

# Metallic nanoparticles and ions accelerate the uptake of extracellular antibiotic resistance genes through transformation

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## Research

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# Abstract

**Background:** Antibiotic resistance genes (ARGs), heavy metal ions and nanoparticles (NPs) are emerging and ubiquitous contaminants in the environment. However, little is known about whether heavy metal-based NPs or ions could facilitate the dissemination of ARGs through natural transformation. This study evaluated the contributions of heavy metal-based NPs (Ag NPs, CuO NPs and ZnO NPs) and their ion forms (Ag<sup>+</sup>, Cu<sup>2+</sup> and Zn<sup>2+</sup>) to the transformation of extracellular ARGs in *Acinetobacter baylyi* ADP1.

**Results:** We found that these commonly-used NPs and ions from environmentally relevant concentrations can significantly promote the natural transformation frequency of ARGs by a factor of 11.0-folds, which is comparable to the effects of antibiotics. The enhanced transformation by Ag NPs, CuO NPs, Ag<sup>+</sup> and Cu<sup>2+</sup> was primarily associated with reactive oxygen species (ROS) over-production and cell membrane damage, which was also evident from up-regulations of both transcription and translation of ROS and outer membrane-related genes. Additionally, transmission electron microscope imaging revealed the roughened cell membrane after Ag NPs, CuO NPs, Ag<sup>+</sup> and Cu<sup>2+</sup> exposure. ZnO NPs and Zn<sup>2+</sup> might increase the natural transformation rate by stimulating the stress response and ATP synthesis. All tested NPs and ions resulted in up-regulating the competence and SOS response-associated genes.

**Conclusions:** Our results demonstrate that Ag, CuO and ZnO-based NPs/ions from environmental concentrations could promote the natural transformation of plasmid-encoded ARGs into naturally competent *A. baylyi*. Our findings provide insights into the contributions of heavy metals and NPs to the spread of antibiotic resistance.

## Background

The dissemination of antimicrobial resistance (AMR) is posing a progressing global health crisis. Disquietingly, based on the scenario analysis, the annual deaths induced by AMR-provoked infection are predicted to reach 10 million by 2050 [1]. One of the primary drivers responsible for the increasing prevalence of AMR is the horizontal gene transfer (HGT) of AMR among various bacteria, which is consisted of three mechanisms: i) Conjugation: the dissemination of antibiotic resistance genes (ARGs) from a donor cell to a recipient cell via pilus-driven physical contact; ii) Transformation: recombination of foreign ARGs after bacterial uptake of exogenous genetic materials; and iii) Transduction: phage-mediated DNA transfer upon infection [2].

In particular, transformation can take place in more than 80 naturally competent bacteria with distant phylogenetical backgrounds, even consisting of human pathogens [3]. Although these bacteria share a wide phylogenetic distribution, the key steps involved in transformation among these species are similar, which consist of: capture of exogenous double-stranded DNA (dsDNA) via type IV transformation pilus or type II secretion systems, degradation of dsDNA into single-stranded DNA (ssDNA), internalization of ssDNA via a DNA translocase complex at the cytoplasmic membrane and lastly, the recombination of exogenous DNA after the homology search [4, 5]. In the clinical aspect, antibiotic stressors such as

aminoglycoside and fluoroquinolone could induce the competence for natural transformation in the human pathogen (e.g., *Streptococcus pneumoniae* and *Legionella pneumophila*) [6, 7]. Worryingly, due to the prevalence of extracellular ARGs, antibiotics residual and naturally competent bacteria in the environment, the environmental transformation of ARGs was estimated to be quite frequent, but which has been largely overlooked [8–10].

Apart from antibiotics, recent studies have reported that anthropogenic pollutants such as heavy metals-based nanoparticles (NPs) and the corresponding metallic ions might have contributed to the spread of AMR [11–14]. For instance, heavy metal ions (e.g.,  $\text{Cu}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Hg}^{2+}$  and  $\text{Zn}^{2+}$ ) and nanoparticles (e.g., ZnO NPs,  $\text{Al}_2\text{O}_3$  NPs and Ag NPs) have been reported to induce antibiotic resistance via co-selection or direct mutation [12, 13, 15, 16]. In addition, heavy metal ions (including  $\text{Cu}^{2+}$ ,  $\text{Ag}^+$ ,  $\text{Cr}^{6+}$ ,  $\text{Ti}^{4+}$  and  $\text{Zn}^{2+}$ ) [17–19] and nanoparticles (e.g.,  $\text{Al}_2\text{O}_3$  NPs, CuO NPs, Ag NPs and  $\text{TiO}_2$  NPs) [18–21] could promote the transfer of ARGs via conjugation. However, it is unclear whether heavy metal-based NPs and ions could enhance the dissemination of ARGs via natural transformation. Basically, free-living extracellular ARGs, heavy metal-based NPs and ions ubiquitously co-exist in the same environment (e.g., wastewater treatment plants (WWTPs)) [22, 23]. Thus, it is of significance to evaluate whether the co-existence of heavy metal-based NPs/ions and ARGs could promote the dissemination of ARGs via natural transformation.

In this study, we aim to investigate whether heavy metal-based NPs and ions could promote the dissemination of ARGs via natural transformation. A transformation model was established by using pWH1266 plasmid carrying  $\text{bla}_{\text{TEM-1}}$  and  $\text{tetA}$  as the exogenous ARGs, and *A. baylyi* ADP1 as the recipient, to investigate the effects of heavy metal-based NPs (including Ag NPs, CuO NPs and ZnO NPs) and their ion forms ( $\text{Ag}^+$ ,  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$ ) on natural transformation. The underlying mechanisms were investigated by detecting the oxidative stress, cell membrane permeability and transmission electron microscopy (TEM) imaging, in conjunction with genome-wide RNA sequencing and proteomic analyses.

## Results

### Heavy metal-based NPs and ions increased transformation frequency

A naturally competent opportunistic pathogen *A. baylyi* was used to evaluate the effects of heavy metal-based NPs (including Ag NPs, CuO NPs and ZnO NPs) and their ionic forms ( $\text{Ag}^+$ ,  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$ ) on the transformation of plasmid pWH1266 encoded ARGs. The tested heavy metal and NP concentrations were included the environmentally relevant concentrations (e.g., 0.1 and 1 mg/L). In general, all the tested heavy metal-based NPs and ions could significantly (\*  $p < 0.05$ , \*\*  $p < 0.01$ ) promote the transformation of pWH1266 plasmid into *A. baylyi* at certain exposure levels (Fig. 1A, B and C). For example, the transformation frequencies under 0.1 mg/L  $\text{Ag}^+$  ( $7.5 \pm 1.1 \times 10^{-6}$  per recipient cell, Fig. 1A), 100 mg/L CuO NPs ( $1.5 \pm 0.04 \times 10^{-5}$  per recipient cell, Fig. 1B) and 10 mg/L  $\text{Zn}^{2+}$ -treated group ( $2.9 \pm 0.2 \times 10^{-5}$  per recipient cell, Fig. 1C) were 2.8, 5.6 and 11.0-folds higher than that of the control groups ( $2.7 \pm 0.5 \times$

