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ARTICLE

Exposure to Al_2O_3 nanoparticles facilitates conjugative transfer of antibiotic resistance genes from *Escherichia coli* to *Streptomyces*

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ABSTRACT

The spread of antibiotic resistance genes (ARGs) has become a global environmental issue; it has been found that nanoparticles (NPs) can promote the transfer of ARGs between bacteria. However, it remains unclear whether NPs can affect this kind of conjugation in *Streptomyces*, which mainly conjugate with other bacteria via spores. In the present study, we demonstrated that Al_2O_3 NPs significantly promote the conjugative transfer of ARGs from *Escherichia coli* (*E. coli*) ET12567 to *Streptomyces coelicolor* (*S. coelicolor*) M145 without the use of heat shock method. The number of transconjugants induced by Al_2O_3 particles was associated with the size and concentration of Al_2O_3 particles, exposure time, and the ratio of *E. coli* and spores. When nanoparticle size was 30 nm at a concentration of 10 mg/L, the conjugation efficiency reached a peak value of 182 cfu/10^8 spores, which was more than 60-fold higher than that of the control. Compared with nanomaterials, bulk particles exhibited no significant effect on conjugation efficiency. We also explored the mechanisms by which NPs promote conjugative transfer. After the addition of NPs, the intracellular ROS content increased and the expression of the classical porin gene *ompC* was stimulated. In addition, ROS enhanced the mRNA expression levels of conjugative genes by inhibiting global regulation genes. Meanwhile, expression of the conjugation-related gene *intA* was also stimulated, ultimately increasing the number of transconjugants. Our results indicated that Al_2O_3 NPs significantly promoted the conjugative transfer of ARGs from bacteria to spores and aggravated the diffusion of resistance genes in the environment.

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ARGs; Al2O3 NPs; *Streptomyces*; spore; conjugative transfer

Introduction

Subsequent to the discovery of penicillin in 1929, the application of antibiotics has become increasingly extensive. Such widespread use has contributed to the rapidly emerging problem of antibiotic resistance, causing difficulties in the treatment of bacterial diseases and thereby threatening human health (Pruden et al. 2006). Antibiotic resistant bacteria and antibiotic resistance genes (ARGs), brought about by the widespread production, use, and misuse of antibiotics (Baquero, Martinez, and Canton 2008, Calfee 2012, Pruden et al. 2006, Udwadia et al. 2012), will continue to pose serious threats to public health in the future (Ashbolt et al. 2013). ARGs are disseminated among microbes through horizontal gene transfer. Genetic materials can transfer between bacteria of the same species as well as between different species via transduction, transformation, and conjugation (Dodd 2012, Massoudieh et al. 2007). Conjugation occurs when direct cell-cell contact transpires, though there is no need for the cells to cling together (Sorensen et al. 2005, Sota and Top 2008). The accumulation of ARGs on plasmids is the basis of the development and diffusion of resistance genes (Qiu et al. 2015).

Previous studies have shown that many factors in the environment affect bacterial antibiotic resistance, such as metals (Baker-Austin et al. 2006). Metals affect resistance mainly by means of co-selection process (include co-resistance or cross-resistance). While for Nanoparticles (NPs), the mechanism is different. NPs are newly classified pollutants with unique physical and chemical properties, and the impacts of NPs on human health

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Supplemental data for this article can be accessed here.

and the environment are receiving increasing attention. The cell membrane is a selective, semi-permeable membrane that constitutes an important barrier for the horizontal transfer of ARGs between microbes (Thomas and Nielsen 2005, Alekshun and Levy 2007). Nanoparticles can cause physical damage to bacterial cell walls and membranes, generating intracellular reactive oxygen species (ROS) (Tan, Fu, and Seno 2010). These ROS are apt to maintain such damage and alter membrane permeability, both of which influence the uptake of plasmids encoding antibiotic resistance (Choi and Hu 2008, Jiang, Mashayekhi, and Xing 2009, Sadiq et al. 2009, Simon-Deckers et al. 2009).

As one of the most commonly used nanoparticles, aluminum oxide (Al_2O_3) NPs are widely used in water treatment, powder coating, insulator layers, and fluorescent lamp material (Martínez Flores, Negrete, and Torres Villaseñor 2003, Afkhami, Saber-Tehrani, and Bagheri 2010, Bhatnagar, Kumar, and Sillanpaa 2010, Hernández et al. 2014). The inevitable release of Al_2O_3 NPs into the environment requires that we seek a basic understanding of their interactions with biological systems, as such interactions may pose potential risks to various ecosystems (Baun et al. 2008, Eduok et al. 2013). Al_2O_3 NPs have been shown to promote horizontal transfer of multi-resistance genes mediated by plasmids across two types of bacteria by inducing intracellular ROS to damage cell membranes and improve the permeability of bacteria (Qiu et al. 2012, Ding et al. 2016). However, certain bacterial species such as *Streptomyces* conjugate with other species via spores instead of mycelium to uptake plasmids. Prior to this study, there have been no reports that demonstrate whether nanomaterials influence this kind of conjugation.

The actinobacterium *Streptomyces* produces two-thirds of the clinically used antibiotics of natural origin. The model strain *Streptomyces coelicolor* M145 can generate various industrially important secondary metabolites (Borodina et al. 2008), hence *Streptomyces* induces important environmental and economic implications. The chromosomes of *Streptomyces* are among the largest bacterial chromosomes, with sizes in the range of 8 Mb to over 10 Mb (Kirby and Chen 2011). In contrast to most bacteria that divide by binary fission, *Streptomyces* have complex life cycles that resemble the growth of eukaryotic filamentous fungi (Flärdh and Buttner 2009, Flärdh 2010). It is noteworthy that under laboratory conditions, stimulation such as heat shock is necessary to promote the transfer of plasmids into *Streptomyces* spores (Moraga et al. 2017). Such stimulation promotes the germination of spores and increases cell membrane permeability, thereby enhancing the propensity to ingest foreign plasmids. Given the stringent requirements of standard experimental conditions, spontaneous initiation of the transfer process is difficult in natural environments. As NPs can also change the permeability of cell membranes, it is thought that exposure to Al_2O_3 NPs facilitates the ability of cells or spores to uptake ARGs through plasmids even without heat shock, allowing the process to occur easily in the natural environment.

Engineered strains such as methylation-deficient *E.coli* ET12567 carrying the non-transmissible pUZ8002 are often used in the study of ARG transfer. The plasmid pUZ8002 is not self-transmissible but instead supplies a mobilization function for other plasmids. After modifying the mobilization system, conjugation of plasmids from *E.coli* ET12567 to *Streptomyces* also worked for non-replicative plasmids such as pSET152 via site-specific integration into the chromosome (Bierman et al. 1992). In a previous study, we demonstrated that the size and concentration of NPs affects the ROS level, thus impacting the permeability of cells (Liu et al. 2018). Consequently, the present research was conducted to confirm whether Al_2O_3 NPs could promote the spread of ARGs from *E. coli* to *Streptomyces* in the environment. We also investigated changes in gene expression related to conjugative transfer in an attempt to understand the underlying mechanisms of this process.

Methods

Characterization of Al_2O_3 particle

Al_2O_3 NPs 30 nm and 80 nm in size or bulk particles were purchased from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). Morphologies and sizes of Al_2O_3 particles were evaluated using a HT7700 transmission electron microscope (TEM; Hitachi, Beijing, China). Suspensions of NPs and bulk particles (BPs) were prepared by adding dry particles

to 2 × YT (yeast extract: 10 g/L; tryptone: 20 g/L and NaCl: 20 g/L) in the following serial dilutions: 1000, 100, 10, and 1 mg/L). Then all suspensions were sonicated (100 W, 40 kHz) for 30 min to facilitate dispersion. A Malvern Zetasizer Nano ZS^{TM} (Malvern Panalytical, Malvern, UK) was used to measure the hydrodynamic diameter and electrophoretic mobility of the particles. Data were collected in triplicate at 25 °C.

