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Natural sphalerite nanoparticles can accelerate horizontal transfer of plasmid-mediated antibiotic-resistance genes

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ABSTRACT

Minerals and microorganisms are integral parts of natural environments, and they inevitably interact. Antibiotic-resistance genes (ARGs) significantly threaten modern healthcare. However, the effects of natural minerals on ARG propagation in aquatic systems are not fully understood. The present work studied the effects of natural sphalerite (NS) nanoparticles on the horizontal transfer of ARGs from *Escherichia coli* DH5α (CTX) (donor) to *E. coli* C600 (Sm) (recipient), and from *E. coli* DH5α (MCR) (donor) to *E. coli* C600 (Sm), and their underlying mechanisms. NS particles (0.5–50 mg L^{-1}) induced an NS-concentration-dependent increase in conjugative transfer frequency. The underlying mechanisms associated with the facilitated ARG transfer included the production of intracellular reactive oxygen species, the SOS response, changes in bacterial cell morphology, and alteration of mRNA levels of bacterial cell membrane protein-related genes and genes associated with conjugative ARG transfer. The information herein offers new mechanistic understanding of risks of bacterial resistance resulting from NS.

1. Introduction

Minerals are naturally occurring chemical compounds. They are an integral part of the natural environment, alongside microorganisms; thus, there are inevitable physicochemical interactions between minerals and microorganisms (Lu et al., 2012). For example, microbes can trap K^+ from the environment and supply K^+ for illitisation through metabolic activity (Aubineau et al., 2019). Some microbes can mediate the Earth's Fe cycle (Melton et al., 2014); for example, primary Fe(III) minerals can be generated when Fe(II) is oxidized by *Rhodovulum iodosum* (Wu et al., 2014). In return, mineral nanoparticles can be adsorbed onto bacterial cells with faster adsorption rates for smaller nanoparticles (Zhang et al., 2011). The absorbed mineral nanoparticles may have different impacts on microorganisms. For example, they may encrust the surface, preventing the microorganisms from taking up nutrients and excreting waste (Nordhoff et al., 2017; Saini and Chan, 2013). Mineral nanoparticles can also noticeably deform bacterial cells, possibly disrupting flagella and making them hardened or stiffer (Zhang et al., 2012), inducing DNA breaks (Gonzalez-Tortuero et al., 2018), or inhibiting DNA replication (Li et al., 2013). This may be why some minerals exhibit antibacterial activity against various bacteria (including pathogenic bacteria (Caflisch et al., 2018) and antibiotic-resistant bacteria [ARB]) (Williams et al., 2011; Zarate-Reyes et al., 2018), or drive bacterial community structure (Liu et al., 2019; Svensson et al., 2017).

Development of antibiotic resistance in bacteria significantly threatens global health (Jiang et al., 2017; Mendelson et al., 2017; Sanderson et al., 2016; Zhang et al., 2019). The development and propagation of antibacterial resistance may be promoted by the recruitment of antibiotic-resistance genes (ARGs) into bacteria, through *de novo* mutation or horizontal transfer of mobile genetic elements, including plasmids, transposons as well as integrons (Bellanger et al., 2014; Dang et al., 2017). In the environment, ARG development mainly involves horizontal gene transfer between bacterial cells, rather than gene function modification through accumulation of point mutations (Aminov, 2011; Bellanger et al., 2014; Huddleston, 2014; Kelly et al., 2009). Horizontal transfers between closely-related bacterial species or strains are common. Transfer happens at an extremely low frequency between bacterial genera (Heuer and Smalla, 2007), although these transfers have greater medical importance. Research has revealed that water and wastewater (pools for ARB and ARGs) appear to be significantly responsible for bacterial antibiotic resistance through

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horizontal gene transfer (Guo et al., 2015; Liu et al., 2012). Aquatic systems as well as water treatment processes, in turn, can influence the frequency of ARG transfer (Guo et al., 2015; Jutkina et al., 2016; Zhang et al., 2017).

Previous evidence suggested that disinfectants and antibiotics can induce enhanced intracellular reactive oxygen species (ROS) generation as well as the SOS response, which can lead to horizontal transfer of ARGs (Beaber et al., 2004). In addition, nanomaterials (Qiu et al., 2012; Wang et al., 2018), ionic liquids (Wang et al., 2015) and disinfectants (Jutkina et al., 2017; Zhang et al., 2017) have also been reported to facilitate conjugative transfer of genes by increasing the permeability of the bacterial cell membrane and affecting conjugation-related gene expression. Therefore, we hypothesize that some minerals can promote the horizontal transfer of ARGs between bacterial strains through intracellular ROS generation, inducing oxidative stress as well as the SOS response. This damages the structures of the cell membrane and alters the expression of genes associated with conjugative ARG transfer. Nevertheless, there are few reports on the effects of natural minerals on ARG transfer in aquatic environments.

To test our hypothesis, the present work studied the influence of natural sphalerite (NS) on conjugative ARG transfer between strains of *Escherichia coli*. *E. coli* is important opportunistic biohazard that causes respiratory tract and intestinal disease. The underlying mechanisms associated with the promoted ARG transfer were also investigated. The information obtained offers new mechanistic understanding of bacterial antibiotic resistance risks resulting from NS.

2. Materials and methods

2.1. Preparation of NS and ZnS suspensions

The NS used in this study was provided by Professor Po Keung Wong (Chinese University of Hong Kong) and was obtained from Huangshaping deposit in Hunan Province, China. The NS was crushed mechanically and milled. We ground NS particles into powder and sieved through 340-mesh; the resulting samples had particle size $\leq$40 μm (Chen et al., 2011; Yang et al., 2011). The main constituent of NS is ZnS nanoparticles (Li et al., 2006). The surface morphology of NS was observed using a scanning electron microscope (SEM, FEI/Nova NanoSEM 450) (Fig. S1). ZnS was purchased from Sigma-Aldrich. The X-ray diffraction profile of NS and ZnS was recorded using a DMAX-2400 (Rigaku, Japan, Cu Kα, λ = 0.15406) with a secondary graphite crystal monochromator (Fig. S2). NS and ZnS suspensions were diluted using ultrapure water. To prevent clustering, before addition to cell culture the suspensions were ultrasonically dispersed in sterilized Eppendorf tubes. The concentrations of NS and ZnS nanoparticles used in the experiments were 0, 0.5, 5, 10, 20, 50, 100 and 500 mg L^{-1}. The ZnS nanoparticles were used as a control.

2.2. Bacterial strains and growth procedures

E. coli is a widely used model microorganism and was selected to evaluate the conjugative transfer frequency of ARG. *E. coli* strains used for assays in the present work are listed in Table S1. Plasmids and bacteria were used as described in previous studies (Chen et al., 2019). Both bacterial strains were cultured in Luria-Bertani (LB) medium with added polymyxin B (PB, 8 μg mL^{-1}) or cefotaxime (CTX, 16 μg mL^{-1}). *E. coli* C600 (Sm), the recipient strain, was cultured in LB medium with added streptomycin (Sm, 3000 μg mL^{-1}). Before the ARG transfer experiments, *E. coli* C600 (Sm) was tested to ensure that it could not grow on plates containing cefotaxime and/or polymyxin B. All donors and recipients were cultured overnight in LB medium supplemented with the appropriate antibiotics, at 180 rpm, 37 °C.

