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A Carotenoid- and Nuclease-Producing Bacterium Can Mitigate *Enterococcus faecalis* Transformation by Antibiotic Resistance Genes

Chong-Yang Ren, Qiu-Jin Xu, Jacques Mathieu, Pedro J. J. Alvarez, Lizhong Zhu, and He-Ping Zhao*

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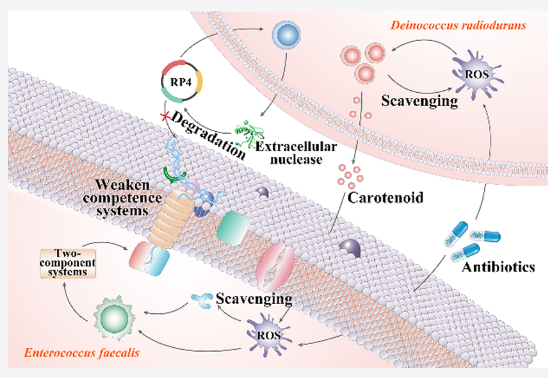
ABSTRACT: Dissemination of antibiotic resistance genes (ARGs) through natural transformation is facilitated by factors that stabilize extracellular DNA (eDNA) and that induce reactive oxygen species (ROS) that permeabilize receptor cells and upregulate transformation competence genes. In this study, we demonstrate that *Deinococcus radiodurans* can mitigate this ARG dissemination pathway by removing both eDNA and ROS that make recipient cells more vulnerable to transformation. We used plasmid RP4 as source of extracellular ARGs (*tetA*, *aphA*, and *blaTEM-2*) and the opportunistic pathogen *Enterococcus faecalis* as receptor. The presence of *D. radiodurans* significantly reduced the transformation frequency from $2.5 \pm 0.7 \times 10^{-6}$ to $7.4 \pm 1.4 \times 10^{-7}$ ($p < 0.05$). Based on quantification of intracellular ROS accumulation and superoxide dismutase (SOD) activity, and quantitative polymerase chain reaction (qPCR) and transcriptomic analyses, we propose two mechanisms by which *D. radiodurans* mitigates *E. faecalis* transformation by ARGs: (a) residual antibiotics induce *D. radiodurans* to synthesize liposoluble carotenoids that scavenge ROS and thus mitigate the susceptibility of *E. faecalis* for eDNA uptake, and (b) eDNA induces *D. radiodurans* to synthesize extracellular nucleases that degrade eARGs. This mechanistic insight informs biological strategies (including bioaugmentation) to curtail the spread of ARGs through transformation.



KEYWORDS: *ARG, ROS, Deinococcus radiodurans, carotenoid, extracellular nuclease*

■ INTRODUCTION

Antimicrobial resistance (AMR) poses a significant threat to public health.[1,2] Globally, infections caused by multidrug-resistant bacteria resulted in approximately 1.27 million deaths in 2019,[3] and could increase to more than 10 million annual deaths by 2050 if no corrective action is undertaken.[4] Although the need for a multipronged approach to stop the dissemination of antibiotic resistance genes (ARGs) is recognized, continued use and misuse of various antibiotics and other factors are exacerbating their spread through various mechanisms. Many previous studies have proven that antibiotic resistance can emerge in hospital environments and natural or engineered ecosystems, such as wastewater treatment plants (WWTPs), especially under sublethal antibiotic stress.[5] A common view is that antibiotics and environmental contaminants can exert selective pressure for ARG dissemination by promoting horizontal gene transfer (HGT). Nevertheless, there is no effective method to inhibit the HGT of ARGs in those contaminated environments.

There are three main ways in which ARGs are disseminated by horizontal gene transfer (HGT): conjugation, transformation, and transduction.[6] Transformation requires bacteria to take up extracellular DNA (eDNA), an important source of ARGs, and subsequently integrate it into their chromosome or convert it into an autonomous extra-chromosomal replicon.[7] Two important factors in the transformation process are a competent physiological state, which is often induced under adverse environmental conditions, and stable eDNA. Over 80 species of bacteria, both Gram-positive and Gram-negative, have been demonstrated to be naturally competent, and the trait is likely much more widespread.[8] Additionally, eDNA is continually released from dead and damaged bacteria, and can persist for long periods of time depending on environmental conditions and DNA fragment morphology,[9] making it abundant and ubiquitous in most environments.[10] For example, eDNA bound to mineral surfaces or humic substances can remain stable for five months, while plasmid-borne eDNA is more resistant to degradation than chromosomal eDNA.[9,10] Therefore, removing eDNA would

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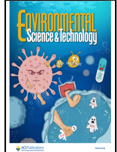

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cut off a source of ARGs and reduce their dissemination through transformation.

Reactive oxygen species (ROS), which can be induced by antibiotics and disinfectants, can also enhance the dissemination of ARGs through transformation.[6,11−14] ROS can enhance transformation frequency of ARGs by increasing cell competence, membrane permeability, pilus generation, and expression of related genes.[6,11−14] In addition, intracellular ROS may contribute to the development of ARGs by inducing mutagenesis.[15,16] Therefore, scavenging excess ROS may be another effective approach to mitigate ARGs spread.

Various microorganisms have the capacity to both degrade eDNA and neutralize ROS. For example, bacteria belonging to the *Deinococcus*, *Myxococcus*, *Streptomyces*, *Flectobacillus*, *Exiguobacterium*, and *Sphingomonas* genera are known to defend against oxidative stress by producing reducing substances such as carotenoids, exopolysaccharides, and small-molecule antioxidants that efficiently scavenge ROS.[17] We previously showed that antioxidant-producing bacteria can scavenge excess ROS (induced by antibiotics) in activated sludge,[18] although the underlying mechanisms and genetic basis were not discerned. In addition, many bacteria are separately capable of synthesizing extracellular nucleases that degrade eDNA.[19] However, until now, no publications have explored whether ARG dissemination through transformation could be biologically mitigated by simultaneous promoting eDNA degradation and ROS scavenging.

We hypothesize that *Deinococcus radiodurans* can mitigate environmental ARG dissemination through transformation, since this species can produce both extracellular nuclease[19] for eliminating eDNA, and reducing substances (e.g., carotenoids)[20,21] for scavenging intracellular ROS in ARG recipient cells. Moreover, nonpathogenic *D. radiodurans* is ubiquitous in water and soil environments and can utilize a wide range of carbon sources for growth,[22] making it attractive for potential application in a wide range of environmental and engineered systems.

In this study, we offer proof of concept that *D. radiodurans* can mitigate ARG uptake by the common opportunistic pathogen *Enterococcus faecalis*, and elucidate the underlying mechanisms. Quantitative PCR was used to monitor the degradation of extracellular ARGs, and intracellular ROS accumulation and superoxide dismutase (SOD) activity of *E. faecalis* were measured to investigate ROS scavenging by *D. radiodurans*. Transcriptomic analyses were also performed to corroborate that *D. radiodurans* produces nucleases that degrade eARGs, and to very that ROS scavenging down-regulates the competence system of *E. faecalis*, and thus, its propensity for transformation.

