

Degradation pathway and molecular mechanism of a novel oxytetracycline-degrading strain *Brucella* sp. F-6

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Abstract: Oxytetracycline (OTC) is widely used in mariculture as a prophylactic and feed supplement, leading to its frequent detection at high concentrations in coastal environments and posing risks to marine ecosystems and human health. To address this issue, a novel OTC-degrading strain, *Brucella* sp. F-6, was isolated from estuarine sediments. It was first verified to degrade OTC into small molecules with a degradation rate of $92.06 \pm 0.98\%$ within 48 h. The OTC degradation pathway was explored, and Glycine betaine monooxygenase (Gbs) and sarcosine oxidase (Sox) were proposed as candidate degradation proteins based on transcriptomic analysis and degradation product identification. The genes encoding candidate degradation protein Gbs were significantly upregulated even at an environmentally relevant concentration of 0.1 $\mu\text{g/L}$ OTC. The Cu-OTC chelate was suggested to be involved in OTC detoxification. Strain F-6 resisted OTC through multiple mechanisms, including drug efflux, outer membrane protection, ribosomal protection, and DNA repair. Meanwhile, nitrogen metabolism, one-carbon metabolism, and the antioxidant system were affected or inhibited. However, only drug efflux was activated under environmental concentrations of OTC. These findings have important implications for research on antibiotic removal and environmental toxicity assessment.

Key words: Oxytetracycline; Biodegradation; Molecular mechanism; Degradation pathway

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1. Introduction

Antibiotics are essential for disease prevention and treatment in humans and animals, but they are usually incompletely absorbed by mammals and thus are excreted and dispersed into the environment^[1]. As a result, the concentration of antibiotics entering aquatic ecosystems has also increased^[2]. The sustained presence of antibiotics in the environment leads to the occurrence and accumulation of antibiotic resistance genes (ARGs) and antibiotic-resistant bacteria (ARB). ARGs and ARBs are closely related to human health; hence, the occurrence and removal of antibiotics in the environment are highly important.

Tetracyclines (TCs) are among the most commonly used antibiotics for the treatment of bacterial infections and for

improving agricultural growth and feed efficiency because of their low cost and broad-spectrum bactericidal properties^[3]. TCs are among the most frequently detected antibiotics in mariculture environments, with detection frequencies ranging from 17% to 83% in typical aquaculture areas surrounding the Yellow Sea^[4]. Oxytetracycline (OTC) is one of the most commonly used TCs. It binds to the 30S subunit of the bacterial ribosome to block protein synthesis. OTC is widely used as a disinfectant in mariculture. In addition, it is also used as a growth promoter and feed additive, with OTC contents reaching mg/kg levels^[5]. The extensive use of OTC, together with its high excretion rate in active form and environmental persistence (>40 days)^[6], has led to its frequent detection at high concentrations in mariculture ponds, adjacent coastal waters, and even aquaculture organisms. Chen et al. detected OTC with the highest concentration of 15,163 ng/L in sea shrimp pond^[7]; OTC was also detected at high concentrations in biofilms (up to 1478.29 ng/g) in typical marine aquaculture farms surrounding the Yellow Sea^[8]. Han et al. also found OTC was the main antibiotic in organism samples in marine aquaculture farms surrounding Laizhou Bay, Bohai Sea^[9]. The frequent detection of OTC in mariculture and coastal environments causes the accu-

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mulation of ARGs in the environment, disrupting the structure and function of marine microbial communities. Furthermore, human intake of OTC via seafood poses a direct threat to human health.

The pervasiveness of OTC has drawn worldwide attention to take necessary action and develop technologies for its remediation to protect the environment. Bacteria, as decomposers, are important candidates for organic pollutant removal and for OTC removal^[10]. Compared with physical and chemical degradation methods, bacterial degradation offers several advantages, such as environmental friendliness, cost-effectiveness, and convenience. Studies on bacterial degradation have focused on two main aspects: the bacterial community (e.g., sludge, sediment, and compost) and a single strain. The effects of the bacterial community on OTC are more complicated and involve a combination of biosorption, biotransformation, and biodegradation^[11]. However, the single strain has more advantages in mechanistic research on OTC degradation. In recent years, several strains with the ability to degrade OTC have been isolated, like *Marinobacter hydrocarbonoclasticus* RAD-2^[12], *Ochrobactrum* sp. KSS10^[13], and *Planococcus* sp. strain pw2^[14], and so on. However, most of the current studies are focused on OTC degradation by the bacterial community, and exploration and supplementation of novel bacterial strains that can degrade OTC are still lacking. OTC-degrading bacteria in special regions such as coastal waters and estuaries have barely been exploited.

In this study, we isolated a novel strain, F-6, identified as *Brucella* sp., which can efficiently degrade OTC from estuary water. The degradation efficiency and influencing factors of F-6 on OTC were investigated. In addition, the OTC degradation pathway, key degradation proteins, resistance and response of strain F-6 to OTC were explored. Moreover, the responses of degradation-, resistance- and metabolism-related genes under mild OTC contamination (0.1 µg/L), heavy OTC contamination (100 µg/L), and OTC bacteriostatic concentration (10 mg/L) were investigated. To the best of our knowledge, this is the first report on the OTC degradation capacity and mechanism of *Brucella* sp.

2. Materials and methods

2.1. Isolation and identification of OTC-degrading bacterial strains

A total of 10 g of sediment from the Jiahe Estuary, Yantai, China, was collected, and 1 g of sediment was added to 100 mL of the minimum medium (MM) culture medium; 50 mg/L OTC was added as the sole carbon source, and the mixture was cultured at 30°C and 160 rpm. After 3 days, 5 mL of the cultured suspension was transferred to 100 mL of fresh MM with 50 mg/L OTC, and the culture was continued for another 3 days. This process was repeated five times. Then, 1 mL of the culture medium was collected and filtered through a 0.22 µm membrane, and the OTC concentration was detected via high-performance liquid chromatography (HPLC). When the OTC concentration was much lower than 50 mg/L, the cultured suspension was diluted and coated on an LB culture plate. Through repeated screening, the strain with the highest OTC degradation efficiency was selected and numbered F-6. The 16S rRNA gene sequence of strain F-6 was amplified via the primers 27F and 1492R and then sequenced and aligned against the Gen-

Bank and EzBioCloud databases. The phylogenetic tree of strain F-6 was constructed via MEGA5.0 software. Strain F-6 was cultured, immobilized, and scanned by scanning electron microscopy (Hitachi S-4800, Japan Tokyo). The configurations of all the culture mediums and buffer solutions used in this study and all sequences of primers used in this study are shown in **Table S1 and S2**.

2.2. OTC degradation experiments

Strain F-6 was cultured for 12 hours in LB medium, and the cells were collected via centrifugation at 8000 rpm for 3 min, and washed with sterile PBS for 3 times. The washed cells of strain F-6 were added to MM medium containing 10 mg/L OTC at a concentration of 10⁸ CFU/mL and then incubated in a shaking incubator at 30°C and 160 rpm as the experimental group. A 1 mL suspension was collected every 12 h, filtered through a 0.22 µm membrane, and detected by HPLC. A 1 mL suspension was collected at 24 h, and the OTC products were analyzed by HPLC-ESI-MS/MS. MM medium containing 10 mg/L OTC without strain F-6 as the control group was collected and detected like the experiment group. Each treatment had three repetitions. The OTC degradation rate was calculated via the following equation:

$$R = \frac{C_0 - C_t}{C_t} \times 100$$

where R represents the degradation rate of OTC (%); C_0 represents the initial concentration of OTC (mg/L); and C_t represents the remaining concentration of OTC at time t (mg/L).

