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DNA phosphorothioate modification facilitates the dissemination of *mcr-1* and bla_{NDM-1} in drinking water supply systems☆

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ABSTRACT

The mechanism driving the dissemination of antibiotic resistance genes (ARGs) in drinking water supply systems (DWSSs) with multiple barriers remains poorly understood despite several recent efforts. Phosphorothioate (PT) modifications, governed by *dndABCDE* genes, occur naturally in various bacteria and involve the incorporation of sulfur into the DNA backbone. PT is regarded as a mild antioxidant *in vivo* and is known to provide protection against bacterial genomes. We combined quantitative polymerase chain reaction, metagenomic, and network analyses for the water treatment process and laboratory-scale experiments for chlorine treatment using model strains to determine if DNA PT modification occurred in DWSS and facilitated the dissemination of mobilized colistin resistance-1 (*mcr-1*) and New Delhi metallo-β-lactamase-1 (bla_{NDM-1}) in DWSS. Our results indicated that the relative abundance of *dndB* increased in the effluent, compared with the influent, in the water treatment plants. Presence of *dndB* copies had a positive correlation with the concentration of chloramine disinfectant. Network analysis revealed *Bdellovibrio* as a potential host for *MCR* genes, *NDM* genes, and *dndB* in the DWSS. *E. coli* DH10B (Wild-type with the *dndABCDE* gene cluster and *ΔdndB*) model strains were used to investigate resistance to chlorine treatment at the concentration range of 0.5−3 mg/L. The resistance of the wild-type strain increased with increasing concentration of chlorine. DNA PT modification protected *MCR- and NDM*-carrying bacteria from chloramine disinfection during the water treatment process. The higher relative abundance of ARGs in the effluent of the water treatment plants may be due to the resistance of DNA PT modification to chloramine disinfection, thereby causing the enrichment of genera carrying *MCR*, *NDM*, and *dndB*. This study provides a new understanding on the mechanism of ARG dissemination in DWSS, which will help to improve the performance of drinking water treatment to control the risk associated with antibiotic-resistant bacteria.

1. Introduction

Antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs), associated with the widespread use of antibiotics and their uncontrolled release to the aquatic environment, pose a serious health threat to humans (Manaia et al., 2016; Surette and Wright, 2017). ARB and ARGs have been detected in different aquatic environments such as surface river water (Pruden et al., 2006), municipal wastewater treatment plants (Li et al., 2010), drinking water treatment plants (DWTPs) (Xu et al., 2016), and water supply reservoirs (Huerta et al., 2013). Several studies have reported the presence of ARB and ARGs in treated water (tap and bottled water) (Wang et al., 2016) and in untreated water (wells, rivers, and lakes) (Jiang et al., 2013; Zhang et al., 2015a, 2015b). Treated water intended for consumption is supplied to the local population; hence, the prevalence, proliferation, and persistence of ARB in drinking water can be a potential threat to human health.

Several human infections caused by multidrug-resistant bacteria are treated with carbapenems and polymyxins, which are regarded as last-resort antibiotics (Kaye et al., 2016; Paterson and

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Harris, 2016). However, the presence of plasmid-mediated colistin resistance gene *mcr-1* in members of the family *Enterobacteriaceae* challenged this paradigm (Liu et al., 2016). *E. coli* carrying *mcr-1*, the EBSL-encoding gene, $bla_{\text{CTX-M}}$ (Haenni et al., 2016; McGann et al., 2016; Zurfuh et al., 2016), and $bla_{\text{NDM-1}}$, the carbapenemase-encoding gene (Delgado-Blas et al., 2016; Zheng et al., 2016) show resistance to last-resort antibiotics.

The spread of ARB in drinking water can be induced by biotic and abiotic factors (Wu et al., 2018). Abiotic factors, such as disinfectants, may result in bacterial genetic mutations, which confer resistance to antibiotics (Zhang et al., 2017a; 2017b). Horizontal gene transfer (HGT) plays a major role in the spread of ARGs among bacterial communities (Wang et al., 2019). In a previous study, the occurrence and dissemination of *mcr-1* and $bla_{\text{NDM-1}}$ in a drinking water supply system in Shanghai has been reported (Khan et al., 2020). However, comprehensive studies are needed for elucidation of the mechanism of ARG spread in drinking water treatment and distribution systems and for determination of the health risks associated with antibiotic resistance in bacteria present in drinking water.

The main sources of drinking water in Shanghai are the Qingcaosha and Jinze reservoirs (receiving water from the Yangtze and Taipu rivers), which supply 70% and 30% of the drinking water requirement to the city, respectively. These reservoirs are contaminated with various pollutants and agents, including antibiotics, due to the discharge from upstream cities; apart from pollution of the water sources, this also leads to dissemination of antibiotics, ARB, and ARGs. Chlorination is the most commonly used disinfection method in DWTPs. Several earlier studies on chlorine treatment reported the enrichment of ARB and ARGs in DWTPs due to the co-selection of resistant bacteria (Bai et al., 2015; Guo et al., 2014; Shi et al., 2013; Xu et al., 2016).