Conjugative transfer between Escherichia coli ET12567 and Streptomyces coelicolor M145

S. coelicolor M145 and ET12567/pUZ8002 carrying chloramphenicol and kanamycin resistance (Cm^R, Km^R) were purchased from China General Microbiological Culture Collection Center (CGMCC; Beijing, China). The Cm^R gene was located on genomic DNA and the Km^R gene was located on plasmid pUZ8002. None of these resistance genes participated in the process of conjugation. To obtain adequate spore quantities, M145 was cultivated on mannitol soya (MS) agar plates (soya flour: 20 g/L; mannitol: 20 g/L and agar: 20 g/L) for 5–7 days at 30 °C and harvested in 20% (v/v) glycerol, then stored at -80 °C (Sigle et al. 2016). Plasmid pSET152 carrying the apramycin resistance gene (Apr^R) was transformed into ET12567/pUZ8002. ET12567 (Km^R,Cm^R, Apr^R) was cultured in Luria broth (LB) medium containing the following antibiotics: kanamycin, 50 mg/L; chloramphenicol, 25 mg/L; and apramycin, 50 mg/L) at 37 °C with shaking at 200 rpm over-night, then 500 μL of culture fluid was added to fresh LB medium with the concentrations of antibiotics listed above. When ET12567 grew to OD_{600} values of 0.4–0.5, it was washed twice with 0.9% NaCl and resuspended in 0.9% NaCl. Then 5 mL TES {N-[tris(hydroxymethyl)-methyl]2-aminopropanesulfonic acid} (0.05 M, pH 8.0) was added to 1 mL of spores (10^8 cfu/mL) and 1 mL ET12567 to activated cells. Five milliliters of 2× YT medium with the serial dilutions of Al_2O_3 described above or pure 2 × YT medium was added to the mixture system then incubated on an orbital shaker at 150 rpm at 30 °C for 2.5 h. After washed three times with 0.9% NaCl, cells were resuspended in 0.9% NaCl and spread on solid MS plates (Sigle et al. 2016). In order to exclude false positives, a control group containing the same volume of spores and ET12567 was mixed and spread on MS plates without exposure to Al_2O_3 particles. After incubation for 11–16 h at 30 °C, these plates were overlaid with 1 mL of water containing 25 mg/L nalidixic acid and 50 mg/L apramycin and incubated at 30 °C for 2 d. Nalidixic acid inhibits the growth of gram-negative bacteria and prevents the contamination of *E. coli*; also, given that pSET152 carries the apramycin resistance gene, apramycin was used to screen out the transconjugants. Three replicates per treatment were conducted and the number of colonies on each plate was calculated, with the average value taken as the number of transconjugants.

To verify whether pSET152 had been integrated into the M145 chromosome, one pair of primers: AMF and AMR (Table S1) were designed to amplify a ~700 bp fragment in the *aac*(3) VI gene. Positive transconjugants acquired from conjugative transfer and negative controls were inoculated in YBP medium (yeast extract: 2 g/L; beef extract powder: 2 g/L; peptone: 4 g/L; NaCl: 15 g/L; glucose: 10 g/L and $MgCl_2$:1 g/L) and shaken at 150 rpm for 24 h at 30 °C. Genomic DNA was extracted from mycelium using a TIANamp Bacteria DNA Kit (TIANGEN Biotech Co., Ltd., Beijing, China) according to the manufacturer's protocol. PCR conditions were as follows: initial denaturation at 96 °C for 2.5 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 40 s, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min.

Bacterial cytotoxicity assessed by high-throughput screening

Bacterial viability was assessed using a Live/Dead^{TM} *Bac*Light^{TM} Bacterial Viability Kit (L-13152; Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA) according to the method described by Liu et al. (2018). Cells were stained with two kinds of fluorescent nucleic acid stain (SYTO 9 and PI) to distinguish the integrity of cell membranes (Pelletier et al. 2010, Li et al. 2011, Binh et al. 2014). Specifically, 1 mL of spores (10^8 cfu/mL) was added to 5 mL TES (0.05 M, pH 8.0). Thereafter, 5 mL 2× YT medium with the serial dilutions of Al_2O_3 described above or pure medium were added and incubated on an orbital shaker at 150 rpm at 30 °C for 2.5 h. We then followed the assay protocol described in

our previous study (Liu et al. 2018, Tong et al. 2013).

Intracellular reactive oxygen species (ROS) assay

The assessment of intracellular ROS induced by Al_2O_3 particles was performed according to our previous research with some changes (Liu et al. 2018). Our method of exposing spores to nanomaterials was consistent with the assessment bacterial cytotoxicity. After exposure for 2.5 h, 1 mL spores were centrifuged and washed three times with 0.9% NaCl then suspended with 1 mL 10 μmol/L 2′,7′-dichlorofluorescein diacetate (DCFH-DA) and incubated at 30 °C in the dark for 30 min. After washing three times with 0.9% NaCl and resuspended in 1 mL 0.9% NaCl, 100 μL of solution was added to each well of the 96-well microplates per treatment with three replicates. The fluorescence intensity was measured using a Synergy™ H4 Hybrid Microplate Reader (BioTek, Winooski, VT, USA) (excitation 485 nm and emission 530 nm). The relative ROS level was represented as the fluorescence intensity ratio of the exposure group to the control group.

Spores were cultured in different kinds of media: one pure 2× YT medium as the control and others with 10 mg/L and 1000 mg/L Al_2O_3 NPs (30 nm or 80 nm) and shaking at 150 rpm at 30 °C. To clarify the role of ROS in conjugative transfer, the ROS eliminating agent N-acetylcysteine (NAC) (Ding et al. 2016) was added to the interaction system. All treatments were conducted in triplicate and the concentrations of ROS were detected.

Permeability assay

Permeability of spores after exposure to Al_2O_3 NPs was assessed by fluorescein diacetate (FDA), which is membrane permeable and non-fluorescent. When FDA entered cells, it was hydrolyzed by intracellular esterases, resulting in the production of fluorescein which was not membrane permeable. The degree of cell permeability was indicated by the amount of fluorescein detected. This method of exposing spores to NPs was consistent with that of bacterial cytotoxicity assessment. After exposure for 2.5 h, spores were centrifuged and washed three times with 0.9% NaCl, then suspended with 1 mL of 10 μmol/L FDA and incubated at 30 °C in the dark for 30 min. After washing three times with 0.9% NaCl and resuspended in 1 mL 0.9% NaCl, 100 μL of solution was added to each well of the 96-well microplates per treatment with three replicates. The fluorescence intensity was measured using the Biotek microplate reader (excitation 485 nm and emission 521 nm). The relative permeability was represented as the fluorescence intensity ratio of the exposure group to the control group.

Detection of gene expression related to conjugative transfer

After exposure to NPs, mixed bacteria were centrifuged at 4000 rpm for 10 min and washed three times with 0.9% NaCl. RNA isolation was performed using TRIzol™ (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Then RNA was transcribed to cDNA with M5 Super qPCR RT Kit (Mei5Bio, China). Polymerase chain reaction (PCR) assays were used to target seven genes, including three global regulatory genes (*korA*, *korB* and *trbA*), two conjugative genes *(trbB* and *trfA*) of plasmid pUZ8002, and an integrated gene (*intA*) of pSET152, as well as one outer membrane protein gene (*ompC*) from the genome of *E.coli* (Wang et al. 2015). Sequences of the primers used are listed in Table S1. Quantitative PCR assays were performed on a Roche LightCycler® 480 thermal cycler (Roche, Basel, Switzerland) with Top Green qPCR SuperMix (TransGen Biotech, Ltd., Beijing, China) to detect gene expression levels. Reactions were carried out under the following conditions: 95 °C for 1 min followed by 40 cycles of 95 °C for 5 s, 60 °C for 20 s and 72 °C for 30 s. PCR analysis was performed in triplicate and 16 s rDNA was used as an internal control for M145.

Results

Characterization of Al_2O_3 particles

Based on TEM images of Al_2O_3 particles of various sizes (Figure S1), their diameters were consistent with those specified by the manufacturer of the NPs (Table S2). The size distributions in YBP medium were measured by DLS analysis, which showed that the aggregate size of NPs was between 835 ± 23–872 ± 31 nm (Table S2). For BPs, little difference was detected between particle sizes

in powder and particle sizes in solution, indicating that the large particles did not form aggregates in the medium. And with the decrease of particle size, the electrophoretic mobility of particles decreased (Figure S2), which indicated that the migration rate of small particles in electric field was fast.

