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Comprehensive analysis of transcriptional and proteomic profiling reveals silver nanoparticles-induced toxicity to bacterial denitrification

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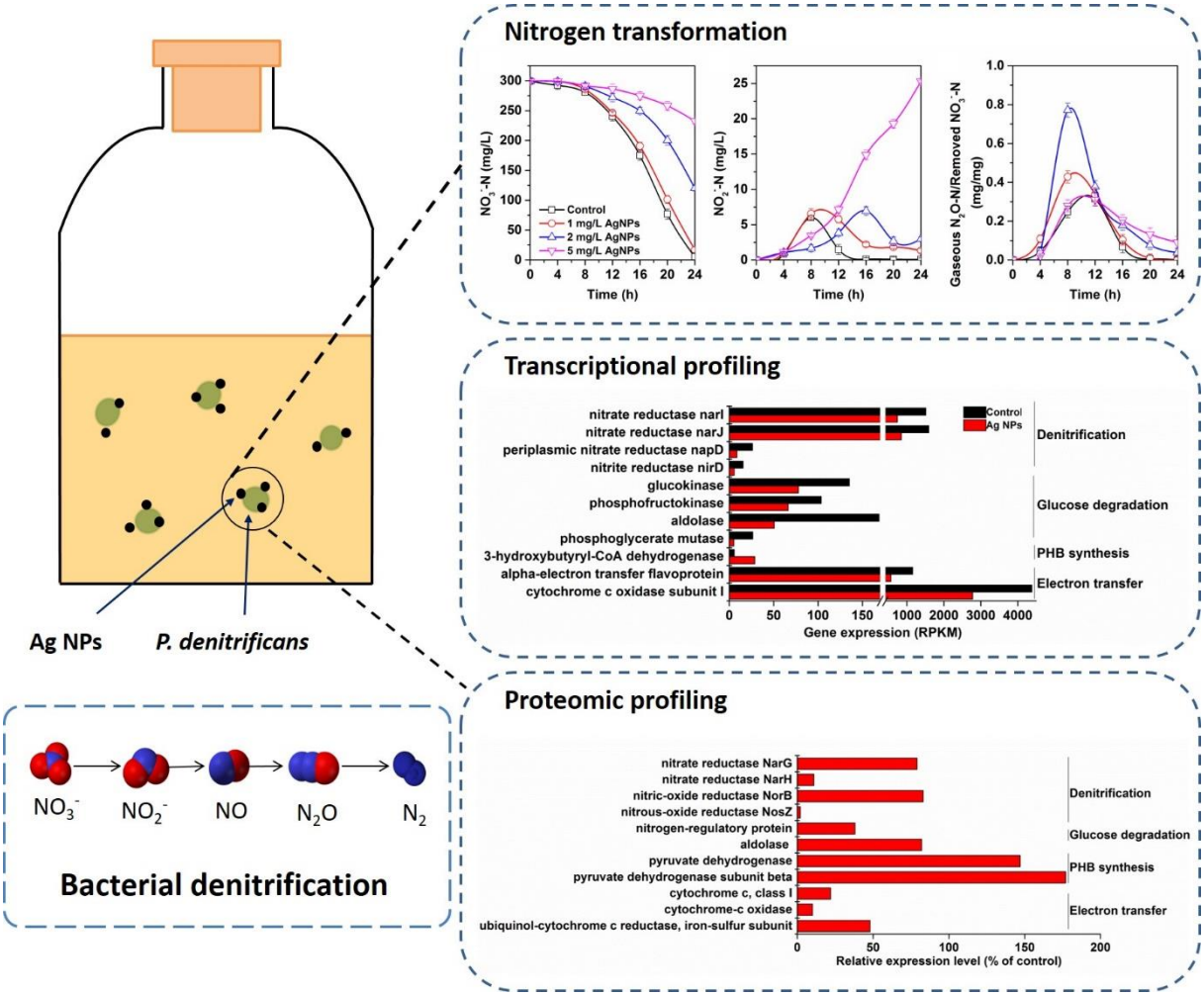
Graphical Abstract



Highlights:

- The effects of Ag NPs on *Paracoccus denitrificans* under anoxic conditions were investigated.
- The mechanism of toxicity of Ag NPs was explored via whole-genome sequence-based analysis and proteomic profiling.
- Ag NPs significantly affected the bacterial denitrification, cell structure and growth.
- Ag NPs changed the expressions of key genes and proteins involved in denitrification.
- Ag NPs suppressed the activities of nitrate reductase and nitrite reductase.

Abstract

Although the toxicity of silver nanoparticles (Ag NPs or nanosilver) to model bacteria has been reported, the effects of Ag NPs on microbial denitrification under anoxic conditions and the mechanism of Ag NPs induced-toxicity to denitrification remain unclear. In this study, the effects of Ag NPs on *Paracoccus denitrificans* under anoxic conditions were investigated, and the mechanism was explored by analyzing the transcriptional and proteomic responses of bacteria to Ag NPs. The presence of 5 mg/L Ag NPs led to excessive nitrate accumulation (232.5 versus 5.3 mg/L) and increased nitrous oxide emission. Transcriptional analysis indicated that Ag NPs restrained the expression of key genes related to denitrification. Specifically, the genes involved in denitrifying catalytic reduction and electron transfer were significantly down-regulated. Moreover, the expression of the genes responsible for polyhydroxybutyrate synthesis was enhanced, which was adverse to denitrification. Proteomic profiling revealed that the syntheses of the proteins involved in catalytic process, electron transfer, and metabolic process were inhibited by Ag NPs. The activities of nitrate reductase and nitrite reductase in the presence of 5 mg/L Ag NPs were only 42% and 61% of those in the control, respectively, indicating the inhibition of denitrifying enzymes. These results improve understanding of the inhibitory mechanism of Ag NPs toward bacterial denitrification.