DNA Phosphorothioate (PT) modification occurs naturally in various bacteria, and it involves the incorporation of sulfur into the DNA backbone governed by *dndABCDE* genes (Wang et al., 2007; Zhou et al., 2005). Since their original identification in *Streptomyces lividans*, the *dnd* gene cluster has been reported in more than 1300 taxonomically diverse bacterial strains (He et al., 2007; Tong et al., 2018). PT DNA is selectively sensitive to oxidative stress (HOCl), suggesting reduced bacterial fitness (Kellner et al., 2017). PT modification mediates overall cellular redox balance in *Pseudomonas fluorescens* pf0-1 (Tong et al., 2018). Cellular oxidative stress level can be increased by PT modification deficiency, which consequently induces the strain to reconfigure its metabolic networks instead of upregulating common antioxidant genes to fend off excess reactive oxygen species in the cell (Tong et al., 2018).

Besides the role of HGT in the spread of ARGs in water supply systems (WSSs), no other mechanism has been reported to provide further insight into the dissemination of ARGs. This study reports for the first time the role of DNA PT modification in facilitating the spread of ARGs, which provide resistance to last-resort antibiotics in the water supply system. It has been demonstrated that *dndB* protects ARB from chloramine disinfection during the water treatment process. This finding will provide a basis for further studies and in-depth investigation of the mechanisms responsible for ARG spread in WSSs.

2. Material and methods

2.1. Sample collection

Samples from seven DWTPs and their respective distribution systems were collected (Fig. 1). Two of the plants receive water from the Qingcaosha reservoir and the remaining five plants receive water from the Jinze reservoir. From each sampling point, a total of four samples were collected (influent of the treatment plant, effluent from the treatment plant, primary water supply system, and secondary water supply system). For metagenomic analysis, one complete WSS was studied, including three samples from the source (Taipu River and Jinze reservoir), two samples from the DWTP (influent and effluent), and four samples from its respective distribution system (two from the primary water supply system and two from the secondary water supply system).

The treatment process of the waterworks comprised coagulation, sedimentation, sand filtration, ozonization, biological activated carbon (BAC) filtration, and chloramine disinfection. The disinfection method used in all the plants comprised chloramine disinfection, except for one plant, (D), where UV/chloramine disinfection was used. Samples were collected and preserved according to the standard examination methods for drinking water (GB/T 5750.2—2006) under the supervision of waterworks engineers. Sterilized bottles were used to collect 5 L of water samples from each site and were kept on ice until their transport to the laboratory. Standard examination methods for drinking water (GB/T 5750.11—2006) were adopted to measure the total chlorine content in the samples.

2.2. DNA extraction and quantitative polymerase chain reaction (qPCR)

A 0.22-μm microporous membrane (Millipore, USA) was used to filter out bacterial cells from the collected samples, due to the reduced bacterial biomass in treated drinking water. The cetyl trimethylammonium bromide extraction method was used to extract total genomic DNA (Huang et al., 2009). Agarose gel and microspectrophotometry were used to measure the concentration and purity of DNA (NanoDrop_ND-2000, NanoDrop Technologies, Wilmington, DE), and qualified DNA was adjusted to 50 ng/μl and stored at −20 °C for further analysis.

All qPCR reactions were performed using the Applied Biosystems 7500 system with the Hieff™ qPCR SYBR® Green Master Mix (YEASEN). A total of four primer sets were used to quantify the gene targets in the present study, including the two ARGs (*mcr-1* and $bla_{\text{NDM-1}}$), which provide resistance to last-resort antibiotics, one *dndB* gene, and the 16S rRNA gene. Primer sequences used for 16S rRNA, *dndB*, *mcr-1*, and $bla_{\text{NDM-1}}$ were the same as those published in previous studies (Ahammad et al., 2014; He et al., 2015; Liu et al., 2016; Zhu et al., 2013). qPCR reaction conditions and data processing were performed as per previously described methods (Chen et al., 2017; Wang et al., 2014). The sequences of primers used in this study and the qPCR reaction conditions are listed in Table S1 in the Supplementary Material. The detection limit of the cycle threshold (CT) was set to 31. The detection of genes was considered positive when values of all the three technical replicates of each sample were above the detection limit. Relative gene abundance was calculated based on the $2^{-\Delta CT}$ method, where $\Delta CT = CT_{(\text{detected ARG})} - CT_{(\text{16S rRNA gene})}$ and was normalized to the 16S rRNA gene as described in a previous study (Schmittgen and Livak, 2008).

2.3. Metagenomic sequencing and bioinformatics analysis

The HiSeq 2000 platform was used to prepare and sequence metagenomic shotgun sequencing libraries (Shanghai Majorbio Bio-pharm Technology Co., Ltd., China). The raw metagenomic reads were pre-processed using Cutadapt (v2.3) for the removal of adapter sequences, low-quality bases ($Q \leq 20$), and ambiguous bases (Ns) (Martin, 2011). High-quality reads were co-assembled using MEGAHIT (v1.1.4—2) with *k*-mers ranging from 37 to 137 and a minimum contig length of 200 bp (Li et al., 2015b). From the co-assembled contigs, genes were predicted using Prodigal (v.2.6.3)

