a clear, sterile PCR film, and cell growth was monitored using absorbance (450 nm and 600 nm) as described below.

3. O_2 consumption assay calibration and measurement

The O_2 partial pressure (in terms of percent air saturation) per well with time was calculated using the methods described by the Oxoplate™ manufacturer. Briefly, fluorescence measurements for an O_2 sensitive indicator dye (excitation: 540 nm, emission: 650 nm) and a reference dye (excitation: 540 nm, emission: 590 nm) are incorporated into equation 1.

$$pO_2 = (100 \times \left(\frac{k_0}{I_R} - 1\right))/(\frac{k_0}{k_{100}} - 1) \qquad (1)$$

Where, I_R is the sample fluorescence intensity reading at 650 nm divided by the fluorescence intensity at 590 nm, k_0 is the I_R average of 4 wells loaded with 300 µL of 0.01 g Na_2SO_3/ml, and k_{100} is the I_R average of 4 wells loaded with 200 µL of 50 mM MES + 1.25% glucose shaken for 2 m before loading. Prior to the experiments, both k_0 and k_{100} were determined after allowing the solutions to incubate in a sealed Oxoplate™ for 1 h at 28 ˚C before measuring fluorescence intensity.

To calibrate O_2 consumption readings, cells were prepared to an OD_{600nm} of 1.15 as described above and serial diluted by 10% to a cell density <0.1% of the starting cell density using 50 mM MES in a clear, sterile 96 well plate. Then, 100 µL of the cells were transferred to an Oxoplate™ containing 50 µL 50 mM MES (loaded ~1 h prior). A sham exposure (an exposure simulating the actual exposure procedure but without any metals or streptomycin) was performed as above, and fluorescence intensity measurements at 540 nm/650 nm and 540 nm/590 nm were recorded every 5 m over a 90 m period. O_2 consumption curves for each well (8 wells/dilution point) were then constructed and the areas under the curves (AUC) were calculated using Sigmaplot Software package (Systat Software, California, USA). The non-linear relationship between cell density (as a proportion) and log(AUC) was determined using JMP® Version 10 (SAS Institute Inc., North Carolina, USA). The mathematical model derived from this relationship (root-mean-square error or NRMSE = 0.032) was used as a calibration equation to determine percent O_2 consumption. One caveat of the method, along with all common microbial respiration assays, is that dead cells may provide substances that are toxic to, or nutritive for, the remaining viable bacteria or serve as a sink for the chemicals being assessed for toxicity. This could influence respiration and/or growth, and will be captured in the measurements.

4. Viability assay calibration and measurement

Cells were prepared to the desired OD_{600nm} as described above and serial diluted (100 µL cell suspension: 100 µL 50 mM MES) in a clear 96 well plate (8 dilution points, 8 wells/dilution point). An Oxoplate™ was loaded with 50 µL of 50 mM MES ~1 h prior to transferring 100 µL of cells/well of the Oxoplate™. A sham exposure was performed by adding 25 µL of 50 mM MES followed by 25 µL 10% glucose + 50 mM MES. Once the Oxoplate™ was brought to 200 µL/well, the plate was sealed with a clear, sterile PCR film and placed in a Wallac Victor 2 microplate reader (Perkin-Elmer Life Sciences, MA, USA) for 1.5 h at 28 ˚C using the Oxoplate™ exposure protocol described below.

After the sham exposure the cells were diluted 500x using 50 mM MES to a volume of 100 µL. Then 100 µL of 2x TY + 50 mM MES was loaded to each well (resulting in 1000x dilution at 200 µL), the plate was covered with a clear, sterile PCR film, and placed in the plate reader at 28 ˚C. Dilution of cells provides the benefit of also diluting any added substrates. Hazan et al.[1] used a similar method and found the estimates of viability gathered from the microtiter plate corresponded well with CFU counts. Every 15 m the plate was shaken for 15 s and allowed to sit for 10 s before absorbance was measured at 450 and 600 nm. Dual-wavelength absorbance measurements were used to overcome the problem of spectral interference resulting from the formation of condensation on the PCR film[2]. We found the dual wavelength method (abs = 450 nm – 600 nm) yielded low well-to-well variation in estimated absorbance of media controls (TY without metal or bacteria) over time (mean $abs_{450nm-600nm}$ = 0.02, standard deviation = 0.002).