Keywords: Silver nanoparticles; Denitrification; Transcriptomics; Proteomic profiling; *Paracoccus denitrificans*

1. Introduction

Silver nanoparticles (Ag NPs or nanosilver) have been widely used as biocides in products, because of their excellent antibacterial property [1, 2]. It is estimated that the average global consumption of Ag NPs was approximately 55 tons/year [3]. However, researchers have recently pointed out that the

production, use and disposal of Ag NPs containing products could lead to the release of Ag NPs into the environment [4]. According to the literature, nearly one-third of Ag NP containing products on the market had the potential to disperse Ag NPs into the environment, and the Ag NPs released from commercial products might lead to serious environmental impacts [5]. Previous studies indicated that Ag NPs could significantly decline the growth parameters and photosynthetic pigments of pea seedlings [6] and showed negative impacts on plants at the proteomic level [7]. Moreover, the continuous release of Ag NPs was observed to hamper many physiological and biochemical processes in living organisms, including autotrophs and heterotrophs [8, 9]. Specifically, Ag NPs could cause risks to wastewater treatment plants, and were observed to affect the performances of conventional biological treatment processes [10-12]. Therefore, it can be deduced that once Ag NPs enter the environment, these nanoparticles might pose potential risks to environmental bacteria and wastewater treatment.

Microbial denitrification is an essential method by which nitrogen in the environment (land, water and organisms) goes back to the atmosphere, completing the nitrogen cycle [13, 14]. Incomplete denitrification might lead to global warming and stratospheric ozone destruction via induction of high emissions of nitrous oxide (N_2O) [15-17]. Because bacterial denitrification is largely responsible for nitrate reduction and N_2O production, the effects of Ag NPs on denitrifying bacteria might influence the efficiency of nitrate removal and the emission of N_2O. A previous study showed that Ag NPs exerted a high inhibitory effect on a biological nitrogen removal process because of its small size [18], but other researchers pointed out that the toxicity of Ag NPs to denitrification might not be correlated with either coatings or particle size [19]. *Yang et al.* observed the negative effects of Ag NPs (with amorphous carbon coatings) on a denitrifier, *Pseudomonas stutzeri* [20]. However, the possible effects of Ag NPs on bacterial denitrification especially nitrate removal and N_2O emission under anoxic conditions have not been

clarified to date.

It is well-known that transcription regulation is the basis of bacterial growth and metabolism. Genome-wide transcriptional profiling can improve our understanding of the influencing mechanism [21]. Furthermore, as the terminal product of gene expression, protein plays an important role in many biological processes, such as catalyzing metabolic reactions and transporting substances [22, 23]. Accordingly, changes in the protein can lead to variations in the microbial growth, and metabolism would be changed. Hence, the performance of protein synthesis needs to be considered. Clearly, the analysis of transcriptional and proteomic profiling might be an effective way to comprehensively explore the possible mechanisms. Unfortunately, previous studies showed that the toxicity of Ag NPs was attributed to the generation of reactive oxygen species (ROS) [24, 25]. Although *Yang et al.* reported the effects of Ag NPs on the expressions of several denitrifying genes [20], the complete transcriptional profile of denitrifying bacteria exposed to Ag NPs remains unclear. Therefore, to explore the underlying mechanism, it is necessary to investigate the effects of Ag NPs on denitrification via a comprehensive analysis of transcriptional and proteomic profiling.

This study was conducted to investigate the effects of Ag NPs on bacterial denitrification by a comprehensive analysis of transcriptional and proteomic profiling. To accomplish this, the transformations of denitrification intermediates, including NO_3^--N, NO_2^--N, and gaseous N_2O, during exposure to Ag NPs were investigated. The relative lactate dehydrogenase (LDH) release and OD_{600} in the presence of Ag NP were then tested to explore the effects on bacterial structure and growth. Finally, the contribution of released silver ions to Ag NP induced effects was determined. To explore the underlying mechanism, a large set of differentially expressed genes (DEGs) involved in denitrification, glycolysis, polyhydroxybutyrate (PHB) synthesis and electron transfer, and the differentially expressed proteins

(DEPs) involved in vital functions closely related to denitrification were identified. The catalytic activities of key denitrifying enzymes were then measured. The results of this study will help improve the understanding of the inhibitory mechanism of Ag NPs on denitrifying bacteria.

2. Materials and methods

2.1. Silver nanoparticles

In this study, Ag NPs (with no coating or capping agent) were purchased from Alfa Aesar (USA). X-ray diffraction (XRD) analysis using a Rigaku D/Max-RB diffractometer equipped with a rotating anode and a Cu Kα radiation source was employed to characterize the Ag NPs (Fig. S1, Supplementary Material). Additionally, transmission electron microscopy (TEM) was conducted using a Philips Tecnai F20 microscope at an accelerating voltage of 200 kV to view the Ag NPs (Fig. S1, Supplementary Material). Before the experiment, a stock suspension was prepared by dispersing 5 mg of Ag NPs in 50 mL of Milli-Q water, followed by 1 h of ultrasonication (20 kHz and 500 W) at room temperature. Using a Malvern Autosizer 4700 (Malvern Instruments, UK), dynamic light scattering (DLS) was conducted and the results showed that the main sizes of Ag NPs in the stock suspension were approximately 65 nm.

