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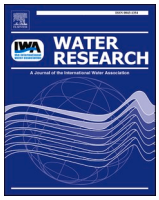

Simultaneous antibiotic removal and mitigation of resistance induction by manganese bio-oxidation process

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ABSTRACT

Microbial degradation to remove residual antibiotics in wastewater is of growing interest. However, biological treatment of antibiotics may cause resistance dissemination by mutations and horizontal gene transfer (HGT) of antibiotic resistance genes (ARGs). In this study, a Mn(II)-oxidizing bacterium (MnOB), *Pseudomonas aeruginosa* MQ2, simultaneously degraded antibiotics, decreased HGT, and mitigated antibiotic resistance mutation. Intracellular Mn(II) levels increased during manganese oxidation, and biogenic manganese oxides (BioMnOx, including Mn(II), Mn(III) and Mn(IV)) tightly coated the cell surface. Mn(II) bio-oxidation mitigated antibiotic resistance acquisition from an *E. coli* ARG donor and mitigated antibiotic resistance inducement by decreasing conjugative transfer and mutation, respectively. BioMnOx also oxidized ciprofloxacin (1 mg/L) and tetracycline (5 mg/L), respectively removing 93% and 96% within 24 h. Transcriptomic analysis revealed that two new multicopper oxidase and one peroxidase genes are involved in Mn(II) oxidation. Downregulation of SOS response, multidrug resistance and type IV secretion system related genes explained that Mn(II) and BioMnOx decreased HGT and mitigated resistance mutation by alleviating oxidative stress, which makes recipient cells more vulnerable to ARG acquisition and mutation. A manganese bio-oxidation based reactor was constructed and completely removed tetracycline with environmental concentration within 4-hour hydraulic retention time. Overall, this study suggests that Mn (II) bio-oxidation process could be exploited to control antibiotic contamination and mitigate resistance propagation during water treatment.

1. Introduction

The spread of antimicrobial resistance (AMR), due in part to the misuse and overuse of antibiotics, is an increasing global threat to public health (Wang et al., 2023b). Antibiotic consumption is on the rise worldwide, and the global antibiotic consumption in 2030 is projected be 200% higher than that in 2015 (Klein et al., 2018). This selective pressure increases the risk of antibiotic resistance development and dissemination (Baym et al., 2016; Levin-Reisman et al., 2017). Microbial degradation is a major process for the removal of antibiotics in natural environments (Gothwal and Shashidhar 2015) and wastewater treatment plants (Dong et al., 2016). However, biological treatment of antibiotic pollution faces the critical challenge of AMR dissemination due horizontal gene transfer (HGT) of antibiotic resistance genes (ARGs), enhanced by selective pressure from residual antibiotics (Grenni et al., 2018; Larsson and Flach 2022; Mao et al., 2015; Wang and Chen 2022). It is therefore important to develop biological processes that both degrade antibiotics and mitigate AMR dissemination for water treatment.

Mn oxides can oxidize many organic compounds (Li et al., 2021), and are frequently used for water treatment and environmental purification (Guan et al., 2022). Mn oxides were also reported to mediate the oxidative transformation of complex humic substances (Sunda and Kieber 1994), polycyclic aromatic hydrocarbons (Zhang et al., 2021a), carbamazepine (Wang et al., 2023a), and antibiotics (Du et al., 2020). Biological oxidation of Mn(II) occurs several orders of magnitude faster than abiotic processes and have gained increasing attention with regard to their environmental applications (Zhou and Fu 2020). Biogenic manganese oxides (BioMnOx) can mediate the degradation of ofloxacin through catalyzing the heterogeneous photo-Fenton (Du et al., 2020), degradation of 17α-ethinylestradiol by direct oxidation (Tran et al., 2018) and degradation of 2,4-dimethylaniline through peroxymonosulfate activation (Zhang et al., 2019). However, BioMnOx is usually separated from cells to abiotically degrade antibiotics, which

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could be a missed opportunity to synergize such chemical processes with antibiotic biodegradation during wastewater treatment.

In addition to degrading antibiotics, BioMnOx could also protect bacteria from environmental stressors, such as UV radiation, predation, viral attack or heavy metal toxicity by coating the outside of the bacteria (Tebo et al., 2005). Antibiotics usually contain electron-donating groups and easily form complexes with metal ions such as manganese (Liao et al., 2023). Mn oxides adopt various crystal forms, and poorly crystalline BioMnOx exhibits high sorption capacity for many organic compounds, including antibiotics (Jiang et al., 2010; Stuckey et al., 2018). The effects of this sorption on selection pressure exerted by antibiotics is unknown. Moreover, Mn(II) could also protect bacteria from reactive oxide species (ROS) by acting as an antioxidant (Archibald and Fridovich 1981a,b). For example, manganese oxidation in *Pseudomonas putida* GB-1 offered oxidative stress protection through increased intracellular manganese (Banh et al., 2013). *Deinococcus radiodurans* R1 also enhanced its resistance to ionizing radiation by accumulating high intracellular manganese (Daly et al., 2004). Oxidative stress induced by pollutants is a key factor that induces mutation and facilitates horizontal gene transfer of ARGs (Shi et al., 2020; Wang et al., 2023b). We previously demonstrated that carotenoids mitigate enrichment and horizontal gene transfer of ARGs by decreasing oxidative stress that makes recipient cells more vulnerable (Ren et al., 2018, 2021, 2022; Ren et al., 2023). Whether Mn plays a similar role as other antioxidants, such as carotenoids, in AMR mitigation is worth investigating.

In this study, a newly isolated Mn(II)-oxidizing bacterium (MnOB), *Pseudomonas aeruginosa* strain MQ2, was shown to oxidize Mn(II) by two multicopper oxidases (MCOs) and a manganese-containing superoxide dismutase (MnSOD). Morphology and distribution of BioMnOx were characterized by scanning electron microscopy (SEM), Thin-section transmission electron microscopy (TEM), X-ray photoelectron spectroscopy (XPS) and X-ray diffraction (XRD). Degradation of tetracycline and ciprofloxacin was quantified, and resistance mitigation was investigated by determining changes in minimum inhibitory concentrations of *P. aeruginosa* strain MQ2 and conjugal transfer frequency of ARGs. ROS was measured to assess oxidative stress protection by Mn(II) oxidation. Transcriptomic analyses were also performed to identify genes that contribute to Mn(II) oxidation and infer mechanisms underlying antibiotic degradation and resistance mitigation.

2. Materials and methods

2.1. Isolation and identification of Mn(II)-oxidizing bacteria (MnOB)

Soil samples were collected from the courtyard of the Anqing Yangtze Delta Future Industry Institute, China. Soil (5 g dry weight) was added to a 250 mL Erlenmeyer flask containing 50 mL Luria-Bertani (LB) medium (pH 7.0) supplemented with 200 mg/L manganese chloride. The flask was incubated aerobically at 35 °C with vigorous shaking at 180 rpm. A 5 mL suspension was transferred to 45 mL fresh LB medium every 48 h and incubated under the same conditions. Pure culture was obtained by plating the enrichment culture onto LB solid medium containing 200 mg/L manganese chloride. Dark brown colonies were preliminarily identified as Mn(II)-oxidizing bacteria. Isolated strains were identified by 16S rRNA gene sequencing using universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The sequences were aligned and compared with available sequences in the GenBank database using BLAST accessed through the National Center for Biotechnology Information (NCBI). The screened Mn(II)-oxidizing strain was belonged to *Pseudomonas aeruginosa*, and we designated it strain MQ2.