Stability curves were plotted for the bacterial strains in normal saline to evaluate their viability and growth during the experimental process (An et al., 2017). Subsequently, the prepared bacterial strains were used for conjugation transfer and mechanistic investigations.

2.3. Assessment of conjugative ARG transfer on exposure to NS and ZnS nanoparticles

Here, horizontal ARG transfer between *E. coli* strains was evaluated based on the optimized conjugation model. The method used was similar to that in a previous study investigating ARG transfer between ARB under light irradiation (Chen et al., 2019). Briefly, suspension of bacterial donor and recipient was exposed to different concentrations of NS or commercial ZnS nanoparticles as described above, or neither (in controls). After a 4-h exposure at 37 °C, the transconjugants of *E. coli* DH5α (CTX) (donor) were screened on LB plates containing Sm (3000 μg mL^{-1}) and CTX (16 μg mL^{-1}). The transconjugants of *E. coli* DH5α (MCR) (donor) were screened on LB plates with addition of PB (8 μg mL^{-1}) and Sm (3000 μg mL^{-1}). Plates were incubated at 37 °C for 24 h. The detailed culture conditions of the bacteria for conjugation are described in the supporting information (SI). The conjugative transfer frequency was assessed according to the equation:

$$\text{Conjugative transfer frequency} = \frac{\text{Number of transconjugants}}{\text{Number of recipents}}$$

In this expression, the unit of bacterial cells is defined as colony-forming units (CFU) mL^{-1}.

2.4. Bacterial growth inhibition

We evaluated the inhibitory effects of NS and ZnS on the growth of donor and recipient bacteria. Overnight-grown cultures of donor and recipient strains were exposed to different concentrations of NS or ZnS. Control samples, without exposure to NS or ZnS nanoparticles (incubation in LB medium only), were also assessed. The treatment period was 4 h. Bacterial concentrations after the exposure and in the controls were assessed using the LB-agar plate counting method described above.

2.5. Determination of intracellular ROS levels

DCFH-DA dye (Invitrogen, Carlsbad, USA) was used to stain cells and measure intracellular ROS generation in recipient and donor cells. Bacterial cultures (10^8 CFU mL^{-1}) were incubated with DCFH-DA (10 μM) for 0.5 h at 37 °C. The samples were gently shaken every 3–5 min, and after treatment were washed three times in phosphate-buffer saline to remove unbound DCFH-DA. The bacterial cultures were then exposed to different concentrations of NS or ZnS nanoparticles at 37 °C for 2 h. After that, each sample was transferred into a 96-well plate (300 μL per well) to measure the fluorescence intensity (excitation 488 nm, emission 525 nm) with a microplate reader (Thermo Fisher Varioskan LUX). The fluorescence intensities of the treatment groups were divided by the values for the control to demonstrate enhanced ROS formation as the "relative ROS content". All samples and controls were analysed in triplicate.

2.6. Enzymatic activity assays

To test the enzymatic activities of catalase (CAT), superoxide dismutase (SOD) as well as glutathione peroxidase (GSH-Px), cells were harvested from 1.5 mL of bacterial cell suspension. The suspension was lysed with B-PER® Bacterial Protein Extraction Reagent (100 μL; Pierce Biotechnology, USA), and then the supernatant was collected by centrifugation for 2 min at 12,000 rpm. The activities of CAT, SOD, and GSH-Px were assayed using a Catalase Assay Kit (S0051, Beyotime Institute of Biotechnology, China), Superoxide Dismutase Assay Kit (Item No. 706002, Cayman Chemical, USA), and Glutathione Peroxidase Assay Kit (A005, Nanjing Jiancheng Bioengineering Institute, China), respectively. Detailed procedures are provided in SI.

The experiments were replicated three times, and the data are represented as means ± standard deviations.

2.7. Assessment of mRNA expression of stress response-coding genes and outer membrane (OM) protein-coding genes

Bacterial cells exposed to different doses of NS or ZnS nanoparticles were harvested by centrifugation for 15 min at 10,000 rpm. Total RNA was extracted from each sample using an RNAiso Plus Kit (TaKaRa, Dalian, China). Then, cDNA was produced using a Reverse Transcription Kit (TaKaRa). The expression of various genes, such as target ARGs, oxidative stress response genes, cell repair genes, DNA repair genes, conjugation-related genes, and OM protein-coding genes was quantitatively evaluated by real-time polymerase chain reaction (RT-PCR). The 16S rRNA gene was also tested as an amplification internal control. Information about these genes and primers for qPCR is in Table S2. Recipes and protocols for qRT-PCR are provided in SI.

2.8. Evaluation of cell membrane permeability

2.8.1. Assessment of OM permeability

OM permeability caused by exposing bacterial cells to NS and ZnS nanoparticles was determined using an *N*-phenyl-1-naphthylamine (NPN) assay. Bacterial cells grown to the logarithmic phase in LB medium at 37 °C were harvested, washed twice, and then resuspended in 0.9% NaCl solution (10^8 CFU mL^{-1}) in the presence of 10 μmol L^{-1} NPN. The suspension was divided into three parts. At 0 min, various amounts of NS or ZnS nanoparticles were added to final doses of 0 (blank control), 0.5, 5, 10, 20, 50, 100 and 500 mg L^{-1}. Then, the fluorescence of the samples was recorded at 30-min intervals (PE LS 45 fluorescence spectrophotometer, Perkin Elmer; excitation 350 nm, emission 420 nm). The fluorescence of NS and ZnS without bacterial cells was also assessed.

2.8.2. Assessment of inner membrane (IM) permeability

IM permeability caused by exposing bacterial cells to NS and ZnS nanoparticles was assessed by recording the leakage of cytoplasmic β-galactosidase from bacterial cells into the culture medium using substrate *O*-nitrophenyl-β-D-galactopyranoside (ONPG). Logarithmic phase bacterial cells cultured in LB medium at 37 °C in the presence of lactose (2%) were centrifugated, washed twice, and then resuspended in 0.9% NaCl solution (10^8 CFU mL^{-1}) with ONPG (1.5 mmol L^{-1}). The suspension was divided into three parts as described above. Then, different amounts of NS or ZnS nanoparticles were added; the final levels were 0 (blank control), 0.5, 5, 10, 20, 50, 100 and 500 mg L^{-1}. The samples were then incubated with gentle rocking at 37 °C. The generation of *O*-nitrophenol (ONP) in samples collected at different time points was evaluated using a spectrophotometer (GE Ultrospec6300 pro) at 420 nm.

2.9. Statistical analysis

After exposure to different concentrations of NS and ZnS nanoparticles, significant differences in conjugative transfer frequency were assessed using one-way analysis of variance at a significance level of $p = 0.05$. If $p < 0.05$, the value was determined as significant; if $p < 0.01$, the value was determined as highly significant (Zhang et al., 2017).