2.2. Exposure experiment

A model denitrifying microorganism, *Paracoccus denitrificans*, was obtained from ATCC (Manassas, VA, USA) and grown in Difco nutrient broth at 30 °C and 160 rpm. After the typical optical density at 600 nm (OD_{600}) reached 0.8-1.0, the *P. denitrificans* was centrifuged at 4754 g for 10 min, then washed three times with 0.1 M phosphate buffer solution (PBS) (pH 7.4). The bacterial cells were resuspended in PBS and used in the exposure experiment. *P. denitrificans* was exposed to 0, 1, 2, and 5 mg/L Ag NPs in a mineral medium to examine the influences of Ag NPs on microbial denitrification, and the sample with no

added Ag NPs (0 mg/L) was used as the negative control. According to a previous publication [26], the mineral medium (per liter) contained 3.0 g KH_2PO_4, 5.704 g Na_2HPO_4, 1.717 g NH_4NO_3, 0.5 g $C_6H_5Na_3O_7 \cdot 2H_2O$, 0.1 g $MgSO_4 \cdot 7H_2O$, 10 g glucose, and 50 μL trace element feed. *P. denitrificans* was inoculated into 100 mL serum bottles containing 50 mL mineral medium and different concentrations of Ag NPs, while its initial OD_{600} value was controlled at 0.05. Next, pure argon gas was purged into each bottle for 10 min to replace the air inside the bottle, after which it was sealed with a rubber stopper to maintain an anoxic condition throughout the entire incubation period. All bottles were placed in a shaker (160 rpm) and held at a constant temperature of 30 ℃. A needle was used for sampling, and the concentrations of NO_3^-, NO_2^- and gaseous N_2O were measured at intervals of 4 h for a total time of 24 h according to our previous study [27] (see Supplementary Material).

2.3. Effect of the released silver ions on bacterial denitrification

The dissolution of Ag NPs in the medium was examined in this study. Simply, several serum bottles contained 50 mL of mineral medium with 1, 2 and 5 mg/L Ag NPs, respectively. Pure argon gas was purged into each bottle for 10 min, and then the bottles were sealed with rubber stoppers to maintain an anoxic condition throughout the entire period. All bottles were shaken at 30 ℃ and 160 rpm. At set intervals, Ag NPs were removed through high speed centrifugation (13148 g) for 10 min, after which 0.5 mL of supernatant was mixed with 4.5 mL of Milli-Q water (containing 2% ultrahigh purity HNO_3). Using inductively coupled plasma mass spectrometry (ICP-MS, Agilent Technologies, USA), the concentration of silver ions in the above solution was estimated. Following the method described in the exposure experiment section, *P. denitrificans* was exposed to the measured concentration of released silver ions. The final concentrations of NO_3^- and NO_2^- were estimated to investigate the possible effects of released silver ions on the denitrification process.

2.4. Transcriptional analysis

RNA-Seq technology was used in this study to reveal the transcriptional profile of *P. denitrificans* in the absence (the control) and presence of 5 mg/L Ag NPs because it was considered an accurate technology for quantification of transcriptional levels [28, 29]. Briefly, cells were harvested after exposure to Ag NPs for 16 h, then centrifuged at 6708 g (4 °C) for 10 min, after which the pellet was treated with TRIzol reagent (Invitrogen) to extract the total RNA. Next, a MICROBExpress Bacterial mRNA Enrichment Kit (Ambion) was employed to isolate the mRNA according to the manufacturer's protocols, and the purified mRNA was prepared for Illumina sequencing using an mRNA-Seq Sample Preparation Kit (Illumina). Finally, the libraries were prepared and sequenced on an Illumina HiSeq 2000 sequencer. All sequencing data have been submitted to the National Center for Biotechnology Information (NCBI) short-read archive (SRA) under accession numbers SRX2718218 and SRX2718219.

The sequences were filtered using a quality control (QC) toolkit v2.2.1 with following criteria: (1) sequence adapters, (2) more than 5% 'N' bases [30], and (3) more than 50% QA ≤ 15 bases. The clean reads were then aligned to the reference genome using SOAP2 [31], after which the gene expression level was evaluated by the RPKM (reads per kilobase of exon region per million mappable reads) [32]. The differentially expressed genes (DEGs) were identified with the criteria of fold change > 2 and false discovery rate (FDR) < 0.05. Finally, the enrichment analyses of gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were conducted using Blast2GO with the default annotation parameters [33].