■ MATERIAL AND METHODS

2.1. Bacterial Strains and Cell-Free Plasmid. A commensal and nosocomial pathogen, *Enterococcus faecalis* strain ATCC29212 was used as the extracellular ARG recipient in this study.[11,23] A carotenoid and extracellular nuclease producer, *D. radiodurans* strain R1, was used as the coadjutant to remove ARGs (amended as purified DNA) and protect the recipient from oxidative damage by antibiotics.[19,21,24,25] Both strains were purchased from the China General Microbiological Culture Collection Center, CGMCC. The plasmid RP4, which encodes resistance to ampicillin (bla_{TEM-2}), tetracycline (*tetA* and *tetR*), and kanamycin (*aphA*), was extracted from *Escherichia coli* DH5α using a plasmid DNA extraction kit (BioMed, China), and used as the cell-free gene transfer agent.[11]

2.2. Determining Transformation Frequencies in the Presence of *D. radiodurans*. Suspensions of *E. faecalis* (1×10^9 cfu/mL) and *D. radiodurans* (1×10^8 cfu/mL) were prepared as described in Supporting Information (SI) Text S1. The free RP4 plasmid was extracted and suspended in elution buffer to a nucleic acid concentration of about 50 ng/μL. The transformation system of the *E. faecalis*−*D. radiodurans* combination (Ef-Dr set) contained 100 μL of the above two bacterial suspensions, and plasmid RP4 about 4 ng/μL (SI Figure S1). Ampicillin, tetracycline, and kanamycin were added at 0, 0.1, 1, 5, or 10 mg/L (each) to apply selective pressure for transformation. These concentrations are higher than the minimum inhibitory concentration (MIC), but did not exceed tolerance levels and were thus sublethal, thus allowing possible reversion to competence.[26] The transformation system of *E. faecalis* alone (Ef-I set) was similar to that of Ef-Dr set except that *D. radiodurans* suspension was replaced with 100 μL PBS. The transformation systems were mixed by vortexing and incubated at 37 °C without shaking for 2 h. After that, 50 μL of the mixture from each transformation system was spread onto NB agar selection plates containing 50 mg/L ampicillin, 20 mg/L tetracycline and 40 mg/L kanamycin to count the number of transformants. The total number of recipients was also counted by spreading the transformation systems onto NB agar without antibiotics. The transformation frequency was calculated as the number of transformants divided by the total number of recipients based on triplicate transformation systems. Transformants were quantified by transformant-selective plates and confirmed by amplifying plasmid genes *tetR* and *traG* as described in SI Text S2 and Table S1. Since *E. faecalis* lacks these two genes in its genome, PCR analysis was able to identify transformants containing the *tetR* and *traG* genes. The PCR products were visualized using 2% agarose gel electrophoresis (SI Figure S2).

To further prove the role of carotenoid on reducing dissemination of ARGs, a transformation system without *D. radiodurans* was established in which carotenoid powder was introduced as the protective agent (Ef-Ca set). Carotenoid was extracted as described in SI Text S3, and recovered under nitrogen to generate a solid powder.

2.3. Enumeration of Damaged Bacteria. Within each transformation system, damaged *E. faecalis* were enumerated using selective plates as described previously.[27] Briefly, appropriate dilutions of bacterial samples in the transformation systems were spread on CATC Agar (Citrate Azide Tween Carbonate, Hopebio, China) plates that select for undamaged *E. faecalis*. The total number of viable *E. faecalis* (damaged and undamaged) were determined by growth on Trypticase soy agar containing 0.3% yeast extract (TSYA), a repair medium for the damaged bacteria.[11] All plate counting tests were performed in triplicate and incubated at 37 °C overnight. Then, the intact rate was calculated as the ratio of undamaged *E. faecalis* (grown on CATC medium) divided by the total number of *E. faecalis* (grown on TSYA medium), and the damage rate was calculated as 100% minus by intact rate.

Cell membrane permeability was tested using the propidium iodide dye (PI) at a concentration of 2 mM (GlpBio, U.S.) using A FACSVerse flow cytometer (Becton, Dickinson and Company, U.S.) as the previously described methods[28] (see details in SI Text S4).

2.4. Measurement of ROS Generation and Superoxide Dismutase (SOD) Activity. To measure ROS generation, the bacterial suspension used for transformation was incubated at 37 °C for 30 min in the dark with 2′,7′-dichlorofluorescein diacetate (DCFDA, at a final concentration of 20 μM, Sigma-Aldrich). In this study, three sets were used to measure ROS generation and SOD activity. The Ef-Dr set contained 90 μL *E. faecalis* suspension and 90 μL *D. radiodurans* suspension, the Ef-I set was inoculated with 180 μL of *E. faecalis* suspension, while the Dr-I set was introduced with 180 μL of *D. radiodurans*. All sets were treated with 0, 1, 5, or 10 mg/L of antibiotics and performed in triplicate. The mixtures were incubated again at 37 °C for 2 h in the dark after completely mixing, then transferred to 96-well plates (Corning, China). Fluorescence was measured (ex 488 nm/em 525 nm) using a microplate reader (TECAN, Austria).[29,30] Calculation of relative fold changes in ROS production were described in SI Text S5.

Superoxide dismutase (SOD) activity was assayed using a commercial kit (BC0170, Solarbio, China) according to the manufacturer's instructions and were performed in triplicate. Briefly, the Ef-Dr, Dr-I, and Ef-I sets were prepared as previously described, but at a larger volume (5 mL) to ensure adequate cell quantities for ultrasonic disruption. Then, the mixtures were sonicated at 20 kHz (150 W) for 10 min to lyse the bacteria using an ultrasonic cell cracker (BILON-150Y, Bilon, China). The lysed mixtures were centrifuged at 5,000 × g, 4 °C for 5 min, and the supernatants were collected to assess the levels of SOD activity using ultraviolet and visible spectrophotometer (UV-8000, Metash, China) at 550 nm. SOD activity of *E. faecalis* in Ef-Dr sets was calculated by subtracting SOD activity of Dr-I sets from the total SOD activity of *D. radiodurans* and *E. faecalis* in Ef-Dr sets.