The initial concentration of OTC, pH, and salinity were selected to investigate their effects on the OTC degradation of strain F-6. The OTC concentrations were set to 0.5, 1, 5, 20, and 50 mg/L. The pH values were set to 5, 6, 7, 8, and 9 according to **Table S3**. The salinity experiment was performed with 0%, 20%, 40%, 60%, 80%, and 100% seawater diluted with phosphate buffer solution, and the salinities were 0, 7, 14, 21, 28, and 35, respectively. The initial OTC concentration for the pH and salinity experiments was 10 mg/L, and the initial cell concentration for all the experiments was 10⁸ CFU/mL. The bacterial suspensions were detected after 1 day. Each treatment had three repetitions.

2.3. Transcriptome analysis

A transcriptome experiment was carried out under 10 mg/L OTC. 5×10⁸ CFU/mL of strain F-6 was cultured in MM medium as the control group, and in MM medium supplemented with 10 mg/L OTC as the experimental group. After 24 hours, the cells were collected for transcriptome analysis. Transcriptome sequencing and analysis were performed by Majorbio (Shanghai, China). Briefly, total RNA was extracted, quantified, and fragmented into ~200 bp fragments. Then, cDNA was synthesized and patched into flat ends by adding End Repair Mix, and an A base was added to the 3' ends. The second strand of cDNA was digested with the UNG enzyme so that only the first strand of cDNA was included in the library. The library was sequenced on the Illumina NovaSeq XP and DNB-SEQ-T7 platforms.

2.4. Key gene responses of *Brucella* sp. strain F-6 to OTC at different concentrations

Based on the known published data, the OTC concentra-

tions in surface water, wastewater, and were set to 0.1 µg/L and 100 µg/L respectively^[6, 15, 16], and its bacteriostatic concentration was set to 10 mg/L^[17]. To investigate the responses of strain F-6 to different concentrations of OTC, cells were inoculated in the MM medium supplemented with 0.1 µg/L, 100 µg/L, or 10 mg/L OTC at an initial cell concentration of 10⁸ CFU/mL, and the same conditions without OTC were used as controls. Each treatment had three repetitions. After 24 hours of culture, the cells in each group were collected, total RNA was extracted via TsingZol Total RNA Extraction Reagent, RNA was reverse transcribed to cDNA via SynScript® RT SuperMix for qPCR, and RT-qPCR was performed via EasyQ SYBR qPCR Mix according to the 2^{-ΔΔCt} method by QuantStudio 5 (Thermo Fisher, America). The primers used in this study are shown in Table S2.

2.5. Analytical methods

The OTC concentration was detected by Agilent 1260 Infinity II HPLC (Agilent, America) equipped with an Agilent reversed-phase SBC18 column (4.6 × 150 mm, 5 µm). The mobile phase consisted of two solvents: acetonitrile and 0.4% (v/v) ethanoic acid at a ratio of 10:90. The flow rate was 1 mL/min, the injection volume was 10 µL, the detection wavelength was 280 nm, and the column temperature was set to 35°C.

The degradation products were detected via LCQ Fleet HPLC-ESI-MS/MS (Thermo Fisher Scientific, America). The mobile phase was the same as that used for HPLC, and the flow rate was 0.3 mL/min. The mass spectrometer was operated in positive ion mode at a full-scan m/z range of 100-1000, an ion spray voltage of 3 kV, a spray voltage of 5 kV and a capillary temperature of 270°C.

2.6. Statistical analysis

For transcriptomic analysis, DEGs were selected by DESeq2 with absolute values of log₂[fold change] > 1 and padjust < 0.05. Group differences in ENR degradation rates and gene responses were evaluated via one-way ANOVA and

Tukey and Waller–Duncan tests via SPSS and GraphPad Prism. All the data are expressed as the mean ± standard deviation.

3. Results and Discussions

3.1. Isolation and identification of OTC-degrading bacteria

After rounds of enrichment and screening, a bacterial strain, F-6, with high OTC degradation efficiency was isolated from estuarine sediment. The 16S rRNA partial sequence of strain F-6 was submitted to GenBank with the accession number PQ461247. Compared with sequences in the GenBank database and in the EZBioCloud type strain sequence database, the 16S rRNA gene sequence of strain F6 was 98.85% similar to that of *Brucella anthropi* HNXS-W4, with accession number of PP092114.1. The neighbor-joining tree based on 16S rRNA gene sequences revealed a closer relationship with *Brucella anthropic* (ATCC 49188) and *Brucella tritici* (SCII24|AJ242584) (Fig. 1a). On the basis of these results, strain F-6 was identified as belonging to the genus *Brucella*. Strain F-6 is a gram-negative bacterium, and scanning electron microscope images revealed that the cells of strain F-6 were non-spore-forming, non-flagellated, and rods (0.55–0.6 × 2.1–4.2 µm), and a few cells longer than 7 µm were also observed (Fig. 1b and 1c).

Some bacterial strains have been reported to have OTC-degrading ability, including *Lysinibacillus* sp. strain 3+^[18], *Klebsiella* sp. stain SQY5^[19], *Marinobacter hydrocarbonoclasticus* strain RAD-2^[12], *Ochrobactrum* sp. KSS10^[13], and *Planococcus* sp. strain pw2^[14], and so on. To the best of our knowledge, this is the first report on OTC degradation by *Brucella* sp. bacteria. Furthermore, it has enriched the microbial resource of oxytetracycline-degrading bacteria in the special environment of estuaries. Now, more studies have focused on OTC degradation by microbial communities rather than on isolated single strains, such as the biodegradation of oxytetracycline by autochthonous microbial communities or activated sludge