Confirmation of Al_2O_3 particles upon conjugative transfer of ARGs from E. coli to spores

After treated with different kinds of nano particles, the efficiency of conjugative transfer can be improved more or less, while that of the corresponding BPs was not obvious (Table S3). Just as Qiu et al. (2012) found that it was the nano-effect not chemical properties of NPs caused the efficiency. Compared with other NPs, the number of transconjugants was most after treated with the same concentration of Al_2O_3 NPs. So in the following work, Al_2O_3 NPs were selected to systematically study the effects of nanomaterials on conjugative transfer.

As shown in Figure 1(A), the number of transconjugants was significantly higher (more than 60 fold higher) than that of controls after treatment with Al_2O_3 NPs (30 nm, 10 mg/L) for 2.5 h. To indicate that the pSET152 plasmid had been integrated into *Streptomyces* chromosomes, primers AMF and AMR were designed to amplify a specific DNA fragment on pSET152. When total DNA of the transconjugants was used as the template and subsequently run on gel electrophoresis, a 700 bp band was detected, indicating the presence of the pSET152 fragment. This fragment was not obtained when total DNA of the controls was used as the template (Figure 1(B)), indicating that the plasmid had been integrated into the chromosomes of *Streptomyces* and that M145 had obtained the ARGs.

Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) was used to determine the content of Al^{3+} in Al_2O_3 NPs of different sizes and concentrations. Results are shown in Table S4. The concentrations of Al^{3+} in all treatments were low and few transconjugants formed after spores were treated with these concentrations of Al^{3+} without heat shock; ultimately, the number of transconjugants present was not significantly different from that of the controls ($p > 0.05$). This indicated that Al^{3+} was not the main reason for the improvement in conjugative transfer efficiency, with the following experiments verifying whether nanoparticles were the cause of the increased efficiency.

Effect of various factors on conjugative transfer and viability of spores

In treatments with different sizes of Al_2O_3 NPs, the number of transconjugants was significantly higher than that of the controls (Figure 2(A)); the highest number was produced by treatment with 30 nm Al_2O_3 NPs at a concentration of 10 mg/L, yielding a 60 fold higher number than that of the controls. The number of transconjugants decreased with increased NP size in treatments at lower concentrations (<10 mg/L). Fewer transconjugants were observed when the concentration of Al_2O_3 rose to 100 mg/L and 1000 mg/L. We also found that conjugative transfer was enhanced with increased particle size at higher concentrations (>100 mg/L). With the prolongation of exposure time (30 nm Al_2O_3 NPs, 10 mg/L), the number of transconjugants increased first to the maximum value of 182 cfu/10^8 spores with an exposure time of 4 h and then decreased when the time increased to 8 h; the number of transconjugants in nano groups were significantly higher than that of controls at each time point (Figure 2(B)). When the ratio of bacteria to spores was 5:1, the number of transconjugants obtained was the highest, with fewer transconjugants for both higher and lower ratios (Figure 2(C)).

To verify whether Al_2O_3 particles were toxic to cells, the viability of spores and *E.coli* was detected after exposure to different sizes and concentrations of Al_2O_3 particles after 2.5 h. Results (Figure 2(D)) showed that when the concentration of nanoparticles was 10 mg/L, there were no significant differences ($p > 0.05$) in viability of spores exposed to different particle sizes of 30 nm and 80 nm and to BPs. When the concentration was higher than 100 mg/L, there were significant differences ($p < 0.05$) in viability when exposed to different sizes of Al_2O_3 particles. For *E.coli*, fatality trend rate was similar to that of spores, but the survival rate was lower with these treatments.

Intracellular ROS levels and permeability changes after treatment with Al_2O_3 NPs

Excessive ROS production induced by nanoparticles causes oxidative stress and bacterial inactivation

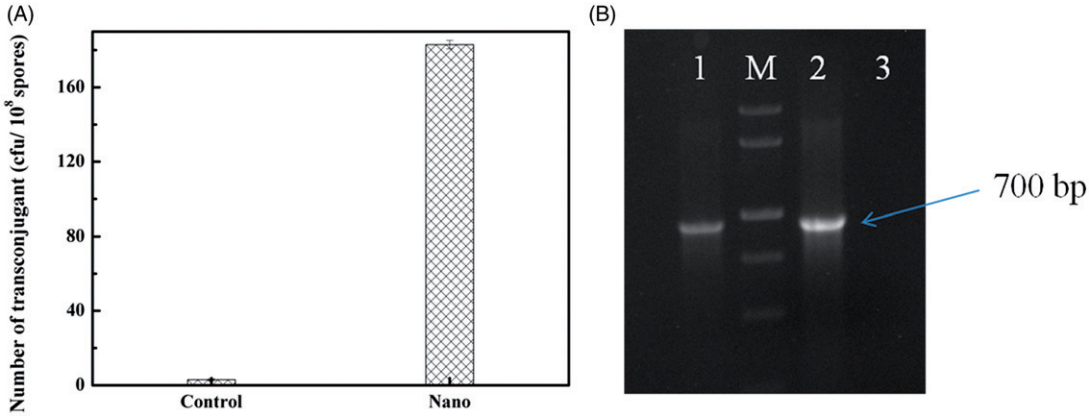

Figure 1. Effect of Al_2O_3 NPs on the formation of transconjugants. (A) The number of transconjugants after spores were treated with 80 nm and 10 mg/L Al_2O_3 NPs for 2.5 h, control means without any nanomaterial treatment. The initial bacteria and spores concentration was 10^8 cfu/mL. (B) PCR identification of transconjugants.1: PCR production of transconjugants, 2: PCR production of plasmid pSET152 (as positive control), 3: PCR production of S. coelicolor M145 (as negative control). 'M' means marker, from 100 bp to 1000 bp.

(Li et al. 2016). In this study, it was found that ROS level was directly proportional to concentrations of Al_2O_3 particles in both spore and in *E.coli* (Figure 3(A,B)). When the concentration was higher than 10 mg/L, there were significant differences ($p < 0.05$) in levels of intracellular ROS upon exposure to particles of 30 nm and 80 nm or to BPs which were inversely proportional to the size of the particles. When the concentration of Al_2O_3 NPs reached 1000 mg/L, the ROS level was ~7-fold higher than that of the controls in spores, while in *E. coli* it was ~5-fold higher than that of the controls. For BPs, the ROS level was much lower, at ~1.5-fold higher than that of the controls even when exposed to a concentration of 1000 mg/L particles.

It was found that Al_2O_3 NPs can damage cell membranes and improve the permeability of bacteria by inducing intracellular ROS; we, therefore, assessed the permeability of cells after treatment with different concentrations and different sizes of Al_2O_3 particles at the same time. Rapidly increase in permeability was found in 10 mg/L Al_2O_3 NPs. This trend was similar in two kinds of cells (Figure 3(C,D)).

ROS elimination result

To verify the roles of ROS in the process of conjugation, N-acetylcysteine (NAC)- ROS eliminating agent was added to the medium with 10 mg/L and 1000 mg/L Al_2O_3 NPs (30 nm or 80 nm), as well as to the pure 2× YT medium control. After the addition of NAC, the effects of ROS were substantially eliminated and there was no significant difference in permeability with that of the control such that the number of transconjugants was reduced to that of the control level (Figure 4(A,B,D)) with 10 mg/L Al_2O_3 NPs. When the concentration of NPs reached 1000 mg/L, after the addition of NAC, the number of spores surviving in this treatment group was more than that without NAC, but less than that of the control (Figure 4(C)). And there were less transconjugants than that without NAC, but more that treated with 10 mg/L NPs (Figure 4(D)).

Effects of Al_2O_3 NPs on cell membranes and conjugative transfer

As the highest number of transconjugants was produced by treatment with 10 mg/L Al_2O_3 NPs, electron microscope methods were used to compare the changes on cell membranes and conjugative transfer after treated with 10 mg/L Al_2O_3 NPs or not.

SEM was used to assess the morphological changes in spores exposed to Al_2O_3 NPs for 2.5 h (Figure 5(A,B)). The controls maintained their ellipsoidal shapes and their outer structures were intact and smooth (Figure 5(A)).Upon treatment with 10 mg/L Al_2O_3 NPs, the morphology of spores did not change significantly (Figure 5(B)).