3. Results

3.1. Bacterial growth inhibition by NS

First, the effect of the minerals on bacterial growth was studied. As demonstrated in Fig. 1, the growth of the *E. coli* strains was dependent on the dose of NS in the range 0.5–500 mg L^{-1}. Nanoscale ZnS concentrations were assessed for comparison under identical conditions. The growth inhibition levels of NS changed relying on *E. coli* strains. For instance, NS least inhibited the growth of *E. coli* C600 (Sm), and most inhibited *E. coli* DH5α (CTX). Similarly, commercial ZnS had the highest inhibitory effect on the growth of *E. coli* DH5α (CTX); this strain thus had the lowest tolerance to mineral damage. The exact trends of the growth curves differed between NS and ZnS treatment. However, the higher the concentration, the higher the inhibition of growth. These results were consistent with previous studies that explored the toxic effect of commercial nano ZnO with different particle sizes on bacteria (Wang et al., 2018). Other engineered nanomaterials have also been found to inhibit bacterial adhesion and proliferation (De Cesare et al., 2019). This may be because the nanoparticles can adsorb onto the bacterial cells and prevent them from absorbing nutrients and excreting waste (Nordhoff et al., 2017; Saini and Chan, 2013). They may also damage the cells by disrupting the cell membrane, DNA, or other components (Gonzalez-Tortuero et al., 2018; Zhang et al., 2012).

3.2. Conjugative transfer frequency of ARGs in the presence of NS

The principal hypothesis to be tested in this study is that some minerals facilitate horizontal ARG transfer among bacteria. Therefore, we evaluated the impact of NS on conjugative ARG transfer from *E. coli* DH5α (CTX) to *E. coli* C600 (Sm), and from *E. coli* DH5α (MCR) to *E. coli* C600 (Sm). Fig. 2a shows that NS particles in the range 0.5–50 mg L^{-1} induced an NS-concentration-dependent increase in conjugative transfer frequencies, of 1.4–3- and 1.5–3.6-fold respectively, compared with controls (without NS or ZnS). However, the conjugative transfer frequencies decreased at an exposure dose of ≥100 mg L^{-1} due to the high inactivation efficiency of the donors and recipients at these high concentrations of particle exposure (Fig. 1). This result was in good agreement with published studies showing that inhibitory effects of some antibiotics, disinfectants and biocides lead to low frequency of conjugative transfer due to the killing of bacteria in these mating systems (Jutkina et al., 2017; Zhang et al., 2017). Furthermore, high particle concentrations disturb contact between cells by being adsorbed onto the surfaces of donor and recipient cells. This results in a decrease in the frequency of conjugative transfer (Zhang et al., 2018b).

There were similar trends in conjugative ARG transfer when the bacteria were exposed to ZnS nanoparticles. The exception was that there was a higher transfer frequency in the presence of 50 mg L^{-1} ZnS compared with 50 mg L^{-1} NS during conjugative ARG transfer from *E. coli* DH5α (CTX) to *E. coli* C600 (Sm) (Fig. 2b). This might be due to differences in the chemical composition, particle size, and toxic effects of NS and ZnS nanoparticles. For example, ZnO and Al_2O_3 may promote ARG transfer because particles are nanoscale (Qiu et al., 2012; Wang et al., 2018). The NS and commercial ZnS nanoparticles selected for this study are widely used in practical settings for bacterial inactivation or pollutant degradation (Chen et al., 2011; Yang et al., 2011). The results of this study suggested that NS potentially enhanced the spread of ARGs between *E. coli*; however, this spread was not as marked as that which occurred on exposure to commercial ZnS. When the mineral concentration reached ≥100 mg L^{-1}, the conjugation transfer frequency of both ARGs was inhibited, dropping below the detection limit. This may also be due to the direct effect of high mineral concentrations on the growth of donor and recipient bacteria at mineral concentrations of 100 mg L^{-1}.

This was similar to the effect of low concentrations of antibiotics and chlorine disinfection on ARG transfer frequency (Guo et al., 2015; Zhang et al., 2017). This is also because the high concentration of mineral particles adsorbed onto the surface of bacterial cells. This then affects the effective contact between donor and recipient bacteria, affecting ARG transfers. The concentration-dependent effect of NS on the transfer of ARGs may be due to the complex composition of NS. In contrast, the nano-ZnS can directly penetrate bacterial cells, and nanoparticles can directly affect the physiological and biochemical

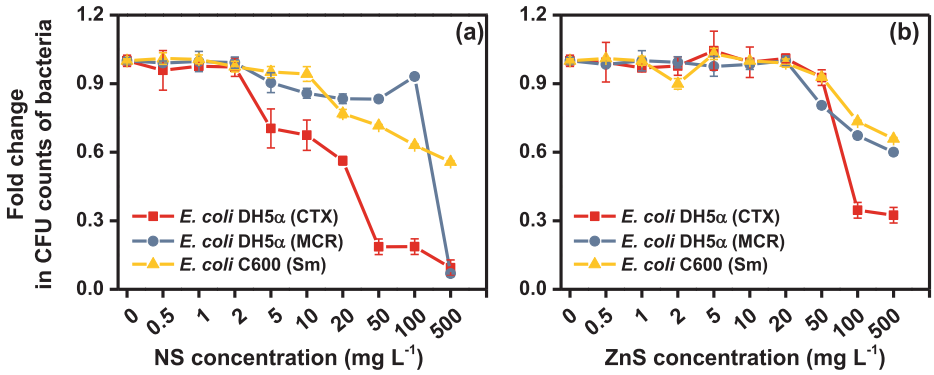

Fig. 1. The dose-dependent growth inhibitory curves of *E. coli* DH5α (CTX), *E. coli* DH5α (MCR), and *E. coli* C600 (Sm) treated with (a) natural sphalerite (NS), (b) ZnS.

reactions of bacteria.

3.3. Conjugative ARG transfer mechanism upon exposure to NS

3.3.1. Effects of NS on ROS and antioxidant systems of ARB

The impact of NS on bacterial activity may be caused by the complex components of NS, which can directly produce high ROS concentrations in cells. ROS in bacterial cells, including superoxide anion radicals ($O_2^{\bullet -}$), $^{\bullet}OH$, and H_2O_2, can cause oxidative stress, damage various bacterial biomolecules as well as interrupt a range of cellular processes (Van Acker and Coenye, 2017). To mitigate ROS accumulation, bacterial cells have evolved various defense mechanisms, including antioxidant systems (Sun et al., 2014). The effect of NS on bacterial ROS generation and the antioxidant system reflects the effect of NS on the bacterial cell membrane, which, in turn, affects bacterial conjugation and gene transfer.

DCFH-DA, a fluorescent probe, was used to monitor intracellular ROS levels in response to nanoparticle treatment. It has been used extensively to detect ROS such as hydrogen peroxide and hydroxyl radicals in cells (Sun et al., 2014). Here, when recipient and donor cells were exposed to NS or ZnS nanoparticles, we found nanoparticle-concentration-dependent increases of ROS levels (Fig. 3). Importantly, when the bacterial strains were exposed to 0.5–50 mg L^{-1} of NS or ZnS nanoparticles, fold-changes of transconjugation activity showed significant positive correlations with ROS levels when the ROS levels were moderate. High levels of ROS elicited by higher concentrations (50–500 mg L^{-1}) of NS and especially ZnS nanoparticles might lead to severe cellular damage or death, and hence a low frequency of conjugative transfer (Fig. 2).

A number of studies have offered convincing proof that, in fine particles, redox cycling organics and transition metals can induce the generation of ROS. This is tightly integrated with oxidative stress, lipid peroxidation, and changes in bacterial cell structure (Jiao et al., 2017; Zhang et al., 2018a). The increased ROS in cells will usually be cleared by antioxidative enzymes, including CAT, SOD as well as GSH-Px. These catalyse the conversion and detoxification of $O_2^{\bullet -}$ and H_2O_2.