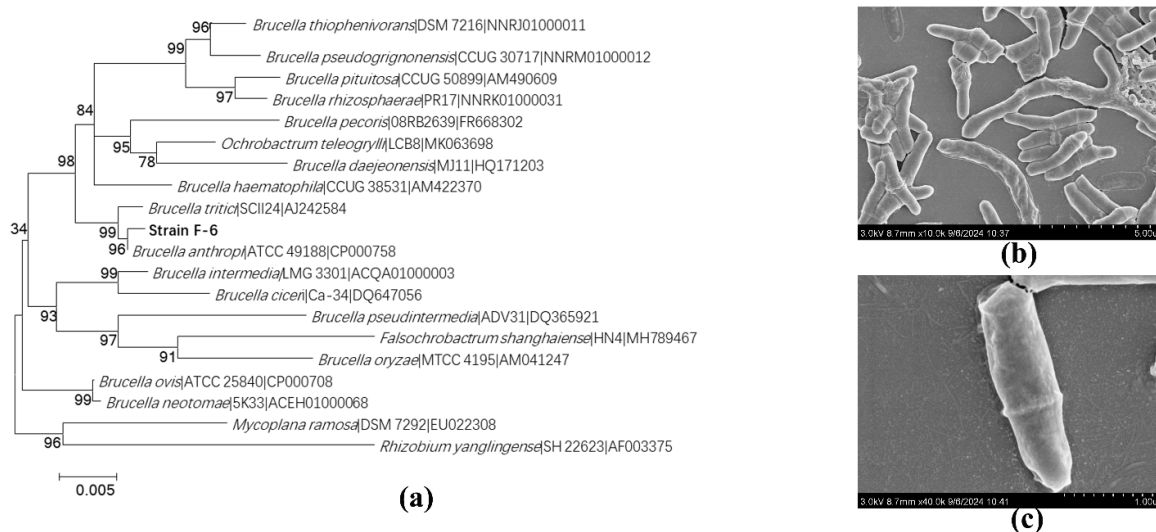


Fig. 1. Phylogenetic tree and scanning electron microscope images of the OTC-degrading bacteria *Brucella* sp. strain F-6. (a): Neighbor-joining tree based on 16S rRNA gene sequences of strain F-6 and related type strains. The numbers at branch nodes indicate bootstrap percentages derived from 1000 replications. Bar, 0.005 accumulated changes per nucleotide position. (b), (c): Scanning electron microscopy image of strain F-6 after 12 h of growth on LB medium at 30°C.

microbial communities. However, the use of single bacteria will be beneficial for research on the molecular mechanism of antibiotic biodegradation.

3.2. Degradation efficiency of OTC by *Brucella* sp. strain F-6

As shown in Fig. 2a, *Brucella* sp. strain F-6 degraded $92.06 \pm 0.98\%$ of 10 mg/L OTC within 48 h by at an initial bacterial concentration of 10^8 CFU/mL, whereas the degradation rate in the control groups was only $35.37 \pm 0.69\%$. For comparison, *Ochrobactrum* sp strain KSS10 could degrade 61.63% of 25 mg/L OTC within 80 h at an initial bacterial concentration of 10^8 CFU/mL^[13]; *Lysinibacillus* strain 3+I could degrade 85% of 10 mg/L OTC within 7 days at an initial bacterial concentration of 10^7 CFU/mL^[18], *Planococcus* sp. strain pw2 could degrade 90.62% of 10 mg/L OTC within 7 days at an initial bacterial concentration of 10^7 CFU/mL^[14]. Overall, strain F-6 shows favorable OTC degradation efficiency. The initial concentration of OTC and salinity significantly affected the OTC degradation rate of strain F-6, whereas the effect of pH was not significant (Fig. 2b-d). Similar to most microbial degradation processes, the higher the initial concentration of OTC, the lower the degradation efficiency of OTC by strain F-6^[20]. The OTC degradation rates for initial OTC concentrations of 0.5 and 1 mg/L for 24 h were $75.26 \pm 2.31\%$ and $74.04 \pm 3.17\%$, respectively, which were significantly higher than $55.42 \pm 2.65\%$ and $51.29 \pm 2.04\%$ for initial OTC concentrations of 5 and 20 mg/L. When the initial OTC concentration was 50 mg/L, the OTC degra-

mentation rate sharply decreased to $21.91 \pm 3.91\%$. Salinity also clearly influenced the OTC degradation efficiency. The lower the salinity was, the greater the OTC degradation efficiency was. When the salinity increased to 7, the OTC degradation rate decreased from $76.96 \pm 4.10\%$ to $49.90 \pm 3.19\%$. When the salinity was greater than 14, the OTC degradation rate decreased to 27–32%. In conclusion, strain F-6 efficiently degraded OTC and was essentially unaffected by pH (5–9). Strain F-6 could degrade OTC at high initial concentrations in seawater, but a lower initial OTC concentration and lower salinity were more conducive to OTC degradation.

3.3. Transcriptomic response of *Brucella* sp. strain F-5 to OTC

A total of 87,620,466 valid reads and 13,156,187,145 bp bases were obtained after the exclusion of low-quality sequences. The clean data were compared with the reference genome sequence of *Brucella anthracis* (NCBI accession number: GCF_016128015.1), and reads mapped successfully accounted for 96.56% of the original reads. Among the mapped reads, uniquely mapped reads accounted for 96.33%. All the transcriptomic data were deposited in NCBI data base (Accession: PRJNA1371707).

Compared with those in the control group, a total of 360 differentially expressed genes (DEGs) were identified in the experimental group (strain F-6 exposed to 10 mg/L OTC). Among all the DEGs, 236 genes were upregulated, and 124 genes were downregulated (Fig. 3a). The transcriptome data were further

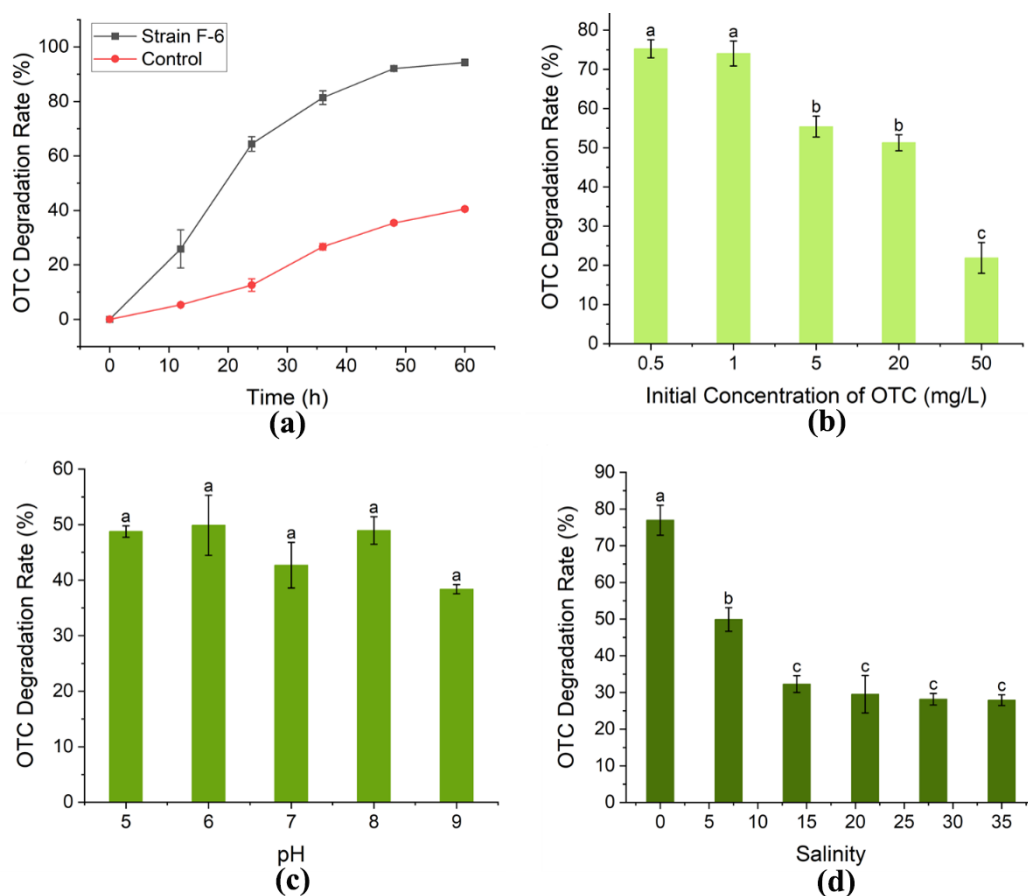


Fig. 2. OTC degradation rates of *Brucella* sp. strain F-6. (a): Time course of OTC degradation rates by strain F-6. (b),(c),(d): Effects of initial OTC concentration (b), pH (c), and salinity (d) on OTC degradation rates by strain F-6. The initial OTC concentration of a, c, and d was 10 mg/L. The degradation time of (b)-(d) was 24 hours.

validated through RT-qPCR, and the comparison results are shown in Fig. 3d. There was a strong positive correlation between them ($R^2 = 0.84$), which could indicate that the expression data from the transcriptome data were reliable.

