Electronic Supplementary Material (ESI) for Environmental Science: Nano.

Supporting Information

Table S1. Enzyme activity fold-changes (with respect to control) of superoxide dismutase (SOD), glutathione S-tranferase (GST), glutathione peroxidase (GPx) and catalase (CAT) in *Pseudomonas* sp. M1 exposed or not to Ag^+ or AgNPs for 90 minutes.

Enzyme	Concentration ($\mu g\ L^{-1}$)	Enzyme activity in fold-change	
		Ag^+	AgNPs
SOD	**50**	3.04*	1.52
	100	6.67*	1.94*
	200	-	2.83*
	300	-	5.83*
GST	**50**	1.35	1.14
	100	1.76	1.23
	200	-	1.38
	300	-	1.59
GPx	**50**	2.95*	1.59
	100	4.18*	2.51*
	200	-	3.11*
	300	-	4.34*
CAT	**50**	1.13	1.16
	100	2.74*	1.99*
	200	-	2.69*
	300	-	1.36

-, not determined; *, treatments that differ significantly from the respective control (Dunnett's test, $P<0.05$).

Protein identification by mass spectrometry

In gel Digestion/Sample Preparation

After denaturation, the proteins were alkylated with acrylamide and subjected to in gel digestion by using the short-GeLC approach (Anjo et al 2014). Briefly, gel pieces were destained using the destaining solution (50 mM ammonium bicarbonate and 30% acetonitrile) following by a washing step with water. Gel pieces were dehydrated on Concentrador Plus/Vacufuge® Plus (Eppendorf). Trypsin (0.01 μg/μL solution in 10 mM ammonium bicarbonate) was added to the dried gel bands and left for 15 min on ice to rehydrate the gel pieces. After this, 10 mM ammonium bicarbonate were added to cover the rehydrated bands and in-gel digestion was performed overnight at room temperature in the dark. The excess solution from gel pieces were collected in a low binding microcentrifuge tube (LoBind®, Eppendorf) and peptides were extracted from the gel pieces by sequential addition of acetonitrile (ACN) in 1% formic acid (FA) (30%, 50%, and 98% of ACN). Then, the tubes were shaken in the thermomixer (Eppendorf) at 1050 rpm for 15 min and solutions were collected to the tube containing the previous fraction. All the peptide mixtures were dried (not completely) by rotary evaporation under vacuum.

Before performing the LC-MS/MS, analysis the peptide mixtures were subjected to SPE using OMIX tips with C18 stationary phase (Agilent Technologies) as recommended by the manufacture. Eluates were dried by rotator evaporation, avoiding to totally evaporate the samples, and peptide mixtures were ressuspended in 30 μL of 2% ACN and 0.1% FA containing iRT peptides (Biognosys AG) as internal standards, followed by vortex, spin and sonication in a water bath (2 min; pulses of 1 sec sonication followed by 1 sec resting, at 20% intensity, in a sonicatorVibraCell 750 watts, Sonics®; Sonics & Materials). In order to remove insoluble material, the peptide mixtures were centrifuged for 5 min at 14000 × *g* and collected into proper vials for LC-MS injection. Five μL of each replicate sample was combined to obtain one pooled sample per experimental condition (in a total of four pools), to be used for protein identification and SWATH-library generation.

SWATH acquisition

Samples were analyzed on a Triple TOF™ 5600 System (ABSciex®) in two phases: information-dependent acquisition (IDA) of the pooled samples, and SWATH (Sequential Windowed data independent Acquisition of the Total High-resolution Mass Spectra) acquisition of each individual sample. Peptides were resolved by liquid chromatography (nanoLC Ultra 2D, Eksigent®) on a MicroLC column ChromXP™ C18CL (300 μm ID × 15cm length, 3 μm particles, 120 Å pore size, Eksigent®) at 5 μL min^{-1} with a multistep gradient: 0-1 min of 2% acetonitrile in 0.1 % FA and, 2-45 min linear gradient from 2 % to 30 % of acetonitrile in 0.1 % FA. Peptides were eluted into the mass spectrometer using an electrospray ionization source (DuoSpray™ Source, ABSciex®) with a 50 μm internal diameter (ID) stainless steel emitter (NewObjective).