2.2. Mn(II) bio-oxidation and characterization of biogenic manganese oxides (BioMnOx)

Strain MQ2 was cultivated aerobically in LB medium 35 °C for 24 h and 48 h and harvested by centrifugation (8000 rpm for 5 min). Cell pellets were washed three times with phosphate buffer solution (PBS) and fixed with 2.5% glutaraldehyde overnight at 4 °C. After washing with PBS, the samples were dehydrated by using increasing concentrations of ethanol. Then, the samples were observed using scanning electron microscopy (SEM; SU8020, HITACHI, Japan) equipped with a HOTIBA EMAX mics2 energy-dispersive X-ray spectroscopy (EDS) system to capture the distribution of the elemental composition of BioMnOx displayed on the cell surfaces. The ultrathin section of the embedding block was obtained by ultramicrotome. Transmission electron microscopy images and EDS were taken using a TEM (Tecnai G2 F30, FEI, USA) equipped with an XPLORE EDS system to capture the distribution of the elemental composition of intra- and extra-cellular BioMnOx. The valence states of Mn in BioMnOx were determined by X-ray photoelectron spectroscopy (XPS, ESCALAB 250XI+, Thermo Fisher Scientific, USA). For X-ray diffraction (XRD) analysis, the sample was scanned over the range of 2θ from 5° to 90° by an X-Ray diffractometer (18 kW, MiniFlex 600, Rigaku, Japan).

2.3. Determination of effects on growth of manganese bio-oxidation

The isolated *P. aeruginosa* strain MQ2 was precultured in LB medium to mid-exponential phase. Then, it was harvested, washed twice with PBS, and transferred into 250 mL Erlenmeyer flasks containing 100 mL of LB medium to an OD_{600} of approximately 0.01. LB medium was supplemented with 100 mg/L $MnCl_2$, 900 μg mL^{-1} ciprofloxacin (Cip) or both $MnCl_2$ and Cip with the same concentration, respectively. Unsupplemented LB medium was used as the control. The flasks were incubated at 35 °C with shaking at 180 rpm, and OD_{600} was measured every 1 or 2 h. All tests were conducted in triplicate.

2.4. Determination of minimum inhibitory concentrations (MICs) and isolation of resistant mutants

The MICs of the ancestor strain to 5 different types of antibiotics: ampicillin (Amp), tetracycline (Tet), ciprofloxacin (Cip), norfloxacin (Nor) and kanamycin (Kana) were determined following standardized protocols of MIC tests (Wiegand et al., 2008) (Table S1). Briefly, *P. aeruginosa* strain MQ2 was precultured in LB medium overnight and diluted to a cell density of $OD_{600} \approx 0.1$, then inoculated into fresh LB medium containing antibiotics at a series of concentrations. The tested cell cultures were incubated at 35 °C for 20 h, and OD_{600} was measured. MIC was defined as the lowest concentration that can completely inhibit cell growth (Kohanski et al., 2010). Because the resistance of the ancestral strain to Cip was the lowest (MIC of 1100 μg/L), Cip was used to determine resistance evolution. LB medium containing 100 μg/L Cip and 100 mg/L $MnCl_2$ was used to determine the effects of Mn(II) on changes in resistance. To collect BioMnOx precipitate, cultures of *P. aeruginosa* strain MQ2 were harvested after 48 h, and washed with ultrapure water five times and centrifuged at 8000 rpm for 15 min to remove impurities. The collected BioMnOx was stored at 4 °C before experiments. To determine the effects of BioMnOx on changes in resistance, 100 mg/L BioMnOx was added to the LB medium. LB medium without $MnCl_2$ or BioMnOx was used as the control. To isolate resistant mutants, diluted cell cultures were spread on LB agar plates containing Cip of 1 × MIC after 250 and 500 generations. Colonies that could grow on the selective plates were considered antibiotic-resistant mutants. Ten mutants were randomly picked up from the control and the manganese oxidation system, and the MICs of these resistant mutants were further determined using gradient antibiotic concentrations as described above.

2.5. Determination of effects of manganese bio-oxidation on conjugative transfer of ARGs

Escherichia coli DH5α, carrying the RP4 plasmid carrying a tetracycline efflux pump gene (*tetA*), an aminoglycoside phosphotransferase

gene (*aphA*), and a β-lactamase gene (bla_{TEM-2}) was chosen as the donor. A mutant strain of *P. aeruginosa* strain MQ2 that can resist 10 mg/L Cip was chosen as the recipient. Considering that *P. aeruginosa* strain MQ2 harbored high resistance to Tet and Amp, 100 μg/L Kana was used to provide the selection stress to the conjugal system. After preculturing in LB medium, the donor and recipient cell pellets were harvested by centrifugation and washed twice with PBS. Next, the cell pellets were resuspended separately in PBS to OD_{600} of 1.0, and mixed at the ratio of 1:1. To determine the effects of Mn(II) on conjugal transfer, 100 mg/L $MnCl_2$ was added to the conjugal system. BioMnOx collected above was added to the conjugal system at a concentration of 100 mg/L to determine the effects of BioMnOx on conjugal transfer. Conjugal transfer system without Mn(II) and BioMnOx was the control, and all the conjugal transfer systems were incubated for 8 h at 35 °C. To count transconjugants, suspensions of conjugal transfer system were spread on LB agar plates containing 50 mg L^{-1} Amp, 50 mg L^{-1} Kana, 40 mg L^{-1} Tet and 10 mg L^{-1} Cip. To count the recipients, suspensions of conjugal transfer system were spread on LB agar plates containing 10 mg L^{-1} Cip. The transfer frequency was calculated by dividing transconjugants over recipients. Conjugative transfer experiments were conducted in triplicate.

2.6. Measurement of ROS generation and determination of the effects of ROS on Mn(II) oxidation

The bacterial suspension of *P. aeruginosa* strain MQ2 was incubated at 37 °C for 30 min in the dark with 2′,7′-dichlorofluorescein diacetate (DCFDA; Sigma-Aldrich) at a final concentration of 20 μM. Then, the bacterial suspension of *P. aeruginosa* strain MQ2 was divided into six equal parts, five of which were treated with 100 mg/L $MnCl_2$, 100 μg/L Cip, both $MnCl_2$ and Cip, 100 mg/L BioMnOx or both BioMnOx and Cip. Bacterial suspension without treatment was used as the control, and all tests were performed in triplicate. After completely mixing, the mixtures were incubated again at 37 °C for 2 h in the dark and transferred to 96-well plates (Corning®, China). A microplate reader (TECAN, Austria) was used to measure the fluorescence intensity with excitation at 488 nm and emission at 525 nm. Relative fold changes of ROS were calculated by dividing the fluorescence intensity of each treated group over the control. To investigate the effects of ROS on Mn(II) oxidation, *P. aeruginosa* strain MQ2 was precultured in LB medium overnight and suspended in 0.9% NaCl solution to a cell density of $OD_{600} \approx 1.0$. The suspension was supplemented with manganese chloride to a final concentration of 100 mg/L, and Cip (100 μg/L) or thiourea, an ROS scavenger (final concentration of 100 μM), were added to increase or decrease ROS, respectively. The concentration of Mn(II) was measured using the potassium periodate spectrophotometric method (Chen and Zhang 2013) every 6 h after filtering with a 0.22 μm filter membrane.