Therefore, the activities of SOD, CAT, and GSH-Px in *E. coli* exposed to NS and ZnS nanoparticles were determined; they are shown in Figs. S3, S4, and 4, respectively. A nanoparticle-concentration-dependent increase in the activities of all the enzymes was observed at higher concentrations (50–500 mg L^{-1}). However, NS and ZnS nanoparticles at concentrations $>$ 50 mg L^{-1} did not promote conjugation and ARG transfer (Fig. 2). This may be because the destruction of the cell membrane directly led to the normal occurrence of conjugation and transfer when there were too many particles.

Separate research found that CAT, SOD, and GSH-Px may be oxidatively damaged, producing protein fragments and protein carbonyl derivatives. This would lead to the loss of their activities when bacterial cells face attack by ROS (Kwon et al., 2000). Here, the rapidly elevated intracellular ROS levels during the nanoparticle exposure probably led to the increased activities of SOD, CAT, and GSH-Px activities to protect the cells against oxidative stress. It may also be that the level of ROS generated overpowered the antioxidative ability of these enzymes. When NS or ZnS nanoparticles interfere with conjugation and ARG transfer as the NS or ZnS concentrations increase, bacterial oxidation and antioxidant reactions also become increasingly intense, and the ROS drive cellular damage.

3.3.2. Effects of NS on gene expression in ARB

Quantitative RT-PCR analysis was conducted to evaluate the levels of mRNA expression of important genes in *E. coli* in response to nanoparticle exposure. These critical genes are related to membrane repair

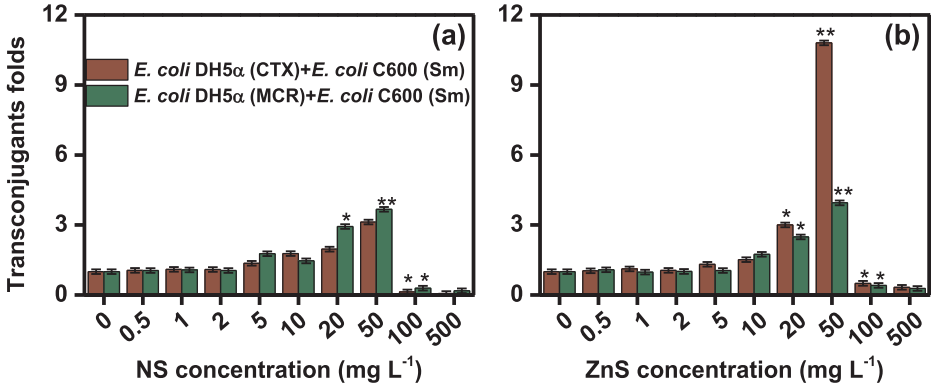

Fig. 2. Impacts of (a) NS and (b) ZnS particles on the ARG conjugative transfer from *E. coli* DH5α (CTX) to *E. coli* C600 (Sm) and *E. coli* DH5α (MCR) to *E. coli* C600 (Sm).

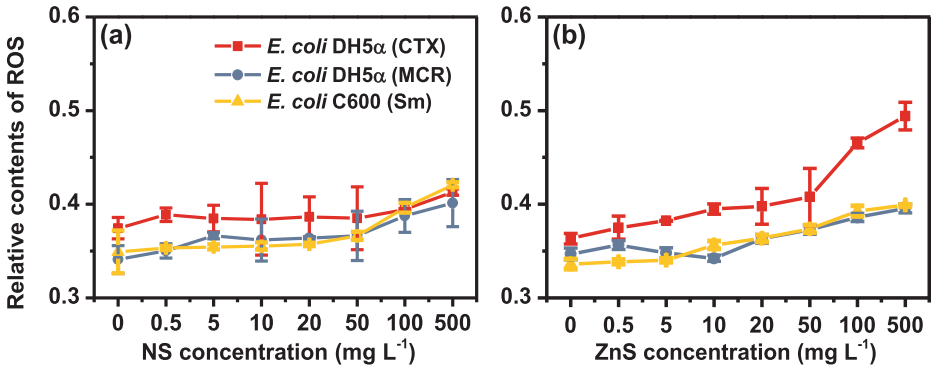

Fig. 3. The effects of (a) NS and (b) ZnS nanoparticles on the levels of intracellular ROSs shown by fluorescent intensity of probe DCFH-DA.

(*basS*, *mdtB*, *cusC*, *motA*, and *yiaD*), oxidative stress (*oxyR*, *rpoS*, *marA*, *ompR*, *soxR*, *soxS*, *osmC*, and *osmY*), DNA repair (*recA*, *recF*, *recJ*, *rpoH*, *rpoD*, *mukB*, *radA*, *lexA*, *ruvB*, and *rcsC*), conjugation relationships (*ftsY*, *tesB*, and *gspE*), and ARGs (bla_{CTX} and *mcr-1*). All these genes have been proven to play critical roles in the horizontal transfer of ARGs as well in as chromosome mutation (Zhang et al., 2018b; Zhang et al., 2017). At the same time, the changes in the genes that regulate the binding and transfer of ARG affect the binding and transfer process. This makes it important to explore the mechanism by which NS promotes the binding and transfer of ARGs.

Fig. 5 shows that the expression levels of stress-related genes in the bacteria were significantly upregulated by exposure to 0.5–500 mg L^{-1} of NS when compared with controls. Stress-related genes are important regulators of gene transcription that mediate the antioxidant defense system and physiological signal monitoring in *E. coli* (Beaber et al., 2004; Cabiscol et al., 2000). This enhancement of expression may contribute to the enhanced conjugative transfer. As mentioned above, NS treatment might produce high levels of intracellular ROSs (Fig. 3) and induce an oxidative stress response (Fig. 5).

The expression of stress-related genes increased 2–8-fold on exposure of cells to 50 mg L^{-1} ZnS nanoparticles. At ZnS concentration $>$ 50 mg L^{-1}, the expression of genes was downregulated, due to bacterial damage and the direct destruction of the bacterial defense system by ROS. In general, the increased gene expression in bacteria is a result of high bacterial activity and is an active response to external stimuli. This is also a reason for the increased ARG transfer efficiency. Previous evidence suggests that the triggered SOS response can irritate horizontal gene transfer, owing to effects on the expression of conjugation-associated as well as conjugational recombination-related genes (Beaber et al., 2004; Guerin et al., 2009; Johnsen and Kroer, 2007).

3.3.3. Effects of NS on the cell membrane of ARB

Increased bacterial membrane permeability is important for accelerating horizontal ARG transfer between two bacterial cells. This is because conjugation transfer requires plasmids to complete transmembrane transport, and high membrane permeability facilitates the process (Qiu et al., 2012). Here, we detected the effect of NS and ZnS nanoparticles on bacterial membrane permeability. Importantly, because Gram-negative bacteria like *E. coli* have and OM and an IM (Mangoni et al., 2004), the changes of permeability of both membranes of the conjugation bacteria exposed with NS and ZnS nanoparticles were analyzed, using NPN and ONPG tests respectively.

