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Antidepressant fluoxetine induces multiple antibiotics resistance in *Escherichia coli* via ROS-mediated mutagenesis

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ABSTRACT

Background: Antibiotic resistance poses a great threat to global public health. Overuse of antibiotics is generally considered as the major factor contributing to it. However, little is known about whether non-antibiotic drugs could play potential roles in the emergence of antibiotic resistance.

Objective: We aimed to investigate whether antidepressant fluoxetine induces multiple antibiotic resistances and reveal underlying mechanisms.

Methodology: Escherichia coli K12 was exposed to different concentrations of fluoxetine (0, 0.5, 5, 50 and 100 mg/L) and the resistant strains were isolated by plating on antibiotic containing plates. Resistant strains were randomly selected to determine the increase of minimum inhibition concentration (MIC) of multiple antibiotics. Genome-wide DNA sequencing was performed on cells cultured in lysogeny broth (LB) without any fluoxetine or antibiotics exposure. RNA sequencing and proteomic profiling of isolated mutants grown in LB with 100 mg/L fluoxetine were analyzed to reveal the underlying mechanisms.

Results: Exposure of *Escherichia coli* to fluoxetine at 5–100 mg/L after repeated subculture in LB for 30 days promoted its mutation frequency resulting in increased resistance against the antibiotics chloramphenicol, amoxicillin and tetracycline. This increase was up to 5.0×10^7 fold in a dose-time pattern. Isolated mutants with resistance to one of these antibiotics also exhibited multiple resistances against fluoroquinolone, aminoglycoside, β-lactams, tetracycline and chloramphenicol. According to global transcriptional and proteomic analyses, the AcrAB-TolC pump together with the YadG/YadH transporter, a Tsx channel and the MdtEF-TolC pump have been triggered to export the antibiotics to the exterior of the cell. Whole-genome DNA analysis of the mutants further revealed that ROS-mediated mutagenesis (e.g., deletion, insertion, and substitution) of DNA-binding transcriptional regulators (e.g., *marR*, *rob*, *sdiA*, *cytR* and *crp*) to up-regulate the expression of efflux pumps, may further enhance the antibiotic efflux.

Conclusions: Our findings for the first time demonstrated that the exposure to antidepressant fluoxetine induces multiple antibiotic resistance in *E. coli* via the ROS-mediated mutagenesis.

1. Introduction

Infections due to multiple antibiotic resistant bacteria (ARB) cause substantial mortality and the spread of antimicrobial resistance (AMR) has become a worldwide threat to public health (Harms et al., 2016). It is estimated that AMR will cause as many as 10 million casualties annually by 2050 if no action is taken now (O'Neill, 2015). More seriously, wastewater, tourism and trade are moving microbes around the globe at an unprecedented scale, resulting in the rapid spread of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) between continents (Zhu et al., 2017).

Overuse or misuse of antibiotics has been believed to be the main cause for the emergence and spread of antibiotic resistance (Harkins et al., 2017). Under selective pressure from antibiotics, microbes will develop resistance through mutation within the genome. In addition, bacteria can acquire antibiotic resistance through horizontal gene transfer (HGT), including transformation, transduction and conjugation (Blair et al., 2015). Both mutation or HGT processes may potentially be

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accelerated with the overuse or misuse of antibiotics (Hwang and Gums, 2016). Recently, non-antibiotic chemicals, e.g. triclosan (Lu et al., 2018), disinfection byproducts (Lv et al., 2014), ionic liquid (Wang et al., 2015) and nanoparticles (Qiu et al., 2012), have been found to promote the spread of antibiotic resistance through mutation or HGT. For example, the chlorine disinfection process in a full-scale wastewater treatment plant (WWTPs) not only increased the abundances of intracellular ARGs, but also enhanced the concentration of extracellular ARGs, suggesting chlorination may contribute to antibiotic resistance (Liu et al., 2018). More seriously, our recent study reported that triclosan found in personal care products that we use daily could induce heritable multi-drug resistance through mutation (Lu et al., 2018). This finding also highlighted that it is essential to evaluate the potential antibiotic-like roles induced by non-antibiotic chemicals.

The antidepressant fluoxetine, as a selective serotonin re-uptake inhibitor, that has rapidly become one of the most heavily prescribed psychotropic drugs used for the treatment of clinical depression (Services, 2012). Adverse effects of fluoxetine on human health also have received considerable concern (Burcu et al., 2017). In addition, fluoxetine could enter into aquatic environment through excretion, showing bioaccumulation in aquatic organisms (e.g., fish) (Schultz et al., 2011) and environmental persistence (Oakes et al., 2010). It has been reported that fluoxetine is mainly excreted in urine with up to 11% of the administered fluoxetine dose being excreted as the unchanged parent compound (Panlilio et al., 2016). Due to ubiquitous occurrence of fluoxetine in wastewater, river systems and groundwater at hundreds or thousands of nanograms per liter (Ford and Fong, 2016; Schultz et al., 2010; Wu et al., 2017), the toxicity and ecotoxicity of fluoxetine has received considerable attention (Brooks et al., 2003). Unfortunately, there is no study about whether fluoxetine could contribute to the emergence of antibiotic resistance.

The objective of this study is to investigate whether the fluoxetine exposure would cause the emergence of antibiotic resistance. *Escherichia coli*, an opportunistic pathogen in the human gut and common in the aquatic environment, was chosen to conduct the experiment. After 30-day fluoxetine exposure, antibiotic resistant mutants were isolated and their resistance to 8 different antibiotics of several types was determined, together with the combined measurements of reactive oxygen species (ROS) production stressed by fluoxetine. The mechanisms of fluoxetine-induced antibiotic resistance were comprehensively investigated by genome-wide DNA sequencing, RNA sequencing and proteomic profiling. This work represents the first investigation of the potential role of fluoxetine for inducing multiple antibiotic resistance. The outcomes would advance our understanding of the dissemination of antibiotic resistance induced by non-antibiotic drugs.

2. Materials and Methods

2.1. Bacteria strains and culture conditions

E. coli K12 (MG 1655) used for antibiotic resistance tests were purchased from the American Type Culture Collection (ATCC 700926). It was grown in lysogeny broth (LB) (10 g/L tryptone (Difco), 5 g/L yeast extract (Difco), 10 g/L NaCl) at 37 °C for 24 h. M-endo agar (BD, USA) plates with 16 mg/L chloramphenicol (Chl), 64 mg/L amoxicillin (Amo) or 4 mg/L tetracycline (Tet), were used to isolate antibiotic resistant mutants of *E. coli*. All the antibiotics in this study were purchased from Sigma-Aldrich (USA).