The adjusted absorbance readings were used to construct bacterial growth curves from each well, where the background absorbance was subtracted from readings at each

time point. Linear interpolation was applied to determine the time (m) at which each growth curve passed an absorbance threshold of 0.005 ($OD_{450nm-600nm}$). A mathematical relationship ($r^2 = 0.98$) between starting cell density (as a proportion) and time to threshold was determined using linear modeling of log(cell density) vs. log(minutes) using JMP® Version 10. This mathematical model is used as a calibration equation to estimate percent viability following exposure.

5. Experimental Design and Data Analysis

A randomized complete block design was used to evaluate the toxicity of Ag^+ (0.5, 1, 2, 3, 4, 6, 8, and 10 µM), Ni^{2+}, and Zn^{2+} (0.63, 1.3, 2.5, 5, 10, 20, 40 and 80 µM) using 7 experimental blocks. A metal free control was also used across all experimental blocks. Treatments were randomized by column and control columns were randomized by row across all experimental blocks to control for variation due to plate placement of treatments and loading order of cells. Technical replicates of each treatment were performed in triplicate in each experimental block. Ions were supplied as either $AgNO_3$, $ZnSO_4$, or $NiSO_4$. Silver was chosen because its toxic effects are well documented and intracellular concentrations are not tightly regulated, whereas Zn^{2+} and Ni^{2+} were chosen because they are regulated essential nutrients that are also toxic at elevated concentrations and both have been shown to display toxicity to *B. subtilis* (a relative of *B. amyloliquefaciens*) at similar concentrations under the same test conditions. Additionally, mechanisms governing intracellular Zn^{2+} concentrations are relatively well understood, while less is known regarding Ni^{2+} homeostasis. Streptomycin (2.6 µM) was used as a positive control, based on a preliminary study, where we found this concentration to significantly, but not

completely, reduce viability. Stock metal concentrations were verified *via* inductively coupled plasma spectrometry (ICP-MS) analysis prior to exposures.

Distribution and variance of residuals were determined following linear regression using distribution plots, Q-Q plots, and Studentized residual plots. Treatment responses were normalized to metal free control wells, averaged within experiments, and the average of experimental means were compared to a hypothetical mean (H_O: μ = 100%, for viability and O_2 consumption measurements) using a two-tailed, one-sample t-test (n = 7, alpha = 0.05, assuming unequal variances). Mean O_2 consumption responses of each experiment were plotted against mean viability measurements and regression curves were fit using SoftMax Pro 6.4 (Molecular Devices, Wokingham, UK).

6. Chemical equilibrium modeling

Exposure media were modeled using Geochem-EZ[3] to determine the free ion activities of the metals of interest (Figures S1-S2). It was necessary to update the ligand database to include stability constants for Zn-MES[4] and Ni-MES complexes[4, 5]. The stability constant for the Ag-MES complex was approximated with the published stability constant for Ag-taurine complexes found using the IUPAC Stability Constant Database[6]. All stability constants were input at infinite dilution. After concentrations of exposure media constituents were entered into Geochem-EZ, pH was fixed at 6, precipitates were allowed to form, and the ionic strength was calculated using an initial “guess” of 0.01 mol, as recommended by the model developers. The standard convergence criteria were implemented.

B. Experiment 2: O_2 consumption and viability assay of PGPR exposed to MNPs

1. Maintenance and Isolation of Bacteria

Maintenance and isolation of bacteria were similar to Experiment 1, but cells were cultured in mini-bioreactors (Corning NY, USA) containing 40 mL of TY + 50 mM MES + 0.01 M $CaCl_2$ at pH 6. After inoculation the bioreactors were placed horizontally on an orbital shaker set at 100 rpm and 28 °C. When cells reached mid-log phase (OD_{600nm} ~0.64), the bioreactors were centrifuged at 2000 rcf and 28 °C for 5 m (3220 rcf, 28 °C, and 10 m for *P. putida*), the medium was removed, and cells were washed 3 times with 10 mL of 50 mM MES using the same centrifugation settings. Cells were then suspended to the desired optical density (reported below) and used for either calibrating the respiration and viability assays or in the toxicological experiments.