2.5. Proteomic analysis

The iTRAQ technology was applied in this study to determine the variations in protein expression induced by Ag NPs. First, protein was extracted from triplicate samples according to the procedure

described in a previous publication [34, 35]. Next, 80 μg of digested peptide was labeled with iTRAQ reagents according to the manufacturer's instructions (Applied Biosystems, USA). All six samples (three for control, and others for samples exposed to Ag NPs) were then reacted with reagents 114, 115, 116, 117, 118, and 119, after which the labeled samples were mixed and desalted on C18 Cartridge (Sigma) before being dried under vacuum. After being reconstituted in 0.1% trifluoroacetic acid, 10 μL of each fraction was injected for nano LC-MS/MS analysis using a Q Exactive MS (Thermo Finnigan) equipped with Easy nLC (Thermo Fisher Scientific). The samples were then loaded onto two Thermo Scientific EASY Columns (0.1 mm × 20 mm, 5 μm - C18; 0.075 mm × 100 mm, 3 μm - C18) successively at a flow rate of 250 nL/min. Peptides were separated with a linear gradient of Buffer B (0.1% formic acid and 84% acetonitrile in MilliQ water) at a flow rate of 200 nL/min.

After separation, samples were analyzed using a Q-Exactive (Thermo Finnigan, USA) mass spectrometer (with a mass range of 300–1800 m/z) in positive ion mode for 240 min. Survey scans were then acquired at a resolution of 70000 at m/z 200, and the resolution for HCD spectra was set to 17500 at m/z 200. The maximum ion injection times were 10 and 60 ms for MS and MS/MS, respectively, and the automatic gain control (AGC) target was set to 3E6. The normalized collision energy was 30 eV and the under-fill ratio was defined as 0.1%. The raw data of MS/MS spectra were analyzed using Mascot 2.2 and Proteome Discoverer 1.4. The MASCOT parameters were set as follows: peptide mass tolerance at 20 ppm; MS/MS tolerance at 0.1 Da; max missed cleavages up to 2. All data were filtered by at least one unique peptide with 99% confidence, and protein identification was determined based on a by false discovery rate (FDR) ≤ 1%.

2.6. Measurements of the catalytic activities of denitrifying enzymes

The activities of key enzymes (nitrate reductase and nitrite reductase) were examined as previously

described the literature [27]. Briefly, cells were collected by centrifugation (5000 rpm for 5 min) after 24 h of exposure, washed three time with 0.1 M phosphate-buffered saline (PBS) (pH 7.4), then resuspended in the same buffer at 4 °C. The suspension was then disrupted by sonication (4 °C, 20 kHz) for 5 min, after which the crude cell extracts were obtained by centrifugation at (12,000 rpm 4 °C) for 15 min. The enzyme activities were determined immediately, after which the protein concentrations of cell extracts were measured using a Protein Assay Kit (Bio-Rad) with bovine serum albumin (BSA) as a standard. The methods used for the transmission electron microscopy analysis and lactate dehydrogenase release assay provided in the Supplementary Material.

2.7. Statistical Analysis

All tests were performed in triplicate, and the results were expressed as the means ± standard deviation. Analysis of variance (ANOVA) was used to identify significant differences among groups, with a $p < 0.05$ considered to be statistically significant.

3. Results and discussion

3.1. Silver nanoparticles inhibit the performance of bacterial denitrification

Although the release of Ag NPs has raised great concerns because of their potential to negatively impact ecosystems in engineered environments, it has been reported that the toxicity of Ag NPs could be significantly lower because of the transformation of Ag NPs into silver(I)-complexes/precipitates in sewage collection networks [10]. The concentration of Ag NPs in wastewater is usually low (e.g., μg/L or lower), but is much higher in sludge treatment systems because of the deposition and biosorption [36, 37], in which denitrification and many other microbial processes occur. Therefore, to explore the possible influences of Ag NPs on bacterial denitrification, this study examined the NO_3^- reduction, NO_2^- variation and gaseous N_2O emission in the absence (negative control) and presence of 1, 2, and 5 mg/L Ag NPs.

After 24 h of exposure, the final concentration of NO_3^--N was obviously higher in the presence of 5 mg/L Ag NPs than its absence (232.5 versus 5.3 mg/L) (Fig. 1a). The accumulated concentration of NO_2^--N reached 25.3 mg/L in the presence of 5 mg/L Ag NPs, while little NO_2^--N was measured in the absence of Ag NPs (Fig. 1b). Since the denitrification rates in the absence and presence of Ag NPs were different, the ratios of gaseous N_2O to removed nitrate throughout the incubation period were calculated to examine Ag NP induced effects on N_2O emissions (Fig. 1c). The results revealed that, when compared with the control, the presence of Ag NPs increased the production of gaseous N_2O per unit of removed nitrogen. Clearly, these results showed that the presence of Ag NPs led to decreased nitrate reduction and caused increased N_2O emissions per unit of nitrogen removed by denitrification.

3.2. Bacterial structure and growth in the presence of silver nanoparticles

To determine the possible influence of Ag NPs on *P. denitrificans*, the growth and metabolism of *P. denitrificans* in the absence and presence of Ag NPs were examined. Transmission electron microscopy (TEM) images revealed that Ag NPs in the medium adhered to the cell membrane of *P. denitrificans* and that some NPs were present inside the bacterial cell (Fig. 2a). Evaluation of the lactate dehydrogenase (LDH) release as an indicator of membrane damage, revealed that the LDH release increased in the presence of 5 mg/L Ag NPs compared to that in the absence of Ag NPs (Fig. 2b), indicating that Ag NPs changed the membrane integrity of *P. denitrificans*. Fig. 2c and 2d illustrate the changes in the bacterial population density and specific growth rate of *P. denitrificans* during exposure to Ag NPs. The presence of Ag NPs inhibited the bacterial specific growth rate, and thus decreased the bacterial population density after 24 h of incubation. Nanomaterials can usually easily induce reactive oxygen species (ROS) production because of their extremely small size and high catalytic activity [38]. This behavior can cause changes in the membrane integrity and cell morphology of living organisms [39, 40], which is consistent

with the observed results.