2.5. Quantification of eDNA Degradation. To monitor the eDNA degradation by *E. faecalis* and *D. radiodurans*, each transformation system (5 mL total volume) was prepared and treated with 1 mg/L antibiotics (ampicillin, tetracycline, and kanamycin). The 5 mL PBS containing 4 ng/μL plasmid RP4 and 1 mg/L antibiotics was used as a control. The thoroughly mixed systems were incubated at 37 °C without shaking and the eDNA was extracted and purified every hour following the previously reported methods (SI Text S6).[9,31] The recovered eDNA were used as templates of qPCR to quantify copies of plasmid RP4. Total DNA of biomass in *D. radiodurans* contained sets were extracted by Soil DNA Kit (Omega, Inc., U.S.) and used for RP4 quantification to rule out the possibility that eDNA adsorbed to *D. radiodurans*. No *traG* gene was detected, which indicated that RP4 was not adsorbed by *D. radiodurans*.

To confirm the role of extracellular nucleases on eDNA degradation, protein enzyme was concentrated by using a centrifugal ultrafiltration tube (3kd, Omega filter, Pall), and concentrated enzyme was used to degrade eDNA. Detailed information is provided in SI Text S7.

To further confirm the role of extracellular nucleases in complex environments, *D. radiodurans* was added into 200 mL sulfamethoxazole-amended activated sludge. eDNA was extracted after incubating at 150 rpm and 37 °C for 8 h following the precipitation method.[32,33] Activate sludge without any exogenous bacteria or with *E. faecalis* were used as the controls. Additional details are provided in SI Text S8.

Conjugal transfer gene *traG* and sulfonamide resistance gene (*sul1*) were used as the representative genes of the plasmid RP4 and activated sludge eDNA, respectively. Establishment of standard curves, primer sets of *traG* and *sul1* and qPCR conditions are shown in SI Table S1 and Text S2, respectively.

2.6. Whole-Genome RNA Sequencing Analysis. To assess gene expression levels during the transformation process, whole-genome RNA sequencing was performed. The same transformation systems as described in Section 2.2 were established and incubated at 37 °C statically. After 2 h exposure to 5 mg/L antibiotics, triplicates of the transformation systems were mixed together and total RNA was extracted using a RNeasy PowerSoil Total RNA Kit (QIAGEN, Germany) according to the manufacturer's instructions. The amount and quality of the extracted RNA were determined with a BioAnalyzer (Agilent). Qualified RNA was used for cDNA synthesis and library construction using an Illumina mRNA sequencing Sample Prep kit, then sequencing was carried out using an Illumina NovaSeq 6000. All RNA sequencing raw data were deposited in the NCBI Sequence Read Archive (accession number PRJNA795314). Trimmomatic,[34] Sortmerna,[35] and Salmon[36] were used to remove the low-quality reads and rRNA as well as to calculate gene expression levels. The measure of "Transcripts Per Kilobase of exon model per Million mapped reads" (TPM) was applied to quantify gene expression.

Differentially expressed genes (DEGs) between the control (Ef-I and Dr-I) and the Ef-Dr sets were analyzed using EdgeR (Version 3.15, https://bioconductor.org/packages/release/bioc/html/edgeR.html). False discovery rate less than 0.05 and absolute value of log2 fold change (log2FC) ≥ 1 were used as the threshold to assess the significance of gene expression difference.[37] DEGs then subjected to clusters of orthologous genes (COG) analysis (http://www.ncbi.nlm.nih.gov/COG/) and KEGG pathway enrichment analysis (http://www.genome.jp/kegg/-pathway.html). Protein−protein interaction (PPI) analysis was performed based on the STRING database (https://string-db.org/) and visualized with Cytoscape software.

2.7. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Validation. Total RNA was extracted from the same transformation systems as RNA-seq and cDNA was synthesized using a Reverse Transcription Kit (TaKaRa). Fifteen and two randomly selected DEGs from the genome of *E. faecalis* and *D. radiodurans*, respectively, were used to assess the expression by qRT-PCR. The housekeeping genes *gdh* and *hpi* in *E. faecalis* and *D. radiodurans* were used as internal controls, respectively. Information on these genes, primers and expression calculation for RT-qPCR is in SI Table S1 and Text S9.

2.8. Statistical Analysis. Differences in transformation frequency, bacterium damage rate, fold changes of ROS generation, SOD activity, and cell membrane permeability were tested by independent-sample *t* test using SPSS version 22.0. Statistically significant differences were judged based on the 95% confidence interval ($p < 0.05$) between treated sets (*E. faecalis* with *D. radiodurans* or carotenoid extracts) and control (*E. faecalis* alone). Data were expressed as mean ± SD of triplicate data for all the experiments.

RESULTS AND DISCUSSION

3.1. *D. radiodurans* Decreased the Transformation Frequency of *E. faecalis* by RP4. The transformation frequency of *E. faecalis* significantly decreased by more than 2.5-fold ($p < 0.05$) when in the presence of *D. radiodurans* and

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a combination of three antibiotics (Figure 1). The transformation frequencies were approximately 6.4 ± 1.5 × 10^{-7}, 5.9 ± 1.8 × 10^{-7}, and 7.4 ± 0.6 × 10^{-7} per recipient in the Ef-I (*E. faecalis* without *D. radiodurans*), Ef-Dr (with both *E. faecalis* and *D. radiodurans*) and Ef-Ca (*E. faecalis* with carotenoid) sets without antibiotics, respectively, which is consistent with previous observations.[11] These frequencies increased to 9.4 ± 2.0 × 10^{-7}, 1.4 ± 0.3 × 10^{-6}, 2.5 ± 0.7 × 10^{-6}, and 2.4 ± 0.5 × 10^{-6} in Ef-I sets with dosages of 0.1, 1.0, 5.0, or 10.0 mg/L for each of the three antibiotics. However, these frequencies were always less than 7.4 ± 1.4 × 10^{-7} and 7.3 ± 0.3 × 10^{-7} in Ef-Dr and Ef-Ca sets with corresponding antibiotic dosages, significantly lower than that of Ef-I sets ($p < 0.05$). This demonstrates that the transformation frequency of *E. faecalis* by plasmid RP4 can be decreased by the presence of *D. radiodurans*, and carotenoid played an important role.

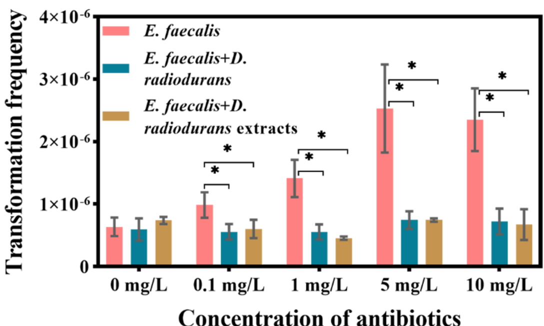

Figure 1. Reduction of *E. faecalis* transformation frequency by combining with *D. radiodurans* and carotenoid under concurrent exposure to ampicillin, tetracycline, and kanamycin ($n = 3$; mean ± SD). The given concentrations were equal for each of these antibiotics. * represents statistically significant difference ($p < 0.05$).