2.2. Fluoxetine exposure and isolation of mutants against antibiotics

An *E. coli* suspension of 40 μL, which has been incubated at 37 °C for 15 h and reaching a cell density of 10^8–10^9 CFU/mL was added to 3.96 mL LB broth containing either 0.5, 5, 50 or 100 mg/L of fluoxetine (Sigma-Aldrich, USA). The bacteria numbers were determined with the pour plate method (W. E. Federation and Association, 2005). After incubation at 37 °C for 24 h, 40 μL of the above *E. coli* cultures were then transferred into 3.96 mL fresh LB containing the responding concentration of fluoxetine and incubated again for 24 h. This subculturing was repeated for 30 days. During the subculturing period, 100 μL of these fluoxetine exposed *E. coli* cultures were plated on the above M-endo agar containing Chl or Tet or Amo, respectively, to screen for antibiotic resistant bacteria. This was performed on the subcultures on Day 1, 3, 5, 10, 20 and 30 and the inoculated plates were incubated for 24 h at 37 °C. The mutation frequency was calculated by dividing the number of antibiotic-resistant colonies by the total bacterial count, which was enumerated from plating cultures without antibiotics onto LB agar. Then, 5–20 resistant mutants were randomly picked from each antibiotic-selected culture and were then grown in 2 mL of LB for 12–16 h at 37 °C. In parallel, *E. coli* cultured in the LB medium without fluoxetine exposure were also transferred for 30 subculture cycles and used to spread on the antibiotics-selective M-endo agar as described above. All the experiments were performed in biological triplicates at least.

2.3. Determination of minimum inhibitory concentrations (MICs)

MICs of *E. coli* against the antibiotics Chl, Amo, Tet, Nor, Lev, Amp, Kan and Cep were determined as previously described (Li et al., 2016). Briefly, *E. coli* suspensions with a final concentration of 10^5–10^6 CFU/mL, determined with the pour plate method (W. E. Federation and Association, 2005), was prepared using 0.1% (*v*/v) overnight *E. coli* culture into fresh LB. Then, 135 μL of this suspension was added to wells in 96-well plates containing 15 μL of serially 2-fold diluted antibiotics (from 0.1 mg/L to 80 mg/L depending on antibiotic types). After 24 h of incubation at 37 °C, the optical density (OD) of each well was measured using an ELISA reader device (Tecan, Swiss) at 600 nm. For each batch, the negative control well without bacterial inoculums and the positive control well free from antibiotics were used to calculate the inhibition of bacterial growth. The MIC was determined as the concentration of antibiotics that inhibits 90% of bacterial growth in LB. Each strain was tested in triplicate.

2.4. ROS measurement

To investigate whether oxidative stress plays a role in promoting fluoxetine-induced mutation, ROS in bacteria was quantified by flow cytometry using the 2′,7′-dichlorofluorescindiacetate (DCF-DA)-cellular ROS detection assay Kit (Abcam, USA) according to the manufacturer's instruction. Briefly, a bacterial suspension at the concentration of 10^5–10^6 CFU/mL in the LB media were prepared in a 1.5 mL tube and then pre-incubated with 20 μmol/L DCF-DA for 45 min at 37 °C. After incubation, DCF-DA loaded cells were treated with fluoxetine (100 mg/L) or Tert-Butyl Hydrogen Peroxide (100 μmol/L) for 2 h at 30 °C. To observe the background fluorescence intensity of *E. coli* responding to DCF-DA, a blank control was set with the addition of sterile distilled water. All treated samples were scanned by the Flow Cytometer (BD Accuri™, USA), under conditions of excitation at 488 nm and emission

at 535 nm. Intracellular levels of ROS were calculated using the BD Accuri C6 Software. The ROS measurements were carried out in biological triplicates.

2.5. DNA and RNA extraction and whole-genome DNA and RNA sequencing

Bacterial DNA was extracted from wild-type *E. coli* K12 or mutant variants cultured in LB broth for 10 h at 37 °C using FastDNA™ SPIN Kit (MP Biomedicals, CA, USA) according to the manufacturer's instructions. DNA samples (A260/A280, 1.8–2.0) were then submitted to Australian Genomics Research Facility (AGRF) for the sequencing on the MiSeq instrument (Illumina) with 150 bp paired-end sequencing, to a coverage of over 100-fold. For the mutant variants isolated from the same condition (labeled as Flu_{1d}-Chl^r, Flu_{1d}-Tet^r, Flu_{30d}-Chl^r, Flu_{30d}-Tet^r and Flu_{30d}-Amo^r), two clones were chosen to DNA sequencing.

10 mL of LB broth with 100 mg/L fluoxetine was inoculated with 10^7–10^8 CFU/mL of fresh cultured wild-type *E. coli* K12 or mutant variants. Following incubation at 37 °C for 10 h with shaking (150 rpm), bacteria were harvested by centrifugation at 8000 × *g* for 10 min. Bacterial RNA was extracted from the prepared cells using the miRNeasy Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. RNA samples (RIN ≥ 7) were then submitted to Macrogen Co. (Seoul, Korea) for strand-specific cDNA library construction and Illumina paired-end sequencing (HiSeq 2000, Illumina Inc., San Diego, CA). The wild-type *E. coli* K12 were sequenced triplicate and three mutant variants isolated from the same exposure condition (labeled as Flu_{1d}-Chl^r, Flu_{1d}-Tet^r, Flu_{30d}-Chl^r, Flu_{30d}-Tet^r and Flu_{30d}-Amo^r) were chosen to RNA sequencing.

2.6. Protein extraction and Sequential Window Acquisition of all Theoretical Spectra-Mass Spectrometry (SWATH-MS) measurement

Ten mL of LB with 100 mg/L fluoxetine was inoculated with 10^7–10^8 CFU/mL of freshly cultured *E. coli*. After growth at 37 °C for 10 h with shaking (150 rpm) bacteria coming from the same cultures for RNA extraction were harvested by centrifugation at 8000 × *g* for 10 min. Bacterial protein was extracted from the cell pellets, then reduced, alkylated and digested according to the procedure described in the Supplementary Material Text1. For each sample, 1 μg of the digested protein was used for subsequent SWATH-MS analysis in triplicates. Additionally, 5 μg aliquots of each triplicate sample were pooled for MS library construction by information dependent analysis (IDA), which was performed in duplicate. Peptides were directly analyzed on a Triple-Tof 5600 instrument (Sciex, USA) equipped with a Nanospray III interface (Supplementary material Text 2).

2.7. DNA-Seq data processing and mutation analysis

Genome alignments and mutation analysis were performed according to the modified methods (Li et al., 2016), in which the genome of *E. coli* strain K12 (Genbank Accession, U00096.3) was selected as the reference. Briefly, Trimmomatic (version 0.36) was used to trim the Illumina paired-end raw data and only properly paired reads were kept for further downstream analysis. To study small variants, these high read-depth datasets were aligned back to the reference sequence using BreqSeq 0.29.0 and highly divergent loci were then subjected to further analysis. Data visualization tools BRIG and Mauve were utilized for comprehensive comparisons and graphical plots.