NPN is a hydrophobic fluorescent probe. It has weak fluoresce in water, but strong fluorescence in hydrophobic environments such as the interior of the bacterial cell membrane. NPN uptake is usually blocked by the undamaged OM; however, the probe can be taken up by cells when this membrane is damaged. On the basis of this principle, the OM permeability of bacterial cells exposed to minerals was assessed using NPN. Fig. S5a and b show that with addition of NS and ZnS nanoparticles to bacterial cell suspensions led to a concentration-dependent increase in NPN fluorescence, particularly at mineral concentrations $\geq$ 50 mg L^{-1}. The intensity of the fluorescence of solutions with various mineral doses, but no *E. coli* cells, was also monitored as a control; NS or ZnS alone did not influence the fluorescence intensity. Therefore, in this study, increased uptake of NPN was associated with increased mineral concentration. This means that NS and ZnS nanoparticles could cause permeabilization of the OM.

The chromogen ONPG is an artificial substrate of β-galactosidase, and is applied for evaluation of IM permeabilization. It is colorless, but its hydrolysis product, ONP, is yellow (λ_{max} 420 nm). Hence, the enzymatic activity can be assessed by the appearance rate of the yellow color. Fig. S5c and d displays the relative absorbance at 420 nm [relative $Abs_{420\ nm}$ = ($Abs_{420\ nm}$ with NS or ZnS nanoparticles and ONPG) – ($Abs_{420\ nm}$ with NS or ZnS without ONPG)]. $Abs_{420\ nm}$ of the blank control (without NS or ZnS) was approximately 0.1. This indicates that some β-galactosidase could naturally leak from the cells. There was no difference in $Abs_{420\ nm}$ compared with the blank for NS or ZnS

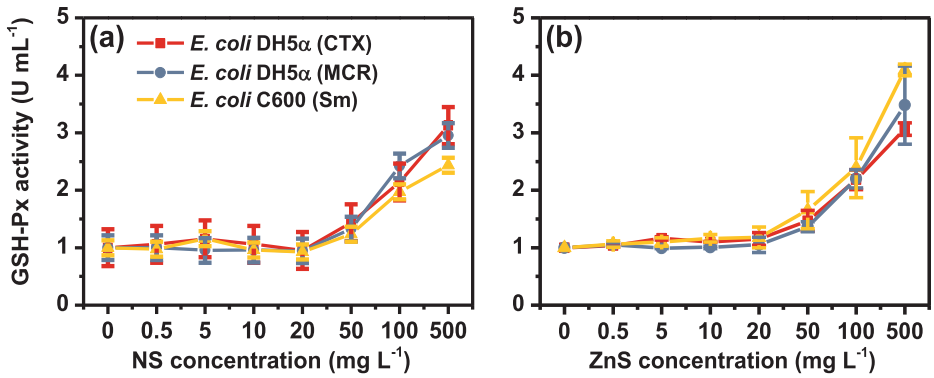

Fig. 4. The effects of (a) NS and (b) ZnS nanoparticles on the activity of GSH-Px.

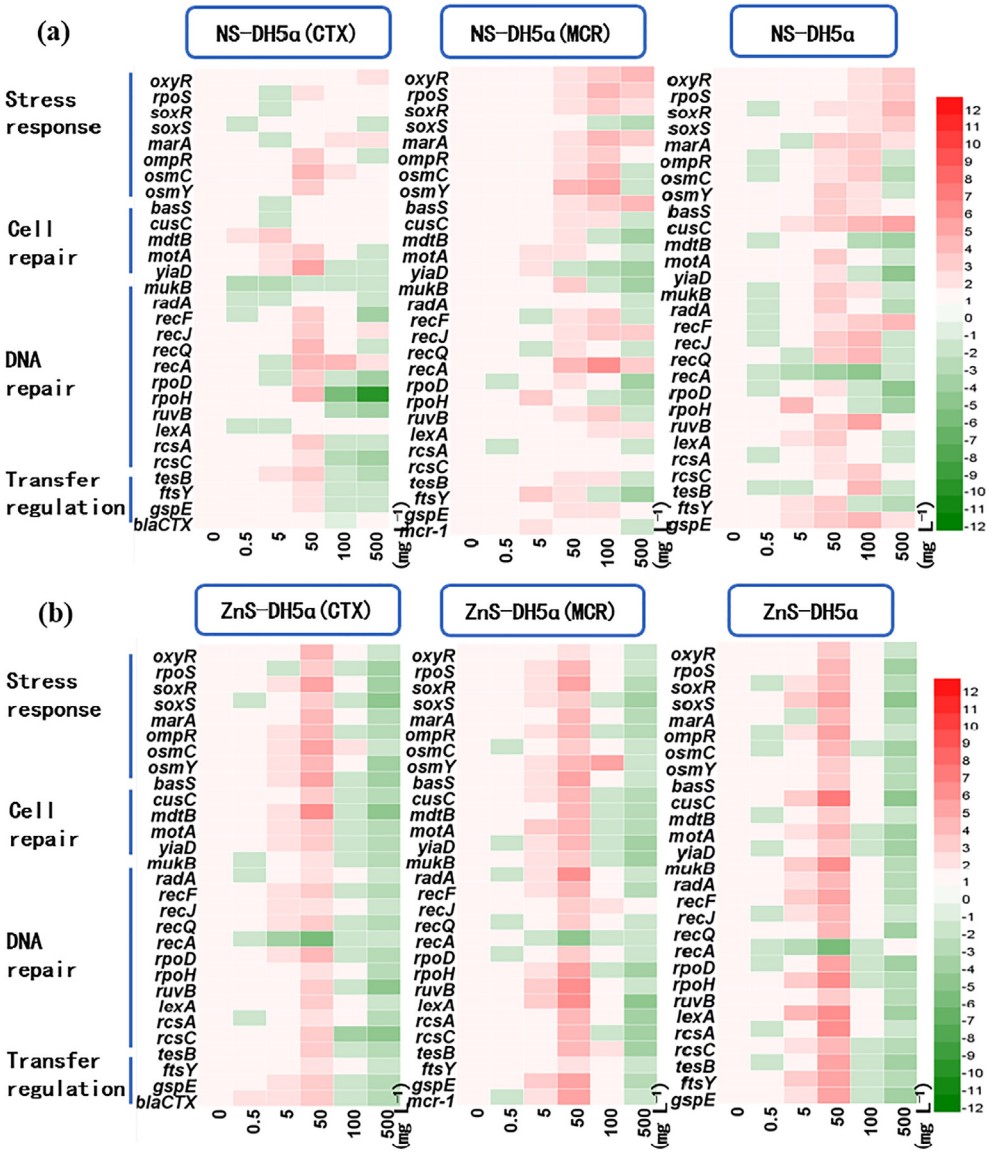

Fig. 5. Gene expression profiles of target genes involved in stress response, cell repair, DNA repair and transfer regulation upon the exposure to (a) NS and (b) ZnS nanoparticles in *E. coli* DH5α (CTX), *E. coli* DH5α (MCR) and *E. coli* DH5α. X-axis: the monitoring time in minutes; Y-axis left: clusters of target genes and list of genes tested, Y-axis right: the figure legend bar (depicted as a green-red colour scale. Red spectrum colour indicates up-regulated expression; green spectrum colour indicated down-regulated expression). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

nanoparticle doses of 0–5 mg L^{-1}. The relative $Abs_{420\ nm}$ increased gradually with NS or ZnS concentration ≥ 50 mg L^{-1}. This suggests a gradual increase in IM permeability with nanoparticle concentration. This may be because a high concentration of NS or ZnS nanoparticles directly damages the bacterial cell membranes, including the IM. Nevertheless, a moderate increase in cell membrane permeability may facilitate the conjugative ARG transfer (Fig. 2); but if the cell membrane permeability becomes too high, the cells may be disrupted, leading to death.

