Exposure to Nanoplastic Particles Enhances Acinetobacter Survival, Biofilm Formation, and Serum Resistance

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Abstract: The interaction between nanoplastics and bacteria remains still largely unclear. In this study, we determined the effect of nanopolystyrene particle (NP) on a bacterial pathogen of Acinetobacter johnsonii AC15. Scanning electron microscopy (SEM) analysis indicated the aggregation of NPs from 10 µg/L to 100 µg/L on surface of A. johnsonii AC15, suggesting that A. johnsonii AC15 acted as the vector for NPs. Exposure to 100–1000 µg/L NPs increased the growth and colony-forming unit (CFU) of A. johnsonii AC15. In addition, exposure to 100–1000 µg/L NPs enhanced the amount of formed biofilm of A. johnsonii AC15. Alterations in expressions of 3 survival-related (zigA, basD, and zur), 5 biofilm formation-related (ompA, bap, adeG, csuC, and csuD), and 3 serum resistance-related virulence genes (lpxC, lpxL, and pbpG) were observed after exposure to 1000 µg/L NPs. Moreover, both CFU and survival rate of A. johnsonii AC15 in normal human serum (NHS) were significantly increased by 1–1000 µg/L NPs, suggesting the enhancement in serum resistance of Acinetobacter pathogen by NPs. In the NHS, expressions of 3 survival-related (zigA, basD, and zur), 9 biofilm formation-related (ompA, bap, adeF, adeG, csuA/B, csuC, csuD, csuE, and hlyD), and 3 serum resistance-related virulence genes (lpxC, lpxL, and pbpG) were affected by 1000 µg/L NPs. Expressions of 1 survival-related (zigA), 5 biofilm formation-related (bap, adeG, csuC, csuD, and csuE), and 3 serum resistance-related virulence genes (lpxC, lpxL, and pbpG) were also altered by 10 µg/L NPs after the addition of NHS. Therefore, exposure to NPs in the range of µg/L has the potential to enhance bacterial virulence by increasing their growth, biofilm formation, and serum resistance.

Keywords: nanoplastic; Acinetobacter; interaction; virulence

1. Introduction

The challenge from global plastic pollution has received the increasing attention [1]. The emerging plastic pollution causes substantial consequences on both the organisms and the environment [2,3]. Weathering of the plastic by certain processes, such as biological process, results in debris formation with the forms of microplastic or even nanoplastic [4,5]. Both microplastics and nanoplastics have been detected in various ecosystems, such as marine and aquatic environments [6,7], which suggests the exposure risk of these materials to human and environmental organisms [8]. Meanwhile, the increasing experimental evidence has demonstrated the potential toxicity of microplastics and nanoplastics on different organisms [9–13].

Besides toxicity of microplastics and nanoplastic themselves, interactions between environmental pollutants and microplastic or nanoplastic have also been received the attentions. Microplastics and nanoplastics can act as vectors for environmental pollutants and even further enhance their toxicity on organisms [14,15]. Moreover, microplastics can further interact with and act as a vector for environmental microorganisms [16,17]. Among microorganisms in the environment, some are bacterial pathogens. Once the bacterial pathogens are available to organisms, they will affect the host health [18,19]. In addition,
microplastic and nanoplastic at high concentration (such as those in the range of mg/L) caused bacterial growth inhibition, and the inhibition from nanoplastic exposure was more severe than that from microplastic exposure [20]. However, the effect of nanoplastics at low concentrations (such as those in the range of µg/L) on microorganisms remains largely unclear.