$10^{-6}$  per recipient cell), respectively. Various NPs and ion types resulted in different trends in terms of transformation frequencies. For Ag and CuO NPs/ions-treated groups, the increments of the natural transformation frequency were concentration-independent when compared to the control groups. For the ZnO NPs/ions-treated groups, a concentration-dependent increase was observed for the natural transformation frequencies when compared to the control groups. From 0.1 mg/L to 100 mg/L, the ZnO NPs/ions-mediated transformation frequencies increased with the increments of ZnO NPs/ions concentrations (Fig. 1C). However, it should be noted that the transformation frequencies of plasmid pWH1266 were significantly (\*  $p < 0.05$ , \*\*  $p < 0.01$ ) decreased under 100 mg/L Ag NPs, 10 and 100 mg/L  $\text{Ag}^+$ , and 10 and 100 mg/L  $\text{Cu}^{2+}$  concentrations (Fig. 1A, B and C).

Multiple approaches were conducted to confirm the uptake of plasmid pWH1266 by *A. baylyi*. Firstly, the minimum inhibitory concentrations (MICs) of recipient wild-type *A. baylyi* and transformants against both Amp and Tet antibiotics were scanned. Since the plasmid pWH1266 encodes resistance genes against ampicillin (Amp) and tetracycline (Tet), transformants should have obtained the resistance against Amp and Tet from the plasmid. As expected, all the transformants exhibited around 15 and 5 folds higher MICs to Amp and Tet, compared to the recipient (Fig. 1D). Secondly, plasmids extracted from the transformants were compared with the donor plasmid by gel electrophoresis. Clear bands from transformants were shown with approximate sizes to the donor (Fig. 1E), indicating that the transformants have received pWH1266 plasmids. Thirdly, the Polymerase chain reaction (PCR) amplification with long amplicons was employed to confirm if the transformants have carried both  $\text{bla}_{\text{TEM-1}}$  and  $\text{tetA}$  genes. All the amplified products from transformants exhibited similar sizes to that of the donor (Fig. 1E), demonstrating that the transformants have harbored plasmid pWH1266.

Collectively, these results confirmed that heavy metal-based NPs and ions could promote the transformation of pWH1266 plasmid into *A. baylyi* at environmentally relevant concentrations.

ROS over-production under the exposure of heavy metal-based nanoparticles and ions

All of the tested Ag, CuO and ZnO NPs/ions were able to increase the intracellular ROS generation of *A. baylyi*. Based on flow cytometer detection results, the recipient strain showed significant (\*  $p < 0.05$ , \*\*  $p < 0.01$ ) concentration-dependent increases of intracellular ROS generation from 0.01 mg/L Ag NPs and CuO NPs, and from 0.1 mg/L  $\text{Ag}^+$  and  $\text{Cu}^{2+}$ , to 100 mg/L Ag and CuO NPs/ions (Fig. 2A and B). More importantly, it was found that the Ag and CuO NPs/ions-increased transformation was correlated with the increase of ROS levels below a certain threshold (approximately around 2-fold of ROS increase compared to the control). For instance, the transformation frequencies of pWH1266 plasmid started to decrease from 10 mg/L of  $\text{Ag}^+$ , Ag NPs and CuO NPs (Fig. 1A and B), at which the ROS generation mediated by the corresponding heavy metal-based NPs and ions was over 2-fold higher than the control groups (Fig. 2A and B). In contrast, the ZnO NPs/ions-facilitated transformation frequencies did not correlate with the ROS generation mediated by ZnO NPs/ions (Fig. 1C and 2C). Moreover, although the ROS generation increased under the exposure of ZnO NPs/ions from 0.01 mg/L, the fold-changes were below 1.3-fold

(Fig. 2C), which is much lower than the increment of ROS mediated by Ag and CuO NPs/ions (e.g., 2.7-fold increase under 100 mg/L Ag<sup>+</sup>, Fig. 2A).

The molecular responses of *A. baylyi* against heavy metal-based NPs/ions were further investigated by using whole-genome RNA sequencing and proteomic analysis. In terms of ROS response, under all heavy metal-based NPs/ions treatments, the expression levels of the 12 known antioxidant system-related genes were mostly up-regulated, especially for the expression of the alkyl hydroperoxide reductase-coding genes *ahpC* and *ahpF* (Fig. 2D, Table S2). To illustrate, in response to the exposures of 1 mg/L Cu<sup>2+</sup>, the expression level of *ahpC* gene were 42.8-fold (i.e., Log<sub>2</sub> fold change (LFC) = 5.42) higher than that of the control. To further validate the oxidative stress response in translational levels, proteomic sequencing was performed and indicated a similar enhancement of the antioxidant system (Fig. 2E, Table S3). Apart from the increased translations of alkyl hydroperoxide reductase *AhpC* and *AhpF*, there is also an upregulation in the translation of catalase *KatA* under all heavy metal-based NPs/ions, in which up to 7.1-fold (i.e., LFC = 2.8) increase was observed when treated with 10 mg/L Zn<sup>2+</sup> (Fig. 2E).

To further verify whether heavy metal-based NPs and ions-mediated ARGs transformations were correlated to the ROS over-production, we then examined the effect of a ROS scavenger, thiourea, on ROS production and ARGs transformation. With 100 μM thiourea added, the ROS production levels were significantly (\* p < 0.05, \*\* p < 0.01) reduced to the extent of control groups across most of the heavy metal-based NPs and ions dosage (Fig. 2F). Correspondingly, the Ag and CuO NPs/ions-mediated ARGs transformation frequencies were significantly (\* p < 0.05, \*\* p < 0.01) decreased to the extent of control groups after thiourea addition, while thiourea did not reduce the ZnO NPs/ions-mediated ARGs transformation to the extent of control groups (Fig. 2G). These results further validated the correlation between ROS over-production and ARGs transformation under the exposure of Ag and CuO NPs/ions.

#### Heavy metal-based nanoparticles and ions increased cell membrane permeability

Cell membrane permeability of heavy metal-based NPs and ions-treated recipient was also evaluated by flow cytometer to verify whether it was associated with transformation enhanced by heavy metal-based NPs and ions (Fig. 3). Similar to the ROS generation, the concentration-dependent increases of membrane permeability could also be detected within Ag and CuO NPs/ions treated *A. baylyi* (Fig. 3A and B). In contrast, there was no significant change across all ZnO NPs/ions-treated *A. baylyi* (Fig. 3C). TEM imaging of *A. baylyi* was also conducted to evaluate the changes in cell membrane morphology under different concentrations of heavy metal-based NPs and ions treatment (Fig. 3D). Compared to the control groups, the increased cell membrane roughness and leakage of cytoplasm could be observed under 1 mg/L Ag<sup>+</sup>, Cu<sup>2+</sup> and CuO NPs dosage. In comparison, no obvious changes in cell membrane morphology could be observed under 10 mg/L ZnO NPs/ions (Fig. 3D).