2. Assay Calibration

The O2 consumption and viability assays were calibrated similarly compared with Experiment 1, with a few exceptions. Cultures were prepared in mini-bioreactors to an OD_{600nm} = 1.19, or 1.045 for *P. putida* (Table S1) before dilution and exposure. Viability curves were gathered by shaking the plate 15 s and measuring absorbance at 600 nm after allowing the plate to rest for 20 s. Strong correlations were found between starting cell density proportions and either the area under O_2 consumption curves or time at which growth curves reached an absorbance threshold of OD_{600nm} 0.09 (after subtracting background absorbance) (Table S2). Cell viability was determined in control conditions (no metal) in terms of CFU/mL as assessed *via* a drop plate method [7] before and after the O_2 consumption assay procedure (Table S1).

3. Exposure Procedure

After culturing to mid-log phase, cells were harvested as described above in 50 mM MES (pH 6) and suspended to OD_{600nm} ~1 (*B. subtilis*: OD_{600nm} = 1.08 ± 0.02; S.

meliloti: OD_{600nm} = 1.06 ± 0.02; *P. putida*: OD_{600nm} = OD_{600nm} = 1.07 ± 0.02, error is one standard deviation). The remainder of the procedure is as in Experiment 1; however, a Spectramax i3 (Molecular Devices, Wokingham, UK) microplate reader was used.

4. Metal and ENM Characterization

Concentrations of stock metals were determined *via* ICP-MS analysis prior to exposure. PVP-AgENMs and sAgENMs were characterized in the exposure medium at ~300 µM total silver and 28 °C using a Zetasizer Nano ZS (Malvern). Hydrodynamic diameter and electrophoretic mobility were estimated using direct light scattering and electrophoretic light scattering, respectively. The particles used were previously characterized and PVP-AgENMs had a primary particle size of 53-58 nm[8, 9], while sAgENMs had a primary particle size of ~65 nm[8], both determined *via* transmission electron microscopy (TEM).

A sham exposure (without bacteria) was performed to examine the abiotic dissolution of PVP-AgENMs and sAgENMs in the exposure medium. Briefly, PVP-AgENM (29, 115, and 230 µM) and sAgENM (~300 µM) were prepared in the exposure medium with glucose and brought to 2 mL in ultracentrifuge tubes. Samples were gently vortexed to ensure mixing and 100 µL was immediately removed for total metal analysis, before centrifuging for 90 m at 28 °C and 246,000 x *g*. According to Stoke's law, these conditions were sufficient to sediment spherical particles ≥ 2 nm. After centrifugation, 1 mL was removed and acidified to 1% HCl for dissolved metal analysis. Samples for total metal analysis were microwave digested at 100 °C and 400 watts for 35 m (20 m ramp, 10 m hold, 5 m cooldown) in 13% HCl/40% HNO_3 before diluting 15 times in double deionized

water, yielding 1% HCl/3% HNO_3. Metal concentrations were determined using an Agilent 7500 series ICP-MS (Santa Clara, Ca.)

5. Chemical Equilibrium Modeling

Free ion concentrations and activities of Ag+ from $AgNO_3$ or the expected dissolved silver from AgENMs were modeled using GeoChem-Ez [3], as in Experiment 1.