Information dependent acquisition (IDA) experiments were performed for each pooled sample. The mass spectrometer was set for IDA scanning full spectra (350-1250 m/z) for 250 ms, followed by up to 20 MS/MS scans (100-1500 m/z for 100 ms each). Candidate ions with a charge state between +2 and +5 and counts above 70 counts per sec were isolated for fragmentation, and 1 MS/MS spectra was collected before adding the ions to the exclusion list for 20 sec (mass spectrometer operated by Analyst® TF 1.6, AB Sciex). Rolling collision energy was used with a collision energy spread of 5. Peptide identification and library generation were performed with Protein Pilot software (v5.1, ABSciex®), using the following parameters: i) search against a database composed by the genus *Pseudomonas* from the SwissProt database (release at June 2015); ii) acrylamide alkylated cysteines as fixed modification; iii) trypsin as digestion type, and iv) allowing biological modifications. An independent False Discovery Rate (FDR) analysis using the target-decoy approach provided with Protein Pilot software was used to assess the quality of the identifications and positive identifications were considered when identified proteins and peptides reached a 5% local FDR (Tang et al 2008, Sennels et al 2009).

For SWATH-MS based experiments, the mass spectrometer was operated in a looped product ion mode (Gillet et al 2012) and the same chromatographic conditions used as in the IDA run described above. The instrument was specifically tuned to allow a quadrupole resolution of 25-m z^{-1} mass selection. Using an isolation width of 26 m z^{-1} (containing 1 m z^{-1} for the window overlap), a set of 30 overlapping windows was constructed covering the precursor mass range of 350-1100 m z^{-1}. A 50 ms survey scan (350-1250 m z^{-1}) was acquired at the beginning of each cycle for instrument calibration and SWATH-MS/MS spectra were collected from 100-1500 m z^{-1} for 100 ms resulting in a cycle time of 3.25 sec from the precursors ranging from 350 to 1100 m z^{-1}. The collision energy for each window was determined according to the calculation for a charge 2+ ion centred upon the window with a collision energy spread of 15.

A specific library of precursor masses and fragment ions was created by combining all files from the IDA experiments, and used for subsequent SWATH processing. Libraries were obtained using Protein Pilot™ software (v5.1, ABSciex®) with the same parameters as described above. Data processing was performed using SWATH™ processing plug-in for PeakView™ (v2.0.01, ABSciex®). Briefly, peptides were selected automatically from the library using the following criteria: i) the unique peptides for a specific targeted protein were ranked by the intensity of the precursor ion from the IDA analysis as estimated by the ProteinPilot™ software, and ii) peptides that shared different protein entries/isoforms were excluded from selection. Up to 15 peptides were chosen per protein, and SWATH™ quantitation was attempted for all proteins in the library file that were identified below 5% local FDR from ProteinPilot™ searches. In SWATH™ Acquisition data, peptides were confirmed by finding and scoring peak groups, which are a set of fragment ions for the peptide.

Target fragment ions, up to 5, were automatically selected and peak groups were scored following the criteria described by Lambert et al (2013). Peak group confidence threshold was determined based on a FDR analysis using the target-decoy approach and 1% extraction FDR threshold was used for all the analyses. Peptide that met 1% FDR threshold in at three of the four biological

replicates were retained, and the peak areas of the target fragment ions of those peptides were extracted across the experiments using an extracted-ion chromatogram (XIC) window of 5 minutes with 20 mDa XIC width. The levels of the proteins were estimated by summing all transitions from all the filtered peptides for a given protein (an adaptation of Collins et al 2013) and normalized to the total intensity.