2.8. RNA-Seq data processing and global transcriptional analysis

The raw sequence reads were processed and then analyzed according to the previous method (Guo et al., 2017). Briefly, following trimming by NGS QC toolkit (version2.3.3), the clean reads of each sample were aligned to the reference genome of *E. coli* K12 (NC_000913.3) using SeqAlto (version 0.5). Cufflinks (version 2.2.1) was used to calculate the strand-specific coverage for each gene and to analyze the differential expression on triplicate cultures from the same condition. Gene expression was calculated as fragments per kilobase of a gene per million mapped reads (FPKM), a normalized value generated from the frequency of detection and the length of a given gene. Differences in fold change values were calculated between wild-type *E. coli* and isolated antibiotic resistance mutants by determining the $\log_2$ fold change (LFC) of the averaged FPKM values of two triplicate experiments. Astringency LFC cutoff of ≥ 1 or ≤ -1 (≥ 2 or ≤ 0.5 absolute fold change (AFC)), with a false discovery rate (FDR) < 0.001 and a *p*-value < 0.05 was adopted to distinguish the differentially expressed genes. Program Circos was used to visualize expression and determine the FPKM values for each gene (Krzywinski et al., 2009).

2.9. SWATH-MS data process and proteomic analysis

The IDA library and SWATH-MS data were loaded into PeakView v 1.2 software for processing using the SWATH micro-processing script, with a confidence level of 99%, the number of peptides set at 5 and the number of transitions used set at 3. A minimum of 2 peptides and 3 transitions was used for quantitative analysis. The R- based program MSstats was used for statistical analysis of the spectral data. Pathway Tools was used for metabolic pathway reconstruction using the identified proteins. Astringency cut-off of LFC at ≥ 0.2 or ≤ -0.2 (i.e., ≥ 1.2 or ≤ 0.9 AFC) with a q value < 0.001 was used to identify the significantly differentially expressed proteins.

2.10. Data availability

DNA Sequencing data are accessible through the Sequence Read Archive (SRA accession number SRP127973). RNA Sequencing data are accessible through the Gene Expression Omnibus (GEO) of NCBI (GSE108190). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD010198 (Vizcaino et al., 2016).

2.11. Statistical analysis

Statistical analyses were performed using Microsoft Excel (Microsoft, Redmond, WA), SPSS 19.0, and SAS9.2.

3. Results and Discussion

3.1. Fluoxetine exposure enhanced the mutation frequency and increased the antibiotic resistance capabilities of E. coli

To test the potential of fluoxetine to induce antibiotic resistance, *E. coli* K12 (MG1655) was exposed to fluoxetine at the concentrations of 0.5, 5, 50 and 100 mg/L and repeatedly sub-cultured in LB for 30 days (Fig. 1A). During the exposure period, the mutation frequencies of *E. coli* culture against Chl, Amo and Tet were evaluated on Days 1, 3, 5, 10, 20 and 30. Our results showed that fluoxetine exposure levels

(ranging from 5 mg/L to 100 mg/L) significantly enhanced the mutation frequency ($p < 0.01$), while the low exposure concentration of 0.5 mg/L did not increase the mutation frequency (Fig. 1B).

$(4.0 \pm 1.0) \times 10^{-1}$ for Chl^r, $(5.0 \pm 1.0) \times 10^{-1}$ for Tet^r and $(4.5 \pm 2.0) \times 10^{-7}$ for Amo^r. These mutation increases were in the order of 5.0×10^7, 3.4×10^7 and 38-fold respectively (Fig. S1), compared to the spontaneous mutation frequency of Chl^r, Tet^r and Amo^r in *E. coli* without fluoxetine exposure.

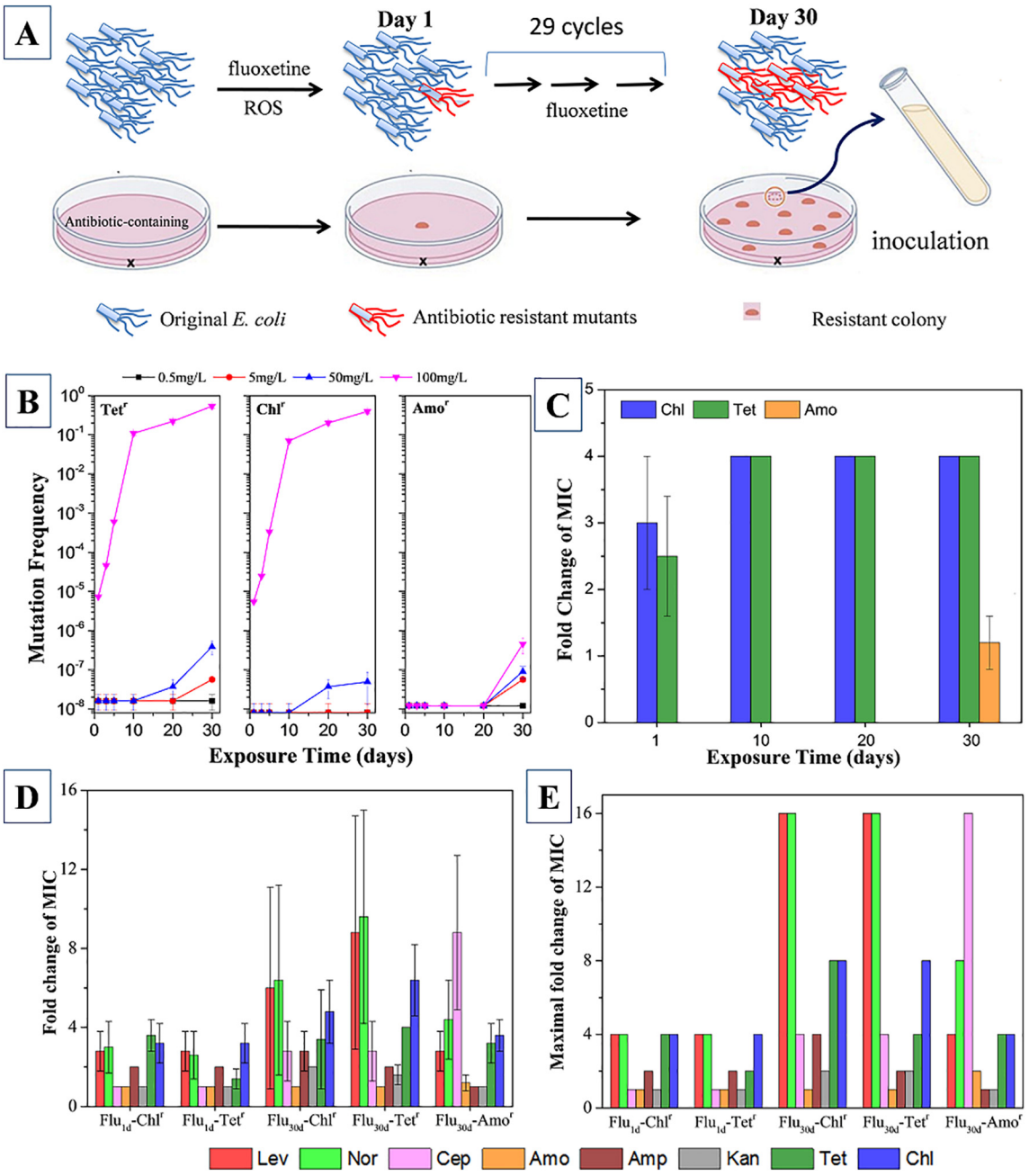


Fig. 1. Effects of fluoxetine exposure on strain *E. coli* K12. A, an illustration to show the production of resistant mutants; B, the mutation frequency of resistance against Chl, Tet and Amo during the exposure period; C, the MIC determined against Chl, Tet and Amo of resistant mutants isolated after different exposure times to 100 mg/L of fluoxetine; D, the fold change of MIC against multiple antibiotics of resistant mutants isolated after 30-days exposure time of 100 mg/L fluoxetine; E, the maximal fold change of MIC for Chl-, Tet- or Amo- resistant *E. coli* mutants isolated after 30-day exposure to 100 mg/L fluoxetine, relative to untreated *E. coli*.