In terms of RNA transcription, most of the outer membrane-related genes (e.g., 20 out of 23 genes under 1 mg/L Ag<sup>+</sup> treatment) were only moderately altered after 2 h of all heavy metal-based NPs and ions treatment ( $0 \leq |\text{LFC}| \leq 1$ ). As exceptions, the outer membrane assembly gene *bamD* were 1.1 to 1.3-fold

(LFC) up-regulated under Ag and CuO NPs/ions treatment, and the ACIAD0121 and adeK genes were down to -1.49 to -2.54-fold down-regulated under all heavy metal-based NPs and ions treatment (Fig. 3E, Table S4). Differently, the translation of outer membrane-related protein (e.g., 19 out of 22 proteins under 1 mg/L Cu<sup>2+</sup>) after 6 h of all heavy metal-based NPs and ions treatment were mainly up-regulated, especially when under CuO NPs/ions treatment (i.e., ACIAD1141, CvpA and OmpA, Fig. 3F, Table S5). Besides, the translation levels of outer membrane proteins were slightly up-regulated under Ag NPs/ions treatment, while fluctuated under ZnO NPs/ions treatment (Fig. 3F, Table S5).

Heavy metal-based NPs and ions stimulated transcription and translation of competence, stress response, SOS response and ATP production-related genes

The key steps involved in transformation of *A. baylyi* ADP1 are consist of type IV transformation pilus systems (pil gene family), translocase complex at the cytoplasmic membrane (com gene family) and recombination of exogenous DNA after the homology search [4, 5]. The heavy metal-based NPs/ions-mediated transcription and translation response of the genes involved in the competence system were further evaluated (Fig. 4A). Firstly, for competence-related genes, three genes associated with type IV transformation pilus and DNA translocase systems (comEA, pilT and pilU) showed increased transcription (e.g., LFC = 1.9 increase of comEA under 1 mg/L Cu<sup>2+</sup>, Fig. 4B), while three proteins (ComP, PilG and PilH) showed enhanced translation (e.g., LFC = 1.4 increase of ComP under 10 mg/L Zn<sup>2+</sup>, Fig. 4C), under all heavy metal-based NPs/ions dosage. Secondly, in terms of recombination-related genes, the transcription of the majority of these genes (e.g., 16 out of 20 genes under 1 mg/L Ag NPs treatment) were up-regulated under all heavy metal-based NPs/ions dosage, particularly for himA gene with LFC = 1.8 upregulation in response to CuO NPs/ions (Fig. 4B). Thirdly, we found that heavy metal-based NPs/ions dosage elevated the transcription of the majority (e.g., 28 out of 34 genes under 1 mg/L Ag<sup>+</sup> treatment) of SOS response-associated genes (Fig. 4B) and the translation of four SOS response-associated proteins (DnaN, HupB, RecR and Ssb, Fig. 4C). More obviously, ZnO NPs/ions considerably enhanced the transcription of the stress response genes (Fig. 4B), in which the transcription of nirD genes were 9.6-fold (LFC = 3.3) higher than the control group when treated with Zn<sup>2+</sup>. Lastly, although the transcriptions of ATP-related genes were not up-regulated (Fig. 4B), the translations of those ATP-related proteins were largely promoted under CuO NPs/ions and Zn<sup>2+</sup> treatment (Fig. 4C).

## Discussion

### Heavy metal nanoparticles and ions promoted ARGs transformation at environmentally relevant concentrations

In this study, we observed that heavy metal-based NPs/ions exposure could boost the natural transformation phenotype in *A. baylyi* at certain concentrations. Under Ag NPs/ions and Cu<sup>2+</sup> treatment, the transformation frequencies increased at low concentrations (e.g., 0.01 to 1 mg/L Ag NPs), but decreased above a certain threshold (e.g., 10 mg/L Ag<sup>+</sup>), due to the decrease of transformants and recipient numbers caused by bactericidal effect from the corresponding NPs/ion. In addition, the

enhanced transformation is concentration-dependent for ZnO NPs and Zn<sup>2+</sup> exposure. The successful transformation of pWH1266 was confirmed using multiple approaches. Our results suggest that heavy metal-based NPs/ions at environmentally relevant concentrations (e.g., 0.01 mg/L CuO NPs/ions) could promote the natural transformation of ARGs to naturally competent *A. baylyi*.

This study employed multiple approaches to elucidate the underlying mechanisms, including flow cytometry to measure membrane permeability and ROS generation, gene expression analysis by whole-genome RNA sequencing, and quantitative proteomic response analysis. Based on the phenotypic and genotypic data, we proposed the mechanisms underlying heavy metal NPs/ions-promoted natural transformation (Figure 5).

The heavy metal NPs/ions-promoted natural transformation was found to be associated with ROS overproduction, as validated by the reversal of the transformation frequency to baseline levels with the addition of a ROS scavenger-thiourea (Figure 2G), with ZnO NPs as an exception. The observed transformation promoted by heavy metal-based NPs/ions was in agreement with our previous findings that CuO and Ag NPs/ions could enhance conjugative ARGs transfer from *Escherichia coli* to *Pseudomonas putida* via over-generation of ROS (18, 19) Likewise, recent studies have also indicated that solar disinfection and water disinfection by-products could increase the natural transformation rates of extracellular DNA in *A. baylyi* via inducing ROS (24, 25).

In addition, we also observed that increased cell membrane permeability was associated with the CuO and Ag NPs/ions-enhanced transformation efficiency (Figure 3). This damaged cell membrane integrity might lead to the formation of cell membrane channels, and enhance the function of the secretion competence systems for an easier ARGs uptake (26, 27). Indeed, we observed up-regulated transcription (*comEA*, *pilU* and *pilT*, Figure 4A) and translation (ComP, PilG and PilH, Figure 4 B) of competence type IV pilus secretion systems genes under heavy metal-based NPs/ions exposure, which could lead to higher natural transformation rate via pilus-mediated DNA capture and translocation (28) Furthermore, the integration of exogenous DNA into the bacterial chromosome depends on the binding to ssDNA and exchange of DNA strands between recombining DNA helices, also called homologous recombination.(29) In *A. baylyi*, it is estimated that only 0.1% of the acquired DNA fragments could be integrated into the genome successfully (30). Our result showed increased transcription for the majority of the *A. baylyi* recombination systems-associated genes under heavy metal-based NPs/ions exposure (Figure 4A), which might indicate the enhanced homologous recombination of ARGs into the *A. baylyi* genome mediated by heavy metal-based NPs/ions.