Three Gorges Reservoir (TGR) is a reservoir located upper stream of Yangtze River in China. In the TGR, plastic pollutions, including the microplastics, have been detected in both sediments and surface water [21,22]. AC15 is an Acinetobacter johnsonii strain isolated from the TGR region [23]. A. johnsonii is an opportunistic pathogen. Using Caenorhabditis elegans as a model host, it was proven that A. johnsonii AC15 is a bacterial pathogen [23]. In this study, we used nanopolystyrene particle (NP) as the example of nanoplastics to determine the effect of NPs at low doses (mainly in the range of µg/L) on A. johnsonii AC15. Our data demonstrated that exposure to NPs in the range of µg/L had the potential to enhance the virulence of bacterial pathogen by increasing both bacterial growth and biofilm formation. The findings highlight the risk of exposure to NPs in the range of µg/L in enhancing virulence of bacterial pathogens. In addition, in this study, we raised some molecular endpoints to assess this effect of NPs in the range of µg/L by identifying dysregulated virulence genes for A. johnsonii AC15.

2. Materials and Methods

2.1. Characterization of NPs

The 100 nm NPs were supplied by Huge Biotechnol. Co. (Wuhan, China). NP properties were evaluated by Zeta potential, dynamic light scattering (DLS), Fourier transform infrared spectroscopy (FTIR) spectrum (Avatar 370, Thermo Nicolet, Madison, WI, USA), and transmission electron microscopy (TEM). The dried NP sample was used for the analysis. Both size distribution and zeta potential of NPs were analyzed using Nano Zetasizer (Nano ZS90, Malvern Instrument, Malvern, UK). The morphology and size of NPs were further observed under the TEM (JEOL Ltd., Tokyo, Japan). FTIR analysis was performed to examine the different peaks of vibrations on NPs. To determine the interaction between NPs and A. johnsonii AC15, scanning electron microscopy (SEM) was further performed to analyze the aggregation of NPs (10–1000 µg/L) on surface of A. johnsonii AC15.

2.2. Acinetobacter Strain

AC15 is our isolated Acinetobacter strain in the TGR region [23]. A. johnsonii AC15 is normally stored in broth glycerol at −80 °C. To determine the location of A. johnsonii AC15 in systematic evolutionary tree, A. johnsonii AC15 DNA was extracted. The 16S rRNA was amplified in 25 µL polymerase chain reaction (PCR) reaction system using an upstream primer (515F: GTGCCAGCMGGCGGTAA) and downstream primer (806R: GGACTACHVGGGTWTCTAAT). Reaction conditions: pre-denaturation (94 °C, 5 min), denaturation (94 °C, 30 s), annealing (52 °C, 30 s), and extension (72 °C, 30 s). After 1% agarose gel electrophoresis, PCR product was used for the sequencing. Sequencing data was compared with the information of published sequences in GenBank. With the aid of BLAST in NCBI, a systematic evolutionary tree, including AC15 was constructed (Figure S1). AC15 shows 99% homologous to Acinetobacter Johnson, and thus AC15 was identified as Acinetobacter Johnson (Figure S1).

2.3. Analysis of A. johnsonii AC15 Growth

Single colony of A. johnsonii AC15 was inoculated in 20 mL LB medium and incubated at 37 °C overnight. In 150 mL LB medium, NPs (0.1–1000 µg/L) and A. johnsonii AC15 (1.5 mL) were added, and AC15 was incubated at 37 °C for 24 h. During the incubation, optical density (OD) values of 595 nm were recorded each hour [24]. Meanwhile, colony-forming unit (CFU) of A. johnsonii AC15 was analyzed by counting colony number of A. johnsonii AC15/mL. The experiments were repeated three times.
2.4. Adsorption Curve of NP during Bacterial Growth

*A. johnsonii* AC15 single colonies were inoculated in 20 mL LB liquid medium under the conditions of 37 °C and 150 rpm shaking for 12 h. In 4 vials of 800 mL LB liquid medium, 4 experimental groups (control, NP (1000 µg/L), 1 mL *A. johnsonii* AC15 bacterial solution, and NP (1000 µg/L) + 1 mL *A. johnsonii* AC15 bacterial solution) were prepared and incubated at 37 °C for 24 h. During the incubation, OD values of 600 nm were recorded per hour. The turbidity of each experimental group was recorded every two hours. To measure turbidity, the supernatant was taken after centrifugation of 20 mL of bacterial solution at 5000 rpm for 3 min. Meanwhile, the CFU values of each experimental group were measured. The experiments were carried out three times.