The mutation frequency followed a dose-response pattern, where it increased with higher levels of fluoxetine exposure (Fig. 1B). At the exposure level of 5 mg/L fluoxetine for 30 days, only mutation frequencies of Tet and Amo resistance were seen to increase. This was a 7-fold escalation compared to the spontaneous mutation frequency of Tet^r 1.6×10^{-8} and Amo^r 1.2×10^{-8} in *E. coli* without fluoxetine exposure. In contrast, when *E. coli* was exposed to fluoxetine at 100 mg/L, the mutation frequencies dramatically increased to

Additionally, the exposure time of fluoxetine affected the mutation frequency significantly ($p < 0.01$). Compared to the 30-day exposure, the mutation frequencies were only $(5.5 \pm 0.1) \times 10^{-6}$ for Chl^r and $(7.3 \pm 0.1) \times 10^{-6}$ for Tet^r after 1-day exposure of 100 mg/L. However, the mutation frequencies increased to $(1.0 \pm 0.0) \times 10^{-1}$ for Chl^r and Tet^r after 10-day exposure of 100 mg/L. It was evident that

the higher the exposure concentration was, the faster the mutation frequency increased with time. The resistant *E. coli* began to grow on Tet^r or Chl^r selecting-plates on Day 1 for 100 mg/L, Day 20 for 50 mg/L and Day 30 for 5 mg/L. In contrast, no colonies were found on the Amo^r selective plates until the exposure time was prolonged to 30 days for 100 mg/L fluoxetine. The results indicate a time and dose-dependent effect on the resistance mutation due to the fluoxetine exposure, indicating the accumulative effect of fluoxetine might induce antibiotic resistance after a long-term exposure in sewage treatment.

MICs against Chl, Tet and Amo were monitored for 8 mutants randomly isolated from each of the Chl-, Tet- and Amo-containing plates. This showed that MICs of Chl and Tet increased significantly for the resistant mutants that were generated by the 100 mg/L fluoxetine exposure ($p < 0.01$, Fig. 1C). Compared to wild-type *E. coli* whose MICs against Amo of 8 mg/L, Chl of 16 mg/L and Tet of 16 mg/L, MICs increased by 2.5–3 folds for the isolated mutants after one-day exposure, and up to 4-fold for all the resistance mutants (labeled as Flu_{nd}-Chl^r or Flu_{nd}-Tet^r, where *n*d means the number of exposure days) isolated after 10-day exposure or more. However, for the Amo resistant mutants, no significant increase of MIC against Amo was observed ($p > 0.05$).

3.2. Fluoxetine exposure increases resistance to multiple antibiotics in E. coli

To further confirm the development of antibiotic resistance, the resistant strains ($n \geq 5$) chosen against each of the three antibiotics (Chl, Tet and Amo) were tested to determine MIC against 8 types of antibiotics: Chl, Tet, Amo, norfloxacin (Nor), levofloxacin (Lev), ampicillin (Amp), cephalexin (Cep) and kanamycin (Kan). Three types of resistant mutants selected by fluoxetine exhibited multiple-drug resistant activities (Fig. 1D, E and Fig. S2, Table S1). For mutant types Flu_{1d}-Chl^r and Flu_{1d}-Tet^r, their average MICs against Nor, Lev, Amp, Chl and Tet increased 2–4 folds compared to the wild-type *E. coli*, but their resistance to Cep, Amo and Kan did not increase. However, once the exposure time was extended to 30 days, the Chl- and Tet-resistant mutants showed stronger resistant capabilities to all tested antibiotics except for Amo, in terms of their MICs (Fig. 1D). In particular, the MICs for Lev and Nor increased by up to 16-fold and their maximal MICs reached 1 mg/L and 2 mg/L, respectively. In comparison, the Amo-resistant mutants isolated after 30-day exposure had increased resistance against Lev, Nor, Cep, Tet and Chl by 2–16 folds ($p < 0.01$), but they had no significantly improved resistance to Amp or Kan ($p > 0.05$).

Remarkably, exposure to fluoxetine, a non-antibiotic drug results in significant increase in antibiotic-resistant mutants compared to the control without exposure to fluoxetine. More seriously, the mutants induced by fluoxetine exposure exhibited multiple resistances to the antibiotics fluoroquinolone, aminoglycoside, β-lactams, tetracycline and chloramphenicol.