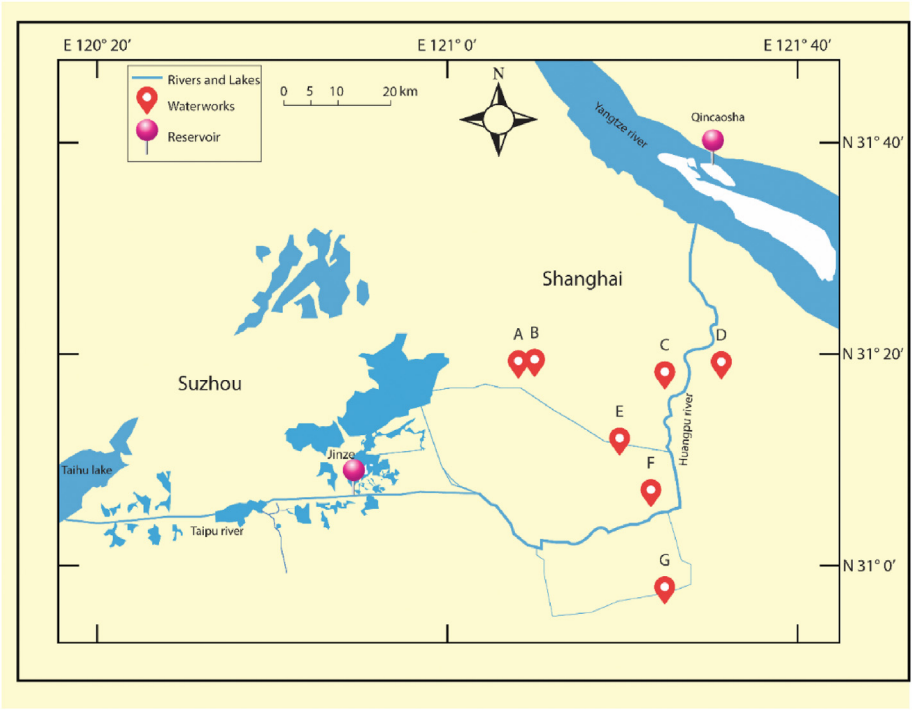

Fig. 1. Map showing the sampling sites in the water supply system. The Qingcaosha reservoir: the source of C and D; the Jinze reservoir: the source of A, B, E, F, and G.

with the default settings except "procedure" (-p parameter), which was set to "meta" to enable prediction of genes in the metagenomic mode (Hyatt et al., 2010). Gene sequences smaller than 100 bp were discarded using Seqtk (v1.3) (Li, 2012), and redundancy was removed using the CD-HIT-EST module of CD-HIT (v4.8.1) with the following parameters: n 8, -c 0.90, and -aS 0.9, to generate the non-redundant (NR) gene catalog (Fu et al., 2012). The NR gene catalog was independently aligned with the Comprehensive Antibiotic Resistance Database (CARD, http://arpcard.Mcmaster.ca, Version 1.1.3) along with an in-house database of *dndABCDE* genes using blastx (Blast + v2.9.0, http://blast.ncbi.nlm.nih.gov/Blast.cgi) and an e-value cutoff of 1e-5.

The relative abundance of ARGs and *dnd* genes was estimated using salmon (0.13.1). Taxonomic classification of AR- and *dnd*-annotated reads was performed using Kaiju (v1.7.2) with default parameters (Menzel et al., 2016). Furthermore, custom Python and bash scripts were used for necessary modification (e.g., merge, filter, sort, and so on) of the data tables.

2.4. Network analysis

The associations among ARGs, *dnd* genes, and their respective microbial taxa were investigated using network analysis (Li et al., 2015a; Tian et al., 2016). A co-occurrence network was constructed among the 18 ARG variants (17 MCR variants and 1 *NDM* variant), 5 *dnd* genes (*dndA-E*), and 380 bacterial genera using data obtained from the 9 water samples to visualize the correlation between ARGs, *dnd* genes, and bacterial taxa (Deng et al., 2012). Briefly, a correlation matrix was constructed by calculating all the possible pairwise Spearman's correlation coefficients among the 403 items, including the 18 ARG variants, 5 *dnd* genes, and 380 bacterial genera (Deng et al., 2012, 2016). An online analysis pipeline available at http://ieg2.ou.edu/MENA was used to perform network analysis, and the interactive platform Gephi (version 0.9.1) was used for network visualization.

The relationship between taxa and the genes (ARGs and *dnd* genes) was investigated by constructing a correlation network. Spearman's correlation coefficient (r) > 0.6 and p-value < 0.01 indicated statistically significant Spearman's correlation between two nodes (Junker and Schreiber, 2008).

2.5. Media, strains, and bacterial growth assay for chlorine treatment

Commercially prepared M9 (Sangon Biotech, Shanghai) and Luria Bertani (LB) broth were used for the experiments and prepared according to the manufacturer's instructions. Ampicillin was used at a final concentration of 100ug/mL (Table S2. in the Supplementary Material).

Two bacterial strains were used, namely wild-type (WT) and *ΔdndB* strain. The selected plasmids were pJTU1238 and pSK + *dnd*CDE. pJTU1238 is an SK + -derived plasmid containing *dndBCDE,* a *Salmonella* complete sulfur-modified gene, which can phosphorylate the host DNA; pSK + *dnd*CDE is based on pJTU1238 (Δ*dndB*), and it can phosphorylate the host DNA and increase the abundance by one fold, compared with pJTU1238 (Liang et al., 2007). The two plasmids were separately transformed into the DH10B strain, and the monoclonal plasmids were isolated overnight and stored at −80 °C for further experiments. The details of the strains and plasmids are listed in Table S3 in the Supplementary Material.