2.5. Adsorption Curve of NP under the Condition of Non-Growth of Bacteria

Four bottles of 400 mL LB liquid medium were prepared and cooled down to room temperature. Two of them were added with 1000 µg/L NPs. *A. johnsonii* AC15 single colonies were inoculated in two vials of 400 mL LB liquid medium at 37 °C for 12 h. After the incubation, 5000 rpm centrifugation was performed for 3 min, and the supernatant was discarded. For one of the vials, 400 mL NPs (1000 µg/L) and 400 mL LB liquid medium were added and mixed well. Another one was only added with 400 mL LB liquid medium and mixed well. The vials were standing cultured at 4 °C for 12 h. During the incubation, OD values of 595 nm were recorded per hour. The turbidity of each experimental group was recorded every two hours. CFU values of each experimental group were measured. The experiments were carried out three times. SEM analysis was further performed to analyze the aggregation of NP (1000 µg/L) on the surface of *A. johnsonii* AC15 during the incubation at 4 °C.

2.6. Biofilm Formation of *A. johnsonii* AC15

The 96 well microtiter plate assay was used to evaluate biofilm formation [25]. In each well, 100 µL NPs (0.1–1000 µg/L) and 10 µL *A. johnsonii* AC15 were added. After incubation at 37 °C for 12 h, concentration of *A. johnsonii* AC15 solution was adjusted to OD600 value to 0.1. After the further incubation at 37 °C for 36 h, the solution was removed, and the wells were washed with PBS buffer and dried naturally. Methanol (100 µL) was added to each well to fix biofilm for 15 min. After that, biofilms of *A. johnsonii* AC15 at the bottom of each well were stained with 1% crystal violet (100 µL) for 5 min at room temperature. Again, 33% glacial acetic acid (100 µL) was added to dissolve crystal violet for 30 min at 37 °C. The optical density for samples in each well was detected at 595 nm. SEM was also performed to directly observe the biofilm formation of *A. johnsonii* AC15. The experiments were repeated three times.

2.7. Serum Resistance Analysis

The serum resistance test was performed as described [26]. NPs (0.1–1000 µg/L) and *A. johnsonii* AC15 (100 µL) were added in LB medium, and the final volume was 10 mL. After incubation at 37 °C for 12 h, concentration of *A. johnsonii* AC15 solution was adjusted to OD600 value to 0.5. After washing with PBS buffer 3 times, *A. johnsonii* AC15 was resuspended in 1 mL PBS buffer. *A. johnsonii* AC15 suspension (100 µL) was mixed with 300 µL normal human serum (NHS) and incubated at 37 °C for 3 h. After that, 100 µL serum of each sample was coated on surface of LB agar medium to incubate at 37 °C for 24 h. CFU was analyzed as described above. The survival rate of *A. johnsonii* AC15 was expressed as ratio between CFU of *A. johnsonii* AC15 suspension added with NHS and that of *A. johnsonii* AC15 suspension without NHS addition. The experiments were repeated three times.

2.8. Real-Time PCR (qRT-PCR)

Total RNAs of *A. johnsonii* AC15 were extracted with Trizol. The concentration and purity of obtained RNAs were determined with a spectrophotometer. Reverse transcription
reactions for cDNA synthesis were performed using Mastercycler gradient PCR system. SYBR Green qRT-PCR master mix was used to detect transcriptional alterations of genes in real-time PCR systems. The internal reference gene is 16S rRNA. Three biological replicates were performed. Primer information is given in Table S1.

2.9. Data Analysis

Software of SPSS V26.0 (SPSS Statistics, Version 26.0; IBM, Armonk, NY, USA) was used for the statistical testing. One-way analysis of variance (ANOVA) and multiple comparisons (LSD tests) were used to examine difference between groups. A p-value of <0.01 (**) was considered to be statistically significant.