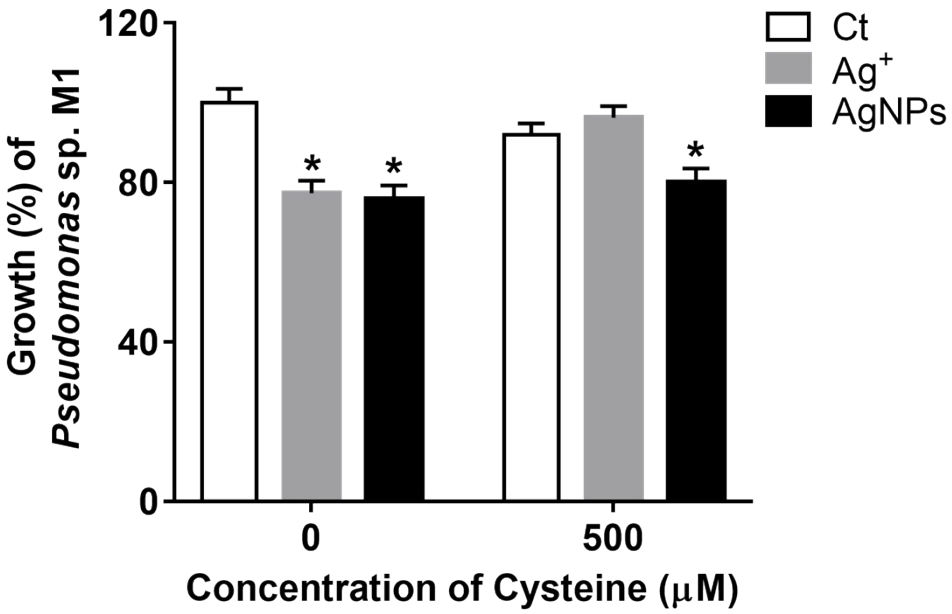

Figure S1 Effects of the Ag^+-ligand cysteine on growth of *Pseudomonas* sp. M1 in MM medium with 0.4% lactate at 30ºC for 90 minutes in the absence (Ct) or presence of Ag^+ (at EC_{20}) or AgNPs (at EC_{20}). Results are expressed as percentage of the unexposed control (unexposed to cysteine or either form of silver). Asterisks indicate significant differences from the unexposed control according to post-hoc Dunnett's multiple comparisons test (P <0.01). Mean ± SEM, n = 3.

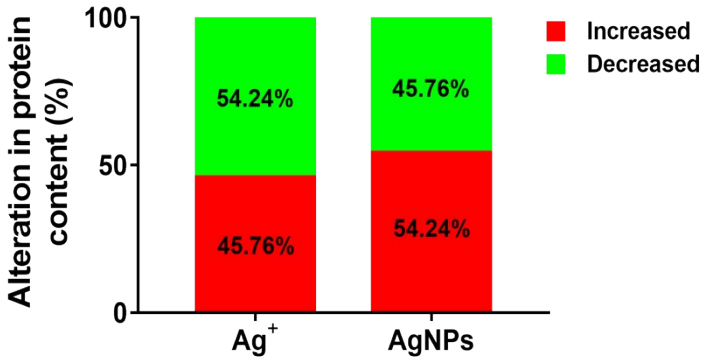

Figure S2 Percentages of significantly altered proteins in exposure to Ag^+ or AgNPs. The contents of proteins increased (in red) or decreased (in green) were calculated in relation to the total number of identified proteins (59 proteins).

Figure S3 Protein networks in *Pseudomonas aeruginosa* adapted from STRING database. The network includes the proteins altered by Ag^{+} and AgNPs in *Pseudomonas* sp. M1 and identified in *P. aeruginosa*. Stronger associations among the proteins are represented by thicker lines. Databases, experiments, textmining, co-expression, neighbourhood, gene fusion and co-occurrence were selected as active interaction sources. Edge confidence: low (0.15), medium (0.40), high (0.70), highest (0.90). The associations between key proteins were highlighted in red.

Table S2. Total identified proteins by SWATH-MS/MS from SDS-PAGE gels with (protein crude) extracts from cells of *Pseudomonas* sp. M1 grown in lactate supplemented mineral medium (MM; as described by Hartmans et al, 1989) in the presence or absence of Ag^+ or AgNPs for 90 minutes. Relative alteration of protein content (average fold change relative to control) of statistically significant proteins ($P<0.05$) and respective cluster inclusion group are shaded in light grey. Positive and negative fold changes suggest increase and decrease in the protein content, respectively.