Single colonies of the two bacterial strains were grown in 5 mL of LB medium overnight at 37 °C. The cells were centrifuged at 3000 × *g* for 8 min at ambient temperature, washed with M9 media, and then re-suspended in fresh M9 medium to obtain an initial optical density of 0.2 at 600 nm (OD_{600}), after which they were allowed to grow at 37 °C and 230 rpm for 60 min. The reaction was carried out in M9 media because LB could quench chlorine (Drazic et al., 2015; Winter et al., 2008). The chlorine solution (100 mg/L) was prepared by adding 0.3 mL of sodium hypochlorite solution to 50 mL of water

and maintained at 4 °C in the dark. The stock was diluted in sterile water immediately before use. Bacterial strains were mixed with the chlorine solution at the desired concentration and were left for 30 min at room temperature. After the reaction was completed, an appropriate amount of the reaction sample was obtained and cultured in LB media, and the growth curve line was monitored in 30-min increments at 600 nm in an EL_X808iu plate-reader (BIO-TEK Instrument Inc.) set at 37 °C. The concentrations of the stock solutions and dilutions were determined before their use by using a Pocket calorimeter II (Hach, USA). The OD of each biological replicate was averaged ($n = 3$) for plotting, and the standard deviation was calculated.

2.6. Data analysis

Microsoft Excel 2016 was used for data organization and GraphPad Prism 7.0 was used to plot and generate graphs (Krzywinski et al., 2009). Spearman's correlation was analyzed using the SPSS 21.0 software, where $p < 0.05$ was considered significant for all statistical tests.

3. Results

The relative abundance of *dndB*, *mcr-1*, and bla_{NDM-1} was observed by qPCR in a total of twenty-eight samples from the seven DWTPs and their respective distribution systems. For metagenomic analysis, nine samples were selected, including samples from the DWTPs showing the highest relative abundance of all genes, and the samples from their respective sources and distribution systems. The relative abundance of *dndABCDE* genes as well as *MCR* and *NDM* variants was determined by metagenomic analysis. Network analysis was conducted to explore potential hosts carrying *dndB*, *MCR*, and *NDM* in the WSS. Furthermore, the positive and negative strains of model *dndB* were used to check the effect of chlorine on their growth.

3.1. Increased relative abundance of dndB in the effluents of DWTPs

3.1.1. Relative abundance of mcr-1, bla_{NDM-1}, and dndB as determined by qPCR

qPCR was used to determine the relative abundance of *dndB*, *mcr-1*, and bla_{NDM-1} in the seven DWTPs and their respective distribution systems. Samples were collected from the influent, effluent, and corresponding primary and secondary water distribution systems.

It was observed that the relative abundance of *dndB*, *mcr-1*, and bla_{NDM-1} increased in the effluents of all the treatment plants, compared with that of the influents (Fig. 2). The relative abundance of *dndB*, *mcr-1*, and bla_{NDM-1} varied in the primary and secondary distribution systems (Fig. 2).

The highest relative abundance, among all treatment plants, was observed in the effluent of one plant i.e., F2 (Fig. 2). The source of water for this plant is the Jinze reservoir, which stores water taken from the Taipu River (Fig. 1). Therefore, for metagenomic analysis, the samples included plant F, its source, and its distribution system.

3.1.2. Metagenomic analysis of the relative abundance of dndB, MCR, and NDM

A total of nine samples spanning a complete WSS were used (three samples from source water, two samples from the influent and effluent of a treatment plant (F), two samples from the primary distribution system, and two samples from the secondary distribution system). Metagenomic analysis revealed seventeen variants of *MCR* and one variant of *NDM* in the WSS (Fig. 4); *dndA-E* were also identified in the samples (Fig. 3a and b). The overall highest relative abundance of *dndA* was observed in all the samples, followed by *dndD*, *dndC*, *dndE*, and d*ndB* in decreasing order of their relative abundance (Fig. 3a).

Among all *dnd* genes, only *dndA* and *dndB* showed increase with regard to their relative abundance in the effluent of the treatment plant, compared with the influent (Fig. 3a and b).

For *MCR* and *NDM* variants, it was observed that their relative abundance was higher in the effluent than that in the influent of the water treatment plant (Fig. 4).

3.2. dndB is positively correlated with MCR, NDM, and chloramine concentration

3.2.1. Correlation among mcr-1, bla_{NDM-1}, and dndB indicated by qPCR

The results of qPCR analysis (from seven treatment plants) were used to determine the correlation among *mcr-1*, bla_{NDM-1}, and *dndB*. The results indicate that the concentration of *dndB* correlated positively with chloramine concentration, *mcr-1*, and bla_{NDM-1} (Table 1). All the *p*-values observed were either 0.001 or less than 0.001, which indicated a markedly strong correlation among the ARGs, *dndB*, and chloramine concentrations used in the water supply systems.