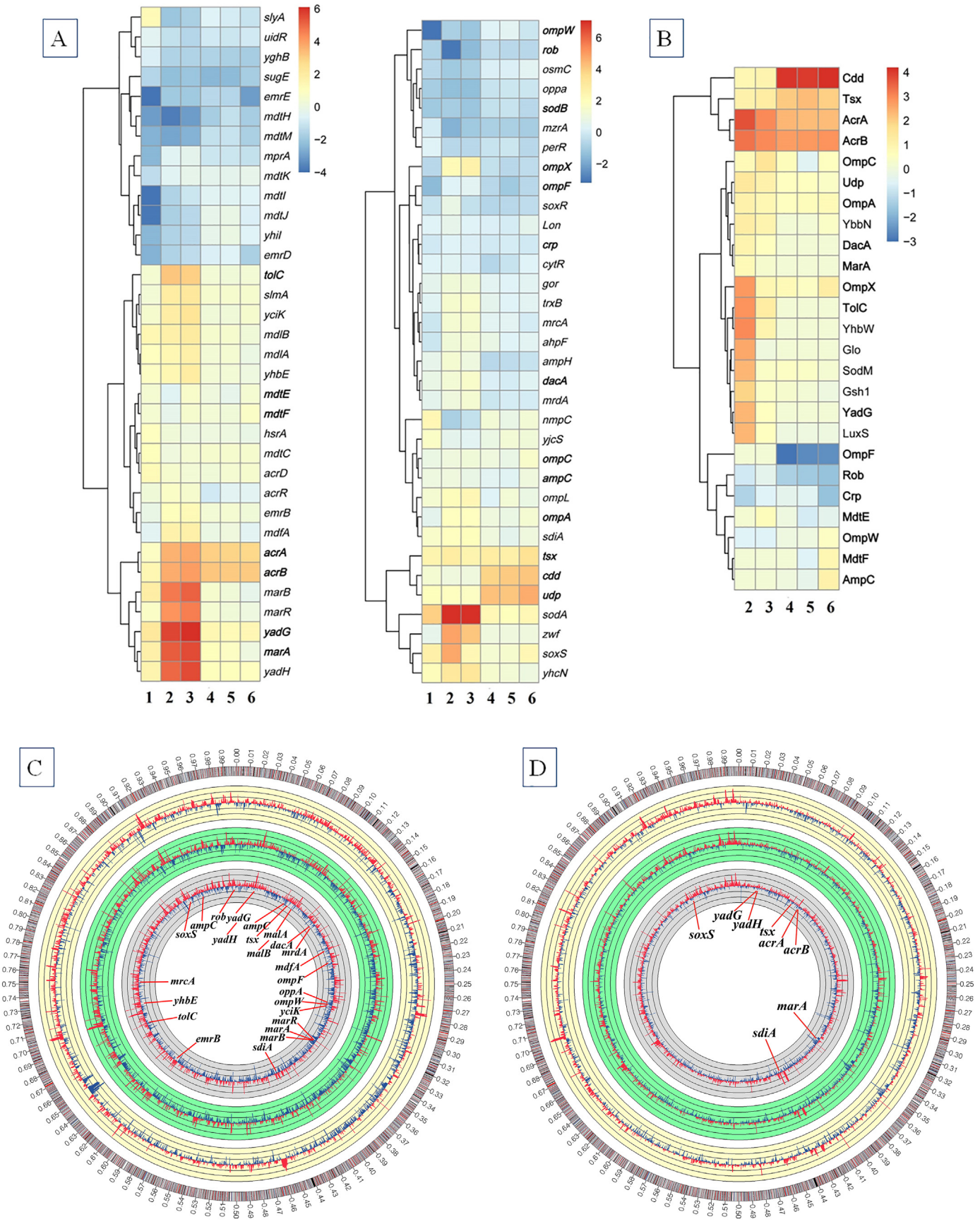
3.3. Fluoxetine triggers overexpression of multidrug efflux pumps based on transcriptomics and proteomics

To reveal the underlying molecular mechanism of fluoxetine-induced antibiotic resistance, global transcriptional and proteomic responses were examined. Both RNA sequencing and protein analyses were conducted on mutant variants (Flu_{1d}-Chl^r, Flu_{1d}-Tet^r, Flu_{30d}-Amo^r, Flu_{30d}-Chl^r and Flu_{30d}-Tet^r, each mutant with biological triplicates, $n = 15$) and wild-type *E. coli* ($n = 3$, set as the acute group) in response to 100 mg/L fluoxetine exposure for 10 h. We focused on the gene expression and protein abundance of multidrug efflux pumps, bacterial membrane permeability, antibiotic resistance enzymes and mobile genetic elements (MGEs) in the resistant mutants (Fig. 2 and Tables S2–S7).

When wild-type *E. coli* was exposed to 100 mg/L fluoxetine for 10 h, the expression levels of multidrug efflux genes *acrB* and *acrD* were increased by 2.5–2.8 folds (AFC), while outer membrane porin genes *ompF* and *ompW* were decreased by 2.8-fold and 9.2-fold (AFC), respectively. Potentially, *E. coli* increased its activity of drug efflux and decreased drug entry to protect itself from the fluoxetine exposure. However, after one-day exposure, both fluoxetine-induced antibiotic resistant mutants Flu_{1d}-Chl^r and Flu_{1d}-Tet^r increased expression of multidrug efflux and porin genes. This included 32–64 absolute folds increases for *yadG* and *yadH*; 8–16 folds increases for *acrA*, *acrB* and *tolC*; 2–8 folds increases for *tsx*, *mdlA*, *mdlB*, *mdfA*, *emrB* and *yciK* when compared with expression in the untreated *E. coli*. The proteomic analysis further confirmed the increased expression for these genes as the corresponding proteins had increased abundances by 8–13 absolute folds for AcrA and AcrB, 2.5–7.5 absolute folds for TolC, 1.5–5.3 absolute folds for YadG, and 2.0–2.5 folds for Tsx. These results suggest the multiple multidrug efflux systems, including resistance-nodulation-division-type (RND) efflux pump AcrAB-TolC, putative ATPase and permease component of ATP-binding cassette-type (ABC) multidrug transporter YadG/YadH, and the membrane channel Tsx, appear to be triggered to enhance the efflux of the antibiotics for the mutants Flu_{1d}-Chl^r and Flu_{1d}-Tet^r.

Compared to antibiotic resistant mutants induced after 1-day exposure, the number of genes/proteins with a significant change in mutants after 30-day exposure was less, indicating mutants (Flu_{30d}-Chl^r, Flu_{30d}-Tet^r and Flu_{30d}-Amo^r) are less sensitive to fluoxetine. Following repeated subculture for 30 days, the expression of genes in isolated mutants Flu_{30d}-Chl^r, Flu_{30d}-Tet^r and Flu_{30d}-Amo^r were increased at 7–8 absolute folds for *acrA*, *acrB*, *tolC* and 6.5–10.6 absolute folds for *tsx*. These increased RNA expression levels after the longer exposure were also concordant with the altered protein levels. These increased levels were absolute 4.9–7.5 absolute folds for AcrA, AcrB and 4–5 absolute folds for Tsx. Additionally, the outer membrane porin protein, OmpF, had significantly decreased abundance by 5.7–8.0 absolute folds, and potentially these lower porin levels would help block antibiotics from entering the cell. In previous studies, the increased presence of the multidrug efflux pump AcrAB-TolC, is reported to cause increased antibiotic efflux and reduced susceptibility to aminoglycoside, β-lactams, fluoroquinolone, tetracycline, and chloramphenicol (Alekshun and Levy, 1999; Fernandez and Hancock, 2012; Kurenbach et al., 2015; Zayed et al., 2015). The protein Tsx functions as the nucleoside-specific channel forming porin, and it is assumed the activity of the channel may facilitate resistance to multiple antibiotics such as Amp and Tet (Xu et al., 2006). Collectively, the RNA expression and protein abundance changes detected in the current study indicate that the multidrug efflux system AcrAB-TolC, membrane channel Tsx together with the membrane porin OmpF are playing vital roles in multiple antibiotic resistance mutants Flu_{30d}-Chl^r, Flu_{30d}-Tet^r and Flu_{30d}-Amo^r induced by fluoxetine.