2.7. Antibiotic degradation experiments

P. aeruginosa strain MQ2 was precultured in LB medium with or without 100 mg/L $MnCl_2$ for 48 h and harvested by centrifuging at 8000 rpm for 5 min. Cell pellets were washed twice with PBS and suspended in carbon-free mineral medium to a cell density of $OD_{600} \approx 1.0$. Cells precultured in LB medium with $MnCl_2$ were used as the BioMnOx group, and cells precultured in LB medium without $MnCl_2$ were used as the control. From each, a 50 mL suspension was transferred to a 250 mL Erlenmeyer flask with Tet and Cip at a final concentration of 5.0 and 1.0 mg/L, respectively. The degradation experiments were conducted in triplicate and incubated at 35 °C with shaking at 180 rpm. To monitor the degradation of the two antibiotics, 1.5 mL samples were taken every 12 h and filtered with 0.22 μm filter membrane to remove biomass. Concentrations of the two antibiotics were analyzed using high performance liquid chromatography (1260 infinity II, Agilent, USA) equipped with a VWD detector. A Poroshell 120 EC-C18 (4.5 × 100 mm, 4 Micon Agilent) was used for Cip detection. The mobile phase consisted of 15% acetonitrile and 85% 0.1% formic acid supplied at a constant total flow rate of 0.4 mL/min. The detection wavelength was set at 275 nm, the injection volume was 50 μL, and the temperature of the chromatographic column was maintained at 35 °C. A Zorbax Eclipse XDB-C18 (4.6 × 250 mm, 5 Micon Agilent) was used for Tet detection. The mobile phase consisted of 82% 0.1% phosphoric acid and 18% acetonitrile supplied at a constant total flow rate of 0.4 mL/min. The detection wavelength was set at 355 nm, the injection volume was 10 μL, and the temperature of the chromatographic column was maintained at 30 °C. Data are reported as the means of the triplicate experiments.

2.8. Genome and transcriptome sequencing

P. aeruginosa strain MQ2 was precultured in LB medium overnight and harvested by centrifuging at 8000 rpm for 5 min. DNA was extracted from cell pellets using a DNeasy PowerSoil Kit (Qiagen) following the manufacturer's instructions. The harvested DNA was visualized using agarose gel electrophoresis and quantified by Qubit® 2.0 Fluorometer (Thermo Scientific). The whole genome of *P. aeruginosa* strain MQ2 was sequenced using an Illumina NovaSeq generating 150-bp paired-end reads. High-quality reads were assembled with SOAP denovo-V2.04 (Li et al., 2008), SPAdes-V3.15.4 (Bankevich et al., 2012) and Abyss-V2.1.5 (Simpson et al., 2009) software. The assembly results of the three softwares were integrated with CISA, and the gap of preliminary assembly results was filled with gapclose. GeneMarkS was used to retrieve the coding sequences (Besemer et al., 2001). Whole genome Blast (Li et al., 2002) search was performed against GO, KEGG, COG, NR, TCDB, and Swiss-Prot databases to predict gene functions. The genome sequencing raw data of *P. aeruginosa* strain MQ2 was deposited in the NCBI Sequence Read Archive (accession number PRJNA967589).

RNA sequencing was used to assess the impact of manganese and antibiotics on transcription. Four exposure conditions were created from a suspension of *P. aeruginosa* strain MQ2 in PBS: the control (without treatment), $MnCl_2$ (with 100 mg/L $MnCl_2$), antibiotic (with 5 mg/L Tet and 1 mg/L Cip) and BioMnOx-antibiotic (with 5 mg/L Tet and 1 mg/L Cip and BioMnOx). The bacterial suspension of the BioMnOx-antibiotic condition was precultured in LB medium with 100 mg/L $MnCl_2$ for 48 h. Each condition had 6 replicates. After treating 2 h, triplicates of each group were mixed together and RNA was extracted using a RNeasy PowerSoil Total RNA Kit (QIAGEN, Germany), resulting in 2 RNA samples for each condition. A BioAnalyzer (Agilent) was used to determine the amount and quality of the extracted RNA. Libraries were generated using NEBNext Ultra RNA Library Prep Kit and sequencing was performed using an Illumina NovaSeq 6000 generating 150-bp paired-end reads. All RNA sequencing raw data were deposited in the NCBI Sequence Read Archive (accession number PRJNA967836). HTSeq v0.6.1 was used to count the reads from each gene. The expected number of Fragments Per Kilobase of transcript sequence per Millions base pairs (FPKM) was calculated based on the length of the gene and read counts. Differential expression analysis of two groups was performed using the DESeq R package (1.18.0). Genes with an adjusted *P*-value <0.05 found by DESeq were assigned as differentially expressed.

2.9. Antibiotic removal in simulated wastewater effluent

A glass reactor with 800 mL working volume was used for investigating antibiotic removal in simulated effluent of wastewater. The reactor was randomly filled with polyurethane sponge cubes (each 2 × 2 × 2 cm^3) to provide substratum for strain MQ2 and prevent rapid loss of biomass. The biomass of strain MQ2 was collected from LB medium and then inoculated into the reactor to a OD_{600} of approximately 1.0. The simulated effluent was prepared with the initial concentrations of chemical oxygen demand (COD), total nitrogen (TN) and total phosphorus (TP) of 50, 15 and 0.5 mg/L, respectively, based on the Integrated Wastewater Discharge standard of P.R. China (Grade I A). The reactor was pre-operated with the simulated effluent containing 100