Previous research suggested that genetic transformation is induced as a global response to stress, which might be a bacterial strategy to acquire extracellular DNA for endogenous DNA repair mediated by SOS response (31). When exposed to heavy metal-based NPs/ions, *A. baylyi* showed increased expression in stress response-associated genes, such as ACIAD0150 and *dnaK* (32), and enhanced transcription (Figure 4A) and translation (Figure 4B) of SOS response-associated genes. Compared to Ag and CuO NPs/ions, ZnO NPs/ions elicited more up-regulating of stress response-associated genes (Figure 4A). Considering

ZnO NPs/ions-mediated transformation did not correlate with ROS overproduction (Figure 2) and cell membrane damage (Figure 3), ZnO NPs and Zn<sup>2+</sup> might facilitate the natural transformation via provoking the stress and SOS response of *A. baylyi*.

Parts of the competent process, such as ComEA, ComF, PilT and PilF facilitated secretion, are ATP-dependent (4, 33). The CuO NPs/ions and Zn<sup>2+</sup>-mediated increase in translation of ATP synthesis-associated protein might fuel the transformation process (Figure 4B), which was consistent with our previous findings showing triclosan could enhance plasmid conjugation via stimulating ATP production (34).

Collectively, Ag, Cu and Zn-based NPs/ions could facilitate the natural transformation of ARGs into *A. baylyi*, but with shared and different aspects among different types of NPs/ions. For Ag and Cu-based NPs/ions, the increase in the natural transformation frequency was found to be mainly associated with the ROS over-production and cell membrane damage. Differently, the provoked stress response and ATP synthesis might contribute to the increased natural transformation under the exposure of ZnO NPs/ions. Similarly, all tested Ag, CuO and ZnO-based NPs/ions could upregulate the transcription and translation of competence and SOS response-associated genes, thus increasing the natural transformation frequency. Simultaneously, heavy metal-based NPs/ions-stimulated transcription and translation of recombination-associated genes could enhance the homologous recombination of ARGs into the *A. baylyi* genome (Figure 5).

## Environmental implications

The extensive applications of heavy metals and heavy metal-based NPs have resulted in the ubiquitous contamination of heavy metals and NPs in the environment. For example, up to sub-mg/L to mg/L levels of Ag, CuO and ZnO-based NPs/ions could be detected in sewer networks, WWTPs and even in rivers (23). Our results demonstrate that Ag, CuO and ZnO-based NPs/ions could promote the natural transformation of ARGs-carrying plasmid pWH1266 from environmental concentrations (0.01 to 1 mg/L) amongst naturally competent *A. baylyi* by a factor of 11-folds, which is comparable to the effect of antibiotics Mitomycin C (19.8-fold at 0.2 mg/L) and Meropenem 12.1-fold at 0.063mg/L) on promoting the plasmid uptake of *Acinetobacter baumannii* (35). Moreover, heavy metal-based NPs/ions were reported to trigger the co-selection of AMR (12, 36), directly induce AMR via mutagenesis (37, 38), as well as facilitate the lateral transfer of ARGs via conjugation (18-20). Considering the co-occurrence of heavy metal-based NPs/ions and ARGs contamination in the environment, heavy metal-based NPs and ions might pose a substantial risk of AMR dissemination among environmental microbial via co-selection, mutagenesis, conjugation and natural genetic transformation.

## Conclusions

In summary, our results demonstrate that Ag, CuO and ZnO-based NPs/ions from environmental concentrations promote the natural transformation of plasmid-encoded ARGs by naturally competent *A.*

baylyi, which is comparable to the effect of antibiotics. The Ag and CuO NPs/ions-induced enhancements of natural transformation was associated with the ROS over-production and cell membrane damage, which could be prevented by the addition of a ROS scavenger. Contrarily, ZnO NPs/ions could increase the natural transformation through provoking stress response and ATP synthesis. Conclusively, all tested NPs and ions might promote the natural transformation of ARGs by up-regulating the competence and SOS response-associated genes. More in situ assessment on the potential risk of heavy metal-based NPs and ions mediated horizontal transfer of ARGs is recommended.

## Materials And Methods

### Bacterial strains, culture media and nanoparticles

In this study, plasmid pWH1266 (8.89 kbps, ATCC<sup>®</sup> 77092<sup>™</sup>) carrying two ARGs, *tetA* against Tet and *bla*<sub>TEM-1</sub> against Amp, was selected as the extracellular ARG donor. Host *E. coli* cells harbor the plasmid pWH1266 were incubated in LuriaBertani (LB) medium (tryptone, 10 g/L; yeast extract, 5 g/L; NaCl, 10 g/L; pH, 7.4) containing 50 mg/L Amp at 37 °C overnight with shaking (150 rpm). The plasmid of was extracted by the Invitrogen<sup>™</sup> PureLink<sup>®</sup> Quick Plasmid Miniprep Kit (Life Technologies, USA) and the concentration was measured by a NanoDrop (Thermo Scientific, Waltham, MA).

The *Acinetobacter baylyi* ADP1 was selected as the recipient. The *A. baylyi* cells were incubated in LB broth to the stable phase. Subsequently, 1 % of cell suspension was transferred into fresh LB broth and incubated at 30 °C for 6 h with 150 rpm shaking. The bacterial solution was dewatered and washed by phosphate buffer solution (PBS, pH = 7.2) twice, and resuspended in PBS containing 40 mg/L Sodium acetate (OD<sub>600</sub> = 1.1).

Tet was purchased from Sigma-Aldrich (USA), while Amp was purchased from Gold Biotechnology (USA). The Ag NPs solution (NM300, 10.16%, w/w) and the ZnO NPs (NM110) was purchased from Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Germany. According to TEM analysis and UV-Vis spectra, the Ag NPs have a spherical shape, and the average particle size is 15 nm (90% < 20 nm). The ZnO NPs have a hexagonal or cubic morphology (~90% in the 20 – 200 nm range). The CuO NPs were purchased from Sigma-Aldrich (USA), which had a primary particle size of < 50 nm. Prior to the transformation experiments, fresh Ag NPs, CuO NPs and ZnO NPs solutions with serial dilutions were prepared and subsequently ultrasonication-treated (80 W) for 30 min in a water bath (20 °C). The AgNO<sub>3</sub>, CuSO<sub>4</sub> and ZnCl<sub>2</sub> (Sigma-Aldrich) solutions were prepared with sterile Milli-Q water.

### Transformation assays

To assess whether heavy metals and NPs could promote the dissemination of ARGs via natural transformation, a transformation model was established by using pWH1266 plasmid as the exogenous ARGs, and *A. baylyi* ADP1 as the recipient. Specifically, 500 µL transformation systems contained the recipient bacteria (10<sup>8</sup> cfu/mL) and plasmid solution (0.8 ng/µL). Both the initial recipient and plasmid

concentrations were optimized in preliminary experiments. Then, Ag, CuO and ZnO NPs/ions were dosed into each of the 500  $\mu\text{L}$  transformation systems to obtain different final concentrations (0, 0.01, 0.1, 1, 10 and 100 mg/L) and incubated for 6 h at 25 °C without shaking. The number of transformant cells was quantified by plating on LB agar plates containing 100  $\mu\text{g}/\text{mL}$  Amp and 5  $\mu\text{g}/\text{mL}$  Tet after the 48 h incubation. Meanwhile, the total recipient cell number was counted on plain LB agar plates. The transformation frequency was calculated by dividing the number of transformants to the total number of recipients. Fold changes in transformation frequencies in NPs/ions-treated groups were normalized to untreated controls. Each assay was conducted with biological triplicates.