In contrast, antibiotic resistance enzymes and MGEs did not seem to contribute to the fluoxetine-induced resistance in all isolated mutants. The mRNA synthesis of the beta-lactamase genes, *ampC* and *ampH*, and of the genes for the penicillin-binding protein, *mrcA*, *mrdA* and *dacA*, were increased by 2.0–3.0 absolute folds for the Flu_{1d}-Chl^r or Tet^r mutants. However, according to the protein changes, only the penicillin-binding protein DacA was found to have higher levels, detected as a 1.5–2.0 absolute folds increase. The expression of MGE-associated genes (e.g., *insB*, *insF*, *ymfD* and *yfjH*) was decreased in all mutants after the exposure of fluoxetine.



(caption on next page)

Fig. 2. Transcriptional and proteomic responses relating to the antibiotic resistance in the *E. coli* strains after 10 h exposure to the 100 mg/L fluoxetine. Heat map of transcriptional (A) and proteomic (B) elements with changed levels that were possibly linked to: cellular antioxidants, multidrug efflux system, bacterial membrane permeability and antibiotic resistance enzymes (LFC of FPKM). Lanes 1 shows changes in gene expression in wild-type *E. coli* set as the acute group; Lanes 2–6 depict changes in gene/protein expression in *E. coli* mutants Flu_{1d}-Chl^r (2), Flu_{1d}-Tet^r (3), Flu_{30d}-Chl^r (4), Flu_{30d}-Tet^r (5), Flu_{30d}-Amo^r (6), respectively. (C, D) A circular representation of the transcriptional profile. The outermost circle represents the full strain MG1655 genome. The gray, green and yellow circles correspond to the expression of each gene in wild-type *E. coli*, and the mutants Flu_{1d}-Chl^r and Flu_{1d}-Tet^r in (C) and the mutants Flu_{30d}-Amo^r, Flu_{30d}-Chl^r, and Flu_{30d}-Tet^r in (D) under exposure to the 100 mg/L fluoxetine, respectively. The red lines in each circle represent of up-regulation gene expression (log2 > 2), while blue represents down-regulation of mRNA expression (log2 < −2). The representative genes are marked at the appropriate genomic position. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Fluoxetine-induced multiple chromosomal mutations in E. coli

To further clarify the genetic mechanisms responsible for multiple antibiotics resistance, genome-wide DNA sequencing was conducted to reveal possible chromosomal mutations in all fluoxetine-induced resistant mutants. Multiple mutations (e.g., deletion, insertion, and substitution) were observed in genes that could be classified into the functional groups belonging to: information storage and processing, metabolism, MGEs (e.g., integrase, transposase and prophage) and hypothetical proteins (Fig. 3; Tables S8–S9).

During the fluoxetine exposure of 30 days, mutations in *cytR* (Q267*, CAG → TAG) and GCN5-related *N*-acetyltransferase (GNAT, 1786333, Δ1 bp) family protein were detected (Fig. 3B). These mutations may enhance the resistance of mutants Flu_{30d}-Chl^r and Flu_{30d}-Tet^r to multiple antibiotics. CytR, an anti-activator of the *cytR-CAP* nucleoside utilization regulon, negatively regulates a small set of nucleoside scavenging and metabolism genes, including *udp*, *cdd*, *tsx*, and *cytR* itself via a CRP-dependent anti-activation mechanism (Barbier and Short, 1992). For *E. coli* under the stress of fluoxetine, significant increases of both mRNA and protein expression w ere found for the genes and corresponding proteins of *udp*, *cdd* and *tsx* except *cytR*. This suggests the mutation in *cytR* may cause derepression of the *cytR-CAP* nucleoside utilization regulon, and promotes the expression of Tsx. Meanwhile, a mutation in the GNAT family protein that determines the bacterial resistance to aminoglycoside antibiotics (Burk et al., 2003) may reduce the susceptibility to aminoglycoside antibiotics. Indeed, we detected that Kan resistance increased by up to 2-fold in the Chl- and Tet–resistance mutants after exposure of 30 days. In contrast, no GNAT mutations were observed in the Amo-resistance mutants. Interestingly, a mutation in cAMP-activated global transcriptional regulator *crp* (V109G, GTA → GGA), which modulates multidrug resistance in *E. coli* through repression of the genes encoding the MdtEF multidrug efflux pump (Nishino et al., 2008), was found in the Amo-resistant mutants (Flu_{30d}-Amo^r). The transcriptional expression level of *mdtEF* did not show any significant changes, however, the proteomic analysis revealed 1.9 absolute folds increased levels of the MdtF protein compared with the wild-type *E. coli*. Therefore, it seems the mutated CRP was less effective in repressing the *mdtEF* regulon and the increased MdtEF-TolC pump levels resulted in increased antibiotic efflux. In addition, quorum-sensing (QS) modulation may be associated with the increased expression of the AcrAB–TolC induced by fluoxetine. We detected a mutation (N217S, AAC → AGC) in the QS system regulator *sdiA* and increased transcription of the gene by 2–4 absolute folds in the fluoxetine-induced resistant mutants (excluding the Flu_{30d}-Tet^r mutant). Recently, increased expression of multidrug transporters induced by QS system regulators, such as SdiA in *E. coli*, has been reported (Tavio et al., 2010). It is also documented that increased expression of *sdiA* in *E. coli* caused the overexpression of the AcrAB–TolC system without decreasing OmpF (Yang et al., 2006).