The DEGs were functionally enriched according to the GO and KEGG enrichment analyses. All the DEGs were significantly enriched in 90 GO terms (P value < 0.05). The most obvious differences with respect to upregulation were focused on the molecular functions of structural molecule activity (GO:0005198), structural constituent of ribosome (GO:0003735), and oxidoreductase activity acting on NAD(P)H (GO:0016652); on the biological processes of tetrahydrofolate metabolic process (GO:0046653), peptide metabolic process (GO:0006518, 0043043), and protein metabolic process (GO:0019538, 0006575); and on the cellular components of intracellular organelles (GO:0043229, 0043232) and ribosomes (GO:0005840) (Fig. 3b). Oxidoreductase enzymes play an important role in antibiotic degradation^[21], and Tian et al. reported that a native laccase from *Pycnoporus* sp. SYBC-L10 could degrade OTC and tetracycline efficiently^[22], peroxidase was also shown to degrade OTC^[23, 24]. The degradation of OTC by

strain F-6 might also be attributed to the upregulation of oxidoreductase activity, which acts on NAD(P)H. The genes associated with the most obviously downregulated genes were related to the molecular functions of antioxidant activity (GO:0016209), oxidoreductase activity (GO:0016491), catalase activity (GO:0004096), inorganic molecular entity transmembrane transporter activity (GO:0015318) and the biological processes of reactive oxygen species metabolic process (GO:0072593), hydrogen peroxide metabolic and catabolic process (GO:0042743, 0042744), pyruvate family amino acid metabolic process (GO:0009078), and monoatomic ion homeostasis (GO: 0055080, 0050801, 0098771), and so on (Fig. 3c). The antioxidant defense system is normally upregulated in response to antibiotic stress, especially in eukaryotes. OTC could also result in an increase in antioxidant activity in the bacterial community^[25], and the antitoxin system of *Bacillus subtilis* was also activated to trigger global regulation against tetracycline pressure^[26]. However, in strain F-6, the antioxidant activity and reactive oxygen species metabolic process were globally downregulated, and OTC could limit this capacity, thus increasing the amount of reactive oxygen species^[27]. This find-

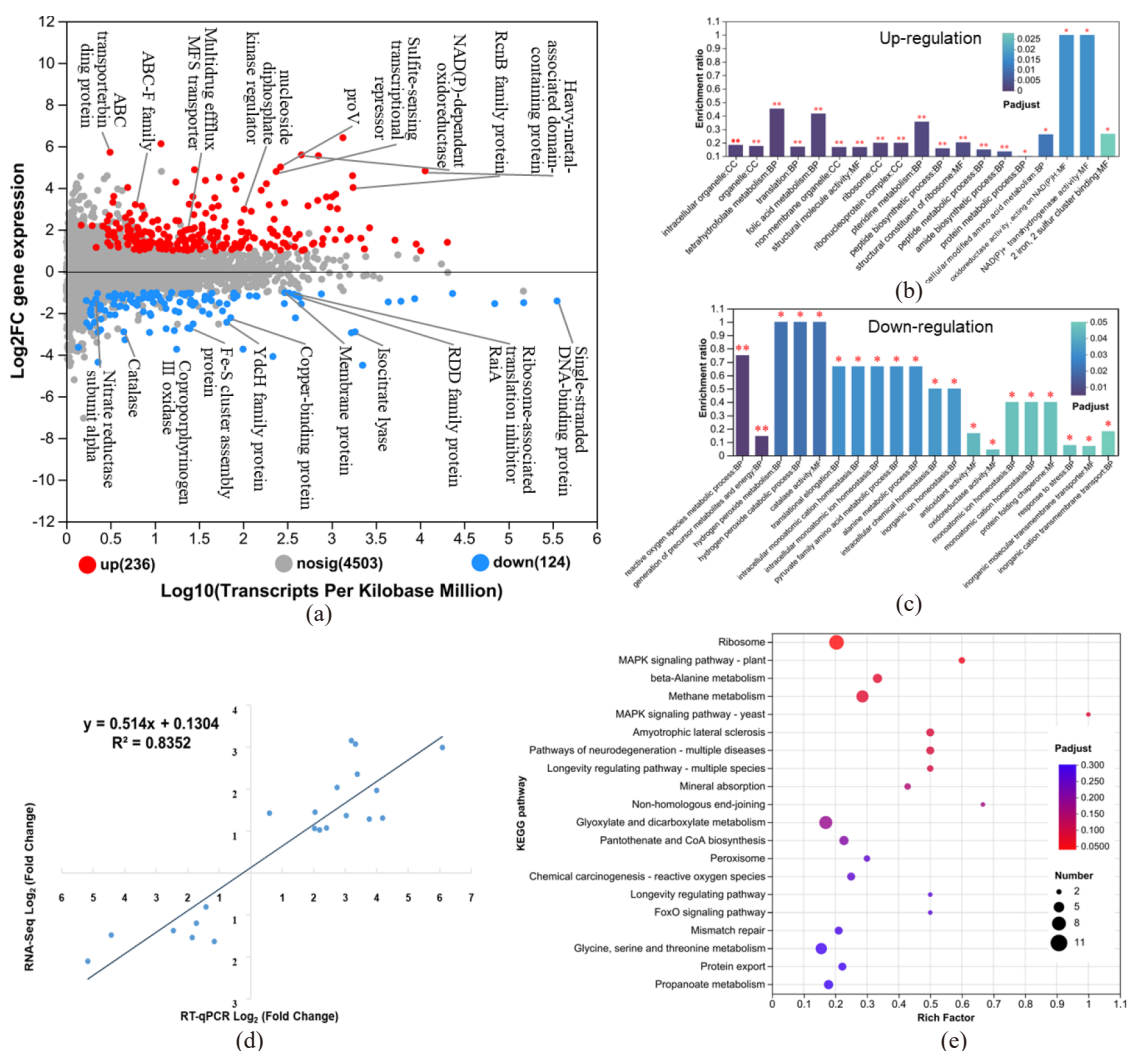


Fig. 3. Transcriptome analysis and RT-qPCR validation. (a): MA plot of expressed genes. Plots in blue, red, and gray separately represent downregulated genes, upregulated genes, and genes without differential expression, respectively. (b): GO enrichment of upregulated genes. (c): GO enrichment of downregulated genes. BP, CC and MF stand for biological process, cellular component and molecular function, respectively. (d): Verification of the transcriptome data via RT-qPCR. The RNA-Seq log2 (fold change) data are the result of the transcriptome. (e): KEGG enrichment analysis. The size of the dot indicates the number of DEGs in this pathway, and the color of the dot corresponds to different p value ranges.

ing also suggested that strain F-6 has alternative defense mechanisms that are different from those of other bacterial species.