Accession number	Protein Name	ANOVA (*P*-value)	Average fold change[a]		Cluster[b]
			Ag^+	AgNPs	
ETM69128.1	30S ribosomal protein S8 RpsH	0.003	-1.077	0.317	1
ETM69126.1	50S ribosomal protein L5	0.003	-0.083	0.210	1
ETM66430.1	30S ribosomal protein S1	0.006	-0.085	0.343	1
ETM69113.1	30S ribosomal protein S10 RpsJ	0.007	-0.254	0.375	1
ETM64203.1	transcriptional regulator	0.007	0.446	1.320	1
ETM69123.1	30S ribosomal protein S17	0.010	-0.336	0.056	1
ETM65842.1	30S ribosomal protein S9	0.015	-0.310	0.067	1
ETM66170.1	membrane protein	0.018	1.648	2.324	1
ETM69133.1	50S ribosomal protein L15	0.018	-0.489	0.124	1
ETM67937.1	30S ribosomal protein S2	0.027	-0.151	0.151	1
ETM64865.1	S-adenosylmethionine synthase	0.045	-0.067	1.450	1
ETM66535.1	transcriptional regulator	0.046	0.139	0.789	1
ETM64930.1	glutamine synthetase	0.046	-0.008	0.454	1
ETM69112.1	elongation factor Tu Tuf	0.000	-0.424	-2.080	2
ETM68814.1	isocitrate dehydrogenase	0.000	0.951	-1.041	2
ETM65642.1	serine hydroxymethyltransferase	0.000	0.142	-2.218	2
ETM67938.1	elongation factor Ts Tsf	0.001	-0.301	-2.539	2
ETM66843.1	succinyl-CoA synthetase subunit alpha	0.001	-0.240	-0.675	2
ETM66664.1	aconitate hydratase B AcnB	0.001	0.370	-0.899	2
ETM64607.1	alkyl hydroperoxide reductase subunit C AhpC	0.002	-0.384	-2.682	2
ETM65247.1	L-lactate permease	0.005	0.266	-0.759	2
ETM65393.1	purine biosynthesis protein purH	0.005	-0.076	-1.470	2
ETM64939.1	preprotein translocase subunit SecB	0.014	-0.141	-0.515	2
ETM63932.1	electron transfer flavoprotein subunit beta	0.019	-0.350	-1.962	2
ETM63915.1	3-ketoacyl-ACP reductase	0.026	-0.150	-1.397	2
ETM65886.1	molecular chaperone GroES	0.027	-0.173	-0.899	2
ETM67957.1	CTP synthetase	0.032	0.153	-0.507	2
ETM65487.1	adenylosuccinate synthetase	0.037	-0.499	-2.017	2
ETM66453.1	isopropylmalate isomerase LeuC	0.000	-1.473	-3.010	3
ETM66454.1	3-isopropylmalate dehydratase small subunit LeuD	0.000	-2.260	-3.138	3
ETM66206.1	hypothetical protein PM1_0208385	0.000	-2.121	-1.807	3

Accession number	Protein Name	ANOVA (*P*-value)	Average fold change[a]		Cluster[b]
			Ag^+	AgNPs	
ETM67686.1	nucleoside diphosphate kinase	0.000	-0.730	-1.943	3
ETM69111.1	elongation factor G	0.000	-0.772	-1.733	3
ETM66456.1	3-isopropylmalate dehydrogenase LeuB	0.000	-1.488	-2.606	3
ETM66285.1	acetyl-CoA synthetase	0.002	-1.797	-1.987	3
ETM67959.1	Enolase	0.004	-0.635	-1.752	3
ETM65137.1	Endoribonuclease	0.017	-1.066	-2.689	3
ETM63983.1	elongation factor P	0.023	-1.170	-3.568	3
ETM65645.1	glutamate dehydrogenase	0.036	-2.063	-1.048	3
ETM69050.1	spermidine/putrescine ABC transporter substrate-binding protein	0.037	-1.549	-3.454	3
ETM66840.1	dihydrolipoamide succinyltransferase	0.001	0.403	0.492	4
ETM65239.1	molecular chaperone DnaK	0.001	1.168	0.454	4
ETM69176.1	outer membrane protein W	0.001	1.230	0.226	4
ETM63384.1	cytochrome CBB3	0.001	1.287	0.560	4
ETM68644.1	phosphoenolpyruvate synthase	0.003	1.157	0.365	4
ETM64990.1	arginine deiminase	0.003	0.981	0.534	4
ETM66534.1	Peptidase	0.005	1.165	0.780	4
ETM67390.1	H-NS histone	0.005	0.741	0.598	4
ETM68804.1	isocitrate lyase	0.008	1.960	0.848	4
ETM65887.1	molecular chaperone GroEL	0.008	0.761	0.407	4
ETM68650.1	Porin	0.009	0.936	0.379	4
ETM68113.1	Porin	0.009	0.846	0.218	4
ETM65223.1	peptidase M54	0.012	0.336	0.264	4
ETM63387.1	cytochrome oxidase subunit I	0.015	1.021	0.700	4
ETM69141.1	catalase/hydroperoxidase HPI(I) KatG	0.017	1.578	0.739	4
ETM68466.1	membrane protein	0.021	1.791	1.300	4
ETM64574.1	F0F1 ATP synthase subunit alpha	0.024	0.499	0.373	4
ETM64558.1	DNA polymerase III subunit beta	0.042	1.039	1.218	4
ETM66851.1	heat shock protein 90	0.043	1.145	0.102	4
ETM65482.1	30S ribosomal protein S18	0.056			
ETM67796.1	30S ribosomal protein S16	0.061			
ETM66001.1	adenine methyltransferase	0.061			
ETM66837.1	succinate dehydrogenase flavoprotein subunit	0.061			
ETM69130.1	50S ribosomal protein L18	0.066			
ETM69124.1	50S ribosomal protein L14	0.067			
ETM69119.1	50S ribosomal protein L22	0.073			
ETM66842.1	malate--CoA ligase subunit beta	0.075			
ETM63981.1	Major outer membrane lipoprotein	0.077			