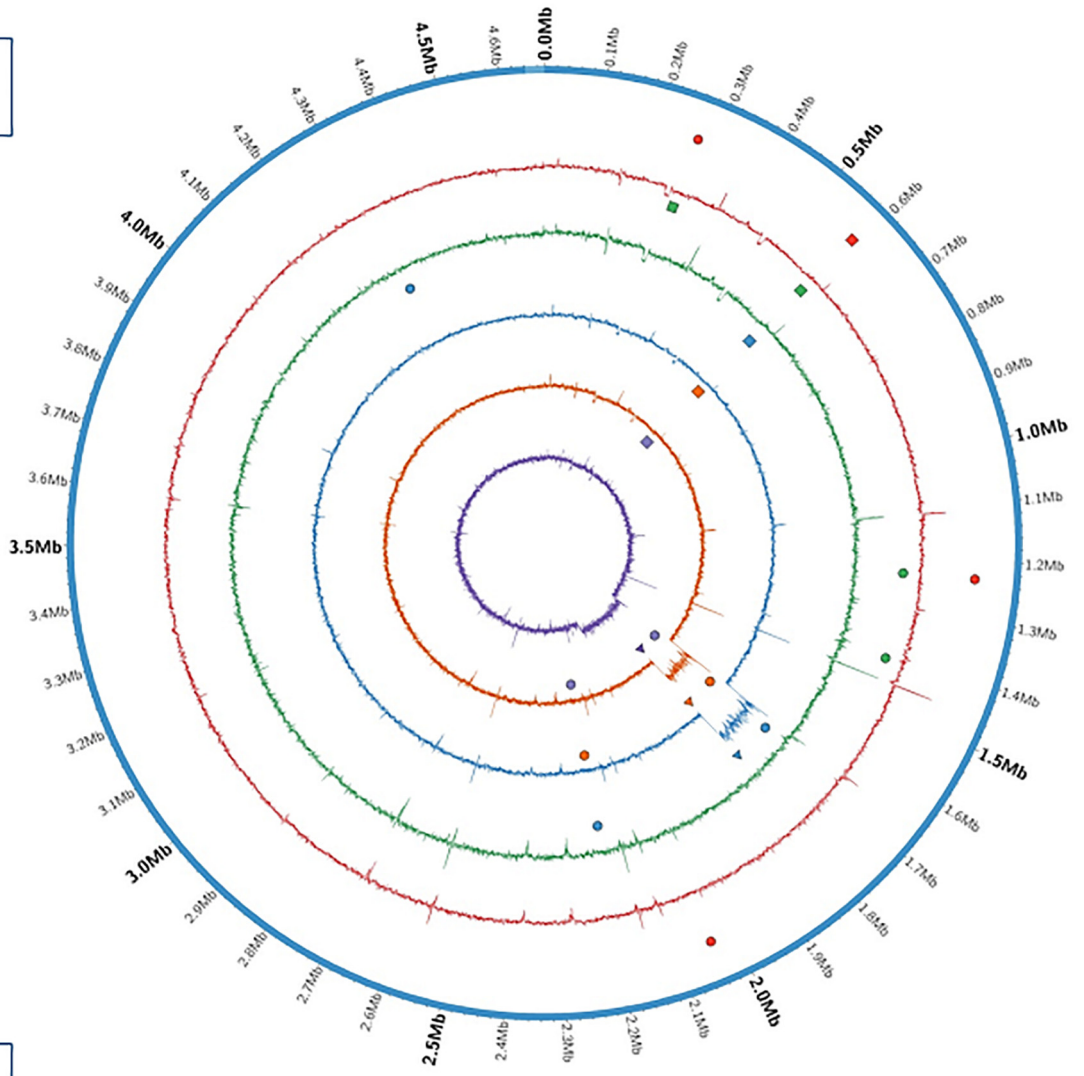
Additionally, some interesting mutations require further characterization. A single base insertion (+G) in the genes of oligopeptide-binding protein (*OppA*) was found in all resistance mutants isolated after 1-day exposure, while a substitution site occurred at *rob* (F163C, TTC → TGC) in all resistant mutants isolated after the 30-day exposure. A base insertion in the *oppA* gene should inactivate the oligopeptide permease system, and this inactivation is generally thought to cause aminoglycoside resistance (Acosta et al., 2000). However, for the mutants Flu_{1d}-Chl^r and Flu_{1d}-Tet^r, no evident Kan resistance could be observed even when their gene expression of *oppA* was decreased significantly (2.6 absolute folds). Therefore, *oppA* may play other roles in the resistance against other antibiotics. The product of *rob* acts as a MarA homolog and another regulator of the *acrAB* operon (Nicoloff and Andersson, 2013). In the Flu_{30d}-Chl^r, Flu_{30d}-Tet^r and Flu_{30d}-Amo^r mutants we detected decreased levels of Rob (2.6–3.2 absolute folds) and no significant expression change of other AcrAB-TolC regulators, e.g., *marA*, *soxS* and *acrR*. Consequently, it seems the substitution in the *rob* gene resulted in increased synthesis of the efflux pump AcrAB-TolC, which provided the enhanced resistance to the antibiotics.

3.5. Reactive oxygen species (ROS) mediated mutagenesis

Under the stress of fluoxetine, it was seen that the wild-type *E. coli* K12 and all the isolated mutants produced significantly higher levels of ROS compared to the wild-type *E. coli* K12 without fluoxetine exposure ($p < 0.01$, Fig. S3). For protection against oxidative attack, bacteria can alleviate ROS levels by activating or increasing expression of cellular antioxidant systems (Bae et al., 2011). Indeed, we detected significant overexpression of superoxide dismutase (*sodA*), glutathione oxidoreductase (*gor*), alkyl hydroperoxide reductase (*ahpF*) and thioredoxin reductase (*trxB*) in the bacteria under the stress of fluoxetine (Table S10). The overproduction of ROS in *E. coli* under the exposure of fluoxetine could cause oxidative damaging effects to DNA through nonspecific rapid reactions (Dharmaraja, 2017), and this may play a role in fluoxetine-induced DNA mutation. Similar ROS-mediated mutagenesis has been found to be involved in the DNA damage induced by ultraviolet-C (UVC) (Silva-Junior et al., 2011). Meanwhile, mutations in ROS-sensing transcription factor *soxR* (D137Y, GAC → TAC) or *perR* (L168L, CTG → CTT) were observed in mutants Flu_{1d}-Chl^r or Flu_{1d}-Tet^r. These mutations may strengthen oxidative stress defense to support bacterial survival and eliminate ROS by derepressing *soxR* or *perR* regulons (Dubbs and Mongkolsuk, 2012) and upregulating the transcription of genes with functions related to reducing oxidants (e.g., *sodA* with 2.3–7.0 absolute folds upregulation). The detailed functional effects of these mutations should be further characterized.

Collectively, a model for multi-antibiotic resistance induced by the fluoxetine exposure is proposed (Fig. 4). Exposure to the antidepressant fluoxetine results in the overproduction of ROS, resulting in mutations in transcriptional regulator genes (e.g., *marR*, *rob*, *sdiA*, *cytR* and *crp*). As a consequence, these mutations promoted expression of multidrug resistance regulating genes such as *marAB* and *soxS*, which contributed to increased expression of the AcrAB-tolC pump, the Tsx channel, the YadG/YadH ABC transportor and the MdtEF-TolC pump, as well as decreased abundance of the outer membrane porin protein OmpF. Thereby enhanced antibiotic resistance occurs by blocking the entrance of antibiotics and pumping out the intracellular antibiotics.

A



B

Mutation	Annotation	Gene	Flu_{1d}-Chl^r		Flu_{1d}-Tet^r		Flu_{30d}-Chl^r		Flu_{30d}-Tet^r		Flu_{30d}-Amo^r	
			1	2	1	2	1	2	1	2	1	2
T→G	F163C(TTC→TGC)	Rob										
Δ1 bp	Coding (40/546 nt)	GNAT family protein										
C→T	Q267*(CAG→TAG)	CytR										
C→G	R428P(CGC→CCC)	SpoT										
A→C	V109G(GTA→GGA)	CRP										
C→A	E122*(GAA→TAA)	MarR										
+T	Coding(254/435 nt)	MarR										
G→A	Q635*(CAG→TAG)	Lon										
G→T	N417K(AAC→AAA)	Lon										
G→T	L168L(CTG→CTT)	PerR										
C→A	D137Y(GAC→TAC)	SoxR										
A→G	N217S(AAC→AGC)	SdiA										
+G	Intergenic(-146/-52)	oppA										

(caption on next page)

Fig. 3. Genetic changes induced by fluoxetine. 10 resistant mutants picked from antibiotic-selected culture were sequenced by Illumina. (A) The genetic mutations identified in mutants (inside to outside: Flu_{1d}-Chl^r, Flu_{1d}-Tet^r, Flu_{30d}-Chl^r, Flu_{30d}-$Tet^r$and Flu_{30d}-Amo^r). The outer circle represents the 4.63 Mb of the *E. coli* reference genome (Genbank Accession, U00096.3), ● represents a single nucleotide mutation, ■ represents a nucleotide insertion and ▲ represents the deletion of a nucleotide at the corresponding sites of the genome; (B) A list of representative mutations in the genomes of isolated mutants. The color cell in this table indicates the presence of the corresponding mutation, while the white cell means the absence of the mutation in mutants.