C. Experimental Design and Data Analysis

A randomized complete block design was used to assess toxicity in each of the test organisms, with 3 experimental blocks and 3 replicates per block. Microplate columns were randomized across experiments and controls were randomized across rows. *B. amyloliquefaciens* and *S. meliloti* were exposed to 2.6 µM streptomycin, $AgNO_3$ (0.5, 1, 2, 3, 4, 5, 6, 10 µM), 296 µM sAgENM, and PVP-AgENM (4.6, 37, 55.6, 111, 148, 185, 222, and 296 µM). The same concentrations of $AgNO_3$, streptomycin, and sAgENM, but 4.3, 34.5, 43, 46, 115, 144, 172, and 230 µM PVP-AgENM were used for *P. putida* exposures. Molarity is expressed in terms of total silver concentration. Viability and O_2 consumption estimates were normalized to the no metal controls and were found to be non-normally distributed. The data were ranked and ANOVA was used to examine experiment by treatment interactions. Because no experiment by treatment interactions were found, observations were pooled across experiments (n = 9) and a two-tailed Wilcoxon signed rank test was used to compare the observations to a hypothetical mean of 100% viability or O_2 consumption (H_0: μ = 100%, α 0.05) using JMP.

Sigmaplot was used to generate non-linear, 4-parameter dose response curves and extrapolate LC_{50} values for viability responses to PVP-AgENM and $AgNO_3$. LC_{50} values were compared using an unpaired t-test with a Bonferroni correction. Additionally,

Sigmaplot was used to perform regression analyses on O_2 consumption-viability plots for interpreting O_2 consumption responses in terms of relative viable cell numbers. Dissolution measurements were used to generate predicted viability responses to the dissolved fraction of AgENMs by extrapolating responses from $AgNO_3$ dose response curves for the average dissolution estimate ± one standard deviation (n = 3).

1. R. Hazan, Y.-A. Que, D. Maura and L. Rahme, BMC Microbiology **12** (1), 259 (2012).
2. K. L. Ratzlaff and D. F. S. Natusch, Anal Chem **51** (8), 1209-1217 (1979).
3. J. Shaff, B. Schultz, E. Craft, R. Clark and L. Kochian, (2009).
4. D. Wyrzykowski, A. Tesmar, D. Jacewicz, J. Pranczk and L. Chmurzyński, Journal of Molecular Recognition **27** (12), 722-726 (2014).
5. D. Wyrzykowski, B. Pilarski, D. Jacewicz and L. Chmurzyński, J Therm Anal Calorim **111** (3), 1829-1836 (2013).
6. L. D. Pettit and K. Powell, Chemistry international (2006).
7. C.-Y. Chen, G. W. Nace and P. L. Irwin, Journal of Microbiological Methods **55** (2), 475-479 (2003).
8. D. L. Starnes, J. M. Unrine, C. P. Starnes, B. E. Collin, E. K. Oostveen, R. Ma, G. V. Lowry, P. M. Bertsch and O. V. Tsyusko, Environmental Pollution **196**, 239-246 (2015).
9. A. R. Whitley, C. Levard, E. Oostveen, P. M. Bertsch, C. J. Matocha, F. von der Kammer and J. M. Unrine, Environmental pollution **182**, 141-149 (2013).