3. Results and Discussion

3.1. Characterization of NPs

TEM analysis showed spherical morphology of NPs (Figure 1A). DLS analysis indicated that the NP size was 102.4 ± 5.8 nm, and the zeta potential of NPs was −9.245 ± 0.491 mV. Before the exposure, the NP suspensions were sonicated at 100 W and 40 kHz for 30 min, and no obvious aggregation was observed in the NP suspensions after the sonication. Based on FTIR analysis, C–H telescopic vibration absorption peak on benzene ring was observed at 3026.729 cm⁻¹, and absorption peak of the C–H telescopic vibration of methyl and methylene was observed at 2915.76 cm⁻¹ (Figure 1B). Three larger absorption peaks between 1620 and 1450 cm⁻¹ were backbone vibration absorption peaks of aromatic hydrocarbons, indicating the presence of benzene rings (Figure 1B). Absorption peaks at 758.36 cm⁻¹ and 700.51 cm⁻¹ are single-substituted extra-surface bending vibration absorption peaks of benzene rings (Figure 1B).

Figure 1. Nanopolystyrene particles (NPs) properties. (A) Transmission electron microscopy (TEM) image of NP suspension before the sonication. (B) FTIR spectrum of NPs.

3.2. Interaction between NPs and A. johnsonii AC15

Based on SEM analysis, the size of A. johnsonii AC15 was larger than the examined NPs (Figure 2). After the exposure, a large number of NPs were observed to be adsorbed and aggregated on the surface of A. johnsonii AC15 (Figure 2). Moreover, the aggregation of NPs at concentrations from 10 μg/L to 1000 μg/L on A. johnsonii AC15 was concentration-dependent (Figure 2).

3.3. Effect of NP Exposure on Growth of A. johnsonii AC15

After exposure to NPs (10–1000 μg/L), we did not observe the obvious alteration in morphology of A. johnsonii AC15 (Figure 2). We further examined the effects of NPs at different concentrations on A. johnsonii AC15 growth. Based on the growth curve analysis, exposure to 1000 μg/L NP significantly increased the growth of A. johnsonii AC15 from the time of 12-h (Figure 3A). In addition, exposure to 100 μg/L NP also moderately increased
the *A. johnsonii* AC15 growth (Figure 3A). In contrast, exposure to 0.1–10 µg/L NP did not affect the growth curve of *A. johnsonii* AC15 (Figure 3A).

![SEM images showing the aggregation of nanopolystyrene particle (NP) particles at concentrations of 10–1000 µg/L on surface of *A. johnsonii* AC15. Red arrowheads indicate *A. johnsonii* AC15. Yellow arrowheads indicate the aggregated NPs on surface of *A. johnsonii* AC15.](image)

**Figure 2.** Scanning electron microscopy (SEM) images showing the aggregation of nanopolystyrene particle (NP) particles at concentrations of 10–1000 µg/L on surface of *A. johnsonii* AC15. Red arrowheads indicate *A. johnsonii* AC15. Yellow arrowheads indicate the aggregated NPs on surface of *A. johnsonii* AC15.

Based on the AC15 CFU analysis, at both 12-h and 24-h, exposure to 100–1000 µg/L NPs obviously increased the CFU of *A. johnsonii* AC15 (Figure 3B). Different from this, at both 12-h and 24-h, exposure to 0.1–10 µg/L NPs could not influence CFU of *A. johnsonii* AC15 (Figure 3B).

![Growth curves of A. johnsonii AC15 after exposure to NPs at different concentrations.](image)

**Figure 3.** Effect of nanopolystyrene particle (NP) exposure on growth of *A. johnsonii* AC15. (A) Growth curves of *A. johnsonii* AC15 after exposure to NPs at different concentrations. (B) Effect of NP exposure on colony-forming unit (CFU) of *A. johnsonii* AC15 at different times. **p < 0.01 vs. Control.