According to the KEGG pathway in which the DEGs were significantly enriched, metabolic pathways and signaling pathways associated with OTC resistance and degradation could be identified. The most significantly enriched KEGG pathways were ribosome (map03010), methane metabolism (map00680), glycine, serine and threonine metabolism (map00260), mitogen-activated protein kinase (MAPK) signaling pathway (map04011, map04016), beta-alanine metabolism (map00410), mineral absorption (map04978), peroxisome (map04146), propanoate metabolism (map00640), and ABC transporters (map02010) (Fig. 3e). Many studies have indicated that tetracyclines bind to the RNA component of bacterial ribosomes^[28], and DEGs are also enriched in the ribosome pathway in strain F-6 under OTC stress. DEGs enriched in methane, glycine, serine, threonine, alanine, and propanoate metabolism might be involved in OTC degradation. The DEGs enriched in mineral absorption and ABC transporters might be related to OTC resistance.

3.4. Possible degradation pathway and key genes associated with OTC degradation by *Brucella* sp. strain F-6

To better understand the degradation pathway and mechanism of OTC degradation by strain F-6, LC-MS and transcrip-

tome data were combined. Ten possible degradation products were detected, and their mass spectra structural formulas are shown in Fig. S1 and S2. Two potential degradation pathways and several hypothetical key genes involved in OTC degradation are shown in Fig. 4 and Table S4.

In pathway I, P1 ($m/z = 432$) was generated through decarbonylation, which referred to the loss of CO from the ring structure of OTC. Liu et al. reported that OTC was likely to undergo a-cleavage to generate a diradical intermediate. Through the loss of CO, the formed diradical could produce other diradical compounds that could generate decarbonylation byproducts by ring closure. However, this process has been reported only under UV irradiation^[29]. P2 ($m/z = 413$) likely formed from P1 by dehydration.

In pathway II, P3 ($m/z = 432$) was generated through serial detachments of methyl groups from dimethylamine attached to the benzene ring of OTC; this process was also reported in microbial fuel cells of *Raoultella* sp. strain XY-1^[30] and the OTC-degrading bacteria *Bacillus* sp. and *Escherichia* sp.^[31]. In the present study, transcriptomic analysis of strain F-6 revealed that the genes encoding glycine betaine monooxygenase (Gbs, encoded by *gbsA* [RS03600] and *gbsB* [RS03595]) and sarcosine oxidase (Sox, encoded by *soxA* [RS11025] and *soxB* [RS11035]) were significantly upregulated under OTC stress (Table S4). Gbs can catalyze the oxidative *N*-demethylation. Sox can catalyze both the oxidative *N*-demethylation of sarco-

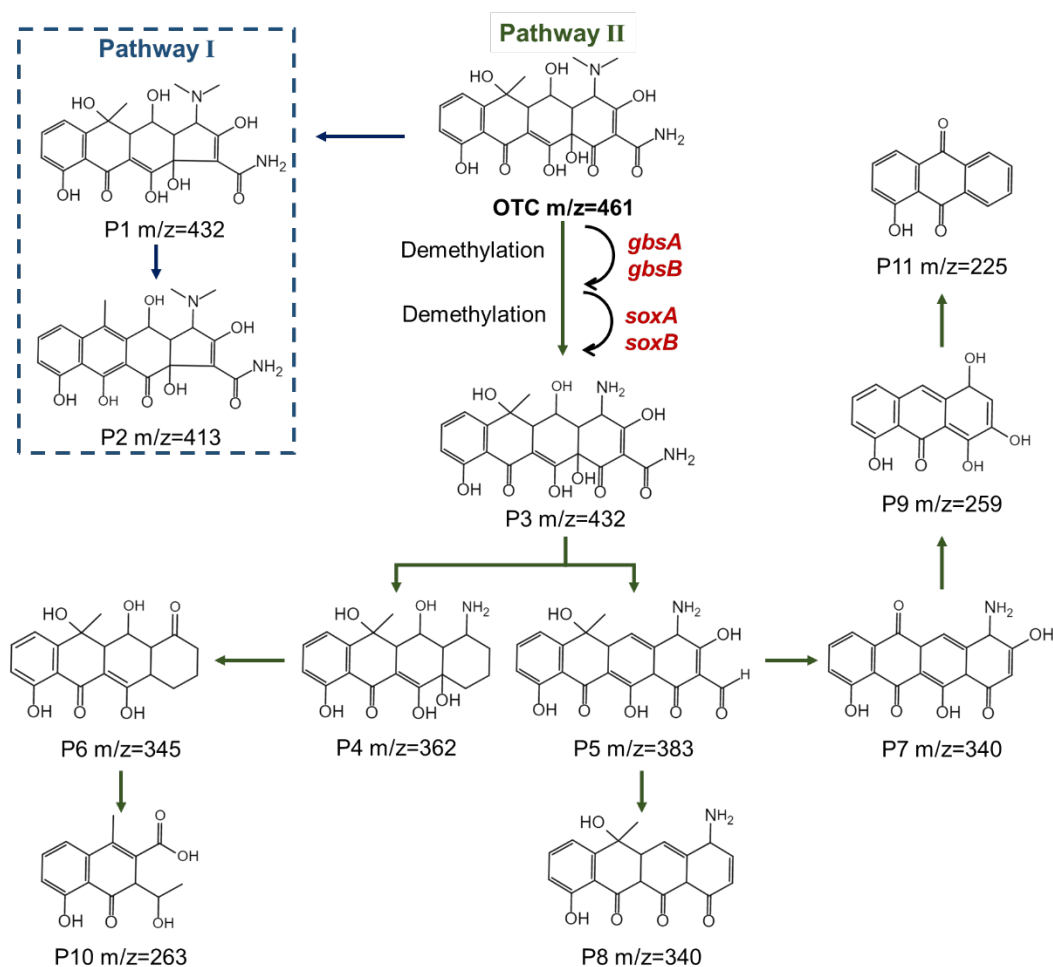


Fig. 4. Potential degradation pathways and candidate genes for OTC degradation by *Brucella* sp. strain F-6. *gbsA/gbsB*: encode glycine betaine monooxygenase subunits A and B; *soxA/soxB*: encode sarcosine oxidase subunit alpha and beta.