3.2.2. Correlation among ARG variants and dndB

Correlation analysis was conducted using the metagenome data from a complete WSS (Fig. 5). It was observed that *dndB* had a positive correlation with some *MCR* variants, including *MCR-1.9*, *MCR-4.3*, and *MCR-5*, but a negative correlation with *MCR-4*. Among the *dnd* genes, *dndB* had a positive correlation with *dndE*, but exhibited a negative correlation with *dndD*. *NDM*-6 had a positive correlation with *MCR-5* and *MCR-6.1,* but exhibited a negative correlation with *dndD*. Furthermore, *dndC* had a positive correlation with *dndE,* but exhibited a negative correlation with *MCR 3.12* and *MCR-7.1*.

However, *dndA* had a negative correlation with *dndC*, *dndD,* and *MCR-7.1*. There was no positive correlation observed between *dndA* and any of the *dnd* genes, *MCR,* or *NDM* variants (Fig. S1 in the Supplementary Material).

3.3. dndB protects bacteria from chlorine treatment

Model strains were used to check the effect of chlorine concentrations on growth. The WT strain harbored all *dnd* genes (*dndA-E*), whereas the Δ*dndB* strain only harbored *dndACDE*, without the *dndB* gene. The two strains were treated with chlorine concentrations in the range of 0–3 mg/L and their growth curves were compared.

The results indicated that at all the concentrations of chlorine, the WT strain showed more growth than the Δ*dndB* strain. The difference in growth was more prominent as the dose increased to 2 and 3 mg/L (Fig. 5).

The standard deviation showed that the observed values were very close to the mean (Table S4 in the Supplementary Material). Most of the values were less than 0.01, and only a few were above 0.01; however, they were less than 1, suggesting a markedly small variation from the mean values shown in Fig. 5.

3.4. Bdellovibrio: the potential host for MCR, NDM, and dndB

For co-occurrence analysis, the ARG data were arranged at the family level and the co-occurrence between the *MCR* phosphorothioate family and *NDM* beta-lactamase family was calculated with their respective genera. The results indicate that *dndA* exhibits a positive correlation with four genera, *dndB* with twenty-one

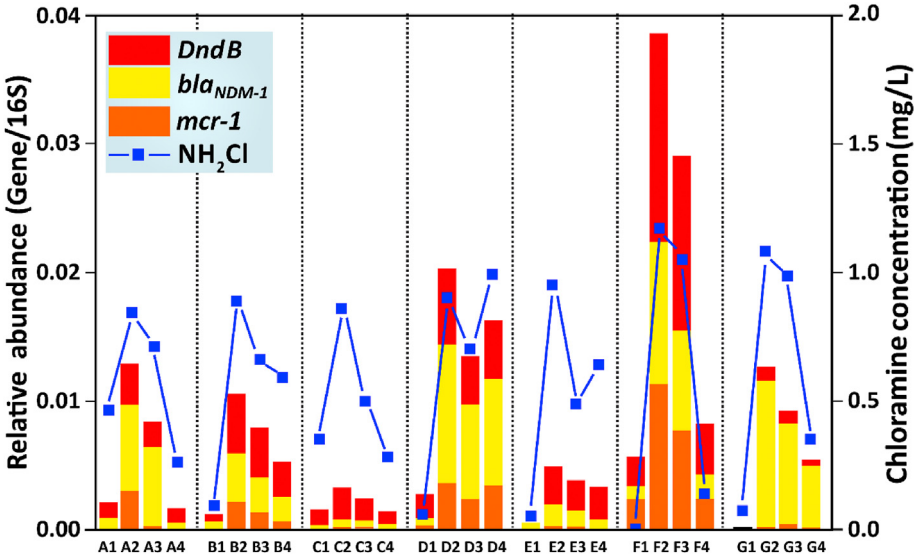

Fig. 2. Relative abundance of mcr-1, bla_{NDM-1}, dndB, and chloramine concentration in different waterworks. A–G: seven different water treatment plants; 1: waterworks influent; 2: waterworks effluent; 3: primary water supply system; and 4: secondary water supply system. The vertical lines in the figure are shown to distinguish between each water treatment plant.

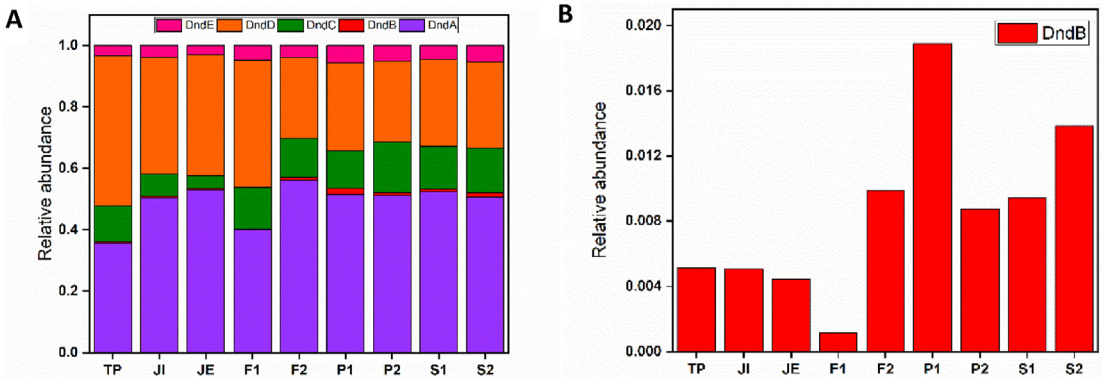

Fig. 3. Relative abundance of dndA-E (A) and dndB (B) in the water supply system as determined by metagenomic analysis. TP: Taipu River; JI: Jinze influent; JE: Jinze effluent; F1: waterworks influent; F2: waterworks effluent; P: primary water supply system; and S: Secondary water supply system.