At the molecular level, changes in the permeability of bacterial cell membranes are regulated by the expression of outer-membrane proteins (OMPs). Some typical OMPs, such as OmpA (34 kDa), OmpC (36 kDa), and OmpF (35 kDa), can affect the the cell membrane permeability of both recipient and donor bacteria. These OMPs may play vital roles in pore formation for, and horizontal transfer of, ARGs. Fig. 6 shows that *ompA*, *ompC* and *ompF* gene expression significantly increased, by 2.7–33-, 4.6–21.9-, and 3.1–25.5-fold, respectively, following 50 mg L^{-1} NS exposure. Comparatively, the mRNA expression of *ompA*, *ompC* and *ompF* rose by 5.3–17.6-, 4–13.6-, and 4–21.1-fold, respectively, following ZnS exposure at the same concentration. Previously, Smith et al. confirmed that *ompA* is mainly regulated at the post-transcriptional level (Smith et al., 2007), and a similar result was obtained in this study (Fig. 6). Bacteria may adapt to the stimulation of external pressure by changing the transcriptional expression levels of stress-related genes. Increase of the level of expression of OM-related genes may also indicate the degree of change in the bacterial cell membrane. ROS production may directly affect OMPs in cell membranes. Enhancing the expression of the membrane protein-coding genes plays a crucial role in forming the OM pores and enhancing cell membrane permeability. This might be related to the antibiotic resistance development via horizontal transfer of ARGs.

4. Discussion

The spread of bacterial antibiotic resistance is an important worldwide concern. Our results show that NS promoted the horizontal transfer of ARG mediated by plasmids between bacteria. The shared nature of NS allows them to enhance the conjugative transfer of

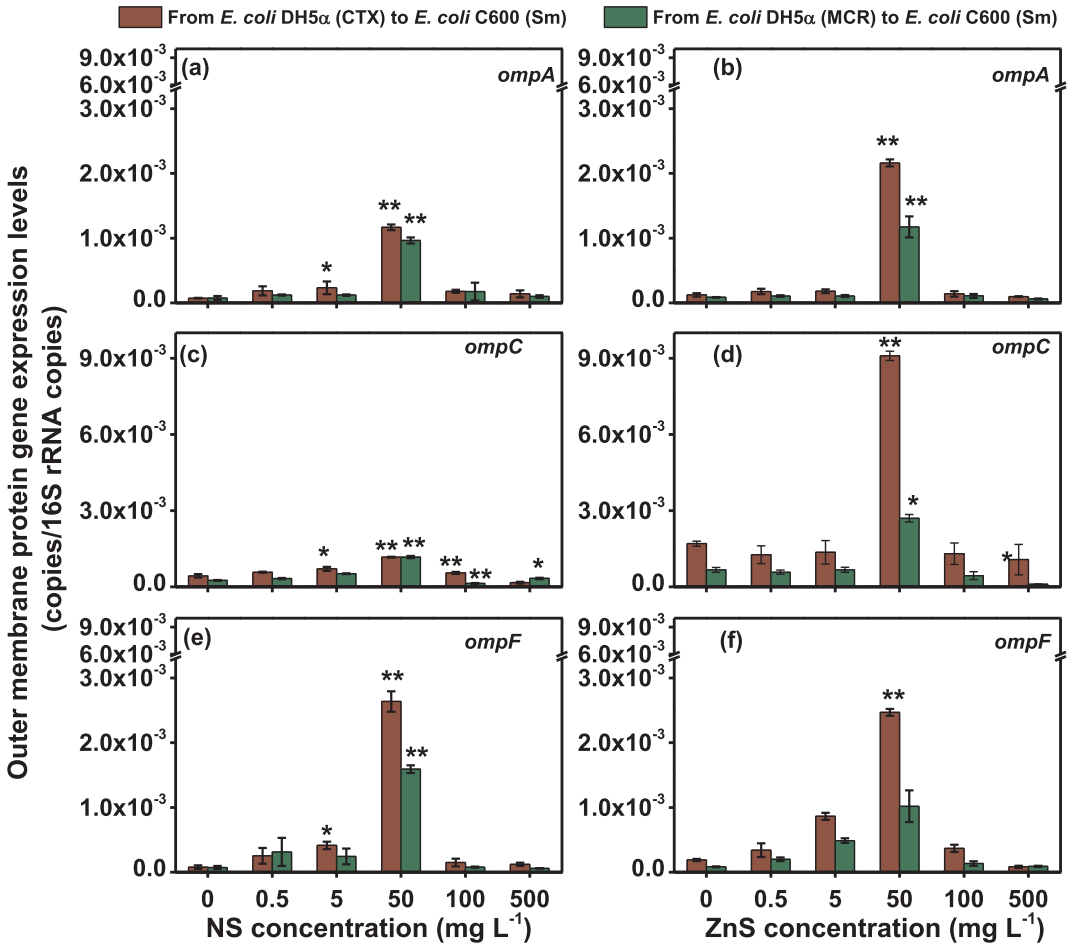

Fig. 6. Effect of NS (a. c. e) and ZnS nanoparticles (b. d. f) on the mRNA expression levels of outer membrane protein genes (*ompA*, *ompC*, and *ompF*) in the mating pairs within *E. coli* (from *E. coli* DH5α (CTX) to *E. coli* C600 (Sm), and from *E. coli* DH5α (MCR) to *E. coli* C600 (Sm)).

resistance-encoding plasmids. Across a number of natural minerals, NS had the most significant effect in promoting the transfer of ARG-carrying plasmids between the same species of bacteria.

In the present work, we concentrated on the effect of exposure of bacterial cells to NS on the plasmid transfers for the following reasons: (1) NS had the same capability as ZnS nanoparticles to promote ARG transfers (≥10-fold compared with controls); and (2) natural minerals regulate the composition of sediment water, soil water, and other water environments (Li et al., 2009). NS has a complex structure with different particle sizes. As such, it has several applications as a catalyst (Li et al., 2008; Li et al., 2006).

We also investigated the mechanisms by which NS promoted the conjugative transfer of ARGs between *E. coli* strains. Study has shown that Gram-positive bacterial cell walls are a barrier to the conjugal transfer of genes from Gram-negative bacteria (Trieu-Cuot et al., 2010). Therefore, we supposed that Gram-negative bacterial cell membranes might also block the conjugal transfer of ARGs. However, oxidative stress is able to damage bacterial cell membranes and might thus accelerate the transfer of nutrients or genes (Farr and Kogoma, 1991; Johnsen and Kroer, 2007). The present work demonstrated that NS led to a stress response in the Gram-negative bacterium *E. coli*, damaged its cell membranes, and promoted the conjugative transfer of ARGs. The expression of stress-related genes was also enhanced by the exposure of cells to minerals. NS may thus also promote horizontal gene transfer by upregulating the stress-related genes involved in conjugation. These results indicate that the stress-related genes are activated and rise these expression, providing the sequential steps that facilitate the frequency of ARG transfer (Guerin et al., 2009).