### 3.4. Adsorption Curve of NPs during Bacterial Growth

According to the growth curve analysis, 1000 µg/L NP could significantly increase the growth of *A. johnsonii* AC15 (Figure 4A). Meanwhile, we observed that exposure to 1000 µg/L NPs could significantly increase the CFU of *A. johnsonii* AC15 at 12 and 24 h.
According to the growth curve analysis, 1000 μg/L NP could significantly increase the CFU of A. johnsonii AC15 at different times. **p < 0.01 vs. A. johnsonii AC15. (Figure 4A). Meanwhile, we observed that exposure to 1–1000 μg/L NPs resulted in the decline in turbidity of A. johnsonii AC15 solution during the growth (Figure 4C).

3.5. Adsorption Curve of NPs under the Condition of Non-Growth of Bacteria

Under the condition of standing culture at 4 °C for 12 h, addition of 1000 μg/L NPs did not have a significant effect on the growth and CFU of A. johnsonii AC15 (Figure S2A,B). According to the turbidity analysis, after the addition of 1000 μg/L NPs, the turbidity remained unchanged during the incubation process (Figure S2C). After addition of 1000 μg/L NP, we did not observe the noticeable accumulation of NPs on the surface of A. johnsonii AC15 (Figure S2D).

The surface charge of the NPs in the LB liquid medium was further analyzed by zeta potential assay. The zeta potential of NP was −9.5 ± 0.56 mV.

3.6. Effect of NP Exposure on Biofilm Formation of A. johnsonii AC15

Biofilm forming is an important virulence factor for bacterial pathogen [25]. Based on crystal violet staining and SEM analysis, exposure to 0.1–10 μg/L NPs did not affect the amount of biofilm formation of A. johnsonii AC15 (Figure 5A–C). In contrast, after exposure to 100 and 1000 μg/L NPs, a significant increase in amount of biofilm formation for A. johnsonii AC15 was observed (Figure 5A–C).

3.7. Effect of NP Exposure on Serum Resistance of A. johnsonii AC15

Serum resistance is another important virulence factor for bacterial pathogens, including Acinetobacter [26]. After the addition of NHS, we found that both CFU and survival rate of A. johnsonii AC15 were not altered by exposure to 0.1 μg/L NPs (Figure 6A,B). In contrast, both CFU and survival rate of A. johnsonii AC15 in NHS were significantly increased by exposure to 1–1000 μg/L NPs (Figure 6A,B).

(Figure 4B). To determine the reason for the interaction between A. johnsonii AC15 and NPs, the turbidity was analyzed to reflect the number of NPs adsorbed by A. johnsonii AC15. Addition with 1000 μg/L NPs resulted in the decline in turbidity of A. johnsonii AC15 solution during the growth (Figure 4C).
**Figure 5.** Effect of nanopolystyrene particle (NP) exposure at concentrations of 0.1–1000 µg/L on *A. johnsonii* AC15 biofilm formation. (A) Crystal violet staining images showing the effect of NP exposure at different concentrations on *A. johnsonii* AC15 biofilm formation. Arrowheads indicate the formed biofilm. (B) Scanning electron microscopy (SEM) images showing the effect of NP exposure at different concentrations on *A. johnsonii* AC15 biofilm formation. Arrowheads indicate the formed biofilm. (C) Effect of NP exposure at different concentrations on the amount of *A. johnsonii* AC15 biofilm formation based on the analysis of OD values of 595 nm. Blank control, without crystal violet staining. **p < 0.01 vs. Control."
The effects of nanoplastics on the expression of virulence genes in Acinetobacter johnsonii AC15 are significant. We selected 17 virulence genes to determine the effects of NP exposure on the expression of these genes in A. johnsonii. **

### 3.8. Effect of NP Exposure on Expression of Virulence Genes in A. johnsonii AC15

With the concern on Acinetobacter survival, biofilm formation, and serum resistance, we selected 17 virulence genes to determine the effects of NP exposure on A. johnsonii AC15 [27,28]. Exposure to 1000 μg/L NPs increased expression of 3 survival-related virulence genes (zigA, basD, and zur) (Figure 7A). Among biofilm formation-related virulence genes, expressions of ompA, bap, adeG, csuC, and csuD were increased by exposure to 1000 μg/L NPs (Figure 7B). Among serum resistance-related virulence genes, exposure to 1000 μg/L NPs increased lpxC, lpxL, and pbpG expressions (Figure 7C).