### **Measurements of MICs, ROS, and cell membrane permeability**

In order to confirm the successful transformation, the MICs of *A. baylyi* recipient and transformants against Amp and Tet were measured. To investigate the effects of ROS and cell membrane integrity on the transformation frequencies, both ROS production and cell membrane permeability of the *A. baylyi* were measured by a CytoFLEX flow cytometer (Beckman Coulter, USA) based on the previous procedure [34]. Each experiment was conducted with biological triplicates.

### **Plasmid extraction, PCR and gel electrophoresis**

For the purpose of confirming the uptake of plasmid by transformants, randomly selected triplicate transformants were cultured in LB broth overnight. Plasmids of transformants was extracted by the Invitrogen™ PureLink® Quick Plasmid Miniprep Kit. The *tetA* genes and *bla*<sub>TEM-1</sub> genes encoded on the pWH1266 plasmid were amplified with long amplicon PCR (Bio-Rad C1000 Touch, USA, Table S1), with an initial denaturation at 95 °C for 10 min followed by 30 cycles of amplification with 30 s denaturation at 95 °C, 30 s annealing at 52 °C and extension at 72 °C for 30 s, ending with a final extension at 72 °C for 5 min. The presence of plasmids and amplified products were tested by 1% agarose gel electrophoresis.

### **Transmission Electron Microscope imaging of NPs/ions-treated *A. baylyi***

To evaluate the effects of Ag, CuO and ZnO NPs/ions on cell membrane morphology, *A. baylyi* cells after 2 h treatment with Ag NPs/ions (1 mg/L), CuO NPs/ions (1 mg/L) and ZnO NPs/ions (10 mg/L) and one untreated *A. baylyi* were observed under a JEM-1010 Transmission Electron Microscope (JEOL, USA) at 80 kV according to the method previously described [18].

### **RNA extraction, genome-wide RNA sequencing and bioinformatics**

The transformation systems were established as described above, with the dosages of 0 mg/L (control), 1 mg/L Ag NPs or Ag<sup>+</sup>, 1 mg/L CuO NPs or Cu<sup>2+</sup> and 10 mg/L ZnO NPs or Zn<sup>2+</sup>. The total RNA was extracted by the RNeasy Mini Kit (QIAGEN®, Germany) after a 2-h mating period. In total 21 RNA samples from the control and heavy metal-based NPs/ions-treated groups (3 biological samples from each of the 7 groups) were performed for strand-specific cDNA library construction and Illumina paired-end sequencing (HiSeq 2500, Illumina Inc., San Diego, CA) at Macrogen (Korea), which generated around 800

Mbp data for each sample. The bioinformatics pipeline was reported in our previous study [34] and described in the supporting information. Differences in gene transcriptional values were calculated between untreated and heavy metal NPs/ions-treated group by determining the LFC of the averaged fragments per kilobase of a gene per million mapped reads (FPKM) values.

### **Protein extraction and proteomic analysis**

Another set of transformation systems the same as the RNA extraction treatment described above was set, only the mating time extent to 6 h. Similarly, 21 bacterial samples were harvested by at 12,000 × g centrifugation for 10 min. Protein was extracted using the B-PER method, and protein data were analyzed by the ProteinPilot software (ABSciex, USA), the R-based program Msstats, and PeakView v2.1 (ABSciex, USA). The detailed proteomic sequencing and bioinformatics procedures were reported in our previous study [34] and described in the supporting information.

### **Statistical analysis and data availability**

Data analysis was used by SPSS 19.0 (IBM, Armonk, USA). Significant differences were performed by Independent-samples *t*-test. A value of  $p < 0.05$  was considered significant. Data were expressed as mean ± standard deviation. All sequencing data in this study have been deposited in publicly accessible databases. RNA sequence data were accessible through Gene Expression Omnibus of NCBI (accession no. GSE139295). The mass spectrometry proteomics data were deposited to the ProteomeXchange Consortium via the PRIDE partner repository (PXD012641).

## **Abbreviations**

ARGs: Antibiotic resistance genes; Amp: ampicillin; AMR: antimicrobial resistance; dsDNA: double-stranded DNA; FPKM: fragments per kilobase of a gene per million mapped reads; HGT: horizontal gene transfer; LB: LuriaBertani; LFC: Log2 fold change; MICs: minimum inhibitory concentrations; NPs: nanoparticles; PBS: phosphate buffer solution; PCR: Polymerase chain reaction; ROS: reactive oxygen species; ssDNA: single-stranded DNA; TEM: transmission electron microscopy; Tet: tetracycline; WWTPs: Wastewater treatment plants;

## **Declarations**

### **Acknowledgements**

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### **Authors' contributions**

SZ, JL and JHG conceived and designed the work. SZ, JL and YW analyzed the data. JL wrote the paper, which has been reviewed, edited, and approved by WY, ZGY and JHG.

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## **Availability of data and materials**

All the RNA sequence data were deposited at the NCBI Gene Expression Omnibus of NCBI (accession no. GSE139295). The mass spectrometry proteomics data were deposited to the ProteomeXchange Consortium via the PRIDE partner repository (PXD012641).

## **Competing interests**

The authors declare no competing financial interest.