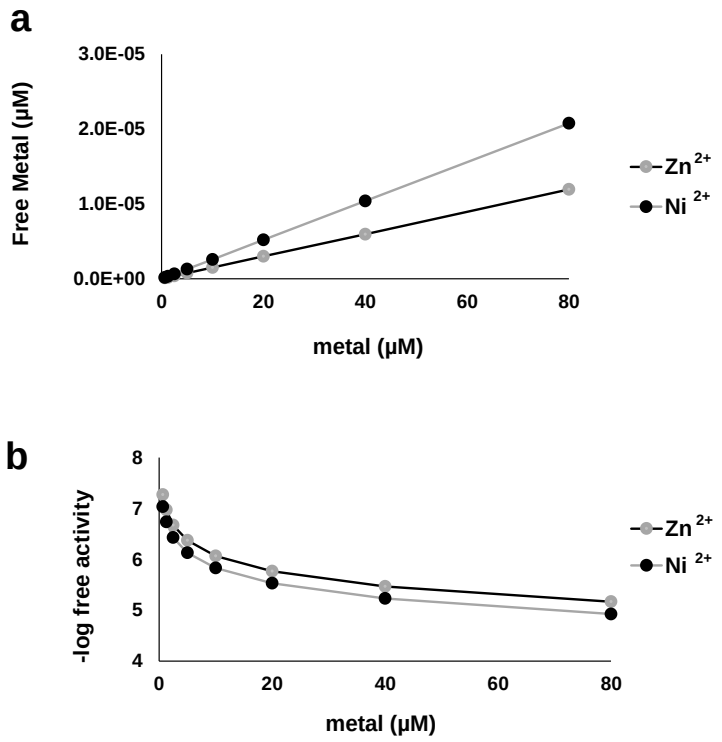

Fig. S1. Predicted $Zn^{2+}$and Ni^{2+} free ion concentrations and activities in exposure media as calculated by GeoChem-Ez. Precipitates were allowed to form, pH was fixed at 6, and ionic strength was calculated as suggested by the developers. a Predicted free Zn^{2+} and Ni^{2+}(µM) in 50 mM MES at exposure metal concentrations used in this study. b –log free $Zn^{2+}$and Ni^{2+}activity in 50 mM MES at exposure metal concentrations used in this study.

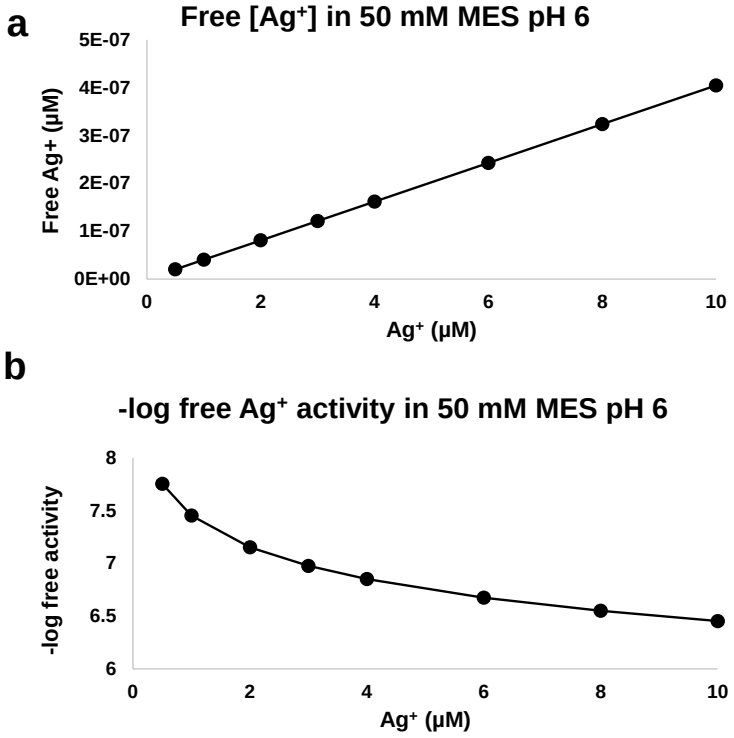

Fig. S2. Predicted Ag^+ free ion concentration and activity in exposure media as calculated by GeoChem-Ez. Precipitates were allowed to form, pH was fixed at 6, and ionic strength was calculated as suggested by the developers. a Predicted free Ag^+ (µM) in 50 mM MES at exposure Ag+ concentrations used in this study. b –log free Ag^+ activity in 50 mM MES at exposure Ag^+ concentrations used in this study.