Accession number	Protein Name	ANOVA (*P*-value)	Average fold change[a]		Cluster[b]
			Ag^+	AgNPs	
ETM64891.1	glucan biosynthesis protein D	0.080			
ETM69106.1	50S ribosomal protein L7/L12	0.081			
ETM64287.1	pyruvate carboxylase	0.085			
ETM64571.1	F0F1 ATP synthase subunit C	0.087			
ETM63916.1	acyl carrier protein	0.088			
ETM69116.1	50S ribosomal protein L23	0.093			
ETM65599.1	50S ribosomal protein L25	0.099			
ETM65822.1	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	0.099			
ETM64991.1	ornithine carbamoyltransferase	0.100			
ETM65218.1	polynucleotide phosphorylase/polyadenylase	0.105			
ETM67644.1	DNA-binding protein	0.106			
ETM65600.1	ribose-phosphate pyrophosphokinase	0.110			
ETM64998.1	dihydroxy-acid dehydratase	0.111			
ETM66300.1	alanyl-tRNA synthetase	0.113			
ETM64556.1	DNA gyrase subunit B	0.113			
ETM68177.1	peptidyl-prolyl cis-trans isomerase	0.114			
ETM67711.1	inosine 5'-monophosphate dehydrogenase	0.124			
ETM66531.1	trigger factor	0.133			
ETM65234.1	carbamoyl phosphate synthase large subunit	0.135			
ETM65177.1	ketol-acid reductoisomerase	0.148			
ETM69137.1	30S ribosomal protein S4	0.160			
ETM67799.1	50S ribosomal protein L19	0.162			
ETM65249.1	4Fe-4S ferredoxin	0.162			
ETM69121.1	50S ribosomal protein L16	0.166			
ETM63881.1	glyceraldehyde-3-phosphate dehydrogenase	0.167			
ETM67675.1	inositol monophosphatase	0.171			
ETM69125.1	50S ribosomal protein L24	0.194			
ETM69139.1	50S ribosomal protein L17	0.195			
ETM65841.1	50S ribosomal protein L13	0.197			
ETM65818.1	organic solvent ABC transporter substrate-binding protein	0.201			
ETM69136.1	30S ribosomal protein S11	0.205			
ETM64827.1	pyruvate dehydrogenase	0.209			
ETM64889.1	amino acid ABC transporter substrate-binding protein	0.211			
ETM69135.1	30S ribosomal protein S13	0.217			
ETM64267.1	phosphoenolpyruvate carboxykinase	0.217			
ETM63814.1	glutamate dehydrogenase	0.222			
ETM64575.1	F0F1 ATP synthase subunit gamma	0.233			