3.3. Effects of the released silver ions on bacterial denitrification

A previous study reported that Ag NPs are slightly soluble in water, and can thus release silver ions under neutral condition [41]. In particular, *Zhang et al.* found that the dissolution of Ag NPs could be thermodynamically favorable under anoxic conditions when nitrate/nitrite were used as terminal electron acceptors [25]. This mechanism suggested that the release of silver ions from Ag NPs might occur during denitrification. As shown in Fig. 3a, the average concentrations of released silver ions in the presence of 1, 2, and 5 mg/L Ag NPs were 0.038, 0.09 and 0.20 mg/L, respectively. Moreover, the presence of 0.09 and 0.20 mg/L silver ions caused increases in the final concentrations of NO_3^--N and NO_2^--N (Fig. 3b), respectively, suggesting that the release of silver ions was an important reason for the adverse effects of Ag NPs on microbial denitrification. Many investigations have indicated that released silver ions were primarily responsible for the toxicity of Ag NPs toward model organisms [42], lacking of the exploration at gene and protein levels. Therefore, it is necessary to study the toxicity of Ag NPs on bacterial denitrification by comprehensive analysis of transcriptional and proteomic profiling.

3.4. Transcriptional profiling of denitrifying bacteria response to silver nanoparticles

To explore the mechanism of Ag NPs-induced effects on microbial denitrification, the genome-wide transcriptional profile of *P. denitrificans* in the absence and presence of 5 mg/L Ag NPs was investigated. It is well-known that high-throughput RNA sequencing (RNA-Seq) is an effective technology for quantification of the expression levels of transcripts under different conditions [28, 29]. Using this method, a total number of 26,774,234 high-quality reads were obtained after raw sequences processing and filtering. After mapping these clean reads to the reference genome of *P. denitrificans*, 93.8% of the total reads matched the reference genome, and 87.9% matched unique genomic locations (Table S1,

Supplementary Material).

In this study, 1240 genes that displayed transcriptional fluctuation (absolute fold change > 2 and FDR < 0.05; differentially expressed genes, DEGs) in response to 5 mg/L Ag NPs were identified, then used for screening analysis. The denitrification process is mainly catalyzed by some essential denitrifying enzymes (i.e., nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR), and nitrous oxide reductase (N_2OR)). Thus, the DEGs closely related to denitrification, such as gene clusters of *narGHJI* (encoding respiratory nitrate reductase), *napABC* (encoding periplasmic nitrate reductase), *nirSECF*, *norCBQDEF*, and *nosRZD*, were analyzed. Previous studies have also reported that microbial denitrification requires degradation of organic materials to supply electrons, which are essential for the complete reduction of nitrate to nitrogen gas [13]. *P. denitrificans* can also accumulate intracellular polyhydroxybutyrate (PHB), which consumes electrons and energy produced by glucose degradation, competing for the electrons used for microbial denitrification [43]. Therefore, in this study, glucose degradation and PHB synthesis played important roles in the reduction of nitrate and N_2O in microbial denitrification. Moreover, the efficiency of electron transfer is important because the denitrification process is based on electron transport [44]. Hence, the transcriptional levels of key genes related to denitrification, glucose degradation, PHB synthesis and electron transfer after exposure to Ag NPs were analyzed.

As shown in Fig. 4, the expression of vital genes directly related to denitrification, such as *narI*, *narJ* and *napD*, was significantly down-regulated in the presence of Ag NPs, suggesting that Ag NPs significantly inhibited the expressions of genes involved in denitrification. The expressions of genes involved in glucose degradation, such as glucokinase (GK) and aldolase, was also down-regulated in the presence of Ag NPs, indicating that the glycolysis process was inhibited by Ag NPs. The presence of Ag

NPs also significantly enhanced the expression of genes involved in PHB synthesis, which was adverse to microbial denitrification. The importance of electron transfer has been reported in the literature [45], and the data of this study indicated that the expressions of vital genes involved in electron transfer, such as alpha-electron transfer flavoprotein and cytochrome c oxidase, were significantly down-regulated in the presence of Ag NPs. These findings suggest that the electron transfer process was simultaneously inhibited by Ag NPs. Previous studies investigated the gene expression of *Daphnia* exposed to Ag NPs and found that changes in the related gene expressions led to inhibitory effects on bacteria [46], which is consistent with the result of the present study. Therefore, the presence of Ag NPs could cause decreased expression of genes related to nitrate reduction and N_2O emissions, and thus inhibition of denitrification.