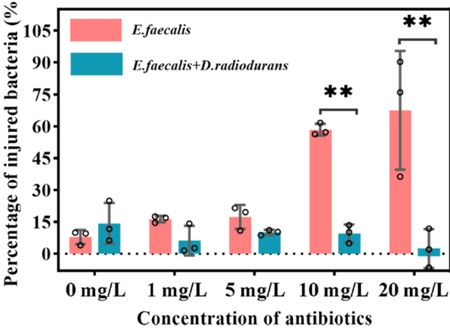

Figure 2. Mitigation of damaged *E. faecalis* cells by *D. radiodurans* under concurrent exposure to ampicillin, tetracycline, and kanamycin ($n = 3$; mean ± SD). The given concentrations were equal for each of these antibiotics. ** represents statistically significant difference ($p < 0.01$).

3.2. *D. radiodurans* Mitigated *E. faecalis* Damage by Decreasing ROS Accumulation. Some damaged bacteria are known to revert and become competent,[26] facilitating their uptake eDNA from the environment. In order to test whether *D. radiodurans* could mitigate the damage of *E. faecalis* induced by antibiotics (thereby mitigating upregulation of the competence system and reducing transformation frequency by plasmid RP4), viable damaged *E. faecalis* were enumerated by selective plates. The percentage of damaged bacteria increased from 16 ± 1.6% at 1 mg/L antibiotics to 58 ± 3% and 68 ± 28% at 10 and 20 mg/L antibiotics in Ef-I sets (only contain *E. faecalis*), respectively (Figure 2). However, the percentage of damaged bacteria was consistently lower than 15% in all Ef-Dr sets (with both *E. faecalis* and *D. radiodurans*). Quantification of damaged *E. faecalis* with these selective plates involves inclusion of sodium azide to which *E. faecalis* damaged by oxidative stress are sensitive.[38] Therefore, the significantly lower percentage of damaged *E. faecalis* in the Ef-Dr than that of Ef-I set at 10 and 20 mg/L antibiotics ($p < 0.01$) reflects the protective effect of *D. radiodurans* against oxidative stress.

Oxidative damage by antibiotics-induced ROS is a common bactericidal mechanism.[39−42] This oxidative stress can also increase cell permeability and the frequency of ARGs dissemination by HGT.[6,11−14,28,43,44] Theoretically, protecting bacteria from oxidative damage can reduce selection pressure for ARG propagation caused by residual antibiotics. To further investigate whether *D. radiodurans* could scavenge ROS accumulating in recipient *E. faecalis*, intracellular ROS and cellular antioxidant system (SOD activity) were measured. Notably, *E. faecalis* ROS accumulation increased in Ef-I sets with increasing antibiotic concentration. The accumulation of ROS was 1.05-, 1.11-, 1.09-, and 1.12-fold higher in Ef-I sets at antibiotic concentrations of 0.1, 1, 5, and 10 mg/L, repsctively, than that of Ef-I controls without antibiotics (Figure 3A), which is consistent with previous reports that antibiotics could induce ROS production.[39−42] In contrast, ROS accumulation in Ef-Dr sets at antibiotic concentrations of 0, 0.1, 1, 5, and 10 mg/L was only 0.91-, 0.92-, 0.87-, 0.92- and 1.00-fold, respectively, than that of controls without antibiotics. These ROS levels are significantly lower ($p < 0.05$) than the corresponding values for the Ef-I sets at antibiotic concentrations of 0.1, 1, 5, and 10 mg/L. The same trend was observed in the carotenoid extracts added set (Figure 3A). Thus, this experiment demonstrates *D. radiodurans*-mediated ROS reduction as an important protection mechanism against *E. faecalis* damage, and thus, decreased susceptibility to transformation.

SOD protects bacteria from superoxide, though it also produces hydrogen peroxide as a byproduct. In tandem with increased ROS accumulation, *E. faecalis* SOD activity increased from 60.3 ± 6.4 to 86.0 ± 6.0, 68.0 ± 3.5, and 91.3 ± 7.4 U/10^8 cfu in Ef-I sets with increasing antibiotic dosage (Figure 3B). In contrast, SOD activity was significantly lower in the corresponding Ef-Dr sets at antibiotic concentrations of 1, 5, and 10 mg/L ($p < 0.05$). This corroborates the antioxidant protection by *D. radiodurans* to neighboring *E. faecalis* and supports the hypothesis that the transformation frequency of *E. faecalis* by plasmid RP4 (which increases with oxidative stress) may be reduced by the presence of *D. radiodurans*.

Cell membrane permeability plays an important role on plasmid transformation, and is easily influenced by ROS induced by environmental stress.[6,11,14,43] During exposure of *E. faecalis* to the antibiotics, the cell membrane permeability increased by 1.14- to 1.34-fold compared to that for the control cells (Figure 3D). While this permeability increased no more than 1.02- and 1.05-fold in the presence of *D. radiodurans* or its carotenoid extracts (Figure 3D). This indicates the protective roles of *D. radiodurans* and its carotenoid to the cell membrane of the adjacent bacteria.

3.3. *D. radiodurans* Removed Extracellular DNA. The exogenous plasmid RP4 in the transformation systems was rapidly degraded by *D. radiodurans* (Figure 4A). Gene *traG* located on RP4 was used to represent the number of plasmid

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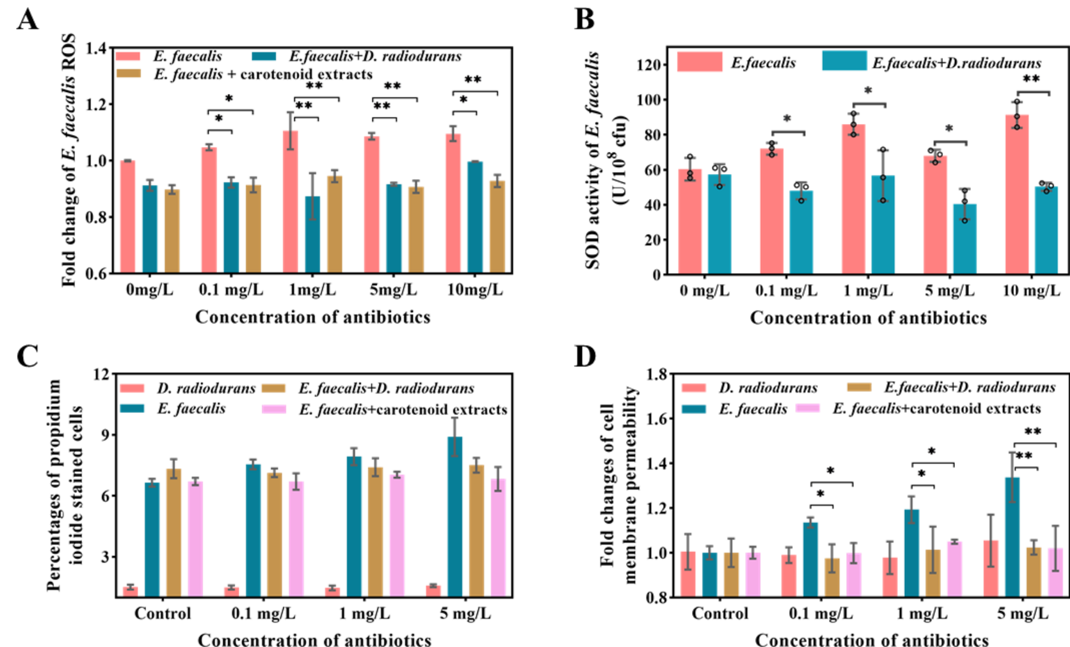