Representative TEM images showed the process of conjugative transfer (Figure 5(C,D)). For control group, there was no bridge between *E.coli* and spores, even close to each other (Figure 5(C)). After the addition of 10 mg/L Al_2O_3 NPs, a suspected

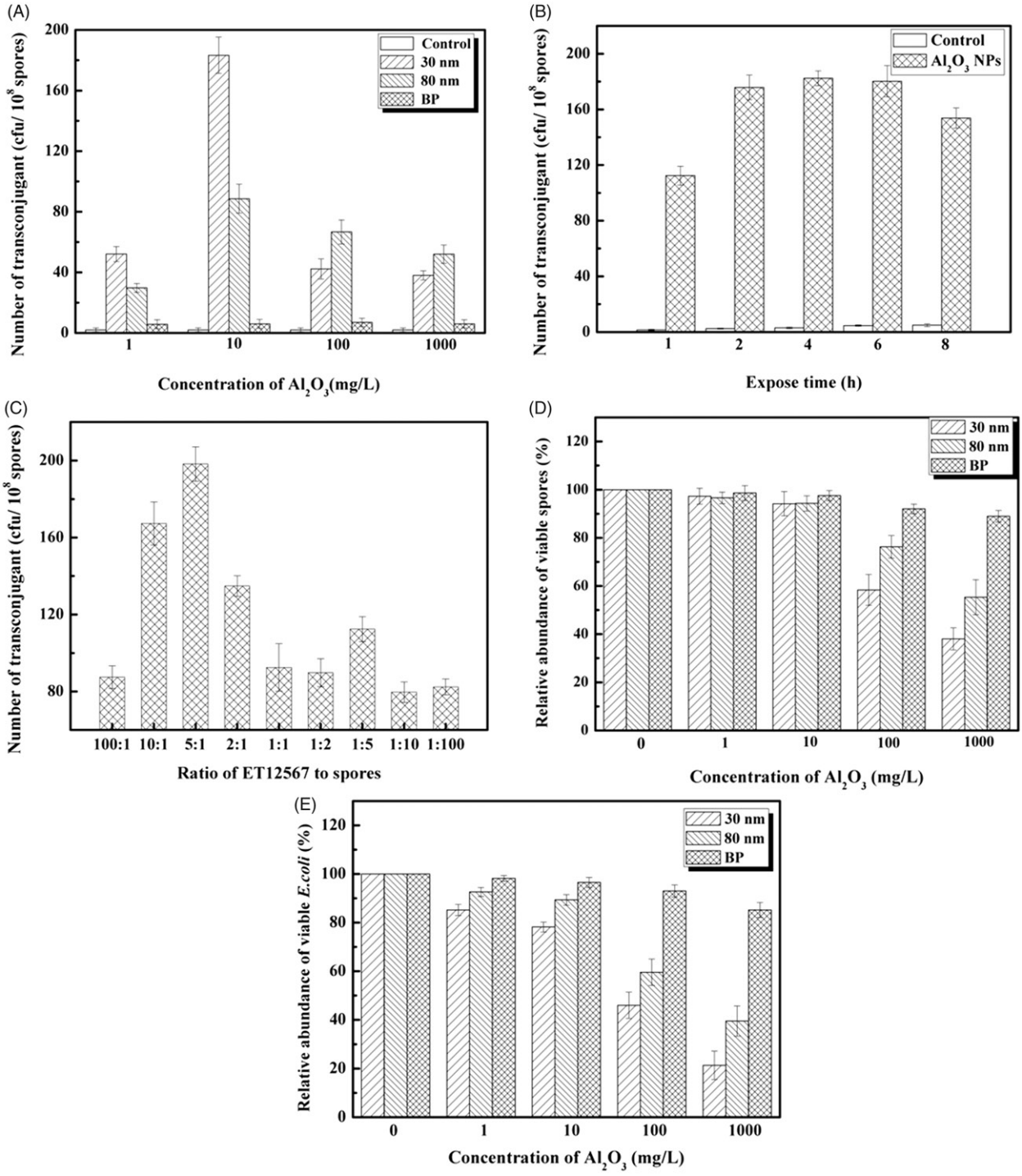

Figure 2. Different factors on the formation of transconjugants induced by Al_2O_3 particles. (A) Effect of different size and concentration of materials. The number of transconjugants after spores treated with different size and different concentration of Al_2O_3 for 2.5 h, and initial bacteria and spores concentration was 10^8 cfu/mL. (B) Effect of contacting time. The number of transconjugants after spores treated with Al_2O_3 NPs (30 nm, 10 mg/L) for different time, and initial bacteria and spores concentration was 10^8 cfu/mL. (C) Effect of different ratio of bacteria to spore. The number of transconjugants after spores treated with Al_2O_3 NPs (30 nm, 10 mg/L) for 2.5 h, and spores concentration was 10^8 cfu/mL, mixed ET12567 with certain rations. (D) The relative abundance of viable spores after treated with different size and different concentration Al_2O_3 particles. (E) The relative abundance of viable ET12567 after treated with different size and different concentration Al_2O_3 particles. Control means without any nanomaterial treatment. Error bar represent standard deviation (n = 3).

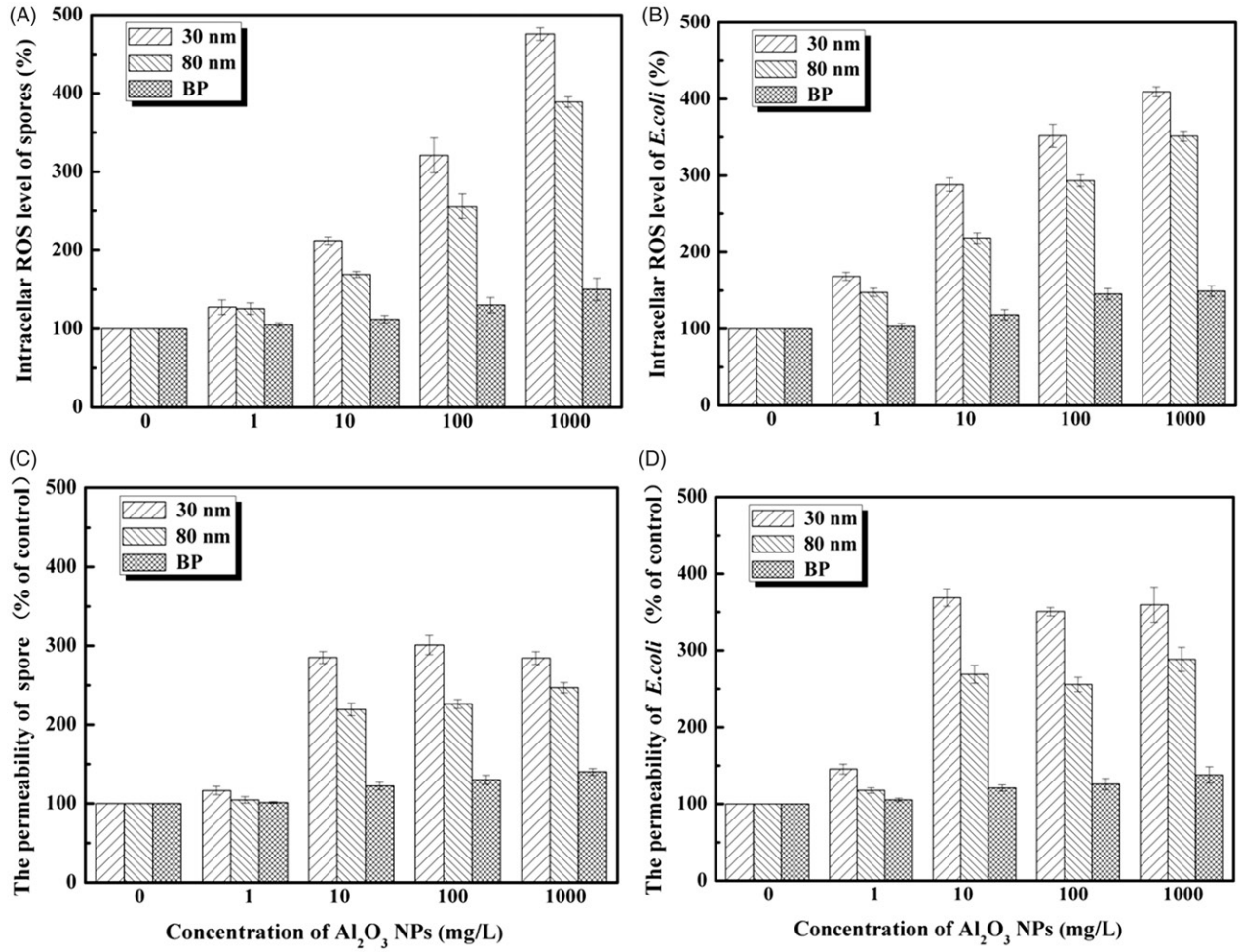

Figure 3. Toxicity of different size and different concentration Al_2O_3 particles to spores of S. coelicolor M145 and E.coli ET12567 in 2× YT medium. (A) The intracellular ROS level of S. coelicolor M145 by different treatments; (B) The intracellular ROS level of E.coli by different treatments; (C) The permeability of S. coelicolor M145 by different treatments; (D) The permeability of E.coli by different treatments. Error bar represent standard deviation (n = 3).

conjugation bridge was created on cell membranes (Figure 5(D)).