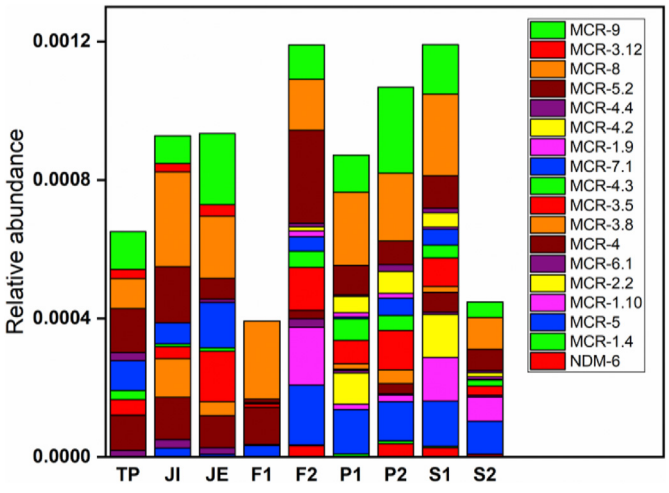

Fig. 4. Relative abundance of MCR and NDM in the water supply system as determined by metagenomic analysis. TP: Taipu River; JI: Jinze influent; JE: Jinze effluent; F1: waterworks influent; F2: waterworks effluent; P: primary water supply system; and S: Secondary water supply system.

genera, *dndC* with eight genera, *dndD* with only one genus, and *dndE* with seventeen genera (Fig. S2 & Fig. S3 in the Supplementary Material).

Based on the taxa, the *MCR* family demonstrated a positive correlation with eight genera, while the *NDM* family had a positive correlation with fifty genera and negative correlation with twenty-eight genera.

The common genera in all the three groups, *dndB*, *MCR*, and *NDM,* were determined, and the results suggested that there were seven common genera for *MCR* and *NDM*, six for *NDM* and *dndB*, and one common genus (*Bdellovibrio*) for all the three genes (Fig. 6).

The relative abundance of the common genera was detected from the source to the distribution system, and it was observed that *Bdellovibrio* could be found in all the samples, with the exception of the Jinze influent. However, it was observed that the relative abundance of *Bdellovibrio* increased in the effluent of the treatment plant and later increased in the primary water distribution system (Fig. 7).

Table 1
Spearman's correlation coefficients among *mcr-1*, bla_{NDM-1}, *dndB*, and chloramine in samples obtained from seven different waterworks and their respective distribution systems (A–G).

		mcr-1	bla_{NDM-1}	*dndB*	Chloramine (mg/L)
mcr-1	R^2	–	0.766	0.823	0.615
	p		<0.001	<0.001	<0.001
***bla*$_{NDM-1}$**	R^2	0.766	–	0.599	0.774
	p	<0.001		<0.001	<0.001
dndB	R^2	0.823	0.599	–	0.580
	p	<0.001	<0.001		0.001