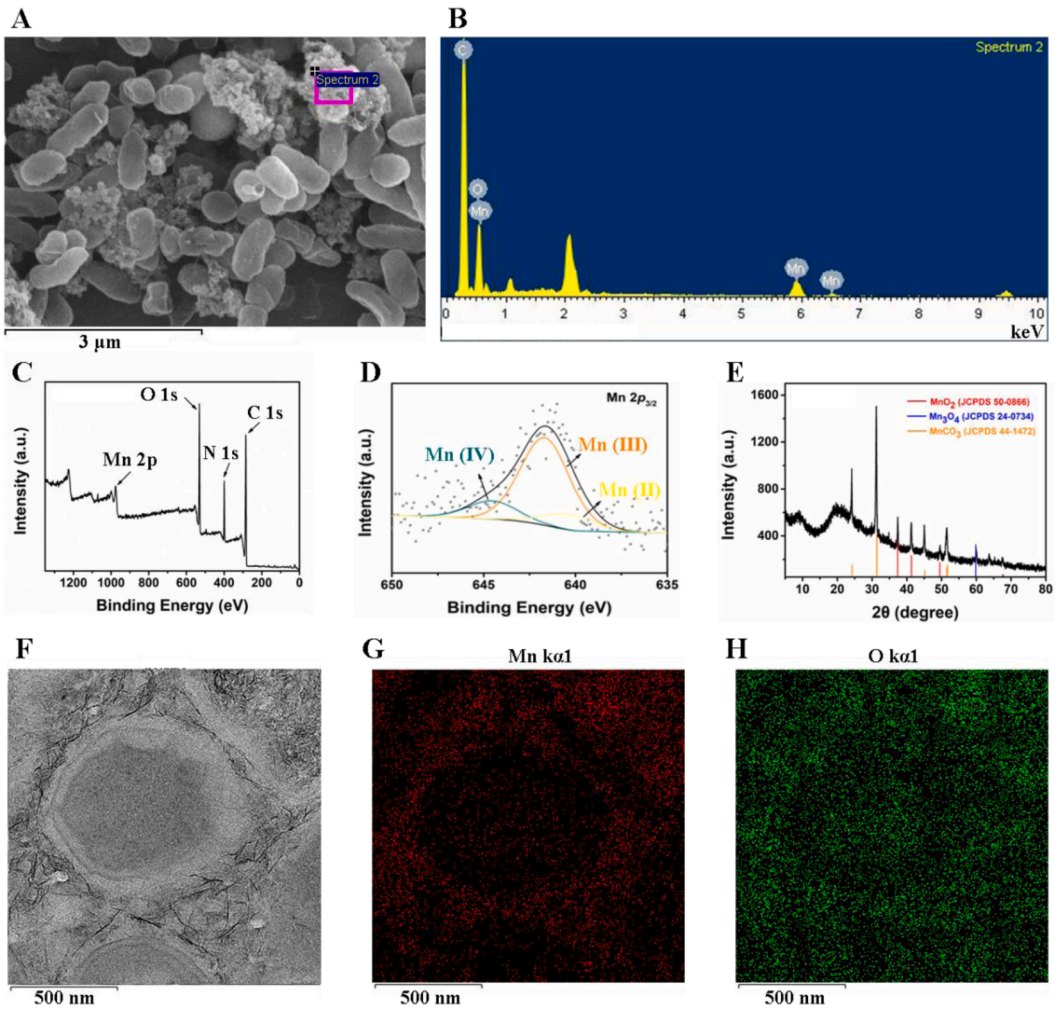

Fig. 1. Characterization of BioMnOx formed by *P. aeruginosa* strain MQ2. (A) SEM image showed the presence of solid among strain MQ2 cells, and EDS spectra of selected area of the solid confirmed the presence of Mn, O and C elements (B). (C) and (D) XPS survey revealed the presence of Mn(II), Mn(III) and Mn(IV) in BioMnOx. (E) XRD pattern revealed the presence of MnO_2, Mn_3O_4 and $MnCO_3$ in BioMnOx. (F) Thin-section TEM image showed that the cells are tightly packed by BioMnOx, and EDS mapping profiles suggested the presence of Mn (G) and O (H) elements in both intracellular and extracellular cells.

mg/L $MnCl_2$ for 48 h to form BioMnOx, then 100 μg/L tetracycline was added to simulate antibiotic pollution in actual wastewater. The hydraulic retention time was set as 4 h with a continuous flow water inlet method. The concentration of COD and tetracycline was measured once a day, and a same reactor without strain MQ2 was used as the control.

3. Results and discussion

3.1. *P. aeruginosa strain MQ2 genome encodes two novel Mn(II)-oxidizing multicopper oxidases*

P. aeruginosa strain MQ2 was isolated from soil by enrichment culture supplementing with a high concentration of Mn(II). The pure culture was obtained by successive plate streaking of dark brown colonies. The whole genome was sequenced to identify Mn(II) oxidation-related genes and compared with other Mn oxidizers. There are no previous reports of *P. aeruginosa* oxidizing Mn(II). The genome size of strain MQ2 was 6.39 Mb with G + C content of 66.45%, and the annotated genome contains 5140 protein-coding sequences. In the genome of the most well-studied model Mn oxidizers *Bacillus* sp. strain SG-1(Dick et al., 2008), *Pseudomonas putida* GB1 (Banh et al., 2013), and *Leptothrix discophora* strain SS-1 (Brouwers et al., 2000a), multicopper oxidases (MCO) are the essential genes responsible for Mn(II) oxidation (Brouwers et al., 2000b). MCOs are a class of Cu enzymes and have substantial amino acid sequence similarity in the regions containing Cu-binding ligands (Geszvain et al., 2012). Strain MQ2 has six MCO-like genes. Two of them exhibited high conservation in the copper-binding sites based on alignment with different MCOs in other Mn oxidizers, although overall sequence similarity was only 25.71–65.64% (Figure S1). Two conserved regions are located near the C terminus and separated by 41 amino acids; the other two are near the N terminus and separated by 42 and 44 residues. Both the N- and C-terminal regions contains a conserved HXH motif, suggesting the two MCOs in strain MQ2 accord with the characteristics of general MCOs in other Mn oxidizers. These two MCOs may be responsible for Mn(II) oxidation.

3.2. *Encapsulation of extracellular BioMnOx and the increase of intracellular manganese levels may mitigate the AMR induction by decreasing oxidative stress*

SEM images of strain MQ2 cultured in Mn(II)-containing medium showed extensive mineral precipitation around cells (Fig. 1A), and Mn, O and C were detected in these solid by EDS (Fig. 1B), suggesting formation of manganese oxide and manganese carbonate. Mn increased over time, as evidenced by comparing samples collected after 24 and 48 h (Figure S2). XPS analysis (Fig. 1C and D) indicated that there were three manganese species in the Mn-containing solid: Mn(II), Mn(III) and Mn(IV). The crystal structures of the Mn-containing solid were