Changes of gene expression after exposure to NPs

Seven genes were chosen to verify whether the addition of NPs affects the expression of genes related to conjugative transfer. OmpC porin was known to play important roles in the membrane transport of genetic information between cells and the environment (Ozkanca et al. 2002). Results showed that the expression of *ompC* was increased after exposure to NPs (Figure 6(A)). And the expression levels of three global regulatory genes *korA*, *korB*, and *trbA* were significantly repressed (Figure 6(B–D)) after exposure compared to those of the control. The degree of gene inhibition was directly proportional to the concentration of NPs and was inversely proportional to particle size. Repression of the three global regulatory genes significantly promoted the expression of *trbB* and *trfA* (Figure 6(E,F)). And the expression of *intA* increased after exposure to NPs.

Discussion and conclusion

Many physical or chemical methods such as metals, ultraviolet exposure, ionic liquids, and chlorination promote the transfer of plasmids between bacteria (Baker-Austin et al. 2006, Guo, Yuan, and Yang 2015, Wang, Mao, and Luo 2015). This research explored a potential relationship between nanomaterials and the spread of ARGs. It showed that the presence of NPs enhances the spread of ARGs from *E. coli* to spores. Concentrations of Al_2O_3 NPs between 1 and 1000 mg/L more or less improved

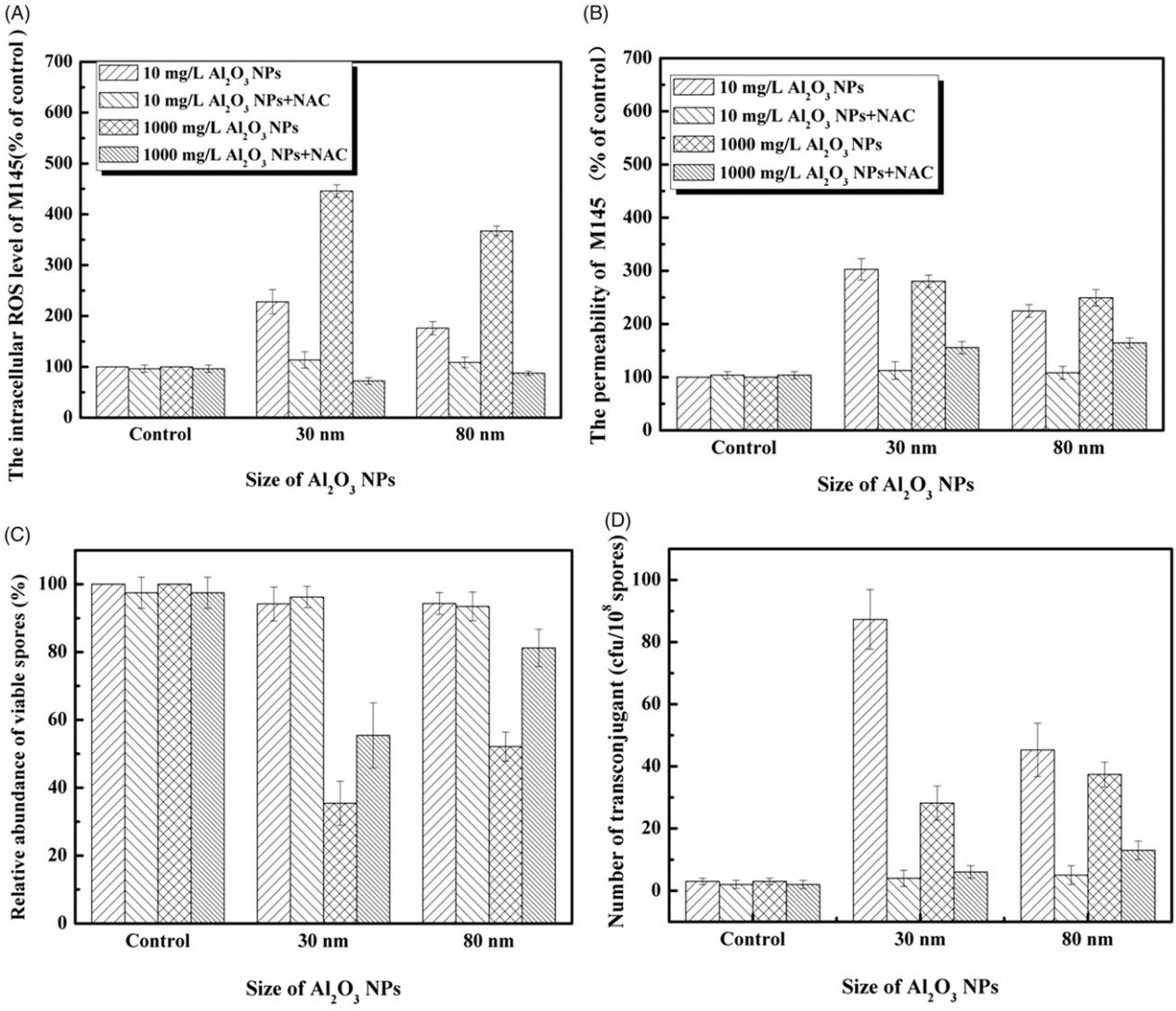

Figure 4. Roles of ROS in Al_2O_3 NPs mediated conjugative transfer, Al_2O_3 NPs used was 30 nm and 80 nm, at the concentration of 10 mg/L and 1000 mg/L. (A) ROS level in spores treated with different size and different concentration of Al_2O_3 NPs and combination of both Al_2O_3 NPs and NAC. ROS production in control cells with NAC treated or not was also shown. The y-axis showed the fold change of ROS in experimental groups over the level of control without NAC, which was set to 100%. (B) Permeability in spores treated with different size and different concentration of Al_2O_3 NPs and combination of both Al_2O_3 NPs and NAC. (C) Survival rate of spores treated with different size and different concentration of Al_2O_3 NPs and combination of both Al_2O_3 NPs and NAC. (D) Number of transconjugants treated with different size and different concentration of Al_2O_3 NPs and combination of both Al_2O_3 NPs and NAC.

the conjugation efficiency. The highest number of transconjugants was obtained after treatment with Al_2O_3 NPs at a concentration of 10 mg/L, even the concentration was 1 mg/L, the number of transconjugants was more than 10 times of control, so the results were environmental relevance.

When the concentration of NPs was less than 10 mg/L, the number of transconjugants was inversely proportional to particle size (Figure 2(A)), while there were no significant differences ($p > 0.05$) in the viability of spores exposed to different particle sizes. This indicated that viability rate was not the cause of the enhanced transconjugation at lower concentrations of nanoparticles. Cells produce a certain amount of ROS after stimulation, causing damage to cell membranes and alterations in membrane permeability. It has been reported that plasmids enter bacteria through conjugation bridges or nanopores created on cell membranes (Tan et al. 2010), which might be the reason that more transconjugants result from treatments with smaller nanoparticles at lower concentrations (Figure 5(D)).