Accession number	Protein Name	ANOVA (*P*-value)	Average fold change[a]		Cluster[b]
			Ag^+	AgNPs	
ETM63869.1	topoisomerase I	0.233			
ETM69108.1	DNA-directed RNA polymerase subunit beta'	0.241			
ETM69189.1	6,7-dimethyl-8-ribityllumazine synthase	0.275			
ETM69022.1	RNA polymerase sigma factor RpoD	0.276			
ETM69127.1	30S ribosomal protein S14	0.276			
ETM68777.1	type III restriction endonuclease subunit R	0.279			
ETM69144.1	single-stranded DNA-binding protein	0.283			
ETM65248.1	L-lactate dehydrogenase	0.301			
ETM67464.1	hypothetical protein PM1_0214880	0.307			
ETM69138.1	DNA-directed RNA polymerase subunit alpha	0.318			
ETM69115.1	50S ribosomal protein L4	0.320			
ETM68502.1	50S ribosomal protein L20	0.336			
ETM69109.1	30S ribosomal protein S12	0.341			
ETM64576.1	F0F1 ATP synthase subunit beta	0.341			
ETM67783.1	phosphoribosylformylglycinamidine synthase	0.341			
ETM65236.1	carbamoyl phosphate synthase small subunit	0.343			
ETM64846.1	glutamate synthase subunit alpha	0.352			
ETM65543.1	leucyl-tRNA synthetase	0.364			
ETM69103.1	50S ribosomal protein L11	0.365			
ETM65788.1	glutamyl-tRNA(Gln) amidotransferase	0.375			
ETM64828.1	dihydrolipoamide acetyltransferase	0.384			
ETM69107.1	DNA-directed RNA polymerase subunit beta	0.396			
ETM63386.1	peptidase S41	0.403			
ETM68119.1	aspartyl-tRNA synthetase	0.421			
ETM65222.1	translation initiation factor IF-2	0.430			
ETM69105.1	50S ribosomal protein L10	0.453			
ETM69117.1	50S ribosomal protein L2	0.456			
ETM66131.1	argininosuccinate synthase	0.462			
ETM69023.1	30S ribosomal protein S21	0.477			
ETM69101.1	preprotein translocase subunit SecE	0.524			
ETM65480.1	50S ribosomal protein L9	0.532			
ETM66834.1	type II citrate synthase	0.539			
ETM65120.1	50S ribosomal protein L28	0.541			
ETM69131.1	30S ribosomal protein S5	0.597			
ETM65488.1	ATP phosphoribosyltransferase	0.601			
ETM65031.1	3-phosphoglycerate dehydrogenase	0.616			
ETM68813.1	isocitrate dehydrogenase	0.635			

Accession number	Protein Name	ANOVA (*P*-value)	Average fold change[a]		Cluster[b]
			Ag^+	AgNPs	
ETM64886.1	DEAD/DEAH box helicase	0.673			
ETM66836.1	succinate dehydrogenase	0.712			
ETM69129.1	50S ribosomal protein L6	0.748			
ETM65870.1	preprotein translocase subunit SecA	0.754			
ETM66301.1	aspartokinase	0.785			
ETM66838.1	succinate dehydrogenase iron-sulfur subunit	0.796			
ETM65179.1	acetolactate synthase	0.803			
ETM64288.1	acetyl-CoA carboxylase subunit alpha	0.821			
ETM65706.1	competence protein	0.836			
ETM69120.1	30S ribosomal protein S3	0.838			
ETM67463.1	hypothetical protein PM1_0214875	0.859			
ETM69102.1	transcription antitermination protein NusG	0.863			
ETM64572.1	F0F1 ATP synthase subunit B	0.863			
ETM66841.1	dihydrolipoamide dehydrogenase	0.867			
ETM64208.1	uroporphyrin-III C-methyltransferase	0.889			
ETM69110.1	30S ribosomal protein S7	0.912			
ETM67885.1	lysyl-tRNA synthetase	0.917			
ETM69104.1	50S ribosomal protein L1	0.924			
ETM65658.1	50S ribosomal protein L21	0.934			
ETM68450.1	ribonucleotide-diphosphate reductase subunit alpha	0.945			
ETM65219.1	30S ribosomal protein S15	0.953			
ETM69114.1	50S ribosomal protein L3	0.988			
ETM66839.1	2-oxoglutarate dehydrogenase E1	0.991			
ETM64883.1	adenosylhomocysteinase	0.992			

[a] Values were calculated as the average data from, at least four independent experiments. Fold changes of statistically significant proteins (ANOVA, $P<0.05$) were determined by Log2 transformation of the ratio values of normalized protein levels obtained using crude protein extracts from cells of *Pseudomonas* sp. M1 after 90 minutes of exposure to Ag^+ or AgNPs at concentrations similar to EC_{20} versus cells grown in control medium.

[b] The unsupervised clustering analysis was performed considering standardization and the 59 statistically significant proteins across the different experimental conditions (Control, Ct; silver ions, Ag^+ and silver nanoparticles, AgNPs) were partitioned into 4 clusters.