5. Conclusions

This study described an increase in horizontal ARG transfer between Gram-negative bacteria when they were exposed to NS nanoparticles. At some concentrations, NS in the water system might increase the horizontal transfer of ARGs given sufficient bacterial density and conjugation time. The mechanism of the acceleration of the transfer of ARGs by NS exposure might be associated with damage to bacterial membranes through oxidative stress. This enhanced the expression of stress-related genes. The findings of this study suggested that mineral–ARB interactions in the natural environment should be carefully evaluated to minimize public health, environmental, and ecological hazards.

CRediT authorship contribution statement

Guiying Li: Supervision. **Xiaofang Chen:** Methodology, Data curation. **Hongliang Yin:** Validation. **Wanjun Wang:** Writing - review & editing. **Po Keung Wong:** Resources. **Taicheng An:** Conceptualization, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2020.105497.

References

Aminov, R.I., 2011. Horizontal gene exchange in environmental microbiota. Front. Microbiol. 2, 158.
An, T., Zhao, H., Wong, P.K., 2017. Advances in photocatalytic disinfection. Springer, Berlin Heidelberg.
Aubineau, J., El Abani, A., Bekker, A., Somogyi, A., Bankole, O.M., Macchiarelli, R., Meunier, A., Riboulleau, A., Reynaud, J.Y., Konhauser, K.O., 2019. Microbially induced potassium enrichment in Paleoproterozoic shales and implications for reverse weathering on early earth. Nat. Commun. 10, 9.
Beaber, J.W., Hochhut, B., Waldor, M.K., 2004. SOS response promotes horizontal dissemination of antibiotic resistance genes. Nature 427, 72–74.
Bellanger, X., Guilloteau, H., Bonot, S., Merlin, C., 2014. Demonstrating plasmid-based horizontal gene transfer in complex environmental matrices: A practical approach for a critical review. Sci. Total Environ. 493, 872–882.
Cabiscol, E., Tamarit, J., Ros, J., 2000. Oxidative stress in bacteria and protein damage by reactive oxygen species. Int. Microbiol. 3, 3.
Caflisch, K.M., Schmidt-Malan, S.M., Mandrekar, J.N., Karau, M.J., Nicklas, J.R., Williams, L.B., Patel, R., 2018. Antibacterial activity of reduced iron clay against pathogenic bacteria associated with wound infections. Int. J. Antimicrob. Agents 52, 692–696.
Chen, X., Yin, H., Li, G., Wang, W., Wong, P.K., Zhao, H., An, T., 2019. Antibiotic-resistance gene transfer in antibiotic-resistance bacteria under different light irradiation: Implications from oxidative stress and gene expression. Water Res. 149, 282–291.
Chen, Y., Lu, A., Li, Y., Zhang, L., Yip, H., Zhao, H., An, T., Wong, P.K., 2011. Naturally occurring sphalerite as a novel cost-effective photocatalyst for bacterial disinfection under visible light. Environ. Sci. Technol. 45, 5689–5695.
Dang, B., Mao, D., Xu, Y., Luo, Y., 2017. Conjugative multi-resistant plasmids in Haihe River and their impacts on the abundance and spatial distribution of antibiotic resistance genes. Water Res. 111, 81–91.
De Cesare, F., Di Mattia, E., Zussman, E., Macagnano, A., 2019. A study on the dependence of bacteria adhesion on the polymer nanofibre diameter. Environ. Sci.: Nano 6, 778–797.
Farr, S.B., Kogoma, T., 1991. Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. Microbiol. Rev. 55, 561–585.
Gonzalez-Tortuero, E., Rodriguez-Beltran, J., Radek, R., Blazquez, J., Rodriguez-Rojas, A., 2018. Clay-induced DNA breaks as a path for genetic diversity, antibiotic resistance, and asbestos carcinogenesis. Sci. Rep. 8, 10.
Guerin, É., Cambray, G., Sanchezalberola, N., Campoy, S., Erill, I., Re, S.D., Gonzalezzorn, B., Barbé, J., Ploy, M., Mazel, D., 2009. The SOS response controls integron recombination. Science 324 1034 1034.
Guo, M.T., Yuan, Q.B., Yang, J., 2015. Distinguishing effects of ultraviolet exposure and chlorination on the horizontal transfer of antibiotic resistance genes in municipal wastewater. Environ. Sci. Technol. 49, 5771–5778.
Heuer, H., Smalla, K., 2007. Horizontal gene transfer between bacteria. Environ. Biosafety Res. 6, 3–13.
Huddleston, J.R., 2014. Horizontal gene transfer in the human gastrointestinal tract: potential spread of antibiotic resistance genes. Infect. Drug Resist. 7, 167.
Jiang, X.L., Ellabaan, M.M.H., Charusanti, P., Munck, C., Blin, K., Tong, Y.J., Weber, T., Sommer, M.O.A., Lee, S.Y., 2017. Dissemination of antibiotic resistance genes from antibiotic producers to pathogens. Nat. Commun. 8, 15784.
Jiao, Y.N., Chen, H., Gao, R.X., Zhu, Y.G., Rensing, C., 2017. Organic compounds stimulate horizontal transfer of antibiotic resistance genes in mixed wastewater treatment systems. Chemosphere 184, 53–61.
Johnsen, A.R., Kroer, N., 2007. Effects of stress and other environmental factors on horizontal plasmid transfer assessed by direct quantification of discrete transfer events. FEMS Microbiol. Ecol. 59, 718–728.
Jutkina, J., Marathe, N.P., Flach, C.F., Dgj, L., 2017. Antibiotics and common antibacterial biocides stimulate horizontal transfer of resistance at low concentrations. Sci. Total Environ. 616, 172–178.
Jutkina, J., Rutgersson, C., Flach, C.F., Larsson, D.G.J., 2016. An assay for determining minimal concentrations of antibiotics that drive horizontal transfer of resistance. Sci. Total Environ. 548, 131–138.
Kelly, B.G., Vespermann, A., Bolton, D.J., Kuiper, H.A., Kleter, G.A., 2009. Gene transfer events and their occurrence in selected environments. Food Chem. Toxicol. 47, 978–983.
Kwon, H.Y., Choi, S.Y., Won, M.H., Kang, T.C., Kang, J.H., 2000. Oxidative modification and inactivation of Cu, Zn-superoxide dismutase by 2,2′-azobis(2-amidinopropane) dihydrochloride. Biochim. Biophys. Acta 1543, 69–76.
Li, K.G., Zhao, X.N., Hammer, B.K., Du, S.Y., Chen, Y.S., 2013. Nanoparticles inhibit DNA replication by binding to DNA: modeling and experimental validation. ACS Nano 7, 9664–9674.
Li, Y., Lu, A., Wang, C., 2009. Semiconducting mineralogical characteristics of natural sphalerite gestating visible-light photocatalysis. Acta Geol. Sin. - Engl. 83, 633–639.
Li, Y., Lu, A., Wang, C., Wu, X., 2008. Characterization of natural sphalerite as a novel visible light-driven photocatalyst. Sol. Energy Mater. Sol. Cells 92, 953–959.