sine and the terminal *N*-demethylation of non-amino-acid compounds^[32]. Flavoprotein monooxygenase has been shown to inactivate tetracycline antibiotics by monohydroxylation^[33]. These results suggest that Gbs and Sox are potential candidate enzymes involved in OTC degradation by strain F-6, although direct enzymatic evidence is still needed. P3 could be hydroxyl detached, demethylated, and subjected to a series of other reactions to generate the product P4 ($m/z = 362$). An intermediate formed by the detachment of the hydroxyl group, and then the amino group was replaced by an aldehyde group involved in the formation of product P6 ($m/z = 345$). P4 might be deaminated by aminotransferase, which could degrade the mycotoxin fumonisin B1 by deamination^[34]. Then, dehydration and a ring-opening reaction followed to form P10 ($m/z = 263$)^[35]. Li et al. reported that *Bacillus brevis* strain MM2 could degrade OTC and produce P4^[20]. P4 was one of the main products of OTC degradation by strain F-6 (**Fig. S1**). P5 was formed with the detachment of hydroxyl groups and amide reduction from P3^[36]. P5 might be further demethylated and deamidated to form P7 ($m/z = 340$) or dehydroxylated and deamidated to form P8 ($m/z = 340$). P7 and P8 were both detected in OTC degradation, and the former was a product from the bacteria *Bacillus* sp. and *Escherichia* sp.^[31], and the latter was from highly dispersed copper single-atom catalysts activated peroxymonosulfate^[36]. P7 was followed by ring opening to obtain P9 ($m/z = 260$)^[37]. P9 underwent de-ethylation and oxidation to obtain P11 ($m/z = 225$).

3.5. Resistance and response mechanism of *Brucella* sp. strain F-6 to OTC

The resistance mechanisms of tetracyclines are categorized into tetracyclines efflux, ribosomal protection, and enzymatic inactivation of tetracyclines^[38]. **Fig. 5** illustrates the overall degradation, detoxication, resistance and response mechanisms of strain F-6 to OTC, with the genes involved shown in the figure, and detailed information about the DEGs and their coding proteins is provided in **Table S4 and S5**. In strain F-6, several cell structural defenses, membrane protection, and drug efflux-related genes, including the ABC transporter, outer membrane synthesis, and RND (resistance-nodulation-division) efflux pump, were significantly upregulated in response to exposure to OTC.

The ABC transporter superfamily plays an important role in multidrug resistance through the efflux of antibiotics^[39]. In strain F-6, the genes of glycine betaine/proline transport system (substrate-binding protein: ProX, permease protein: ProW, ATP-binding protein: ProV) and erythritol transport system (substrate-binding protein: EryG, permease protein: EryF, ATP-binding protein: EryE) were significantly upregulated. Glycine betaine, proline, and erythritol are osmoprotectants and membrane protectants in cells, and OTC promotes cell membrane damage^[40], thus, the delivery of more osmoprotectant protectants to the membrane might be one of the resistance mechanisms of strain F-6 to OTC. Moreover, strain F-6 responded to OTC stress by upregulating genes encoding membrane pro-

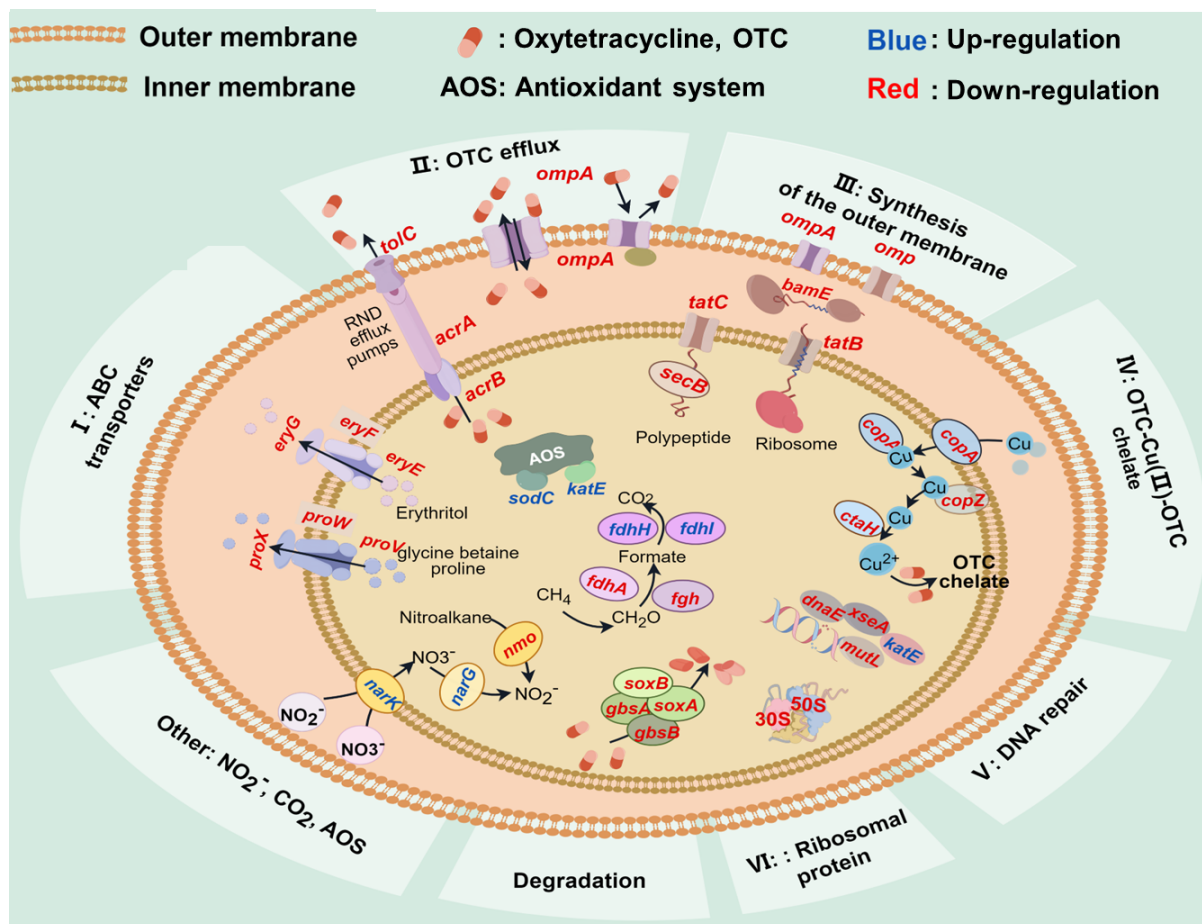


Fig. 5. Overall resistance and response mechanisms of *Brucella* sp. strain F-6 to OTC. The red and blue characters represent upregulated and down-regulated genes, respectively.

teins, such as outer membrane protein (OMP, OmpA) and membrane protein (OM), to strengthen the synthesis of the outer membrane. Nascent OMP is synthesized in the bacterial cytoplasm and can then be delivered through the inner membrane via two mechanisms: delivery via a protein-exporting chaperone (Sec B) to twin-arginine translocase (TatC) or via the ribosome to Sec-independent protein translocase (TatB)^[41]. This is also a protein export process. The outer membrane is an effective permeability barrier for protecting gram-negative bacteria from noxious chemicals in the environment. In addition, OmpA can improve drug efflux from cells and decrease drug accumulation^[42, 43]. RND transporters function as major multidrug efflux pumps in many gram-negative bacteria. These RND efflux pumps consist of three components: an inner membrane RND transporter, a periplasmic adaptor protein, and an outer membrane channel^[44, 45]. Upregulation of the genes encoding the efflux RND transporter periplasmic adaptor (AcrA), efflux RND transporter permease subunit (AcrB), and tol-pal system protein (TolC) indicated that RND-mediated efflux might be an important resistance mechanism of strain F-6 against OTC.