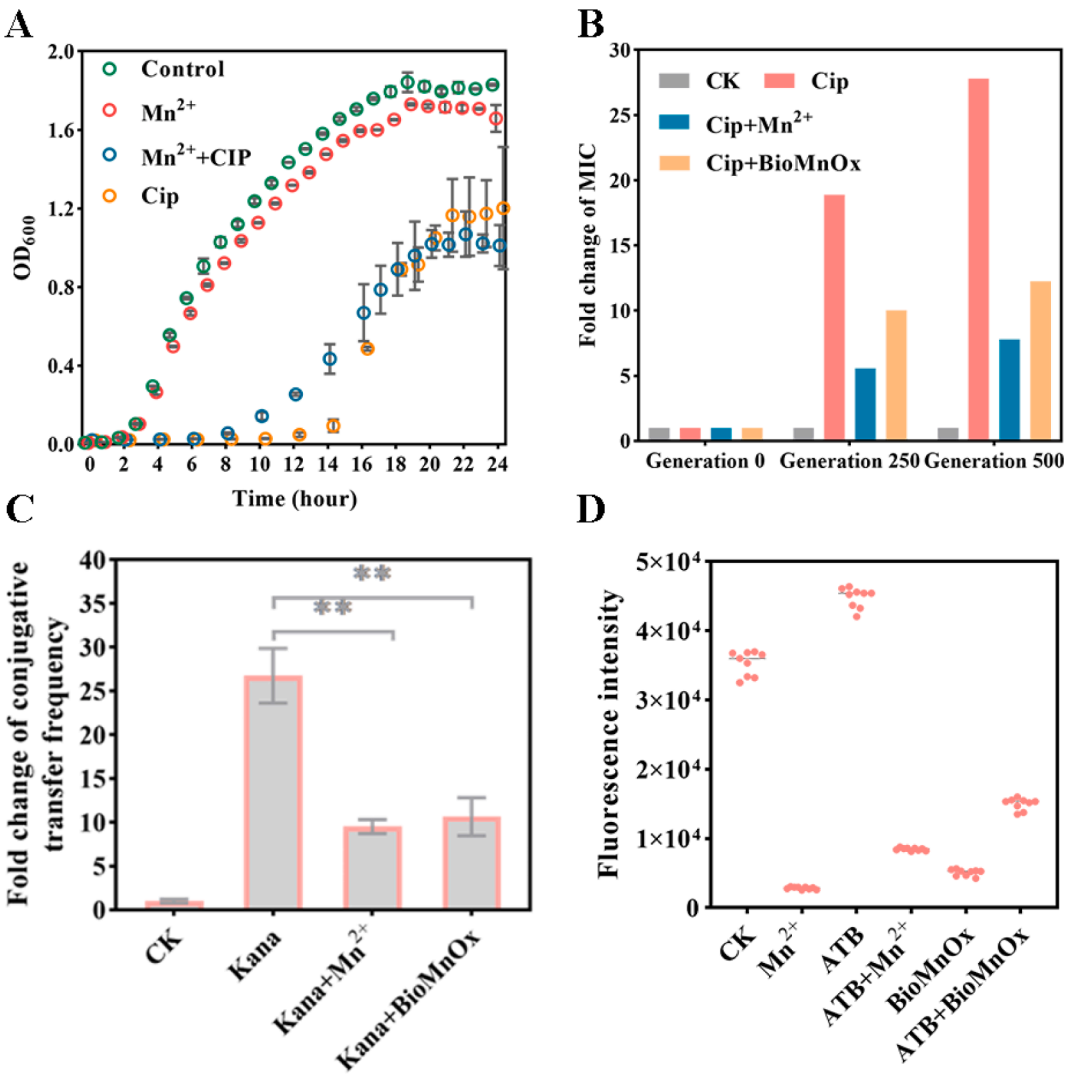

Fig. 2. Manganese bio-oxidation relieved the inhibition of antibiotic to *P. aeruginosa* strain MQ2 growth (A), retarded the increase of minimum inhibitory concentration (MIC) (B), conjugative transfer of RP4 plasmid (C), and ROS generation (D) under antibiotic selective pressure. Cip: Ciprofloxacin; Kana: Kanamycin sulfate; CK: The control without treatment; ABT: Antibiotic.

determined by XRD assay (Fig. 1E). The results exhibited two diffraction peaks (shown with red solid lines) at 2θ of 37.36°, 41.32° and 49.49° that corresponded with the major crystal phase of MnO_2 (JCPDS 50-0866). Two peaks (shown with blue solid lines) at 59.84° and 67.63° corresponded with Mn_3O_4 (JCPDS 24-0734). The remaining four peaks (shown with orange solid lines) at 24.25°, 31.36°, 45.18° and 51.68° correspond with $MnCO_3$ (rhodochrosite, JCPDS 44-1472).

BioMnOx has previously been reported to mediate the degradation of antibiotics via oxidation, and thus has great potential for antibiotic pollution remediation. $MnCO_3$ precipitation may serve as the inorganic carbon pool by coupling the hypervalent MnOx reduction and organic oxidation (Li et al., 2019). TEM (Fig. 1F) revealed that the cells were coated tightly by the Mn-containing solid, in which Mn and O were detected by EDS (Fig. 1G and H). This phenomenon was also reported in the model Mn oxidizer *Bacillus* sp. strain SG-1 (Soldatova et al., 2012). The coated BioMnOx served as a reservoir of Mn(II), which can protect them from oxidative damage (Brouwers et al., 2000b; Tebo et al., 2004). This benefit may shield MnOBs under antibiotic exposure by decreasing oxidative stress. Interestingly, thin-section TEM image revealed that Mn is present both inside and outside the cell of *P. aeruginosa* strain MQ2 (Fig. 1G). It was reported that intracellular Mn could act as an antioxidant to consume ROS and enhanced the resistance of MnOBs to oxidative stress (Archibald and Fridovich 1981a,b; Banh et al., 2013; Daly et al., 2004). As reported previously, oxidative stress is one of the most important driving forces for resistance mutation and HGT of ARGs (Wang et al., 2023b; Zhang et al., 2021b). Therefore, Mn(II) oxidation and the formed BioMnOx have the potential not only to degrade antibiotics but also mitigate the AMR induction by alleviating oxidative stress.

3.3. Manganese bio-oxidation process mitigated AMR inducement and decreased ARG conjugative transfer

To investigate the effects of Mn(II) and antibiotics on the growth of *P. aeruginosa* strain MQ2, the growth curves were measured in the presence of 100 mg/L $MnCl_2$, 100 μg/L Cip treatment or both (Fig. 2A). Mn(II) had little effect on the growth of *P. aeruginosa* strain MQ2. As an Mn oxidizer, *P. aeruginosa* strain MQ2 could shield other microorganisms from the toxic effects of manganese (Gadd and Griffiths 1977). Lag phase of *P. aeruginosa* strain MQ2 induced by Cip (about 12 h) was shortened by the presence of Mn(II) (about 8 h). This suggests that Mn (II) played a protective role for *P. aeruginosa* strain MQ2. Cip resistance can arise in *P. aeruginosa* via mutations in multiple genes, and is expected to do so under prolonged sublethal exposure. To determine

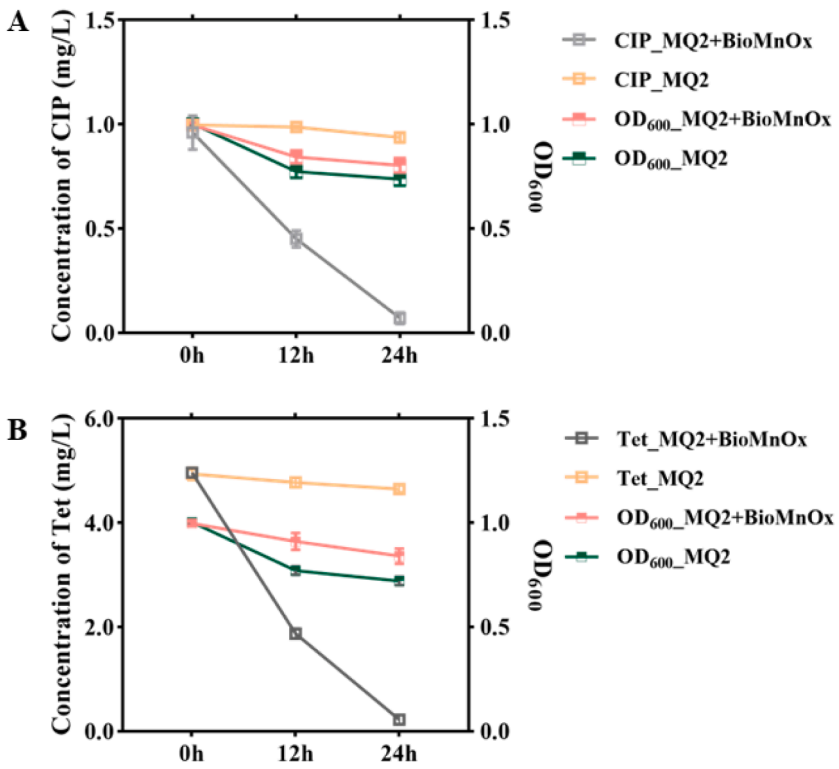

Fig. 3. Removal of (A) ciprofloxacin (Cip) and (B) tetracycline (Tet) mediated by BioMnOx, and more biomass of *P. aeruginosa* strain MQ2 retained in BioMnOx containing groups.

whether Mn(II) can counteract this pressure, changes in the MIC following exposure to 100 μg/L Cip for 11 days (approximately 500 generations; Fig. 2B). The original MIC of *P. aeruginosa* strain MQ2 to Cip was 1100 μg/L. After exposure, the MICs augmented by 18.9- and 27.8-folds at 250 and 500 generations, respectively. However, the resistance against Cip only increased 5.6- and 7.8-fold when cultured with the presence of Mn(II), and 10.0- and 12.2-fold when cultured with the presence of BioMnOx. Similarly, conjugative transfer frequency of plasmid RP4 between *E. coli* DH5α and *P. aeruginosa* strain MQ2 increased 26.7 ± 3.1-fold under the exposure to 100 μg/L Kana (Fig. 2C). It was significantly lower when Mn(II) and BioMnOx were added to the conjugative transfer systems ($p<0.01$) at only 9.5 ± 0.8- and 10.6 ± 2.2-fold compared to the unselected control.