**Figure 6.** Effect of nanopolystyrene particle (NP) exposure on serum resistance of A. johnsonii AC15. (A) Colony-forming unit (CFU) of A. johnsonii AC15 in normal human serum (NHS) after exposure to NPs. (B) Survival rate of A. johnsonii AC15 in NHS after exposure to NPs. **p < 0.01 vs. Control.

**Figure 7.** Effect of nanopolystyrene particle (NP) exposure on expression of virulence genes in A. johnsonii AC15. (A) Survival related genes. (B) Biofilm formation related genes. (C) Serum resistance related genes. **p < 0.01 vs. Control.
3.9. Effect of NP Exposure on Expression of Virulence Genes in A. johnsonii AC15 in NHS

We further used these 17 virulence genes to investigate the effect of NP exposure on survival, biofilm formation, and serum resistance of A. johnsonii AC15 after the addition of NHS. In the NHS, expressions of survival-related virulence genes (zigA, basD, and zur) were upregulated by exposure to 1000 µg/L NPs, and the zigA expression was also upregulated by exposure to 10 µg/L NPs (Figure S3A). After the NHS addition, expressions of biofilm formation-related virulence genes (ompA, bap, adeF, adeG, csuA/B, csuC, csuD, csuE, and hlyD) were upregulated by exposure to 1000 µg/L NPs, and the bap, adeG, csuC, csuD, and csuE expressions were also upregulated by exposure to 10 µg/L NPs (Figure S3B). In the NHS, expressions of serum resistance-related genes (lpxC, lpxL, and pbpG) were upregulated by exposure to 10 and 1000 µg/L NPs (Figure S3C).

During the past several years, more and more attention has been paid to the potential risks of nanoplastic in the environment. Nanoplastics can be accumulated in environmental animals and may affect multiple organs of organisms through the circulatory system [29]. Exposure to nanoplastics can result in several aspects of toxicity, such as oxidative stress, neurotoxicity, reproductive toxicity, inflammatory response, and metabolic disorders [30–34]. The transgenerational toxicity on organisms can be further detected after nanoplastics exposure [35–38]. Nevertheless, compared with the toxicity of nanoplastics on environmental animals or plants, there are few reports on the effects of nanoplastics on microorganisms. Some reports have suggested the role of microplastics as vector of environmental microorganisms [16,17]. In this study, based on SEM analysis, we observed the aggregation of a large number of NPs on the surface of A. johnsonii AC15 (Figure 2), which suggested that the A. johnsonii AC15 acted as the vector of nanoplastics particles. This observation may be very different from the interaction between microplastics and microorganisms.

Previous studies have suggested that nanoplastics at high doses (such as those in the range of mg/L) could cause harmful effects on the physiological processes of bacteria. For example, exposure to 50 nm NPs (200 mg/L) inhibited the anaerobic digestion of sludge and affected the growth [39]. Similarly, the growth of marine bacterium Halomonas alkaliphile was inhibited and the ecological function was interrupted after microplastic exposure [20]. Additionally, the positively charged NPs could further cause lethal effects on yeast cells [40].

In this study, we further examined the effect of NPs at low doses (mainly in the range of µg/L) on bacteria. According to the growth curve and CFU analysis, 100–1000 µg/L NPs could promote the growth of A. johnsonii AC15 (Figure 3). The effect of NPs in the range of µg/L on bacteria was very different from that of high doses. That is, exposure to NPs in the range of µg/L may potentially enhance the risk of bacterial pathogen on environmental organisms by increasing bacterial growth. Therefore, more attention should be paid to the possible adverse effects of long-term and low-dose exposure to nanoplastics on bacterial pathogens in the environment.