Strain F-6 upregulated genes encoding ribosomal proteins to resist OTC. OTC operates by targeting the bacterial ribosome, especially the small subunit (30S), effectively obstructs the attachment of aminoacyl-tRNA to the mRNA-ribosome complex, thereby inhibiting protein synthesis^[46]. Eleven ribosomal genes were significantly upregulated under OTC stress to maintain ribosome structure and function, 8 encoded 50S ribosomal subunit proteins, and 3 encoded 30S ribosomal subunit proteins. This phenomenon also occurred in *Escherichia coli* MG1655 under OTC stress^[47]. Moreover, strain F-6 resisted OTC damage via DNA repair, as evidenced by the upregulation of genes encoding exodeoxyribonuclease (Exo7), DNA polymerase (Dpo3), and DNA mismatch repair endonuclease (MutL). An increase in DNA repair enzymes in bacteria is a common response strategy for coping with antibiotics in bacteria, such as in *Paenarthrobacter* sp. AT5, which responds to sulfamethoxazole^[48], and in the response of the compost microbial community to OTC^[25].

The upregulation of genes encoding a copper chaperone (CopZ) and a P-type Cu(I) transporter (CopA) indicated that strain F-6 increased the absorption of Cu(I) and oxidized Cu(I) to Cu(II) via cytochrome c oxidase subunit IV (CytH). Hassan et al. reported that OTC could form an OTC-Cu(II)-OTC chelate, and this chelation reaction might reduce the toxicity of OTC^[49, 50]. The sites of chelation of oxytetracycline with Cu(II) are shown in **Fig. S3**. The formation of copper-OTC chelates was also a mechanism of OTC detoxification in strain F-6.

In addition to the resistance mechanisms mentioned above, strain F-6 also had other noteworthy responses to OTC. First, the nitrogen metabolism of strain F-6 was affected. The nitrate/nitrite transporter (NarK) plays a central role in nitrate uptake and nitrite excretion across the membrane^[51]; downregulation of *narK* genes might reduce nitrate uptake and nitrite excretion. Moreover, the *narG* gene (encoding nitrate reductase) was downregulated, while the *nmo* gene (encoding nitronate monooxygenase), a key enzyme in the conversion of nitroalkane catabolism to nitrite^[52], was upregulated. Overall, OTC might cause nitrite accumulation, and these nitrites are derived from nitroalkane catabolism rather than nitrate reduc-

tion. Substantial nitrite accumulation has also been observed in the OTC-degrading strain *Ochrobactrum* sp. KSS10^[13]. Second, OTC could affect the one-carbon metabolism of strain F-6. Upregulation of the *fdhA* gene (encoding glutathione-independent formaldehyde dehydrogenase) and the *fgh* gene (encoding S-formylglutathione hydrolase) could promote formate formation, whereas downregulation of the *fdhL* and *fdhH* genes (encoding formate dehydrogenase subunits) could reduce the conversion of formate to CO₂. Formate, a potential biomarker of one-carbon metabolism, might accumulate in strain F-6. Studies on the effects of antibiotics on carbon metabolism have focused mostly on central carbon metabolism, with few studies on one-carbon metabolism^[53]. Specifically, the *sodC* gene (encoding superoxide dismutase, Cu-Zn family) and the *katE* gene (encoding catalase) were downregulated, indicating that OTC restricts the activities of the antioxidant system of strain F-6, which is different from the usual upregulation in response to antibiotic stress^[25, 54].

In conclusion, strain F-6 resisted OTC damage through a variety of mechanisms. First, strain F-6 could increase protein export as well as glycine betaine/proline and erythritol transport to maintain the structure and stability of the outer membrane in response to destruction of the cell membrane by OTC. Second, strain F-6 enhanced RND efflux pumps and the outer membrane protein OmpA to expel OTC from cells or prevent OTC from entering cells. Third, it upregulates genes encoding several ribosomal proteins and DNA repair proteins to maintain ribosome structure and function. Fourth, strain F-6 degraded or chelated OTC to detoxify it through OTC degradation proteins or OTC-Cu(II)-OTC chelation. OTC also affected the physiological processes of strain F-6, such as reducing nitrate and nitrite intake and the conversion of nitrate to nitrite, but increasing nitroalkane catabolism to nitrite, ultimately leading to the accumulation of nitrite. OTC could also affect one-carbon metabolism and restrict the antioxidant system of strain F-6.

3.6. Response of *Brucella* sp. strain F-6 to OTC at different concentrations

OTC is often detected in wastewater and river water at concentrations ranging from ng/L to µg/L and, in some cases, at mg/L levels. Based on the known published data, environmental concentrations of OTC were set to 0.1 µg/L and 100 µg/L respectively, which represented mild OTC contamination and heavy OTC contamination. Meanwhile, bacteriostatic concentration of OTC was set to 10 mg/L. The fold change (log₂FC) was calculated as difference in expression between different levels of OTC stress and the control. In accordance with the results of the transcriptome analysis described above, representative genes for OTC degradation, resistance, and other responses were selected, including *gbsB* for OTC degradation process, *copZ* for Cu-OTC chelate, *tolC* for RND efflux pump, *ompA* for OTC efflux and the outer membrane synthesis, *proV* for ABC transporter of membrane protectant, *rpmF* for ribosome protection, *mutL* for DNA repair, *narK* for nitrogen metabolism, and *katE* for antioxidant system (**Fig. 6A-6I**).

The OTC degradation process of strain F-6 was activated even under mild OTC contamination (**Fig. 6a**), indicating that strain F-6 would degrade OTC at a low OTC concentration (0.1 µg/L), and hold great promise for the treatment of environmen-