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## Figures

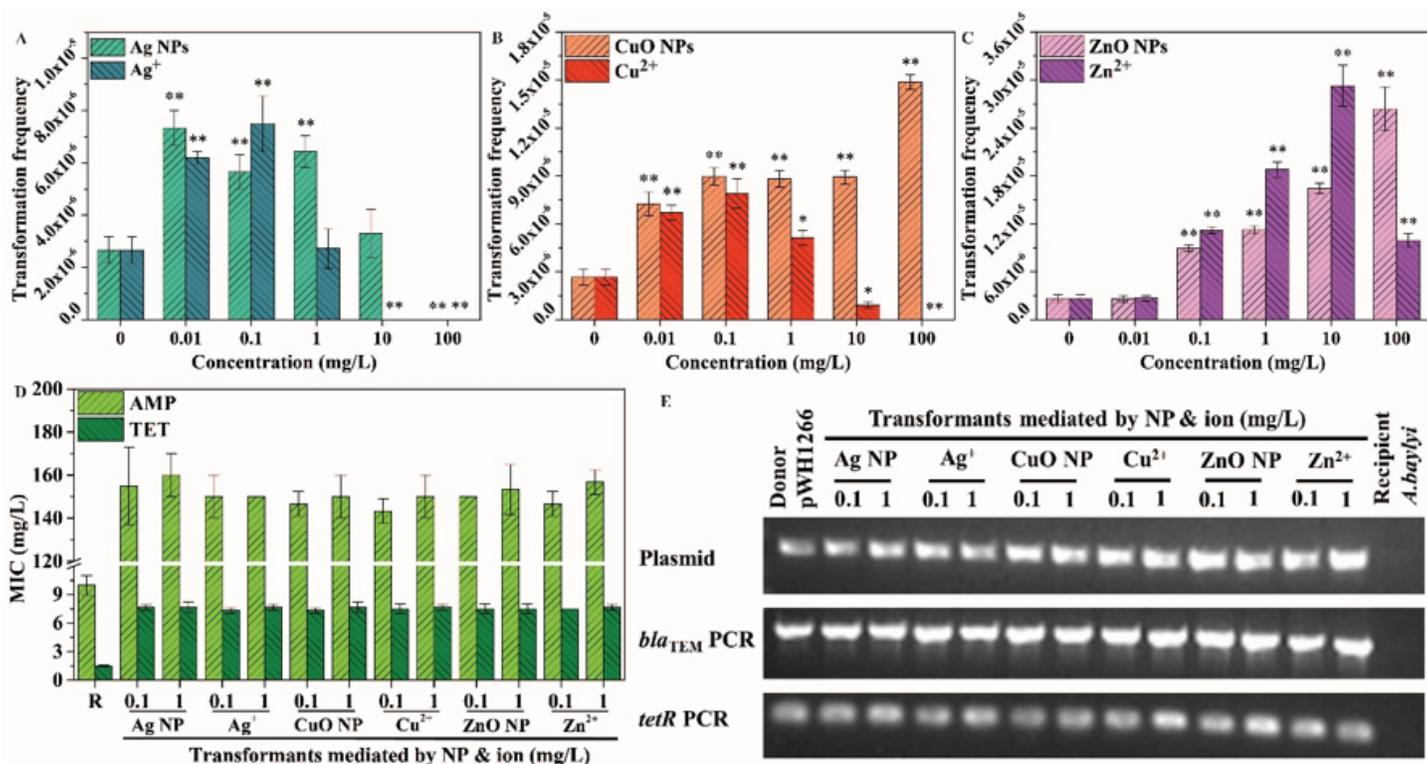
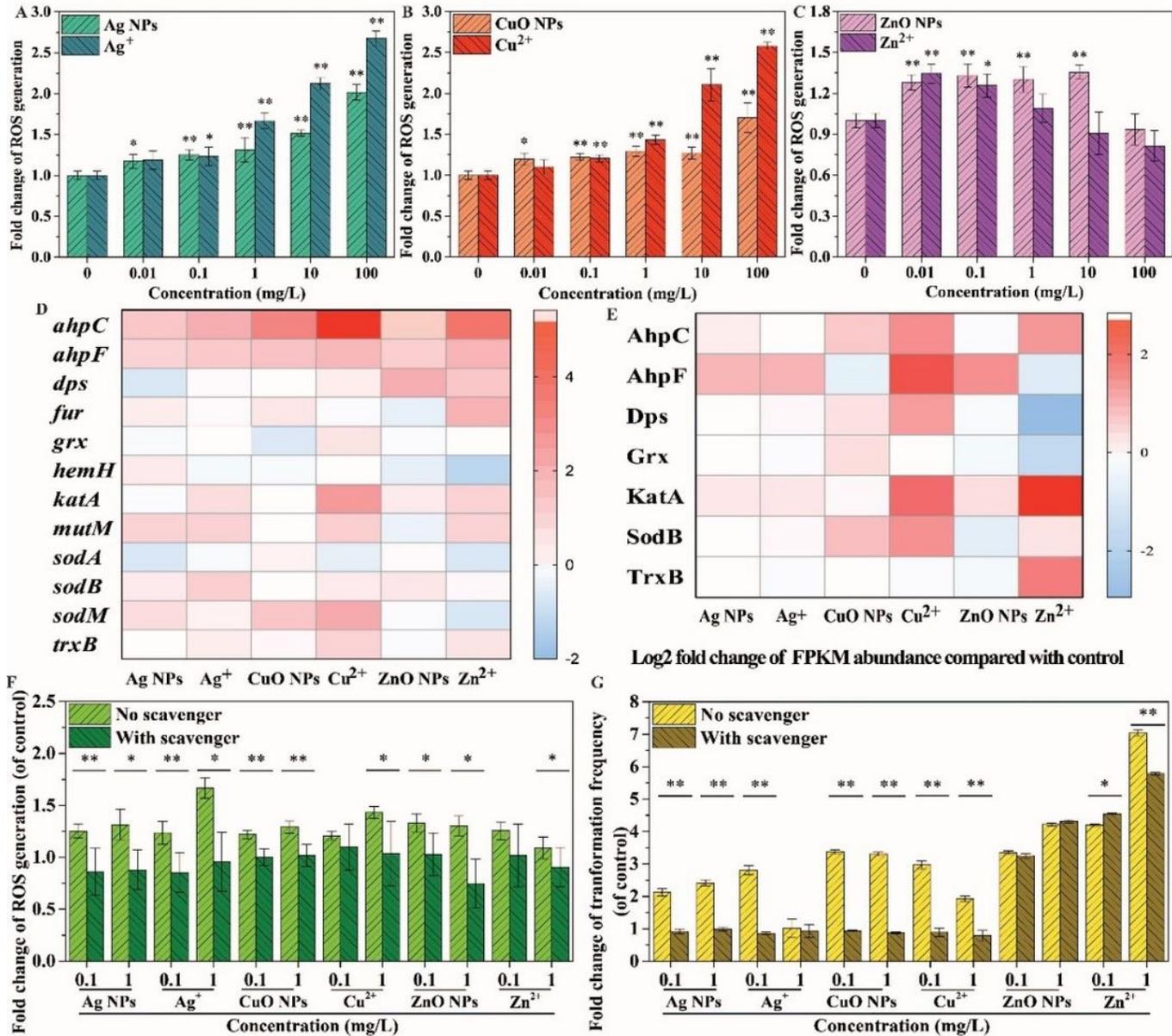


Figure 1

Effects of Ag NPs and Ag<sup>+</sup> (A), CuO NPs and Cu<sup>2+</sup> (B), and ZnO NPs and Zn<sup>2+</sup> (C) on the transformation frequencies of plasmid pWH1266 to *A. baylyi* ADP1 after 6 h. Significant differences between individual heavy metal-based NPs and ions-treated groups and the control groups (0 mg/L) were tested with Independent-sample t test and shown with \* (p < 0.05), \*\* (p < 0.01). D. MICs of the recipient and transformants against ampicillin (AMP) and tetracycline (TET). Results represent the mean ± standard deviation of a minimum of three biological replicate samples. E. Gel electrophoresis of plasmids and PCR on blaTEM-1 and tetA gene on donor pWH1266 plasmid, transformants and recipient.