To evaluate the role of manganese bio-oxidation process on the oxidative pressure, ROS generation was measured (Fig. 2D). The fluorescence intensity was significantly lower in Mn(II) and BioMnOx treated groups than in the control ($p<0.01$), whether antibiotics were present or not. The fluorescence intensity in the antibiotic treated group was 44,806.3 ± 1457.9, 1.27-fold higher than the control, while it was only 0.08- to 0.42-fold of the control in Mn(II) and BioMnOx treated groups. These results indicated that antibiotics increased the oxidative pressure, and both Mn(II) and BioMnOx decreased the pressure by scavenging ROS. These results corroborate previous observations that BioMnOx can protect bacteria from oxidative damage by coating the cells (Brouwers et al., 2000b; Tebo et al., 2004), and intracellular Mn consumes ROS by acting as an antioxidant (Archibald and Fridovich, 1981a,b; Banh et al., 2013; Daly et al., 2004). Mn(II) oxidation experiments were also conducted to investigate the effects of ROS on the Mn (II) oxidation rate (Figure S3). Mn(II) oxidation was the slowest when thiourea (a ROS scavenger) was added, and was the fastest when 100 μg/L Cip (increased ROS) was added. It was previously reported that ROS released by bacteria could mediate secondary Mn(II) oxidation (Jofré et al., 2021), which corroborates conversely that Mn(II) can consume ROS. This suggests that the relief of oxidative stress provided by manganese bio-oxidation alleviates selective pressure exerted by residual antibiotics for mutation and conjugative transfer of ARGs.

3.4. BioMnOx oxidatively degrades antibiotics

In the presence of BioMnOx, Cip and Tet were efficiently degraded (Fig. 3). The removal efficiency of Cip (1.0 mg/L) reached 93% within 24 h. In contrast, only 6% of Cip was removed in the control containing *P. aeruginosa* MQ2 without BioMnOx, which indicates that *P. aeruginosa* strain MQ2 does not directly biodegrade Cip. Similarly, 96% of Tet was removed within 24 h from an initial concentration of 5.0 mg/L, while only 6% of Tet was removed in the control without BioMnOx. There was no Cip and Tet inactivation genes that may be able to degrade antibiotics identified in the genome of *P. aeruginosa* strain MQ2 by searching against the comprehensive antibiotic resistance database (CARD). Only 3 *tetT*-like genes confer *P. aeruginosa* strain MQ2 the tetracycline resistance by antibiotic target protection (Table S2). Four genes are associated with the quinolone resistance, two are formed through antibiotic resistant gene variant or mutant and the other two confer quinolone resistance by antibiotic target protection (Table S2). Thus, strain MQ2 itself could not degrade the two antibiotics, which supports the role of BioMnOx in the degradation of antibiotics.

MnO_2 can oxidize Cip by dealkylation and hydroxylation at the piperazine moiety and degrade Tet by isomerization and oxidation of phenolic-diketone and tricarbonylamide groups (Chen and Huang, 2011; Zhang and Huang, 2005). Products after oxidation by MnO_2 have lower antibacterial activity and thus may be utilized by microorganisms (Chen and Huang, 2011). Based on OD_{600} measurement, more biomass remained in the BioMnOx system after 24 h for both Cip and Tet. Considering that antibiotics were the only organic matter in these systems, BioMnOx-mediated antibiotic degradation may generate byproducts that support the growth of *P. aeruginosa* strain MQ2 to a certain extent. A previous study demonstrated that BioMnOx oxidatively transforms humic substances to low-molecular-weight organic

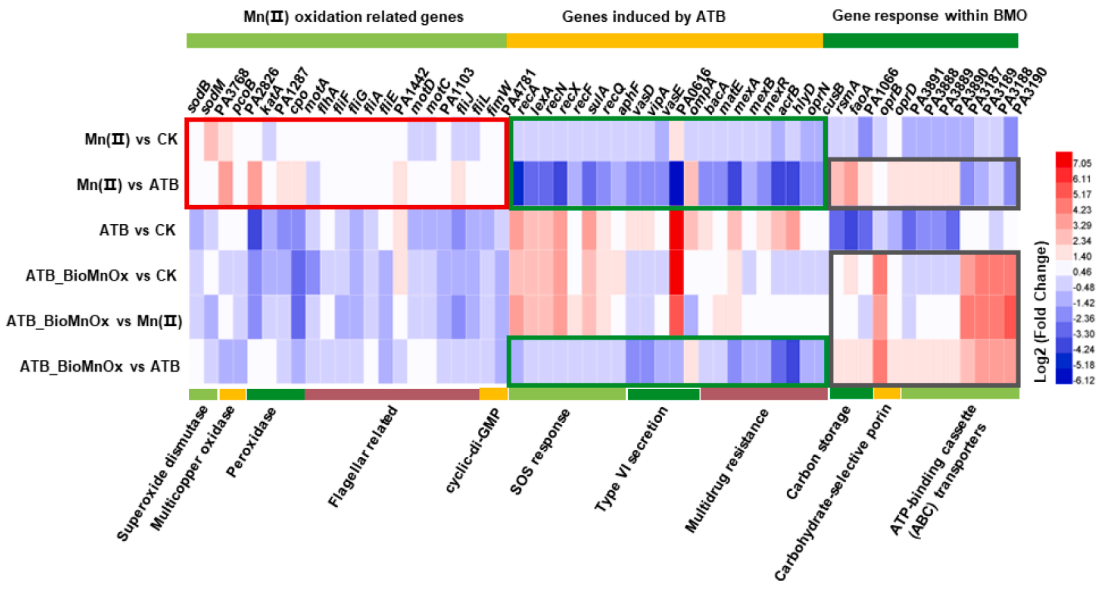

Fig. 4. Transcriptomic response of *P. aeruginosa* strain MQ2 to Mn(II), antibiotic and BioMnOx. Mn(II) induced the upregulation of multicopper oxidase, superoxide dismutase, peroxidase, flagellar and cyclic-di-GMP related genes (red box). Mn(II) and BioMnOx induced the downregulation of SOS response, type IV secretion system and multidrug resistance related genes (green box). BioMnOx induced the upregulation of carbon storage, carbohydrate selective porin and ABC transporter related genes (gray box). Each gene is represented by the log2 of the fold change (log2FC). The row name represented the comparison of transcriptional data between different groups. For instance, Mn(II) vs CK indicates the comparison between *P. aeruginosa* strain MQ2 with 100 mg/L $MnCl_2$ treatment and the control. CK: the control without treatment; ATB: Antibiotic; BMO: Biogenic manganese oxidation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

compounds that can be used as a carbon source by microorganisms (Sunda and Kieber 1994).