As the concentration of particles became higher, the ROS level increased proportionally, but fewer

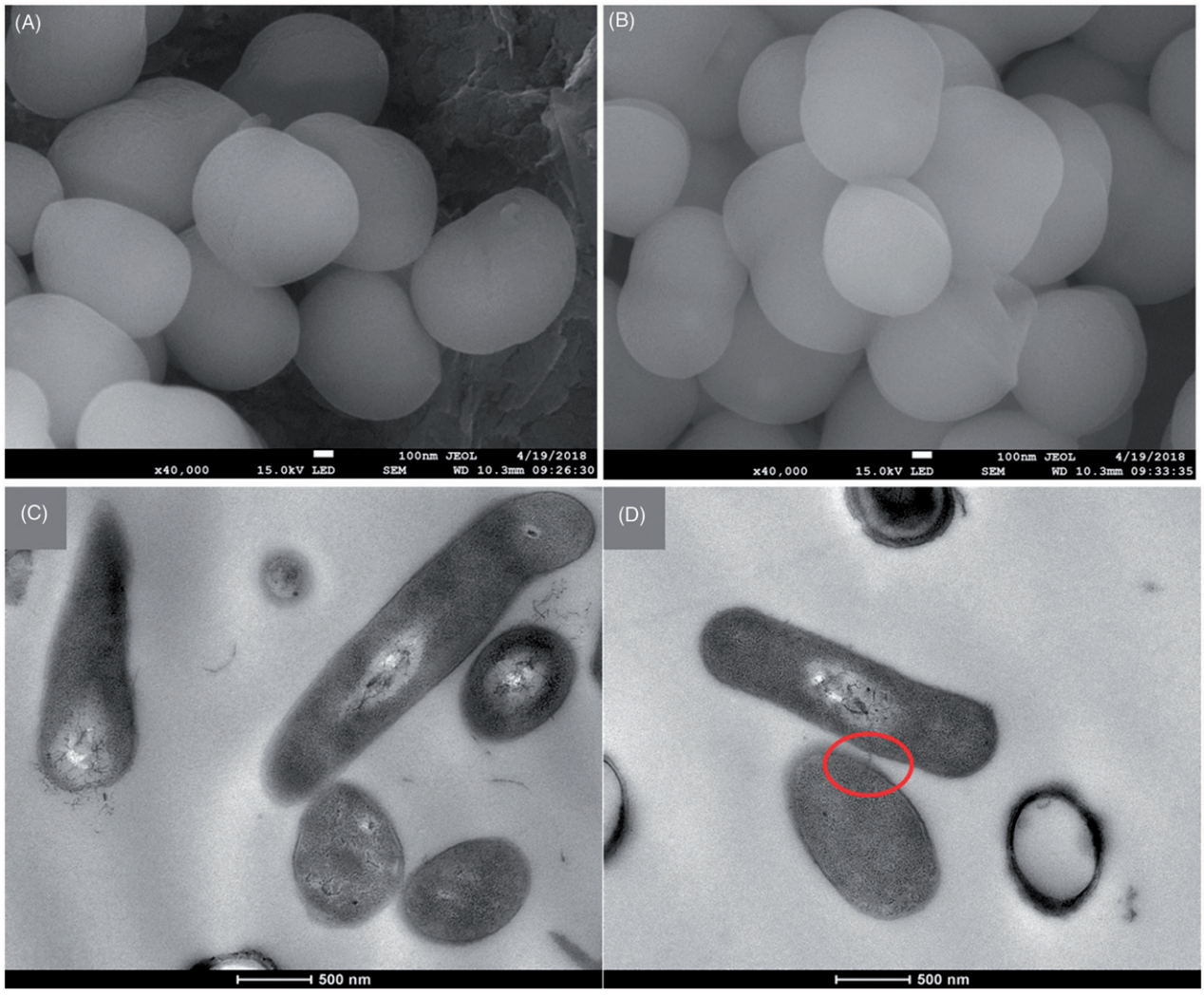

Figure 5. The electron microscopy images of S. coelicolor M145 spores and the process of conjugative transfer by different treatments. A: SEM images of control without any Al_2O_3 particles treatments; B: SEM images of spores were exposed to 10 mg/L Al_2O_3 NPs (30 nm) for 2.5 h; C: TEM images of the process of conjugative transfer without any particles; D: TEM images of the process of conjugative transfer after treated with 10 mg/L Al_2O_3 NPs (The ellipse pointed to the conjugation bridge between E.coli and spore).

transconjugants were found; furthermore, even fewer transconjugants were produced upon treatment with 30 nm particles than with 80 nm. Cells were seriously damaged when the concentration of NPs was too high and some of the cells died or in the least lost membrane function. Lower viability corresponded to fewer transconjugants upon treatment with smaller particles, indicating that viability was responsible for the decrease in conjugative transfer at higher concentrations of Al_2O_3 NPs (>100 mg/L). These results suggested that conjugation bridges and/or nanopores played important roles in transconjugation at lower concentrations of nanomaterials and defunctionalization of membranes caused by high ROS levels was more important at high concentrations of nanoparticles.

To verify the roles of ROS in the process of conjugation, an elimination assay was performed, and it was confirmed that when the concentration of NPs was lower, ROS was responsible for accelerating conjugation. When the concentration of NPs reached 1000 mg/L, after the addition of NAC, the number of spores surviving in this treatment group was more than that without NAC, but less than that of the control. This indicated that there were other factors that caused toxicity to spores besides ROS when the concentration of NPs was higher. In a previously study, it was found that nanoparticles induced ROS and nanoparticles themselves were

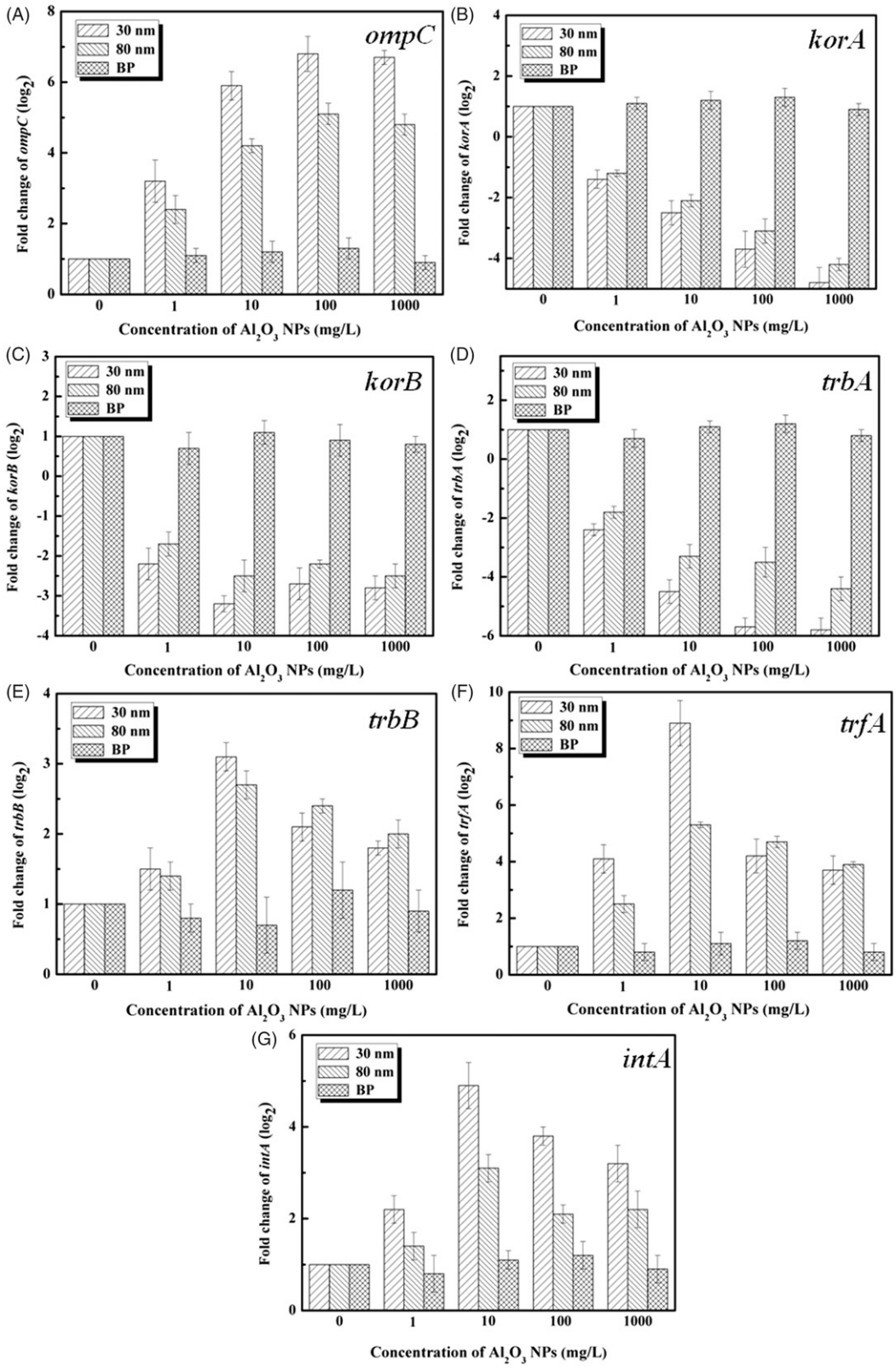

Figure 6. Changes of gene expression after exposed to NPs. The concentration of NPs had a significant effect on the expression levels of outer membrane protein gene (A), the global regulatory genes (B – D), conjugative genes (E – F), and insert gene (G). Error bar represent standard deviation (n = 3).