To determine why a large number of NPs were accumulated on the surface of bacteria, the turbidity of NP in the supernatant of bacteria during growth and non-growth processes were analyzed. NPs caused the decline in turbidity of bacterial solution during the growth (Figure 4C). Different from this, after the NPs addition, the turbidity of bacterial solution during non-growth process remained unchanged (Figure S2C). These observations suggested that the bacteria were more likely to adsorb NPs during the growth process. The zeta potential of NP in LB medium was $-9.5 \pm 0.56$ mV. Previous study has shown that positively charged polystyrene nanoplastics (such as PS-NH$_2$) could efficiently transport across cell membranes, while negatively charged PS (such as PS-COOH) and neutral PS had little or no effect in transport [41]. In this study, our data implied that the accumulation of NPs on surface of bacteria may be not due to electrostatic interactions.

The potential adverse effect of NPs in the range of µg/L on bacterial pathogens was also reflected by the increase in biofilm formation of A. johnsonii AC15. Biofilm is an important virulence factor of Acinetobacter infection, drug resistance, and escape from host immune response [41]. After formation of Acinetobacter biofilm, its ability to resist adverse factors, such as dryness and lack of nutrition, is significantly enhanced, and its survival
ability and spread infection with hosts are enhanced [42]. Some studies have shown that microplastics can provide bacteria with a new adhesion matrix and colonize to form a biofilm [43]. Unlike microplastics, nanoplastics are smaller in size and do not allow bacteria to colonize them. However, based on our observations, nanoplastics could be adsorbed on the surface of bacteria (Figure 2). Meanwhile, both crystal violet staining and SEM analysis have indicated that the amount of biofilm formation of A. johnsonii AC15 was increased significantly by exposure to 100–1000 µg/L NPs (Figure 5). Therefore, exposure to NPs at low doses may further enhance the risk of bacterial pathogen by increasing biofilm formation.

Some studies have suggested that the ability to resist human serum killing is main survival method of Acinetobacter in the host [44,45]. According to serum resistance analysis, we observed that exposure to 1–1000 µg/L NPs could even significantly increase CFU and survival of A. johnsonii AC15 in the NHS (Figure 6). Therefore, besides behavior and effect on Acinetobacter growth in the environment, NPs also affected serum-resistant property of A. johnsonii AC15. In addition, the correlation between high biofilm formation activity and high resistance to NHS has been well demonstrated for bacterial pathogens [46]. That is, the increase in biofilm formation caused by NPs might contribute to the enhancement in serum-resistant property of A. johnsonii AC15. The 1 µg/L is a predicted environmental concentration for NPs [47]. Our data further implied the exposure risk of NPs at the predicted environmental concentration for environmental animals by affecting serum-resistant property of bacterial pathogens.

The observed effects of NP exposure on Acinetobacter survival, biofilm formation, and serum resistance were associated with the alteration in expressions of related virulence genes. Acinetobacter survival and proliferation in the environment and host is the premise of Acinetobacter pathogenesis. Along with the increase in survival of A. johnsonii AC15, expressions of 3 survival-related virulence genes (zigA, basD, and zur) were increased by 1000 µg/L NP (Figure 7A). Similarly, in the NHS, the expressions of these 3 survival-related virulence genes were increased by 1000 µg/L NP (Figure S3A). More importantly, the zigA expression was also increased by 10 µg/L NP after NHS addition (Figure S3A), which suggested that the addition of NHS may enhance the effect of NPs to increase the Acinetobacter survival. zigA gene governs the zinc uptake system [48]. basD gene controls the iron uptake system [49]. Besides the involvement in the absorption of iron under human host iron deficiency conditions, the product of basD is necessary for Acinetobacter to survive and cause apoptosis of human alveolar epithelial cells [50]. zur gene encodes a transcriptional regulator required for control of oxidative stress and zinc metabolism [51].