3.5. Mn(II) and BioMnOx decreased HGT and mitigated resistance development by suppressing conjugative transfer system and relieving oxidative stress

Transcriptome sequencing of *P. aeruginosa* strain MQ2 was conducted to detect the gene expression response to Mn(II), antibiotic and BioMnOx. Mn(II) oxidation related genes were judged by the DEGs by comparing the Mn(II) group with the control. A total of 355 DEGs were detected, including 218 upregulated and 137 downregulated genes. Among the upregulated DEGs, genes encoding superoxide dismutases (SOD) (e.g., *sodB* and *sodM*), MCOs (e.g., PA3768 and *pcoB*), peroxidases (e.g., *katA, cpo*, PA1287 and PA2826), flagellar related (e.g., *motACD, flhA* and *fliAEFGHJL*) and signal receptor (*fimW* and PA4781) were overexpressed under exposure to Mn(II) (Fig. 4 red box). Genes *sodB* and *sodM* in the genome of *P. aeruginosa* strain MQ2 are manganese-containing superoxide dismutase (MnSOD), which has been reported to contribute to the adaptation to oxidative stress of *P. aeruginosa* (Polack et al., 1996). This also explains the enhancement of antioxidant capacity of *P. aeruginosa* strain MQ2 by the presence of Mn(II) by upregulating the MnSOD. Mn(II) induced the expression of MCOs confirmed the speculation in Section 3.1 that MCOs are responsible for Mn(II) oxidation. Peroxidases in *Aurantimonas manganoxydans* and *Erythrobacter* sp. were also reported to oxidize manganese (Anderson et al., 2009). The high expression of both MCOs and peroxidases is consistent with the high Mn(II) oxidation activity. Mn(II) oxidation could also be regulated by flagellar-mediated responses to the surface substrate (Geszvain et al., 2011), which is consistent with our results that a large number of flagellar genes upregulated in the Mn(II) containing group. A second messenger, cyclic di-GMP signaling were reported links to Mn(II) oxidation in *P. resinovorans* (Piazza et al., 2022). Apparently, there were multiple paths in *P. aeruginosa* strain MQ2 to regulate the Mn(II) oxidation by direct oxydasis or maintain homeostasis.

To investigate the effects of antibiotics (Cip and Tet combined) on *P. aeruginosa* strain MQ2 and the protective role of Mn(II) and BioMnOx, the DEGs were defined as their differential expressions in either of the following comparisons between each treated group: antibiotics (ATB) vs control (CK), antibiotics+BioMnOx (ATB_BioMnOx) vs CK and ATB_BioMnOx vs Mn(II). Consistent with previous research, antibiotics induced the upregulation of oxidative stress related genes in *P. aeruginosa* strain MQ2, including SOS response (e.g., *recAFNQX, lexA, aphF* and *sulA*), type IV secretion systems (T4SS) (e.g., *vas DE, vipA, ompA* and PA0616) and multidrug resistance (e.g., *bacA, matE, mexABR, acrB, hlyD, oprN* and *cusB*) (Fig. 4 green box). Antibiotics induce intracellular ROS overproduction that directly damage DNA and further activate the SOS response to counteract and prevent oxidative stress (Ren et al., 2022; Shi et al., 2020). T4SS is an essential system in conjugation and plays an indispensable role in single-stranded DNA (ssDNA) delivery through the membranes of the donor and recipient cells (Guglielmini et al., 2013). The downregulation of T4SS genes in the Mn(II) and BioMnOx conditions corroborates the decrease in conjugative frequency in Section 3.2. Multidrug resistance genes involved in antibiotic efflux are important for resistance in *P. aeruginosa*. The downregulation of multidrug resistance genes in the Mn(II) and BioMnOx conditions corroborates the mitigation of antibiotic resistance development, evidenced by the smaller MIC changes when exposed to Cip in Section 3.2. All these results suggested that Mn(II) and BioMnOx provides oxidative stress defense.

The comparisons between antibiotic and BioMnOx contained groups and others, including ATB_BioMnOx vs CK, ATB_BioMnOx vs Mn(II) and ATB_BioMnOx vs ATB, were used to assess the whether BioMnOx transformation of antibiotic enabled downstream metabolism in *P. aeruginosa* strain MQ2. Carbon storage (e.g., *rsmA, faoA* and PA1066), carbohydrate-selective porins (e.g., *oprB* and *oprD*) and ATP-binding cassette (ABC) transporters (e.g., PA3888, PA3889, PA3890, PA3891, PA3187, PA3188, PA3189 and PA3190) related genes were upregulated in the BioMnOx condition (Fig. 4 gray box). *rsmA* is involved in the regulation of various bacterial processes including carbon metabolism (Sabnis et al., 1995), motility (Wei et al., 2001) and stress response

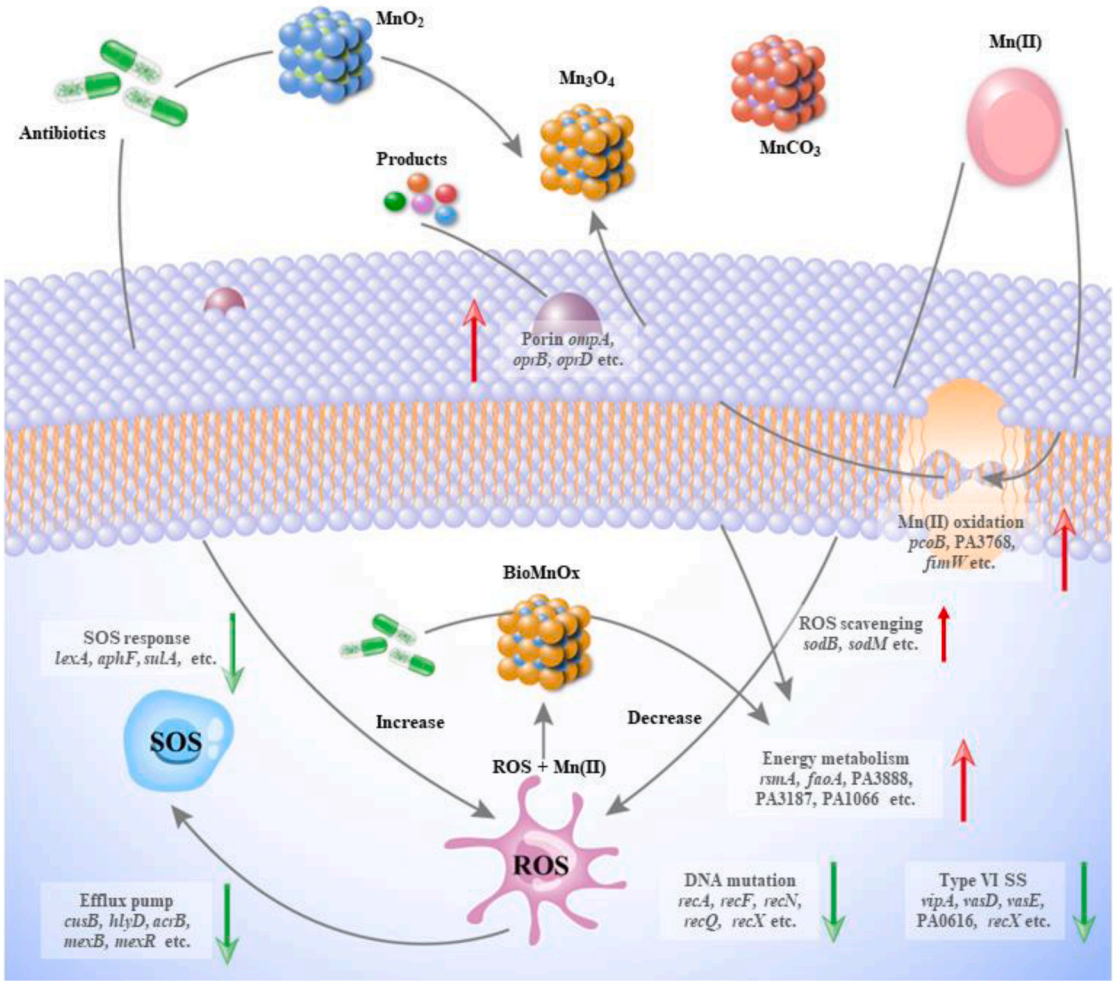

Fig. 5. Proposed mechanisms of *P. aeruginosa* strain MQ2 to metabolize antibiotics assisted by BioMnOx, and resist oxidative stress by the protection from both BioMnOx and Mn(II). Red arrows indicate up-regulated gene expression; Green arrows indicate down-regulated gene expression.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Fields and Thompson 2008). The OprB and OprD family of porins regulate the diffusion of glucose across the outer membrane (Wylie and Worobec 1995) and nutrient uptake, respectively, in *P. aeruginosa* (Tamber et al., 2006). ABC transporters belong to a transport superfamily that couple ATP hydrolysis to the uptake and efflux of nutrient across the cell membrane in bacteria (Davidson and Chen 2004). The upregulation of these genes suggests that BioMnOx may chemically decompose the antibiotics into products that are then used by *P. aeruginosa* strain MQ2 as carbon source, which can explain the higher OD_{600} in BioMnOx condition of the antibiotic degradation experiments in Section 3.4.