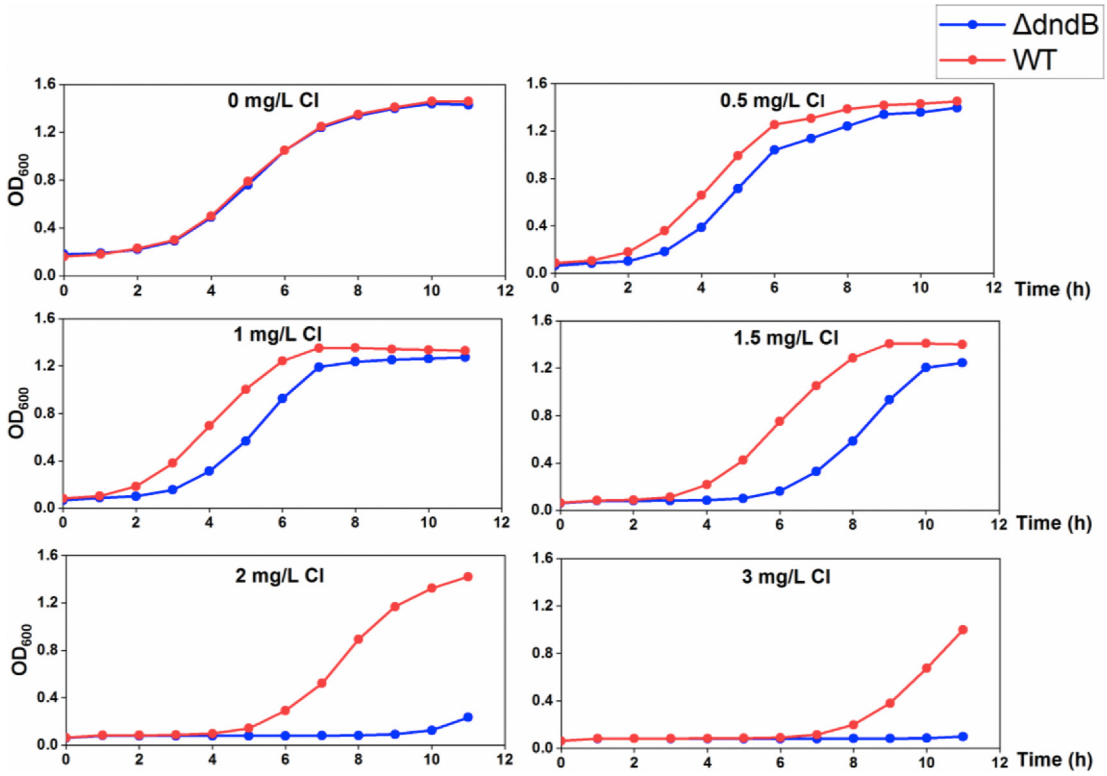

Fig. 5. Comparison of the sensitivities of the wild-type (WT) and ΔdndB strains to chlorine.

4. Discussion

Health authorities in developed countries continue to engage efforts on the improvement and maintenance of drinking water quality in the distribution system. Thus, many international standards have been developed to improve drinking water quality, including disinfection techniques, to prevent harmful bacteria. However, traditional chlorine disinfection cannot eliminate the bacteria in the effluent completely, which results in rapid re-colonization in the WSSs (Bargellini et al., 2011; LeChevallier et al., 1988; Taylor et al., 2009).

4.1. MCR and NDM disseminate in the WSS

Many studies have reported the presence of ARB and ARGs in the DWSS from the untreated water source to the final treated drinking water supply system (Bai et al., 2015; Guo et al., 2014; Jiang et al., 2013; Schwartz et al., 2003; Xu et al., 2016). However, the dissemination of ARGs, which provide resistance to last-resort antibiotics in the DWSSs, has not been explored. It has been reported that *mcr-1* and bla_{NDM-1} are disseminated in the drinking water supply system in Shanghai, which receives water from the Taipu River (Khan et al., 2020). However, this study included samples from seven different points receiving water from the two sources in Shanghai (the Qingcaosha reservoir and the Jinze reservoir), and it was observed that the relative abundance of *mcr-1* and bla_{NDM-1} increased after the water treatment process, regardless of the source (Figs. 2 and 4).

Previous studies have focused on the wide distribution of ARB, which are resistant to a variety of antibiotics, in source water (Coleman et al., 2013; Egervarn et al., 2017; Fernando et al., 2016; Huerta et al., 2013; Jiang et al., 2013). The occurrence and quantification of ARGs in DWTPs were the focus of a study, in which a decrease in the absolute abundance of ARGs was reported; however, the relative abundance was found unchanged (Xi et al., 2009). A study conducted to understand the mechanism of ARG variation in DWTPs in the Yangtze River Delta reported an increased abundance of sulfonamide and tetracycline resistance genes after BAC (Guo et al., 2014).

Among the treatment processes, chlorine is believed to enrich ARGs after utilization in DWTPs (Bai et al., 2015; Guo et al., 2014; Shi et al., 2013; Xu et al., 2016). Globally, chlorination is the most commonly used disinfection technique against bacteria. Free chlorine is either used in low concentration (0.2–0.5 mg/L) to

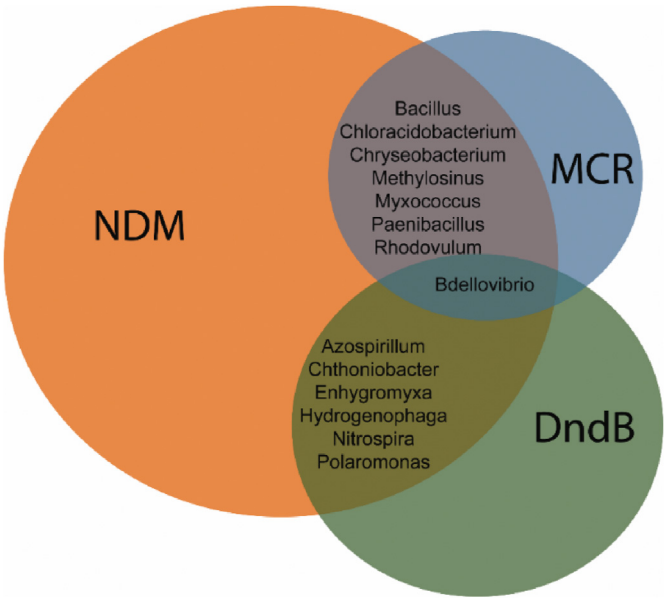

Fig. 6. Network analysis of the common genera as potential hosts for MCR, NDM, and dndB.