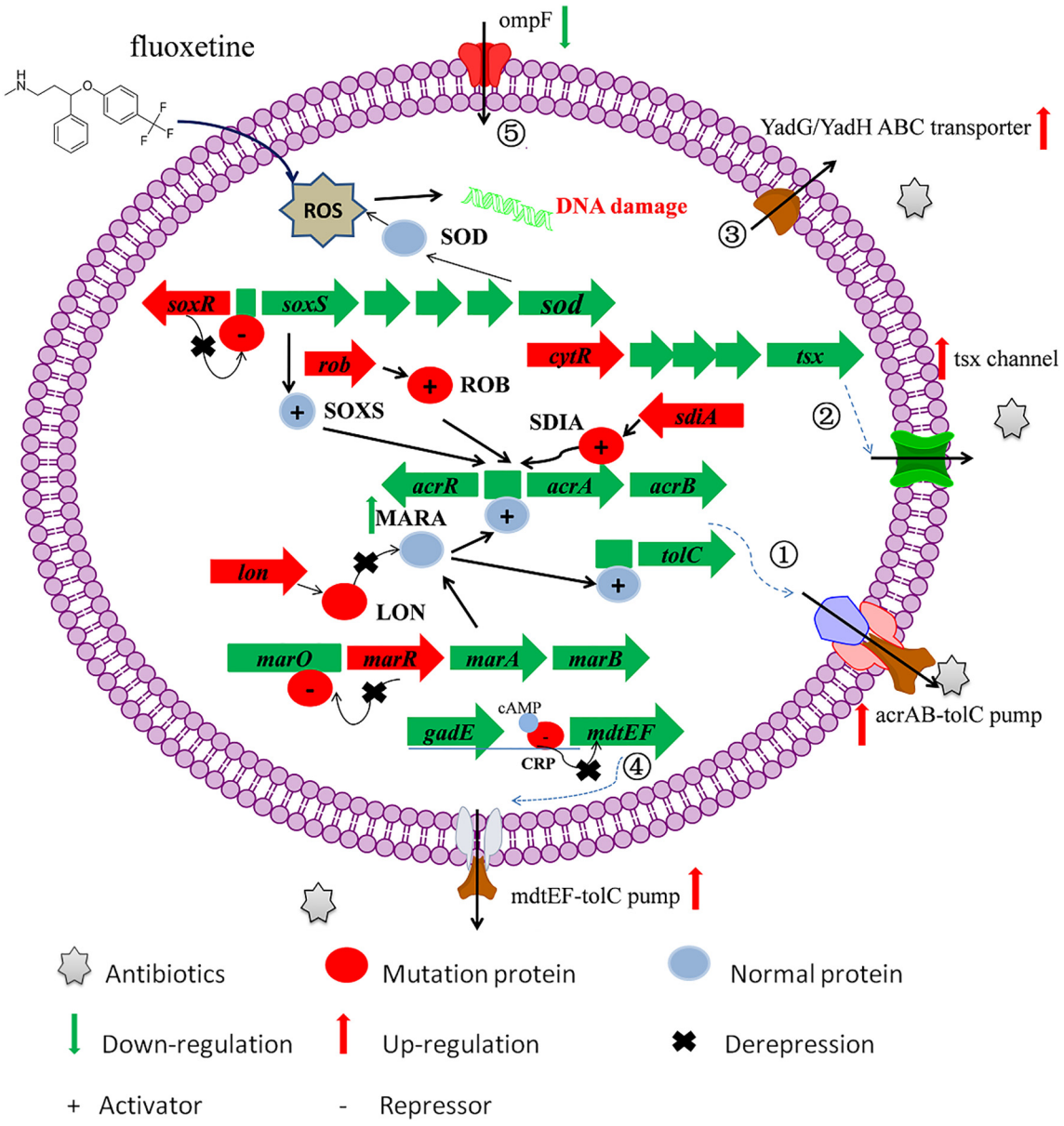


Fig. 4. Hypothetical mechanisms of resistance against multiple antibiotics activated by fluoxetine in *E. coli* mutants. Overproduction of ROS induced by the exposure of antidepressant fluoxetine mediated mutagenesis in DNA-binding transcriptional regulators, e.g., *marR*, *rob*, *sdiA*, *cytR* and *crp*, which may result in the over-expression of the multidrug efflux system and promote antibiotics efflux via the AcrAB-TolC pump ①, Tsx channel ②, YadG/H ABC transportor ③ and the MdtEF-TolC pump ④. Decreased outer membrane porin protein OmpF ⑤ may also help block antibiotics from entering the cell. The supposed resistance pathways involved are for the mutants: Flu_{1d}-Chl^r, Flu_{1d}-Tet^r, ①②③; Flu_{30d}-Chl^r, Flu_{30d}-Tet^r, ①②⑤; Flu_{30d}-Amo^r, ①②④⑤.

4. Conclusions

Our previously common understanding is that sub-MIC of antibiotics pose the dominant selection pressure to cause antibiotic resistance. Differently, our study for the first time demonstrated that as a non-antibiotic drug, the antidepressant fluoxetine induces multi-antibiotic resistance brought about by ROS-mediated mutagenesis, which will change our current understanding of the emergence of AMR. The mutation frequency has been dramatically increased under the exposure of fluoxetine (up to 5.0×10^7 fold in a dose-time pattern). DNA-, RNA- and protein-sequencing helped us identify critical determinants in the emergence of multi-drug resistance induced by the selection pressure of fluoxetine exposure. It is revealed that the frequent exposure of fluoxetine mainly led to chromosomal mutations (including deletion, insertion, and substitution in *marR*, *rob*, *sdiA*, *cytR* and *crp*) that caused altered regulation of genes encoding a multi-efflux pump system (e.g., AcrAB-TolC pump). Clearly, our findings also worsen the already grim perspective of the ongoing antibiotic resistance crisis. It is evident here that we also need to develop efficient control strategies to minimize the emergence of antibiotic resistance due to non-antibiotic

drugs/medicines. Considering the wide application of fluoxetine, and the prevalence of antibiotic-resistant bacteria, our findings are also a wake-up call to start re-evaluating the potential antibiotic-like roles induced by non-antibiotic drugs. Further work is required to investigate effects of fluoxetine on the dissemination of antibiotic resistance in mixed culture with long-term evolution period under environmentally relevant fluoxetine concentrations.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2018.07.046.

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