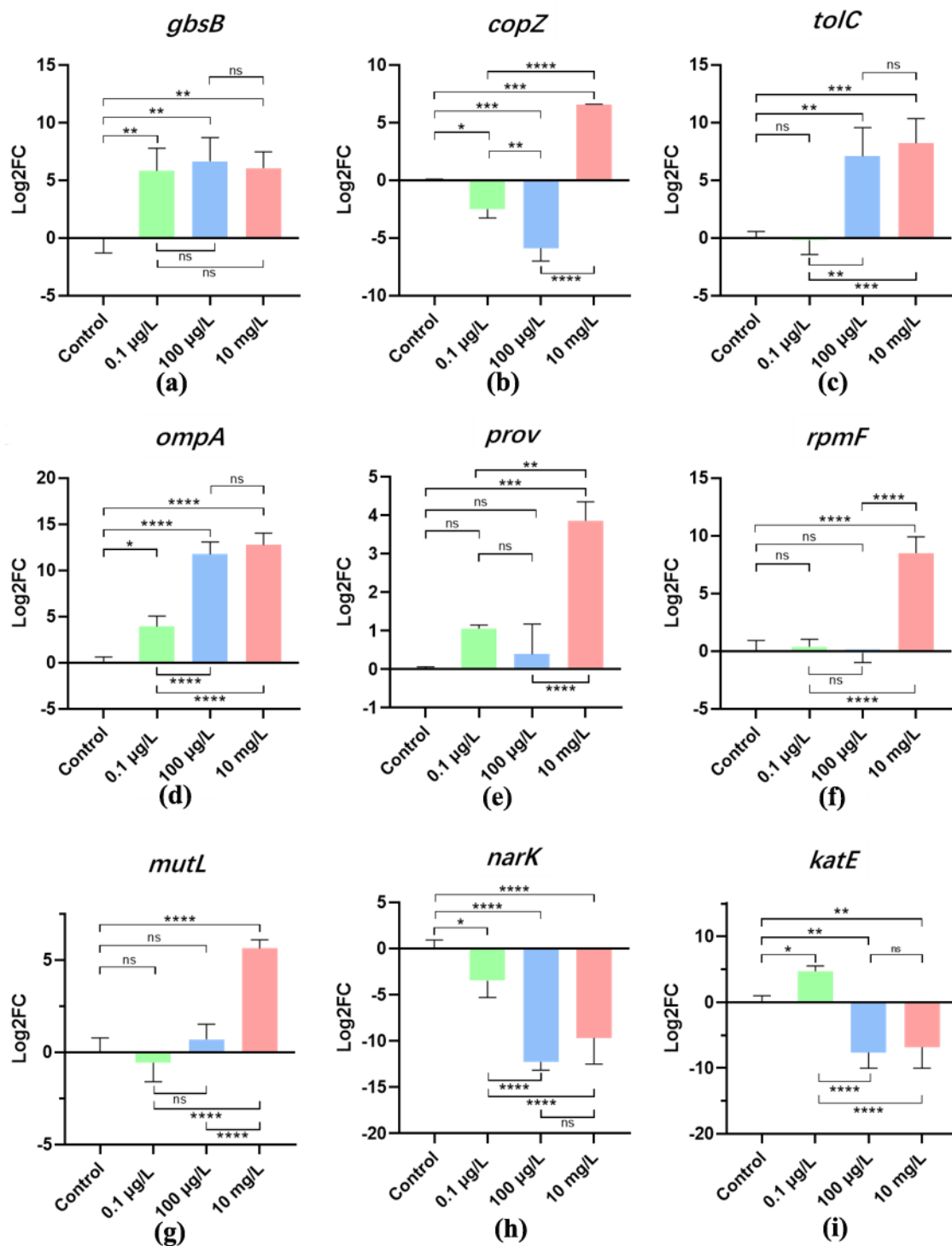


Fig. 6. Effects of OTC at different concentrations on *Brucella* sp. strain F-6, including degradation (a), Cu chelation (b), efflux and outer membrane (c, d, e), ribosome protection (f), DNA repair (g), nitrogen metabolism (h), and antioxidant system (i) related genes. The fold change (log2FC) was calculated as the difference in expression between different levels of OTC stress and the control. Significant differences between groups are marked with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; ns, no significant difference.

tal OTC pollution. In contrast to the bacteriostatic concentration, the environmental concentration of OTC inhibited the absorption and utilization of Cu(II) (Fig. 6b), indicating that OTC was not detoxified through the Cu-OTC chelate at the environmental concentration. However, some studies have shown that only high-OTC treatments (4.6–9.2 mg/L) decrease the Cu absorption of the marine microalga *Isochrysis galbana*^[55].

The drug efflux pump of RND was activated by heavy OTC contamination and was not activated by mild OTC contamination

(Fig. 6c). But drug efflux of OmpA was activated by the mild OTC contamination (Fig. 6d). Therefore, it's speculated that the mild OTC contamination would activate partial drug efflux. The resistance mechanism of OTC is mostly related to drug efflux, and high concentrations of OTC have been shown to spread ARGs through horizontal gene transfer among microbial communities^[56]. Lin et al. reported that 100 µg/L OTC strongly promoted ARGs abundance^[57]. However, few studies have focused on the effects of ng/L OTC on the spread of

ARGs. The drug efflux pump of strain F-6 was not activated, but a functional protein with an efflux function was activated at the ng/L level of OTC, which has important implications for the spread of ARGs in the environment with trace concentrations of antibiotics.

In addition to the drug efflux process, the OTC resistance mechanisms of membrane protection, ribosome protection and DNA repair were activated only at bacteriostatic concentrations of OTC (Fig. 6e–6g). Therefore, they might not constitute the main resistance mechanism of strain F-6 to OTC at environmental concentrations. However, the absorption of inorganic nitrogen was inhibited by mild OTC contamination (Fig. 6h). Several previous studies have shown that antibiotic residues affect nitrogen transformation in the bacterial community^[58], but few studies have focused on the influence of trace antibiotics on the nitrogen mechanism. Notably, the antioxidant system of strain F-6 was activated by mild OTC contamination, in contrast to the inhibition of heavy and bacteriostatic pollution (Fig. 6i). In eukaryotes, catalase activity significantly increases after chronic exposure to 0.3 µg/L OTC^[59]; however, in the bacterial community, catalase activity increases after exposure to 100 µg/L OTC^[60]. To the best of our knowledge, no studies have investigated the response of single bacteria or bacterial communities to ng/L OTC.

In summary, there were both similarities and differences in the responses of degradation-, resistance- and metabolism-related genes under OTC stress between antimicrobial concentrations and environmental concentrations. The degradation process, partial efflux function, and nitrogen metabolism were activated or affected even under mild OTC contamination (0.1 µg/L). The drug efflux pump of RND was activated under heavy OTC contamination (100 µg/L). The antioxidant system was started to be inhibited under 100 µg/L OTC in contrast to the stimulation of 0.1 µg/L OTC. Other OTC resistance mechanisms, including membrane protectant transportation, ribosome protection and DNA repair were activated only at bacteriostatic concentration of OTC.

4. Conclusions

In this study, a novel bacterial strain, *Brucella* sp. F-6, was shown to be able to efficiently degrade OTC into small molecules. Strain F-6 might degrade and detoxify through glycine betaine monooxygenase, sarcosine oxidase, and Cu-OTC chelate and resist OTC through drug efflux, outer membrane protection, ribosome protection, and DNA repair. Some effects related to degradation, resistance and metabolism processes were observed at environmental concentrations of OTC. The results of this study also provide a strategy for research on the environmental behavior and toxicity of trace amounts of antibiotics in the environment. However, since *Brucella* sp. F-6 is a pathogenic bacterium, considerable work is still required before its practical application, including systematic evaluation of biosafety, as well as the screening, heterologous expression and functional verification of key functional proteins.

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Declaration of Competing Interest

Lingxin Chen is an Editorial Board Member of [Environmental Chemistry and Safety] and was not involved in the editorial review or the decision to publish this article. The author declares that there is no conflict of interest regarding the publication of this paper.

Electronic Supplementary Material (ESM)

The online version contains supplementary material available at <https://doi.org/10.26599/ECS.2026.9600047>.

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