Figure 3. Influence of *D. radiodurans* on *E. faecalis* ROS accumulation (A), SOD activity (B) and cell membrane permeability (C and D). (A) Fold changes of ROS generation in *E. faecalis*. (B) SOD activity in *E. faecalis*. (C) Percentages of PI stained cells. (D) Fold changes of cell membrane permeability. The given concentrations were equal for each of ampicillin, tetracycline and kanamycin. The error bar represents three replicates in triplicate independent analyses. * and ** represent statistically significant difference ($p < 0.05$) and ($p < 0.01$), respectively.

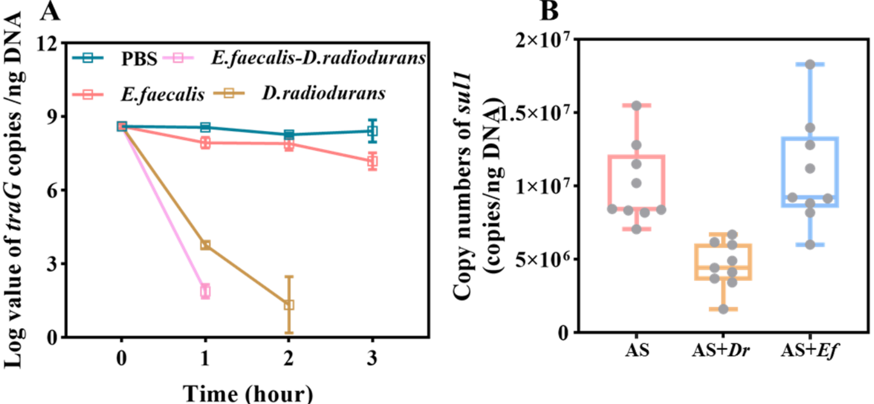

Figure 4. Removal of extracellular DNA (eDNA) by *D. radiodurans*. (A) Quantification of *traG* (contained on plasmid RP4) by qPCR ($n = 3$; mean ± SD). (B) Degradation of eDNA by *D. radiodurans* in activated sludge ($n = 9$).

RP4. There was little change of *traG* concentration in the control (RP4 in PBS) and Ef-I sets after 3 h, which indicated the persistence of eDNA in PBS and an inability of *E. faecalis* to degrade eDNA. However, *traG* copy number dramatically decreased by over 5 orders of magnitude, with its log value (*traG* copies/ngDNA) declining from 8.6 ± 0.1 to 3.8 ± 0.1 and 1.9 ± 0.3, within 1 h in the Dr-I (*D. radiodurans* alone) and Ef-Dr (with both *E. faecalis* and *D. radiodurans*) sets, respectively. Moreover, *traG* was removed below detection (1.5×10^2 copies/μL) within 2 h in the Ef-Dr set. Extracellular nucleases isolated from *D. radiodurans* could degrade eDNA effectively (SI Figure S3). The copy number of *traG* decreased by more than 5 orders of magnitude, with its log value (*traG* copies/ngDNA) declining from 8.9 ± 0.2 to 3.5 ± 0.2, within 2 h in the nuclease-added group, while this value was 9.0 ± 0.1 in the PBS group. Thus, *D. radiodurans* effectively removed eDNA by releasing extracellular nucleases and thus the source of exogenous ARGs, which was reflected in decreased frequency of *E. faecalis* transformation.

D. radiodurans also effectively removed eDNA in activated sludge. As shown in Figure 4B, abundance of *sul1* was $4.6 \pm 0.2 \times 10^6$ copies/ngDNA ($n = 9$) in the eDNA of activated sludge amended with *D. radiodurans*, representing more than 2.2-fold decline compared with that in activated sludge amended with *E. faecalis* ($1.1 \pm 0.4 \times 10^7$ copies/ngDNA ($n = 9$)) or unamended ($1.0 \pm 0.3 \times 10^7$ copies/ngDNA ($n = 9$)). Thus, nuclease-producing *D. radiodurans* played a significant role in removing eDNA in this complex system, while *E. faecalis* (not known for extracellular nuclease synthesis) had no contribution to eDNA removal.

3.4. *D. radiodurans* Mitigates Upregulation of Genes Related to Competence of *E. faecalis*. More than 4 361 751, 5 375 723, and 5 783 795 reads for the Ef-I, Dr-I ,and Ef-Dr sets were obtained from RNA-seq, and approximately 99%, 82.7%, and 83.1% clean reads from the Ef-I, Dr-I,

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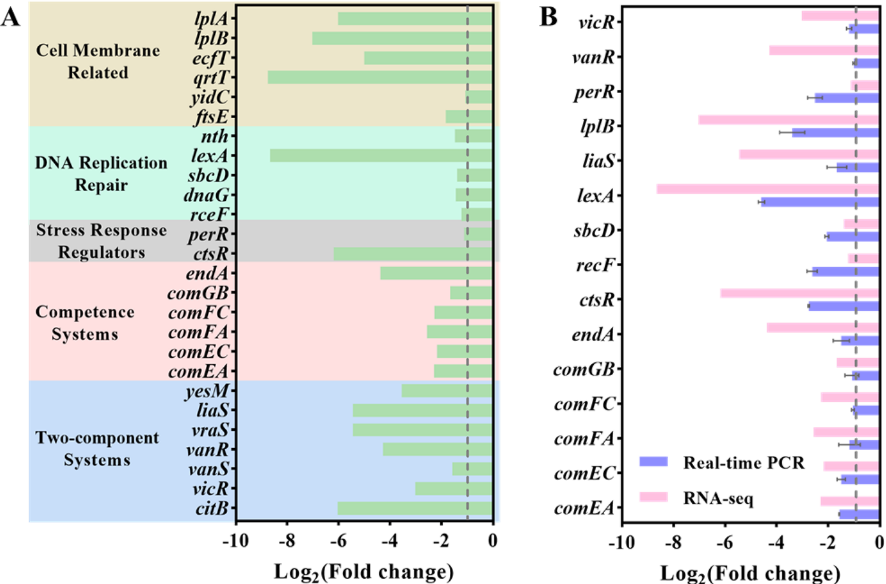

Figure 5. Fold changes of expression of downregulated functional genes in *E. faecalis* based on RNA-seq (A) and the validation of randomly selected DEGs by RT-qPCR ($n = 3$; mean ± SD) (B).

and Ef-Dr sets were mapped to the reference genome, respectively. There were 365 upregulated genes and 275 downregulated genes in *E. faecalis* from the Ef-Dr set compared with Ef-I set, accounting for 12.7% and 9.6% of the total reference genome genes, respectively.