two main factors that affected the toxicity of NPs to *S. coelicolor* (Liu et al. 2018); it was hypothesized that the physical damage to cells by NPs was another cause of cell death. Nanomaterials could not only destroy cell membranes by colliding, but also adsorb on bacterial surfaces, which blocked the transport channels of cell membranes, reacted with some biomacromolecules on cell membranes, and affected their normal physiological functions (Mosaferi et al. 2016). This indicated that when the concentration of NPs was higher, ROS and nanoparticles together caused the defunctionalization of cell membranes and finally influenced the efficiency of conjugative transfer.

It also found that the addition of NPs could affect the expression of genes related to conjugative transfer. After exposure to NPs, the expression of *ompC* was increased and it contributed to the enhancement of cell membrane permeability. This trend of permeability (Figure (3C,4D)) was consistent with that of ompC, facilitating gene transfer of the plasmid carrying ARGs. *trbB* and *trfA* were critical genes that regulated the formation of conjugative bridges between donor and recipient cells, regulated by three global regulatory genes *korA*, *korB*, and *trbA*. korA and korB represses *trfA* expression, while *trbB* was repressed by korB and trbA. And the repression of the three global regulatory genes after exposure to NPs significantly promoted the expression of *trbB* and *trfA*, which increased the conjugative transfer of ARGs. As *trbB* and *trfA* genes regulate the formation of

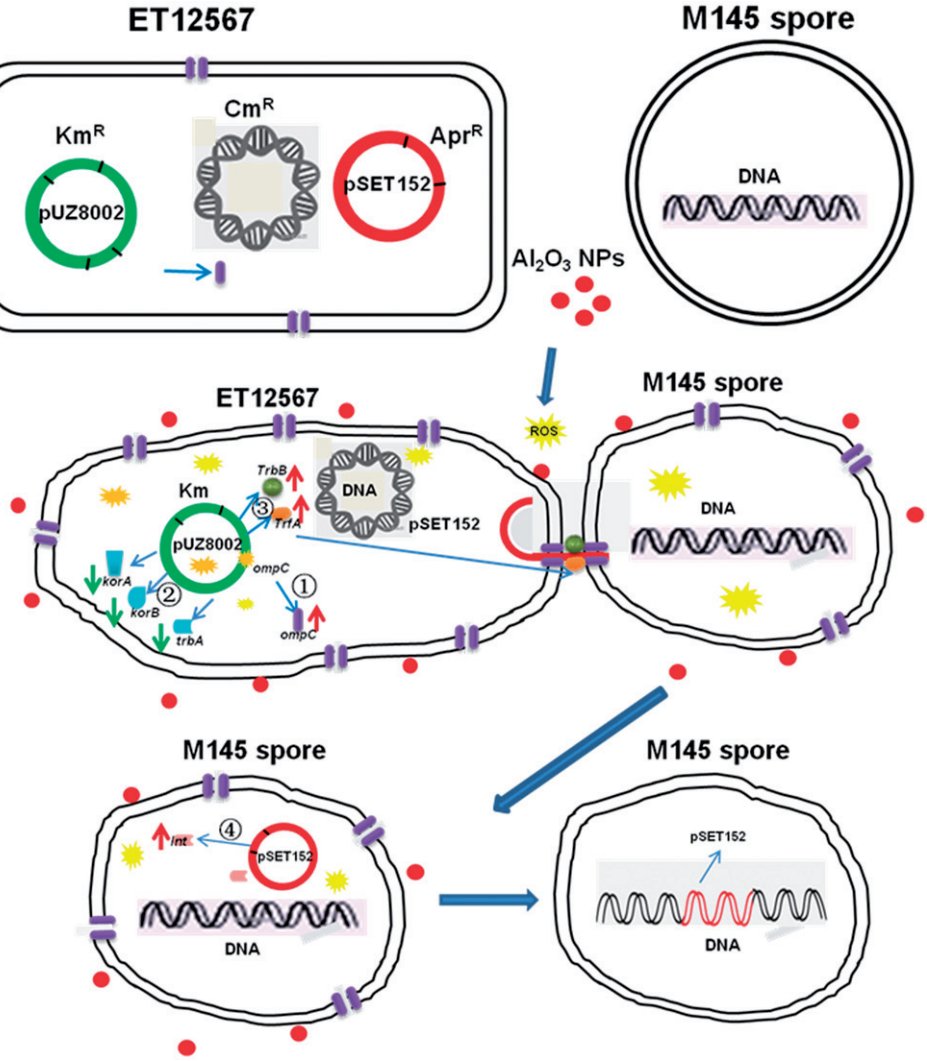

Figure 7. Model of how Al_2O_3 NPs promoted conjugative transfer from E. coli to S. coelicolor M145.①②③④ representing the order of gene expression. Cm^R, Km^R and Apr^R meant chloramphenicol, kanamycin and apramycin resistance genes, respectively. Red arrows represented the up-regulation of genes expression, while green arrows represented the down-regulation of genes expression.

conjugative bridges, they were directly related to the transfer efficiency of plasmids into spores. As shown in Figure 6(E,F), changes in the trends of these two genes were consistent with those of the transconjugants (Figure 2(A)), indicating that these two genes are the most critical genes in the control of conjugative transfer. It has been reported that the expression of the *intA* gene inserts plasmid into chromosomes (Bierman et al. 1992) and the expression of *intA* increased after exposure to NPs, which eventually enhanced the expression of ARGs in M145. Through the above analysis, it can be concluded that the addition of NPs affected gene expression during conjugative transfer, with the cell permeability enhanced, the number of donor-recipient bridges increased, the ability of plasmids to insert receptor chromosomes improved, and eventually the number of conjugators increased.

It was hypothesized that ROS is a bridge linking the effects of extracellular NPs to intracellular gene expression. As shown in Figure 7, after the addition of NPs, the intracellular ROS content increased and expression of the *ompC* gene was stimulated by the destruction of the cell membrane, resulting in the formation of donor-recipient bridges or nanopores on the cell membrane. In addition, ROS would bind to the *korA*, *korB*, and *trbA* genes or their proteins, thereby promoting the expression of *trbB* and *trfA*. Meanwhile, ROS stimulated the expression of the *intA* gene, such that eventually, the number of transconjugants increased.

With the extensive use of NPs, more and more environmental and human health problems caused by the NPs in the environment have received attention. Many studies have shown that NPs have certain biological toxicity (Li et al. 2016, Mosaferi et al. 2016, Liu et al. 2018), and it has been reported that NPs can enhance the ability of antibiotic genes to spread among bacteria (Qiu et al. 2012, Ding et al. 2016). To the best of our knowledge, this study is the first to confirm that NPs can significantly promote conjugative transfer of ARGs between bacteria and spores, facilitating the transfer of ARGs to *Streptomyces* from other bacteria. In this way, their antibiotic resistance is enhanced. Meanwhile, the substantial chemical stability of Al_2O_3 NPs provides selective pressure on environmental bacteria such that the diffusion of ARGs is aggravated, posing environmental and health risks. This study suggests that nanomaterials should be used restrainedly and the safety of them should be evaluated carefully in order to control or reduce the dissemination of resistance genes or the formation of drug-resistant bacteria in the environment caused by nanomaterials. In addition, our results provide a potential method to improve the efficiency of conjugative transfer from bacteria to spores in scientific research.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

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Exposure to Al_2O_3 nanoparticles facilitates conjugative transfer of antibiotic resistance genes from *Escherichia coli* to *Streptomyces*

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Methods

Analysis of concentrations of Al^{3+} in media

To estimate dissolution of Al_2O_3 in the media, the Al^{3+} concentrations were determined after shaking for 48 h at concentrations of 10, 100 or 1000 mg/L Al_2O_3. The media were centrifuged at 14,000 ×g for 30 min, and dynamic light scattering was used to prove no particles existed in the supernatant. 4 mL HNO_3 and 2 mL H_2O_2 were used to digest the supernatant in a microwave digestion system (MDS-15, Sineo, China) at 800 W, 120 °C for 10 min and then 800 W, 160 °C for 20 min. All digested samples were adjusted to the final volume of 50 mL with distilled water.The Al^{3+} concentration was quantified by Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) (IRIS Intrepid II XSP, Thermo Elemental, America).