Biofilm formation plays an important role in Acinetobacter adhesion, infection, drug resistance gene transmission, drug resistance, and escape from host immune response [52]. Accompanied with the increase in biofilm formation, expressions of 5 biofilm formation-related virulence genes (ompA, bap, adeG, csuC, and csuD) were also increased by 1000 µg/L NPs (Figure 7B). Different from this, after the NHS addition, expressions of all the examined biofilm formation-related virulence genes were increased by 1000 µg/L NPs (Figure S3B). In addition, 5 of them could be further increased by 10 µg/L NPs (Figure S3B). These observations implied that, under the NHS addition condition, the role of NP exposure in increasing the Acinetobacter biofilm formation was also enhanced. ompA is essential for adhesion to epithelial cells [53]. Bap contributes to biofilm formation and adhesion to eukaryotic host cells [54]. The biofilm formation of Acinetobacter is related to the overexpression of AdeFGH efflux pump including both adeF and adeG [55]. Csu fimbriae (csuA/B, csuC, csuD, and csuE) play a role in the initial steps of biofilm formation by allowing bacterial cells to adhere to abiotic surfaces and initiating the formation of micro colonies before the biofilm structure is fully developed [56]. hlyD is involved in the protein secretion during biofilm formation [56].

Moreover, after the exposure to 1000 µg/L NP, we observed both the serum resistance and alteration in expressions of 3 serum resistance-related virulence genes (lpxC, lpxL, and pbg) (Figures 6 and 7C). In contrast, after the NHS addition, expressions of these
3-serum resistance-related virulence genes were increased by 10 and 1000 µg/L NPs (Figure S3C). These observations suggested that the responses of serum resistance-related virulence genes to NPs were enhanced under the NHS addition condition. Among serum resistance-related genes, lpxC and lpxL are lipopolysaccharide (LPS) related genes, and Acinetobacter LPS have the function of evading host immune responses and triggering host inflammatory responses [57]. Inhibition of LpxC protects mice from resistant Acinetobacter baumannii by modulating inflammation and enhancing phagocytosis [58]. pbpG encodes putative low-molecular-mass penicillin-binding protein 7/8 (PBP-7/8) and contributes to the Acinetobacter survival [59].

4. Conclusions

Together, we examined the effect of nanoplastic exposure in the range of µg/L on A. johnsonii AC15. The obvious aggregation of NPs on the surface of A. johnsonii AC15 was observed, suggesting the role of A. johnsonii AC15 as the vector of NPs. Meanwhile, after exposure to 100–1000 µg/L NPs, we observed the increased growth and CFU of A. johnsonii AC15, the enhancement in the amount of biofilm formation of A. johnsonii AC15, and the enhanced serum resistance of A. johnsonii AC15. The observed effects of NP exposure on survival, biofilm formation, and serum resistance of A. johnsonii AC15 were associated with the alterations in expressions of survival-related (zigA, basD, and zur), 5 biofilm formation-related (ompA, bap, adeG, csuC, and csuD), and 3 serum resistance-related virulence genes (lpxC, lpxL, and pbpG). In addition, the responses of survival, biofilm formation, and serum resistance-related virulence genes to NPs were enhanced under the NHS addition condition. Therefore, our results demonstrated the potential of nanoplastics in the range of µg/L in enhancing the virulence of bacterial pathogens by increasing their survival, biofilm formation, and serum resistance in the environment. Further investigation on the interaction between NPs in the range of µg/L and certain pathogens in the real environment is needed to be further carried out in the future.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nano12234222/s1, Figure S1: Systematic evolutionary tree including AC15. The form of labeled tree was used. After the sequencing, the data was compared with the published sequences in GeneBank. Using BLAST in NCBI website, the systematic evolutionary tree was constructed. In this evolutionary tree, AC15 was identified as Acinetobacter Johnson; Figure S2: Adsorption curve of NPs under the condition of bacterial non-growth. (A) AC15 growth curve after exposure to 1000 µg/L NP. (B) Effect of 1000 µg/L NP exposure on AC15 CFU at different times. (C) Turbidity analysis of bacterial solutions after cultivation at 4 °C for 12 h. (D) SEM analysis to show NPs accumulation on surface of AC15 under the condition of 4 °C cultivation; Figure S3: Effect of exposure to NPs on expression of virulence genes in AC15 in NHS. (A) Survival related genes. (B) Biofilm formation related genes. (C) Serum resistance related genes. ** p < 0.01 vs. Control; Table S1: Primer information for qRT-PCR.

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