3.6. Postulated mechanisms of antibiotic degradation and antibiotic resistance inducing mitigation by manganese bio-oxidation process

Based on characterization of BioMnOx, MIC measurement, conjugative transfer frequency and ROS, antibiotic degradation, and RNA sequencing, two underlying mechanisms of antibiotic degradation and resistance mitigation are proposed (Fig. 5). First, BioMnOx is formed by the MCOs and other Mn(II) oxidation enzymes, and the formed BioMnOx chemically transforms the antibiotics. Second, Mn(II) decreases the oxidative stress by scavenging the ROS induced by antibiotics, thus mitigating antibiotic resistance development and conjugative transfer of ARGs.

MCOs are a well-known Mn(II) oxidation enzyme in many bacteria (Brouwers et al., 2000b; Geszvain et al., 2012). In the genome of *P. aeruginosa* strain MQ2, there were two novel MCOs sharing low sequence similarity but highly conserved sites with the MCOs in other Mn oxidizers. We propose that these genes are responsible for Mn(II) oxidation. In addition, the intercellular ROS may oxidize the Mn(II) to MnOx, as in *Arthobacter oxydans* (Jofré et al., 2021). Thus formed, BioMnOx coats the outside surface of the cells and shields them antibiotics, which it then oxidatively decomposes. Both the shielding and degradation provide protection for *P. aeruginosa* strain MQ2 against antibiotic toxicity. Under oligotrophic conditions (antibiotics as the sole carbon source), the products of antibiotics could be used by *P. aeruginosa* strain MQ2 to support its growth, as evidenced by optical density and upregulation of energy metabolism genes in BioMnOx conditions. Mn(II) is considered an antioxidant that can scavenge intracellular ROS (Archibald and Fridovich 1981a,b). The presence of Mn(II) offers defense against oxidative stress for *P. aeruginosa* strain MQ2 by increasing the intracellular Mn level, which was visualized by thin-section TEM images. This oxidative stress defense further mitigates the development of antibiotic resistance and conjugative transfer of ARGs in the presence of antibiotics, which would otherwise constitute a selection pressure. ROS is a key factor driving antibiotic- and pollutant-induced mutation and HGT of ARGs, which can be reversed through ROS scavenging (Ren et al., 2021, 2022; Yu et al., 2020).

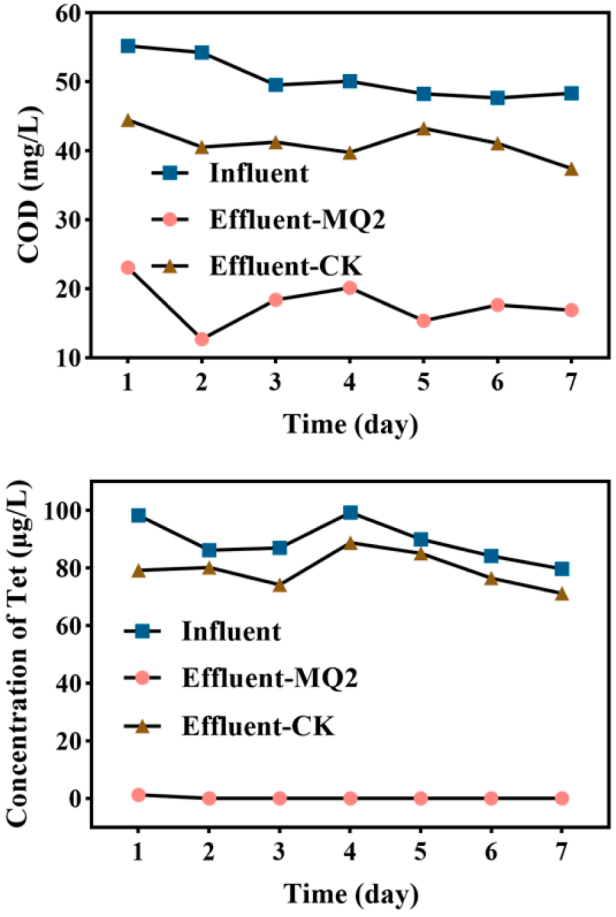

Fig. 6. Performance of COD and tetracycline (Tet) removal in manganese bio-oxidation based reactor. Effluent-MQ2 and Effluent-CK represent the effluent from the strain MQ2 contained and the control reactor, respectively.

3.7. Antibiotic was removed in simulated effluent by manganese bio-oxidation based reactor

A manganese bio-oxidation based reactor was constructed to investigate the practicability of this process for antibiotic removal. As show in Fig. 6, COD and tetracycline were both well removed in the manganese bio-oxidation based reactor. Strain MQ2 as a heterotrophic *Pseudomonas*, could consume carbon source in the effluent, which indicated that the bacterium also has a certain effect on the advanced treatment of wastewater. Tetracycline could be completely removed in the manganese bio-oxidation based reactor, while the removal rate of the control was only 11.0 ± 0.05%. It was suggested that this manganese bio-oxidation process has a big potential in removing the residue antibiotics in effluent of wastewater treatment plants. Wastewater treatment plants have been considered as important reservoirs of antibiotics, and the residue antibiotics will cause big risks on the dissemination of ARGs (Wang et al., 2021). Therefore, it is essential to remove the residue antibiotics from the wastewater effluents before flowing into the natural environments.

4. Conclusion

This study provides a potential biological strategy for removing antibiotics and prevent ARGs contamination with MnOBs by producing BioMnOx and decreasing oxidative stress. MnOx forms mainly from microorganisms and is one of the strongest oxidants that can oxidize a variety of refractory pollutants. Thus, it may be worthwhile to explore whether these microorganisms could be used for antibiotic removal in antibiotic contaminated sites, such as wastewater. However, the biggest problem of microbial degradation of antibiotics is the enrichment and spread of ARGs. MnOBs relieve antibiotic selective pressure via oxidation resistance and chemical oxidation of antibiotics before adaptation and evolution occur. Many studies have demonstrated the use of manganese oxides to remediate organic pollutants chemically or biologically. MnOBs serving as manganese pollution remediation specialists can precipitate soluble Mn, alleviating concerns about downstream manganese contamination. Overall, this study provides an inspiration to explore microbial processes that can be used for antibiotic and ARG contamination control for wastewater treatment, highlighting the advantages of involving nontoxic and inexpensive chemicals at moderate conditions.

Supporting Information

Multiple sequence alignment of MCO genes; Elements of Mn, O and C detection by EDS system; Mn(II) oxidation; Pearson correlation between samples of RNA-seq; The MICs of the ancestor strain MQ2 to 5 different types of antibiotics; Quinolone and tetracycline resistance genes identified by searching against CARD.

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.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2023.120442.

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