**Figure 2**

Effects of Ag NPs and Ag<sup>+</sup> (A), CuO NPs and Cu<sup>2+</sup> (B), and ZnO NPs and Zn<sup>2+</sup> (C) on ROS generation in *A. baylyi* ADP1 after 2 h exposure. Significant differences between individual heavy metal-based NPs and ions treated groups and the control groups (0 mg/L) were tested with Independent-sample t test and shown with \* (p < 0.05), \*\* (p < 0.01). Heat-maps showing the transcription (D) and translation (E) of ROS-associated genes under the exposures of heavy metal-based NPs and ions. Effects of ROS scavenger

(thiourea) on the heavy metal nanoparticles/ions mediated ROS generation (F) and transformation of plasmid pWH1266 (G). Significant differences within individual heavy metal-based NPs and ions treated groups with or without scavenger were tested with Independent-sample t test and shown with \* ( $p < 0.05$ ), \*\* ( $p < 0.01$ ). The asterisk brackets indicate significant differences within each NPs/ions dosage with and without the ROS scavenger. Results represent the mean  $\pm$  standard deviation of biological triplicate samples.

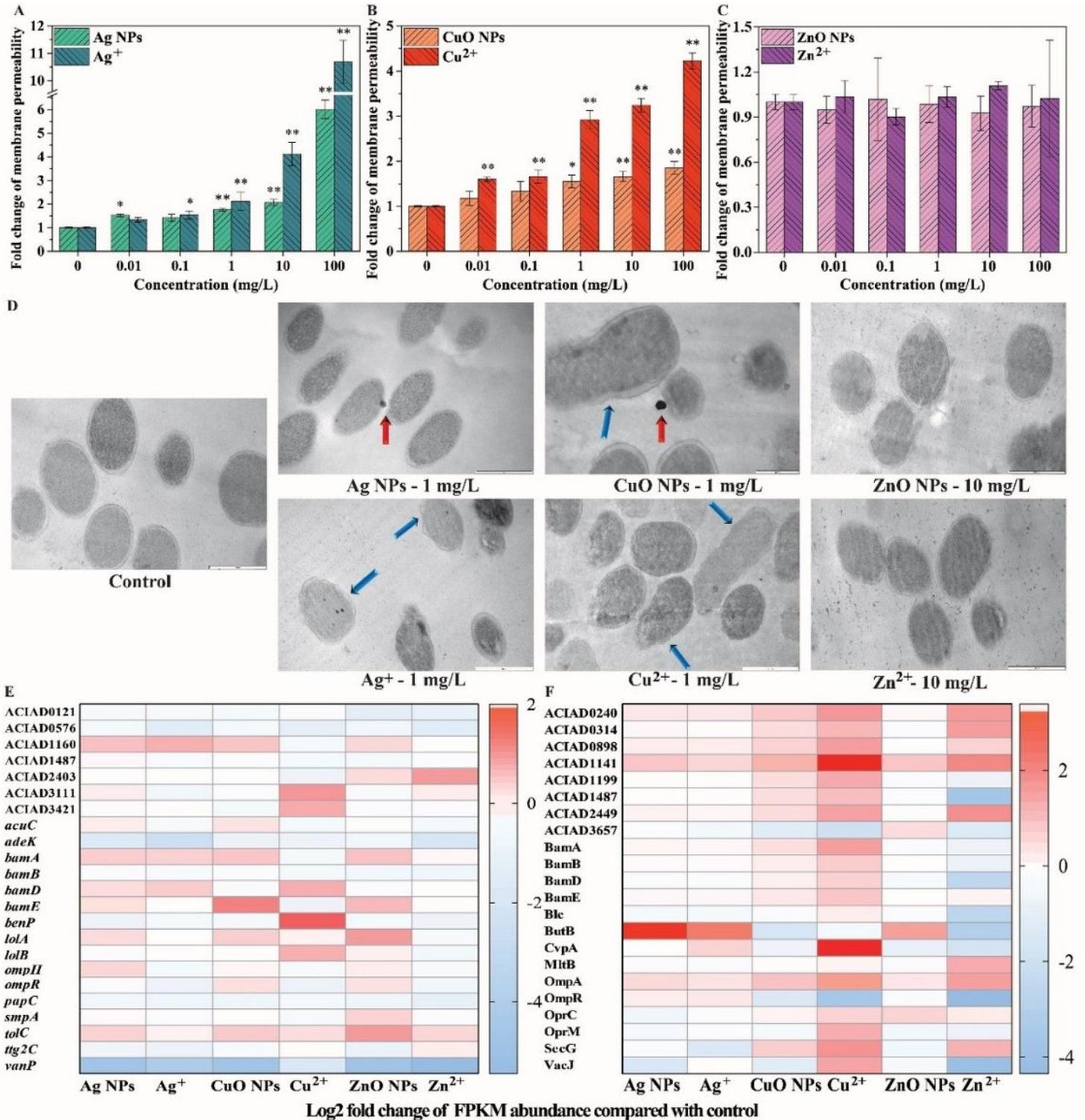
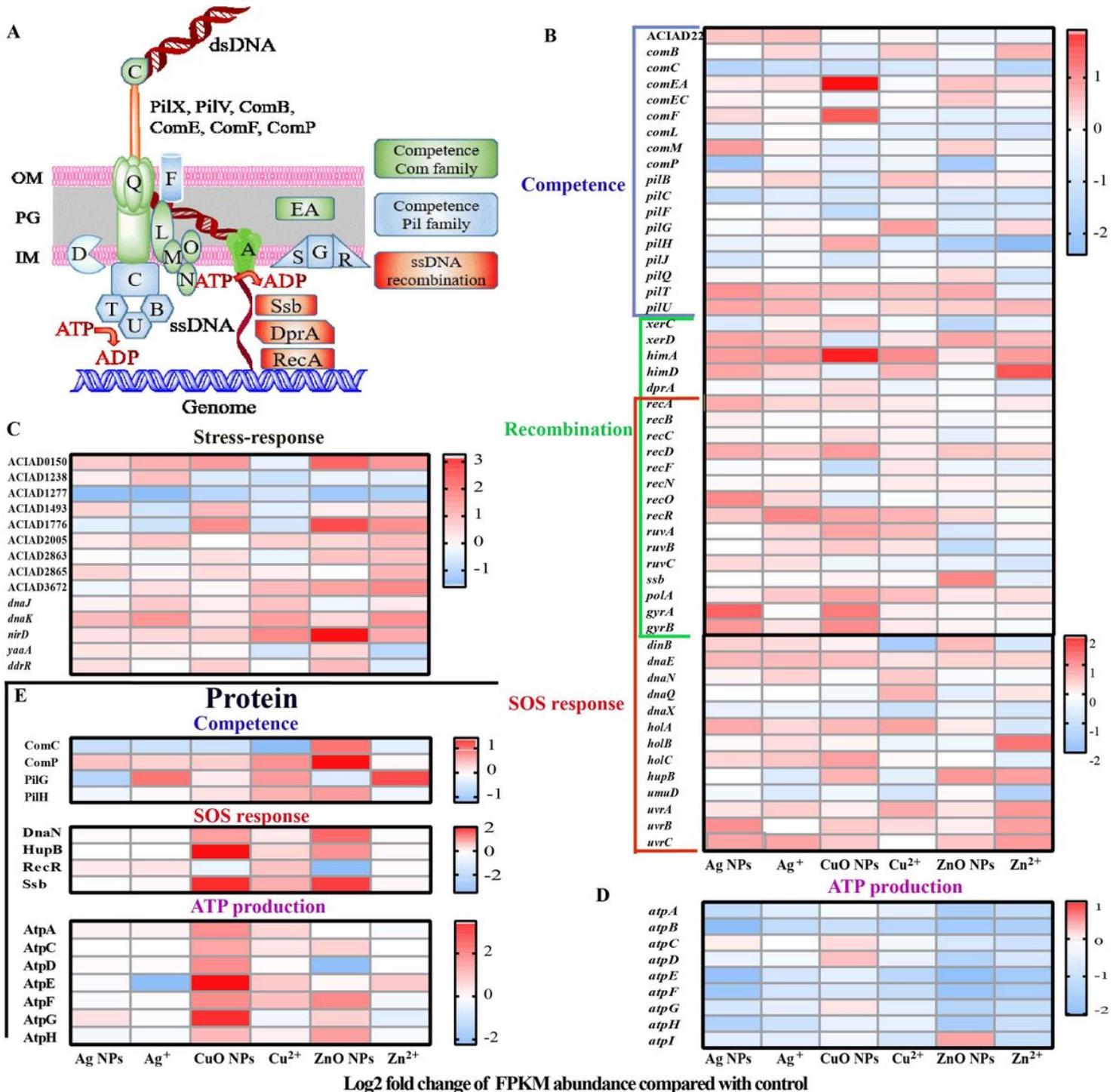