Competence-stimulating peptides (CSP) induced by certain stressors are first sensed by two-component systems, which transduce the signal to competence systems.[45,46] In this study, genes encoding two-component systems associated with the SOS response (*citB*, a typical extracytoplasmic-sensing two-component system regulating the inheritance of plasmids and the SOS response to β-lactam antibiotics[47,48]), genetic competence (*vicR*, the response-regulator component regulating critical genes for cell-wall synthesis, cell-membrane composition[49,50] and genetic competence[51−53]) and antibiotic resistance (*vanSR*, *vraS*, *liaS*, and *yesM*, closely related to antibiotic resistance, cell wall damage, and virulence factor expression[46,54−56]) exhibited a notable downregulation (log2 FC from −1.6 to −6.0, $p < 0.05$) in *E. faecalis* (Figure 5A). After activation of two-component systems, integral membrane proteins (ComEA and ComGB) bind and uptake DNA, followed by change of double-stranded DNA to single-stranded status by membrane nuclease EndA.[57−59] Single-stranded DNA then transfer into cytoplasm through the transmembrane channel formed by channel protein ComEC.[60] The entered single-stranded DNA was protected from degradation by complexes constructed by ComFA, ComFC, and DprA.[61] Herein, all the integrated competence systems (ComEA, ComEC, ComFA, ComFC, ComGB, and EndA) exhibited a notable downregulation (log2 FC from −1.7 to −4.4, $p < 0.05$) in *E. faecalis* (Figure 5A). These results indicate that *D. radiodurans* could reduce *E. faecalis* competence under antibiotic stress.

Stress response regulators of *E. faecalis*, such as *ctsR* and *perR*, were also downregulated (log2 FC of −6.2 and −1.1, $p < 0.05$) in the Ef-I set (Figure 5A). CtsR is a transcriptional regulator associated with environmental stress (e.g., H_2O_2, acid and heat-shock), and PerR is an oxidative stress-response regulator related to the development of genetic competence.[62,63] Downregulation of these genes suggests that *D. radiodurans* could help weaken external oxidative stress.

In addition to genes directly related to competence, genes responsive to antibiotic damage, such as DNA damage repair-related genes (*rceF*, *dnaG*, *sbcD*, *lexA*, and *nth*) and cell-membrane integrity-related genes (*ftsE*, *yidC*, *qrtT*, *ecfT*, *lplB*, and *lplA*) were also downregulated (log2 FC from −1.1 to −8.7, $p < 0.05$) (Figure 5A). Among them, *rceF* and *lexA* regulate SOS response and sense oxidative DNA damage, which were reported associated with HGT of ARGs.[64,65] Genes encoding membrane proteins, specifically *ecfT*, *ftsE*, *yidC*, a0nd *lplB*, play crucial roles in energy-coupling,[66] ATP hydrolysis,[67] protein insertion and folding,[68] and permease components,[69] respectively. Thus, these data indicate that *D. radiodurans* protects *E. faecalis* from DNA and membrane damage that would otherwise result from exposure to these antibiotics.

To validate the RNA-Seq results, qRT-PCR was performed for 15 randomly selected DEGs. The results of qRT-PCR were consistent with the transcriptome data (Figure 5B), indicating the reliability of the comparative analysis of our transcriptomes.

To further characterize the transcriptomic effects of the presence of *D. radiodurans*, PPI network analysis was conducted (SI Figure S4). Two-component systems, DNA replication and repair systems, and stress response regulators were well clustered and correlated to competence systems. Although only one membrane-related gene was shown in the network (*yidC*), it did interact with the competence system. These results further support that down-regulation of genes involved in repairing antibiotic-induced damage could also reduce competence in *E. faecalis*.

3.5. *D. radiodurans* Upregulated Expression of Genes Related to Self-Protection and Carotenoid Synthesis under Antibiotic Exposure, and Secreted Extracellular Nuclease to Remove eDNA. To investigate why *D. radiodurans* impacts *E. faecalis* gene expression as described, the *D. radiodurans* transcriptomes from the Dr-I set without antibiotics and Ef-Dr set with antibiotics were compared.

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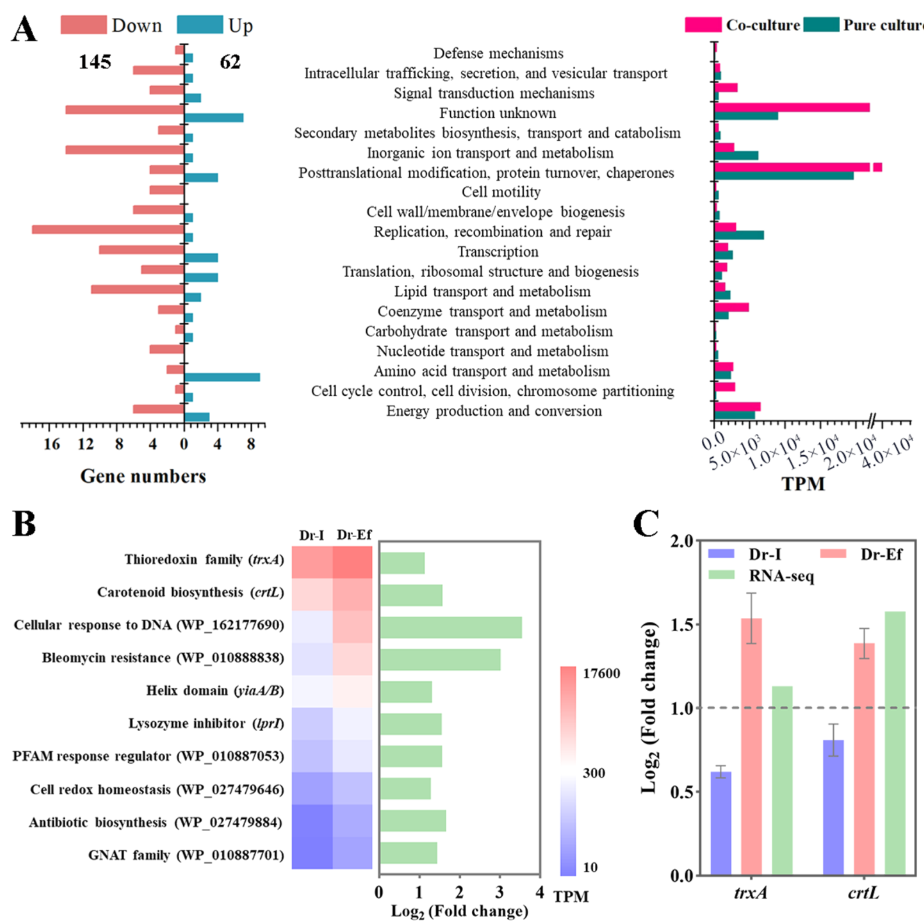

Figure 6. Analysis of *D. radiodurans* differentially expressed genes. (A) Gene numbers and transcripts per kilobase of exon model per million mapped reads (TPM) of clusters of orthologous genes (COG) category; (B) $\log_2$ fold change and TPM of selected upregulated gene; (C) the validation of randomly selected DEGs by RT-qPCR ($n = 3$; mean ± SD).