Li, Y., Lu, A.H., Wang, C.Q., 2006. Photocatalytic reduction of Cr-VI by natural sphalerite suspensions under visible light irradiation. Acta Geol. Sin. - Engl. 80, 267–272.
Liu, J.L., Yao, J., Lu, C., Li, H., Li, Z.F., Duran, R., Sunahara, G., Mihucz, V.G., 2019. Microbial activity and biodiversity responding to contamination of metal(loid) in heterogeneous nonferrous mining and smelting areas. Chemosphere 226, 659–667.
Liu, M.M., Zhang, Y., Yang, M., Tian, Z., Ren, L.R., Zhang, S.J., 2012. Abundance and distribution of tetracycline resistance genes and mobile elements in an oxytetracycline production wastewater treatment system. Environ. Sci. Technol. 46, 7551–7557.
Lu, A., Li, Y., Jin, S., Wang, X., Wu, X.-L., Zeng, C., Li, Y., Ding, H., Hao, R., Lv, M., Wang, C., Tang, Y., Dong, H., 2012. Growth of non-phototrophic microorganisms using solar energy through mineral photocatalysis. Nat. Commun. 3, 768.
Mangoni, M.L., Papo, N., Barra, D., Simmaco, M., Bozzi, A., Di Giulio, A., Rinaldi, A.C., 2004. Effects of the antimicrobial peptide temporin L on cell morphology, membrane permeability and viability of *Escherichia coli*. Biochem. J. 380, 859–865.
Melton, E.D., Swanner, E.D., Behrens, S., Schmidt, C., Kappler, A., 2014. The interplay of microbially mediated and abiotic reactions in the biogeochemical Fe cycle. Nat. Rev. Microbiol. 12, 797–808.
Mendelson, M., Balasegaram, M., Jinks, T., Pulcini, C., Sharland, M., 2017. Antibiotic resistance has a language problem. Nature 545, 23–25.
Nordhoff, M., Tominski, C., Halama, M., Byrne, J.M., Obst, M., Kleindienst, S., Behrens, S., Kappler, A., 2017. Insights into nitrate-reducing Fe(II) oxidation mechanisms through analysis of cell-mineral associations, cell encrustation, and mineralogy in the chemolithoautotrophic enrichment culture KS. Appl. Environ. Microbiol. 83, 19.
Qiu, Z.G., Yu, Y.M., Chen, Z.L., Jin, M., Yang, D., Zhao, Z.G., Wang, J.F., Shen, Z.Q., Wang, X.W., Qian, D., Huang, A.H., Zhang, B.C., Li, J.W., 2012. Nanoalumina promotes the horizontal transfer of multiresistance genes mediated by plasmids across genera. P. Natl. Acad. Sci. USA 109, 4944.
Saini, G., Chan, C.S., 2013. Near-neutral surface charge and hydrophilicity prevent mineral encrustation of Fe-oxidizing micro-organisms. Geobiology 11, 191–200.
Sanderson, H., Fricker, C., Brown, R.S., Majury, A., Liss, S.N., 2016. Antibiotic resistance genes as an emerging environmental contaminant. Environ. Rev. 24, 205–218.
Smith, S.G.J., Mahon, V., Lambert, M.A., Fagan, R.P., 2007. A molecular Swiss army knife: OmpA structure, function and expression. FEMS Microbiol. Lett. 273, 1–11.
Sun, H., Li, G., Nie, X., Shi, H., Wong, P.K., Zhao, H., An, T., 2014. Systematic approach to in-depth understanding of photoelectrocatalytic bacterial inactivation mechanisms by tracking the decomposed building blocks. Environ. Sci. Technol. 48, 9412–9419.
Svensson, S.L., Behroozian, S., Xu, W.J., Surette, M.G., Li, L., Davies, J., 2017. Kisameet glacial clay: an unexpected source of bacterial diversity. Mbio 8, 14.
Trieu-Cuot, P., Carlier, C., Martin, P., Courvalin, P., 2010. Plasmid transfer by conjugation from Escherichia coli to Gram-positive bacteria. FEMS Microbiol. Lett. 48, 289–294.
Van Acker, H., Coenye, T., 2017. The role of reactive oxygen species in antibiotic-mediated killing of bacteria. Trends Microbiol. 25, 456–466.
Wang, Q., Mao, D.Q., Luo, Y., 2015. An ionic liquid facilitates the conjugative transfer of antibiotic resistance genes mediated by plasmid RP4. Environ. Sci. Technol. 49, 8731–8740.
Wang, X., Yang, F., Zhao, J., Xu, Y., Mao, D., Zhu, X., Luo, Y., Alvarez, P.J.J., 2018. Bacterial exposure to ZnO nanoparticles facilitates horizontal transfer of antibiotic resistance genes. Nanoimpact 10, 61–67.
Williams, L.B., Metge, D.W., Eberl, D.D., Harvey, R.W., Turner, A.G., Prapaipong, P., Poret-Peterson, A.T., 2011. What makes a natural clay antibacterial? Environ. Sci. Technol. 45, 3768–3773.
Wu, W.F., Swanner, E.D., Hao, L.K., Zeitvogel, F., Obst, M., Pan, Y.X., Kappler, A., 2014. Characterization of the physiology and cell-mineral interactions of the marine anoxygenic phototrophic Fe(II) oxidizer Rhodovulum iodosum - implications for Precambrian Fe(II) oxidation. FEMS Microbiol. Ecol. 88, 503–515.
Yang, X., Li, Y., Lu, A., Yan, Y., Wang, C., Wong, P.K., 2011. Photocatalytic reduction of carbon tetrachloride by natural sphalerite under visible light irradiation. Sol. Energy Mater. Sol. Cells 95, 1915–1921.
Zarate-Reyes, L., Lopez-Pacheco, C., Nieto-Camacho, A., Palacios, E., Gomez-Vidales, V., Kaufhold, S., Ufer, K., Zepeda, E.G., Cervini-Silva, J., 2018. Antibacterial clay against gram-negative antibiotic resistant bacteria. J. Hazard. Mater. 342, 625–632.
Zhang, H., Chang, F., Shi, P., Ye, L., Zhou, Q., Pan, Y., Li, A., 2019. Antibiotic resistome alteration by different disinfection strategies in a full-scale drinking water treatment plant deciphered by metagenomic assembly. Environ. Sci. Technol. 53, 2141–2150.
Zhang, W., Hughes, J., Chen, Y.S., 2012. Impacts of hematite nanoparticle exposure on biomechanical, adhesive, and surface electrical properties of *Escherichia coli* cells. Appl. Environ. Microbiol. 78, 3905–3915.
Zhang, W., Rittmann, B., Chen, Y.S., 2011. Size effects on adsorption of hematite nanoparticles on *E. coli* cells. Environ. Sci. Technol. 45, 2172–2178.
Zhang, Y., Gu, A.Z., Cen, T., Li, X., He, M., Li, D., Chen, J., 2018a. Sub-inhibitory concentrations of heavy metals facilitate the horizontal transfer of plasmid-mediated antibiotic resistance genes in water environment. Environ. Pollut. 237, 74–82.
Zhang, Y., Gu, A.Z., Cen, T.Y., Li, X.Y., Li, D., Chen, J.M., 2018b. Petrol and diesel exhaust particles accelerate the horizontal transfer of plasmid-mediated antimicrobial resistance genes. Environ. Int. 114, 280–287.
Zhang, Y., Gu, A.Z., He, M., Li, D., Chen, J.M., 2017. Subinhibitory concentrations of disinfectants promote the horizontal transfer of multidrug resistance genes within and across genera. Environ. Sci. Technol. 51, 570–580.