3.5. Proteomic profiling of denitrifying bacteria response to silver nanoparticles

To further explore the mechanism by which the inhibitory effects occurred, the proteomic profiles of *P. denitrificans* in the absence and presence of 5 mg/L Ag NPs were analyzed. In this study, 78 proteins that displayed fluctuations in expression (absolute fold change > 1.5 and FDR < 0.05) in response to 5 mg/L Ag NPs were identified. During bacterial denitrification, the extracellular carbon source needs to be transported into cells before being utilized for microbial growth and denitrification [47-49]. It has been reported that glucose transport is accomplished by the cooperation of several important proteins, including solute-binding protein (GtsA), inner membrane protein (GtsB and GtsC), and ATP-binding protein (MalK) [50]. Moreover, bacterial denitrification is a series of sequential redox reactions that relies on electron transfer. In the electron transfer chain, electrons are sequentially delivered by electron transfer proteins, such as cytochrome bc1, cytochrome c, and denitrifying enzymes [44]. The key denitrifying enzymes sequentially catalyze the reduction reactions from nitrate to nitrogen. Previous studies indicated that changes in electron transport proteins or denitrifying enzymes could affect the performance of

denitrification [46, 51, 52]. Therefore, in this study, the effects of Ag NPs on the DEPs related to the processes of denitrification, glucose degradation, PHB synthesis and electron transfer were explored.

As shown in Fig. 5, key proteins closely related to denitrification, such as *NarH* and *NosZ*, were substantially down-regulated during exposure to 5 mg/L Ag NPs, which could result in the restraint of denitrification. Moreover, proteins related to glucose degradation and electron transfer also decreased significantly, which could enhance the inhibition of denitrification. It should be noted that proteins relevant to PHB synthesis were obviously enhanced, which was adverse to the denitrification process. Hence, the presence of 5 mg/L Ag NPs could cause adverse effects on protein synthesis, which is related to nitrate reduction and N_2O emission. The results of transcriptional and proteomic profiling of denitrifying bacteria response to Ag NPs, were consistent with those of the proteomic analysis.

3.6. Relative activities of the key enzymes response to silver nanoparticles

Microbial denitrification is highly dependent on the expressions and activities of essential denitrifying enzymes, such as nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR), and nitrous oxide reductase (N_2OR). Hence, the relative activities of the key enzymes (NAR and NIR) after exposure to different concentrations of Ag NPs were examined. As shown in Fig. 6, the activities of NAR and NIR decreased with increases in Ag NPs concentration, and the relative activities of NAR and NIR in the presence of 5 mg/L Ag NPs decreased to approximately 42% and 61% of the control, respectively. Therefore, the presence of Ag NPs resulted in a decrease in catalytic activities of key denitrifying enzymes, which finally caused a decrease in nitrate reduction and large N_2O emissions.

4. Conclusions

The potential risks of Ag NPs to bacterial denitrification were investigated and the mechanism was revealed by a comprehensive analysis of transcriptional and proteomic profiling. The reductions of nitrate

and nitrous oxide were significantly inhibited by the presence of Ag NPs. Moreover, Ag NPs were found to attach onto the surfaces of bacterial cells, disrupting the membrane integrity. The released silver ions accounted for the inhibitory effects of Ag NPs on bacterial denitrification. Investigation of the mechanism indicated that the expressions of genes involved in denitrifying catalytic reduction, electron transfer and PHB synthesis were significantly influenced by Ag NPs. However, the expressions of genes responsible for PHB synthesis were up-regulated, and enhancement of the PHB synthesis was unfavorable to denitrification because of competition for carbon sources. Moreover, proteomic profiling indicated that the synthesis of functional proteins involved in the catalytic process, electron transfer, and metabolic process were inhibited by Ag NPs, which was in accordance with the results of transcriptional profiling. Finally, the activities of key denitrifying enzymes were also inhibited by the presence of Ag NPs. The results presented herein improve the understanding of toxicity of Ag NPs to bacterial denitrification.

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Appendix.

Supplementary Material

This file contains additional analytical methods, Table S1, and Figure S1. This information is available free of charge via the Internet at http://www.journals.elsevier.com.

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Figure captions

Fig. 1. - Effects of Ag NPs on the transformations of denitrification intermediates (a: NO_3^--N, b: NO_2^--N, c: gaseous N_2O-N/removed NO_3^--N). The data are shown as mean value ± standard deviation from three independent measurements.

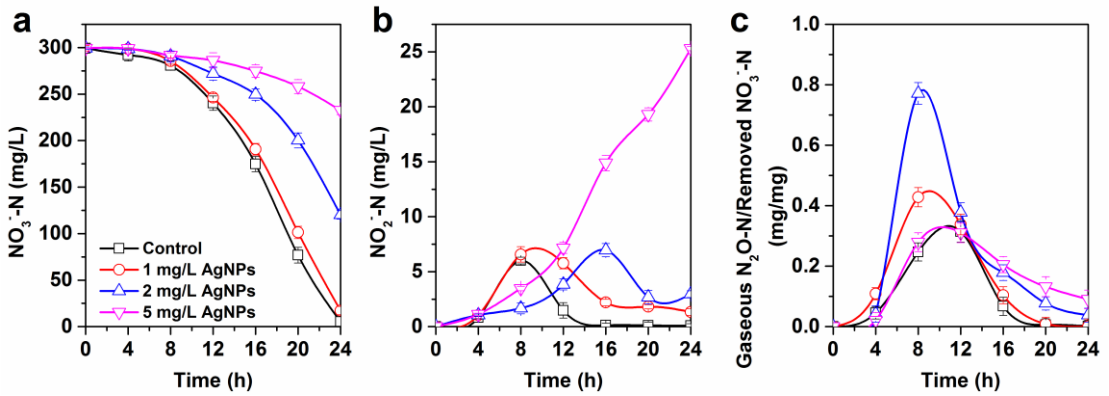
Fig. 2. - Effects of Ag NPs on the denitrifying bacterium *P. denitrificans*. (a) TEM images of *P. denitrificans* in the absence (left) and presence (right) of Ag NPs; (b) Relative LDH release (an indicator of membrane damage); (c) Bacterial growth curve in response to Ag NPs; (d) Specific growth rate in response to Ag NPs; Data are shown as mean value ± standard deviation from three independent measurements.