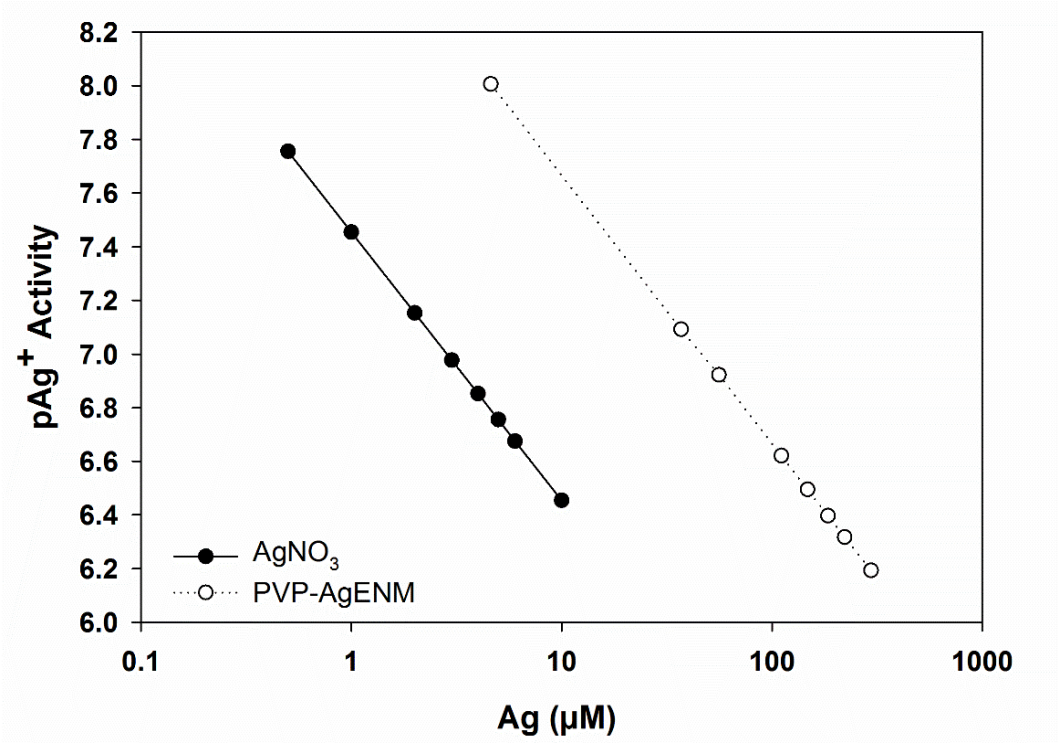

Fig. S3. -log free Ag^+ activities (pAg^+) in $AgNO_3$ and PVP-AgENM in 50 mM MES as predicted using GeoChem-EZ. The pH was fixed at 6, precipitates were allowed to form, and ionic strength was calculated as suggested by the developers.

Table S1. CFU/mL before and after O_2 consumption assay. CFU, colony forming unit; PGPR, plant growth promoting rhizobacteria, S.D., one standard deviation of the mean; OD_{600nm}, optical density at 600 nm. D_{600nm} is the optical density of the isolated cells prior to diluting 1:1 with medium for the O_2 consumption assay. Letters represent significant differences at α 0.05 as determined *via* a Studentized t-test comparing CFU/mL before and after the O_2 consumption assay. All CFU/mL estimates are from n = 6 plates, excluding *B. amyloliquefaciens* (n = 3 plates).

		Mean CFU/mL ± Standard Deviation (S.D.)			
Species	OD_{600nm}	Before	S.D.	After	S.D.
B. amyloliquefaciens	1.159	1.05E+08[a]	8.33E+06	1.01E+08[a]	2.28E+07
S. meliloti	1.189	1.63E+09[a]	1.46E+08	1.98E+09[b]	1.22E+08
P. putida	1.191	1.13E+09[a]	2.13E+08	6.67E+08[b]	1.15E+08
P. putida	1.045	6.35E+08[a]	3.82E+07	6.58E+08[a]	2.96E+07

Table S2. Calibration curve fitting results of the O_2 consumption and viability assays. PGPR, plant growth promoting rhizobacteria. Softmax Pro 6.4 was used to regress O_2 consumption (area under curve) or viability (time to OD_{600nm} = 0.09) against cell density proportion.

	O_2 Consumption Assay		Viability Assay	
PGPR	Curve Fit	R^2	Curve Fit	R^2
B. amyloliquefaciens	4-parameter	0.999	log-log	0.987
S. meliloti	linear	0.998	log-log	0.994
P. putida	4-parameter	0.999	log-log	0.999

Table S3. CFU/mL used in toxicity experiments. CFU/mL, colony forming units in one mL of bacterial suspension; S.D., one standard deviation of colony forming units

CFU/mL is estimated from absorbance measurements

PGPR	Mean CFU/mL	S.D.
B. amyloliquefaciens	1.59E+07	1.60E+05
S. meliloti	1.10E+09	3.31E+07
P. putida	7.17E+08	2.73E+07