Scanning electron microscopy (SEM) analysis

The impacts of NPs on spores' morphology were assessed by scanning electron microscopy (SEM). After 2.5 h exposure, cells were centrifuged at 4,000 ×g for 10 min, and fixed in 2.5% glutaraldehyde (in 0.1 M phosphate buffer, pH 7.0) overnight, then washed three times with phosphate buffer (pH 7.0) before dehydrating in 30, 50, 70, 80, 90, and 100% methanol successively, after naturl air-drying, sputter-coated with gold, and then observed via a scanning electron microscope (SEM, JEOL, Beijing, China) (Pakrashi *et al.* 2011).

Transmission electron microscopy (TEM) analysis

After conjugation, the bacteria were fixed at 4 °C over night with precooled 2.5% glutaraldehyde fixative. The cells were post-fixed in 1% osmium tetroxide for 2 h and washed three times with phosphate buffer (pH 7.0). Subsequently, cells were dehydrated in a graded ethanol series (30%, 50%, 70%, 80%, 90% and 100%); The cells were then embedded in resin, and resin blocks were sectioned using an ultra-microtome (Leica EM UC7, Solms, Germany) with a diamond knife. Ultra-thin sections (70−90 nm) were stained with uranyl acetate (2%, 20 min, 25 °C) for TEM observation.

Results

Table S1. List of primers used in this work

Gene		Primer sequence
AMF		CTCACGTTAAGGGATTTTG
AMR		ATGAGCTCAGCCAATCGA
trfA	F	GAAGCCCATCGCCGTCGCCTGTAG
	R	GCCGACGATGACGAACTGGTGTGG
trbB	F	CGCGGTCGCCATCTTCACG
	R	TGCCCGAGCCAGTACCGCCAATG
korA	F	TCGGGCAAGTTCTTGTCC
	R	GCAGCAGACCATCGAGATA
korB	F	CTGGTCGGCTTCGTTGTA
	R	TGAAGTCACCCATTTCGGT
trbA	F	TGGAAACTCCCCTACCTCTT
	R	CCACACTGATGCGTTCGTAT
ompC	F	GTCGGCGGTTCTATCACTTATG
	R	CGAGTTGCGTTGTAGGTCTG
intA	F	GTTCGAGCCCGACGTAATCC
	R	CCACGCCTGAAGCTCATACC
16sr DNA	F	CCTACGGGAGGCAGCAG
	R	ATTACCGCGGCTGCTGG

Table S2. Characterization of different size of Al_2O_3 (n=100)

	30 nm	80 nm	BPs
Particle size	34.9±3.7 nm	83.7±4.3 nm	2.8±1.0 μm
DLS[a]	842±56 nm	897±43 nm	3.4±0.9 μm

a: scattering intensity-weighted mean

Table S3 Number of transconjugants after treated with different kinds of materials

Material	Size	Concentration（mg/L）	Number of transconjugants
CuO NPs	40 nm	10	59.7±11.9
		100	15.0±5.6
	100 nm	10	46.3±3.5
		100	8.3±3.8
CuO BPs	2.5 μm	10	2.7±1.5
		100	3.3±1.5
Al_2O_3 NPs	30 nm	10	182.0±11.0
		100	55.0±9.6
	80 nm	10	87.6±11.5
		100	66.0±9.5
Al_2O_3 BPs	3.2 μm	10	2.3±1.2
		100	2.7±1.2
Ball-milled biochar	100-1000 nm	10	89.7±12.0
		100	26.7±5.0
Biochar	＞10 μm	10	2.3±1.5
		100	3.7±1.2
Control			3.0±1.0

Table S4. The solubility of Al^{3+} in different sizes (30 nm, 80 nm and BPs) and different concentration (10 mg/L,100 mg/L and 1000 mg/L) Al_2O_3 NPs in 2×YT medium. The number of transconjugants after spores of *S. coelicolor M145* treated with these concentrations of Al^{3+} without heat shocking was listed in the table. The number of transconjugants of control without Al^{3+} and heat shocking was 3.0±1.0 and the numbers of tranconjugants with different sizes and concentration of nanoparticles were all lower than that of control indicating no impact of Al^{3+} on transconjugation.

	10 mg/L (mg/L)	Number of transconjugants	100 mg/L (mg/L)	Number of transconjugants	1000 mg/L (mg/L)	Number of transconjugants
30 nm	0.446±0.047	3.33±1.52	0.527±0.019	3.67±1.53	0.857±0.035	3.00±2.00
80 nm	0.192±0.025	2.33±0.57	0.349±0.046	2.67±0.58	0.381±0.032	2.33±1.15
BPs	0.116±0.034	2.67±1.53	0.188±0.021	2.67±1.15	0.211±0.046	2.33±1.53
Control		3.0±1.0.				

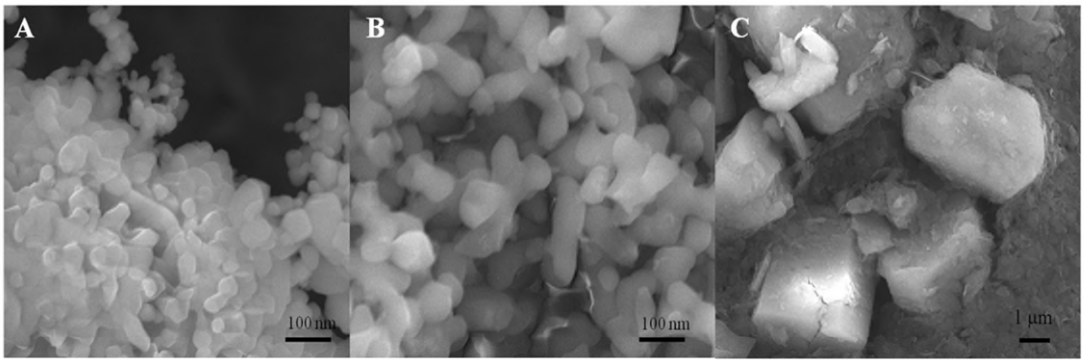

Figure S1: TEM images of Al_2O_3 particles with different size. (A) 30 nm, (B): 80 nm, (C): Bulk particles

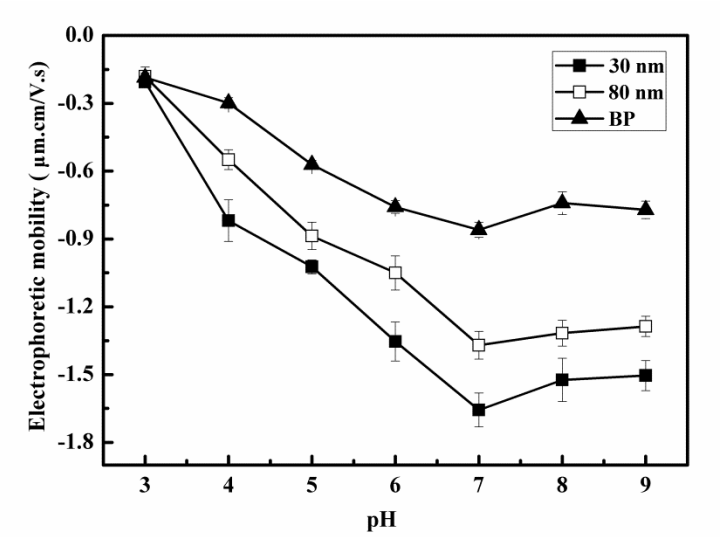

Figure S2: Electrophoretic mobility of different size particles in YBP media.

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