Fig. 3. - Effects of released silver ions on the denitrification due to the dissolution of Ag NPs. (a) Dissolution curves of Ag NPs in the culture medium. (b) Concentrations of NO_3^--N and NO_2^--N in the absence and presence of Ag NPs and its released silver ions at the end of exposure. The data are shown as mean value ± standard deviation from three independent measurements.

Fig. 4. - Effects of 5 mg/L Ag NPs on the expressions of the genes involved in denitrification, glucose degradation, PHB synthesis, and electron transfer.

Fig. 5. - Expressions of differentially expressed proteins (DEPs) involved in denitrification.

Fig. 6. - Effects of different concentrations of Ag NPs on the relative activities of the key enzymes related to denitrification process.



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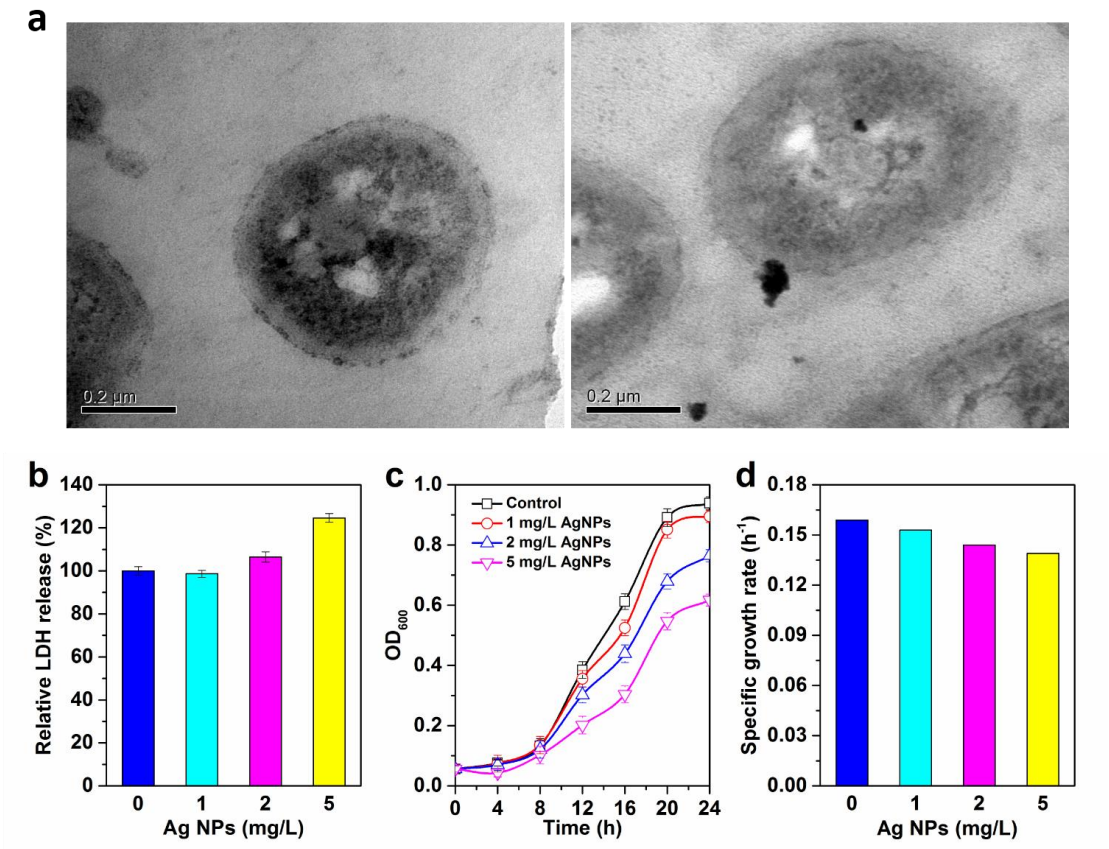

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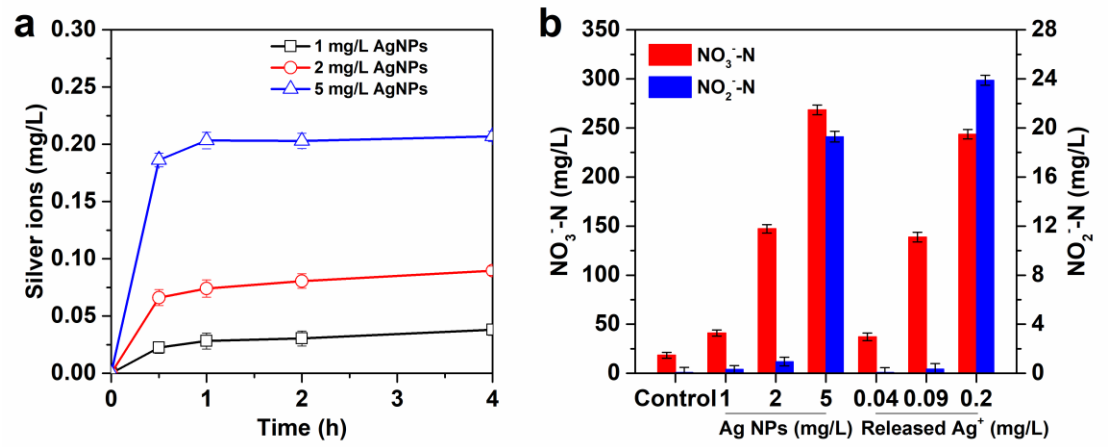