There were 207 DEGs, including 62 upregulated and 145 downregulated genes (Figure 6A left) in the Ef-Dr set. Among these DEGs, genes involved in energy production and conversion, cell cycle control, coenzyme transport, and metabolism, signal transduction mechanisms, and defense mechanisms had higher expression in the Ef-Dr set with antibiotics than Dr-I (Figure 6A right). *D. radiodurans* is an extremophilic organism that harbors powerful systems for resisting oxidative stress, DNA damage, and starvation.[70] Specifically, genes encoding the thioredoxin system and cell redox homeostasis were significantly upregulated (log2 FC of 1.1 and 1.3, $p < 10^{-4}$) in the Ef-Dr set with antibiotics (Figure 6B). Thioredoxin systems are employed by bacteria to defend against oxidative stress and maintain protein function,[71] while redox homeostasis is indispensable for maintaining cellular processes (e.g., ROS response, redox reaction, signaling, xenobiotic removal, and protection of protein thiols).[72] In addition, genes coding for PFAM response regulator (external environment stimulus responses)[73] and aminoglycoside and bleomycin resistance (bleomycin resistance dioxygenase and GNAT family enzymes)[74] were also higher in the Ef-Dr set. These findings indicate that *D. radiodurans* first strengthened its own protection to survive exposure to antibiotics, making it possible to further protect neighboring *E. faecalis*.

Carotenoid synthesis is a defense strategy utilized by some bacteria to protect against ROS-mediated damage.[17,21,22] Note that the expression of the carotenoid synthetase gene *crtL* in the Ef-Dr set with antibiotics exhibited significant upregulation (log2FC of 1.6, $p < 10^{-12}$) (Figure 6B), while the concentration of carotenoid synthesized by *D. radiodurans* under different concentrations of antibiotics was higher than that in the absence of antibiotics (SI Figure S5). Therefore, as *D. radiodurans* is able to secrete carotenoid into the environment,[75] neighboring bacteria would be also benefit from reduced oxidative damage. Transformation frequencies in the sets with carotenoid extracts were significantly lower ($p < 0.05$) than that in Ef-I sets without carotenoid extracts (Figure 1), further confirming that carotenoid plays an important role in mitigating ARG transformation.

Genes involved in the cellular response to eDNA also presented a high level (TPM of 2944) and significant upregulation (log2FC of 3.6, $p < 10^{-49}$) in the Ef-Dr set with antibiotics. It was reported that eDNA could induce a DNA damage response and threaten *D. radiodurans* survival by negatively impacting genome integrity through random DNA integration.[76] As a preventative measure, *D. radiodurans* degrades extracellular DNA via extracellular nucleases.[19] Accordingly, genes encoding extracellular nucleases (ExeM/NucH family extracellular endonuclease) exhibited constitutive expression in the Dr-I set without antibiotics (TPM of 199) and Ef-Dr set with antibiotics (TPM of 119). This result could explain the fast decrease in eDNA concentration in Ef-Dr sets (Figure 4A). Note that some microorganisms may catabolize DNA in the cytoplasm with the help of import systems and

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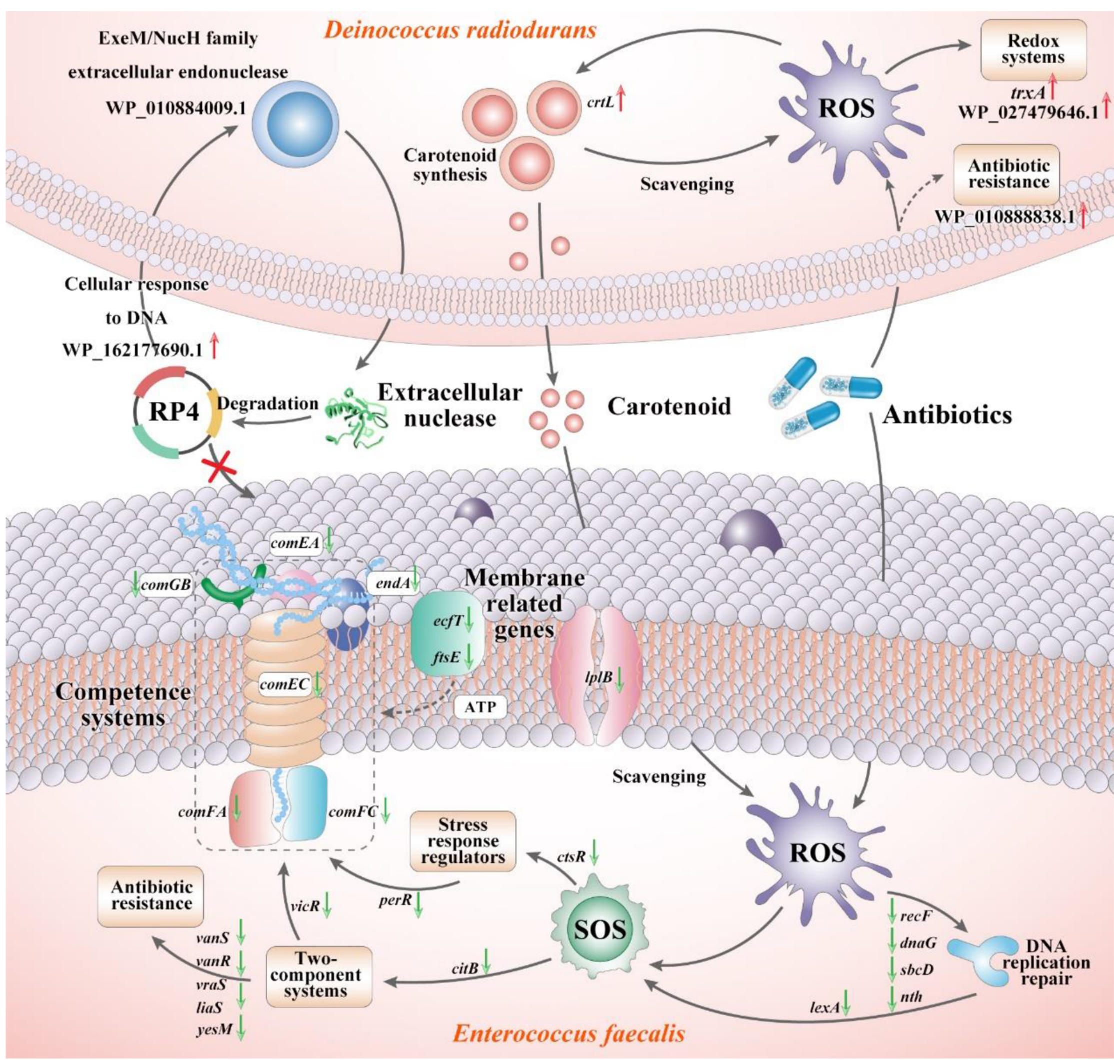