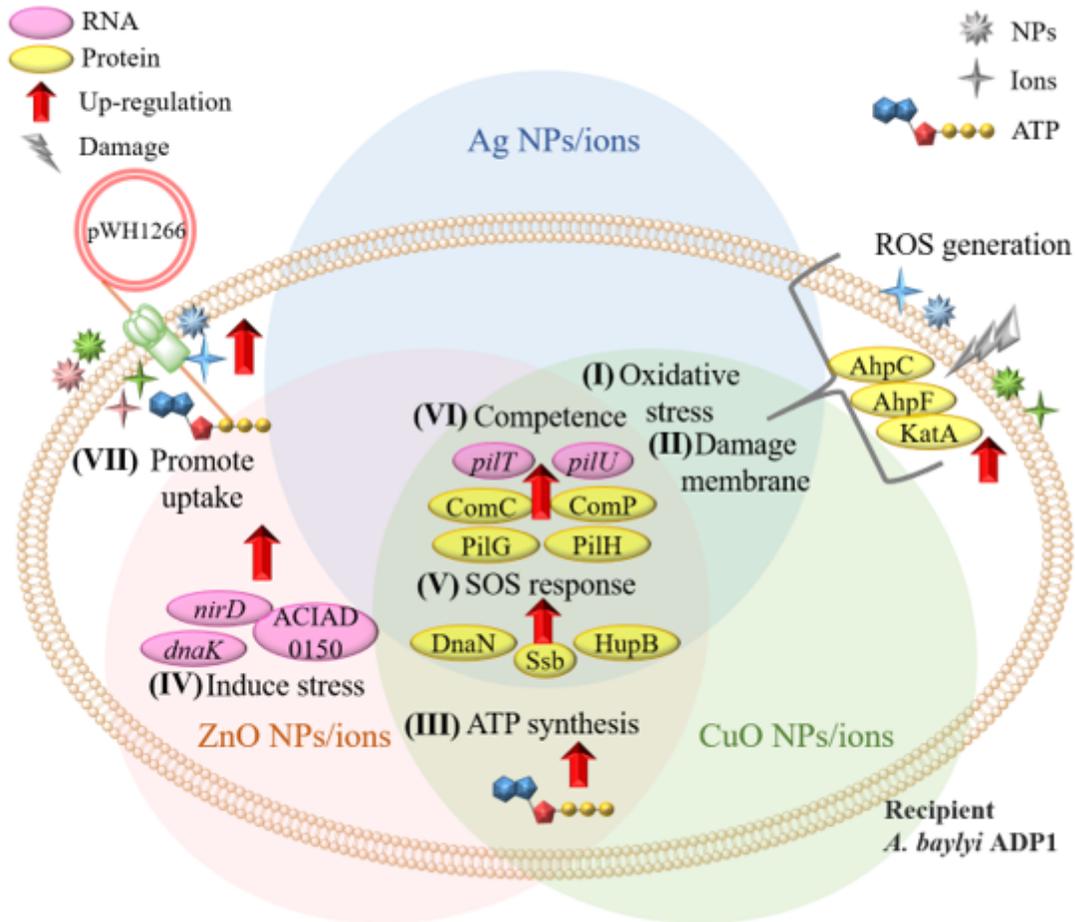
Figure 3

Effects of Ag NPs and Ag<sup>+</sup> (A), CuO NPs and Cu<sup>2+</sup> (B), and ZnO NPs and Zn<sup>2+</sup> (C) on the cell membrane permeability of *A. baylyi* after 6 h, detected by a flow cytometer. Results represent the mean ± standard deviation of biological replicate samples. Significant differences between individual heavy metal-based NPs and ions-treated groups and the control groups (0 mg/L) were determined by Independent-sample t-test and marked with \* (p < 0.05) or \*\* (p < 0.01). D. TEM images of *A. baylyi* in ultrafine slices are shown for the control groups and the cells treated with different concentrations of heavy metal-based NPs and ions for 6 h (scale bars are 1 μm). Red arrows indicate the leakage of cytoplasm and blue arrows indicate the roughened membrane. Heat-maps showing the transcription (E) and translation (F) of outer membrane-associated genes under exposure to various heavy metal-based NPs and ions.



**Figure 4**

A schematic of the *A. baylyi* ADP1 competence system (A). Effects of Ag NPs/ions, CuO NPs/ions and ZnO NPs/ions on the transcription (B) of competence, recombination, SOS response; the transcription of (C) stress response and (D) ATP production-related gene and (E) the translation of competence, SOS response and ATP production-related proteins.



**Figure 5**

Proposed mechanisms of heavy metal-based NPs/ions-promoted natural transformation of pWH1266 plasmid into *A. baylyi* ADP1. Firstly, the exposure to Ag and CuO NPs/ions could induce the (I) oxidative stress to *A. baylyi* that resulted in (II) cell membrane damage, leading to the enhanced plasmid uptake by membrane channels formation. Secondly, CuO and ZnO NPs/ions could (III) stimulate the synthesis of ATP, which can fuel the uptake of plasmid pWH1266. Moreover, ZnO NPs/ions could (IV) induce the stress-response of *A. baylyi* that might initiate the uptake of plasmids. Lastly, all of the three heavy metal-based NPs/ions could up-regulate the transcription and translation of (V) SOS response and (VI) competence-related genes of *A. baylyi*. Consequently, heavy metal-based NPs/ions could (VII) promote the uptake of plasmid pWH1266 by *A. baylyi*.

## Supplementary Files

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