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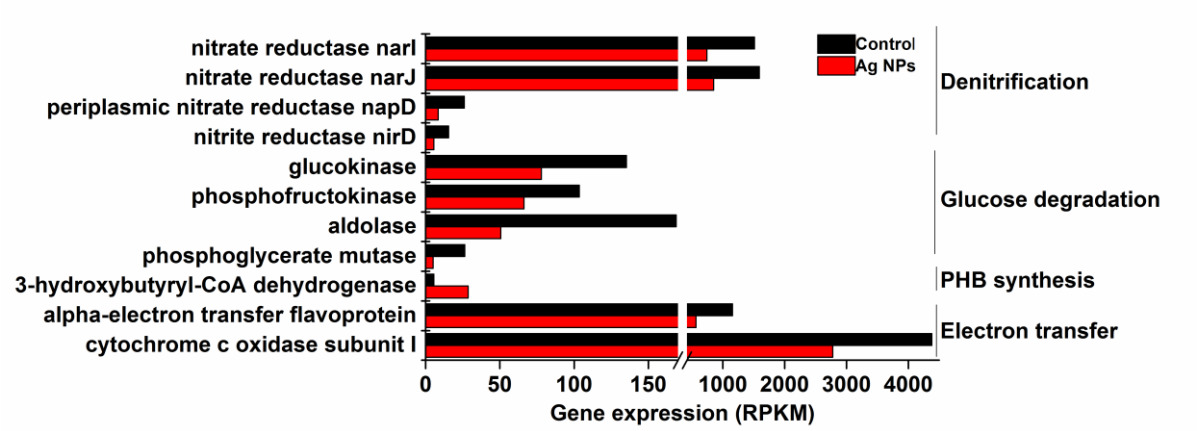

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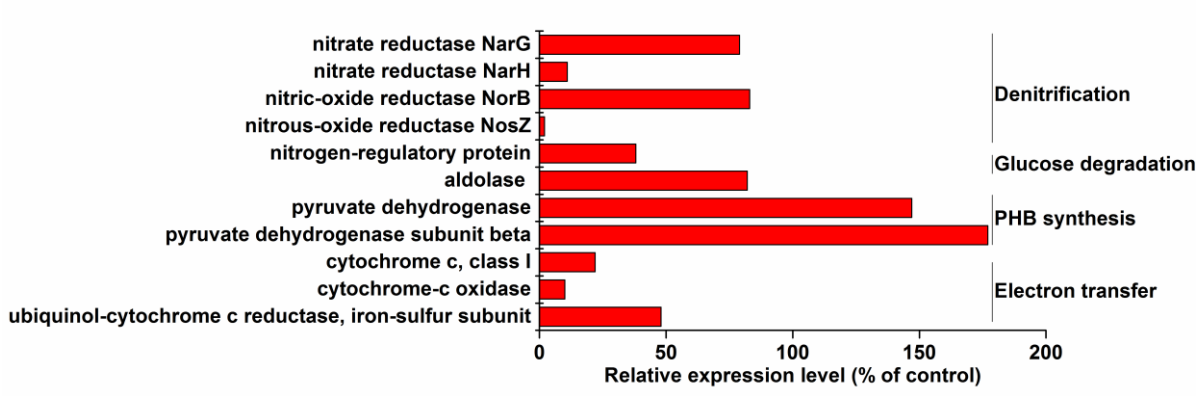

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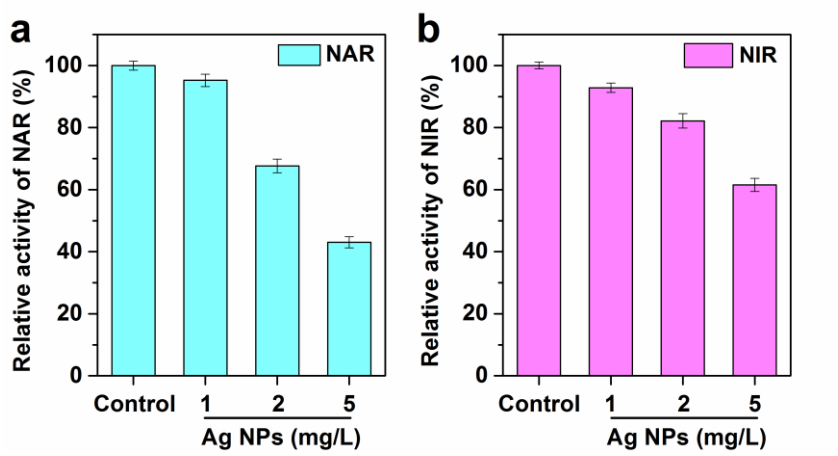

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