Figure 7. Mechanisms by which *D. radiodurans* reduces the transformation frequency of *E. faecalis* by ARGs. Antibiotics induce *D. radiodurans* to produce carotenoids that scavenge ROS, which mitigates upregulation of *E. faecalis* competence system. Extracellular nuclease synthesized by *D. radiodurans* degrade the extracellular RP4 resistance plasmid that was used as ARG source for transformation.

imported subcomponents. However, *D. radiodurans* does not harbor these systems, obviating this mechanism. Thus, *D. radiodurans* extracellular nucleases play an important role in the degradation of eDNA, thereby reducing the transformation frequency of RP4 to *E. faecalis*. The results of qRT-PCR were consistent with the transcriptome data (Figure 6C), indicating the reliability of the comparative analysis of the transcriptomes.

3.6. Mechanisms by which *D. radiodurans* Mitigates *E. faecalis* Transformation.

Based on measurements of transformation frequency, damaged bacteria percentage, ROS generation, eDNA degradation, and RNA sequencing, we propose two underlying mechanisms by which *D. radiodurans* mitigates uptake of exogenous ARGs by *E. faecalis* (Figure 7). First, antibiotics induce *D. radiodurans* carotenoid synthesis and release, which enhances ROS scavenging and downregulates ROS-induced competence systems in *E. faecalis*. Second, eDNA induces extracellular nuclease genes, resulting in increased eDNA degradation and reduction of exogenous ARGs available for transformation.

Apparently, carotenoids released by *D. radiodurans* helped *E. faecalis* scavenge intracellular ROS and reduce its SOS response. Consequently, *E. faecalis* downregulated genes coding two-component systems sensing oxidative stress. Signal feedback from the two-component systems was then weakened, and thus reduced the expression of competence-related genes. Furthermore, ROS reduction mitigated DNA and membrane damage to *E. faecalis*, and downregulated its expression of cell membrane and DNA repair related genes. As a result, ARG transformation frequency to *E. faecalis* was substantially reduced. Genes regulating antibiotic resistance in *E. faecalis* were also downregulated, as *E. faecalis* experienced reduced selective pressure from β-lactam antibiotics due to a reduction in antibiotic-mediated oxidative damage. Considering that there are no antibiotic degradation genes in the *D. radiodurans* genome, and that antibiotic concentrations were not decreased in the Ef-Dr transformation systems, we can exclude the possibility that the decrease in *E. faecalis* ROS was due to antibiotic biodegradation.

Many different bacteria, including *D. radiodurans*, degrade eDNA by secreting extracellular nucleases.[77−81] After eDNA degradation, the byproducts, deoxynucleotide monophosphates (dNMPs), serve as nutrient sources and even enhance bacteria tolerance to oxidative stress.[19,82] Therefore, extracellular RP4 was quickly degraded by extracellular nuclease,

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which decreased the frequency of natural transformation. This result is consistent with a previous report that extracellular nucleases decreased natural transformation of *Vibrio cholerae*.[77]

3.7. Environmental Implications. This study offers proof of concept that *D. radiodurans* can be used to mitigate ARG transfer via transformation, by producing carotenoids and extracellular nucleases that target two critical factors that exacerbate this ARG dissemination mechanism: eDNA and ROS. In addition to *D. radiodurans*, various microorganisms such as *Myxococcus*, *Streptomyces*, and *Vibrio* possess the ability to synthesize antioxidants or extracellular nucleases. Thus, it may be worthwhile to explore whether other microorganisms could thrive in pertinent systems and effectively reduce the transformation frequency. Overall, this study encourages future research into bacterial interspecies interactions and microbiome manipulations that could mitigate horizontal transfer of problematic genes through transformation, and offers mechanistic insight into how bioaugmentation can help mitigate the spread of ARGs in potential hot spots for antibiotic resistance dissemination.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.2c03919.

Bacterial culture; PCR and qPCR conditions; extraction and measurement of carotenoid; detection of cell membrane permeability; calculation of relative fold changes in ROS production; extraction and purification of eDNA; eDNA degradation by extracellular nucleases; eDNA degradation in activated sludge; calculation of mRNA expression; primers and amplicon information for PCR analysis; *E. faecalis* transformation by cell-free plasmid RP4 in the presence of *D. radiodurans*. Electrophoresis of transformant colony PCR products. Removal of eDNA by extracellular nucleases; the protein−protein interaction (PPI) network analysis; carotenoid measurement (PDF)

■ AUTHOR INFORMATION

Corresponding Author

He-Ping Zhao − *MOE Key Lab of Environmental Remediation and Ecosystem Health, College of Environmental and Resource Science, Zhejiang University, Hangzhou, China 310058;* orcid.org/0000-0002-5177-8010; Phone: 0086-571-88982739; Email: zhaohp@zju.edu.cn

Authors

Chong-Yang Ren − *MOE Key Lab of Environmental Remediation and Ecosystem Health, College of Environmental and Resource Science, Zhejiang University, Hangzhou, China 310058*

Qiu-Jin Xu − *MOE Key Lab of Environmental Remediation and Ecosystem Health, College of Environmental and Resource Science, Zhejiang University, Hangzhou, China 310058*

Jacques Mathieu − *Department of Civil and Environmental Engineering, Rice University, Houston, Texas 77005, United States;* orcid.org/0000-0003-1776-8772

Pedro J. J. Alvarez − *Department of Civil and Environmental Engineering, Rice University, Houston, Texas 77005, United States;* orcid.org/0000-0002-6725-7199

Lizhong Zhu − *MOE Key Lab of Environmental Remediation and Ecosystem Health, College of Environmental and Resource Science, Zhejiang University, Hangzhou, China 310058;* orcid.org/0000-0001-5044-277X

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.est.2c03919

Notes

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