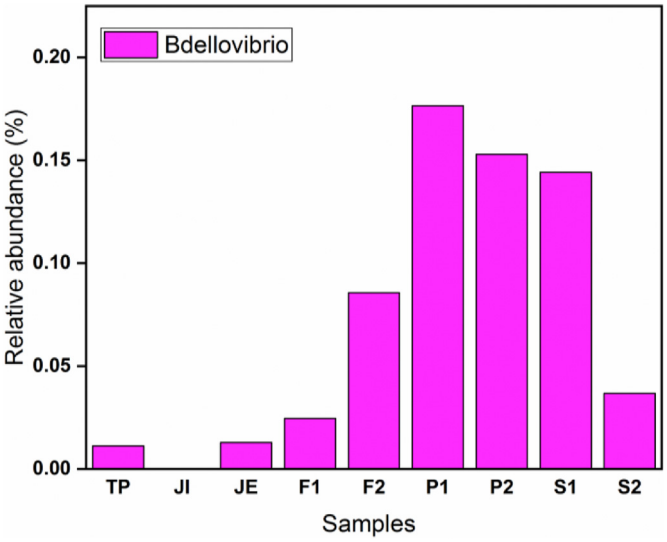

Fig. 7. Relative abundance of Bdellovibrio as a potential host for MCR, NDM, and dndB in the water supply system. TP: Taipu River; JI: Jinze influent; JE: Jinze effluent; F1: waterworks influent; F2: waterworks effluent; P: primary water supply system; and S: Secondary water supply system.

maintain the quality of water in distribution systems as a secondary disinfectant (Deborde and von Gunten, 2008) or as an installation disinfection treatment in higher concentrations known as hyper-chlorination (Garcia et al., 2007; Szewzyk et al., 2000).

Chlorine stress conditions may result in different responses depending upon the dosage, contact time, nature of the chlorination agent, and the nature of ARB and ARGs. A study based on metagenomic sequencing reported the enrichment of *ampC*, *aphA2*, bla_{TEM-1}, *tetA*, *tetG*, *ermA*, and *ermB* using chlorine dioxide as a chlorination agent; however, *sulI* was significantly removed (Shi et al., 2013). Similar results were reported with chloramine as the chlorination agent (Bai et al., 2015).

The transmission of ARGs from environmental ARB to harmful bacteria may increase through the chlorination process. An increase in cell permeability by chlorination has been reported, which may enhance the conjugative transfer of ARGs (Sharma et al., 2016). A low dosage of chlorination not only results in the enhancement of ARB and ARGs, but has also been implicated in multi-drug efflux pump development that mediates co-resistance and plasmid over-replication (Shi et al., 2013). Furthermore, antibiotic resistance co-selection is also mediated by the mutagenic activities of various disinfection byproducts (Lv et al., 2014).

4.2. *dndB facilitates survival of ARB against multiple barriers in WSS*

The *dnd* gene cluster, *dndABCDE,* occurs in diverse bacterial genera, in which *dndBCDE* occurs as a single operon, while *dndA* is either located near *dndBCDE* or elsewhere in the genome (Wang et al., 2007; Zhou et al., 2005). The *dnd* gene cluster incorporates sulfur into the DNA backbone by phosphorothioation (Zhou et al., 2004, 2005). Many studies have explored the functions of the *dnd* genes and their roles in phosphorothioation (He et al., 2015; Hu et al., 2012; Yao et al., 2009; You et al., 2007).

Some studies have demonstrated the indirect role of *dndB* as a transcriptional repressor, compared with *dndACDE*. The *dndB* gene binds the upstream promoter region of the *dndBCDE* gene cluster, thereby negatively regulating the transcription of its own gene and *dndCDE*. The deletion of *dndB* in *S. enterica* serovar Cerro 87 not only aggravated DNA degradation but also resulted in a 2-fold increase in PT modification. The role of *dndB* as a negative regulator helps in the tight control of cellular PT levels (He et al., 2015) because excessive and insufficient PT levels may interfere with PT-related functions.

PT-modified DNA exhibits enhanced resistance to nucleases (Eckstein, 1985). A study reported that *Salmonella enterica* serovar Cerro 87 exhibited a higher survival rate against H_2O_2 exposure than the PT-deficient mutant (Xie et al., 2012). Hydroxy-radical scavenging in the presence of PT modification protected genomic DNA and proteins against oxidative stress (Wu et al., 2017); hence, PT is regarded as a mild antioxidant *in vivo*. Additionally, various environmental stress conditions (low temperature, low salinity level, high pressure, UV radiation, X-ray radiation, and heavy metal stresses) have resulted in the growth of *E. coli* and *Shewanella piezotolerans* in the presence of PT modification (Yang et al., 2017).

Unlike *dndACDE*, *dndB* is not directly involved in PT modification but has been reported as a negative regulator of the *dnd* gene cluster *dndABCDE* (He et al., 2015). PT desulphurization resulting in DNA strand breakage was observed during *in vitro* oxidation with both H_2O_2 and HOCl treatments (Kellner et al., 2017). However, with PT modification, WT *S. enterica* serovar Cerro 87 showed higher sensitivity to HOCl than its Δ*dndB-H* mutant (Kellner et al., 2017).

By performing *in vitro* experiments, we discovered that the growth of WT and Δ*dndB* strains was affected by the increase in chlorine concentration. However, the damage caused by chlorine to Δ*dndB* was much higher compared to that in the WT strain. This phenomenon is consistent with that reported in the literature (Kellner et al., 2017).

There are two possible reasons for the decrease in the chlorine tolerance of the Δ*dndB* strain in comparison with the WT strain. The first possible reason is the increased sulfur modification in the Δ*dndB* strain, and the second reason may be attributed to the protective role of *dndB*, which increases the tolerance of the WT strain to chlorine. Moreover, the reduced tolerance of the Δ*dndB* strain may be attributed to the absence of *dndB* protein. Kellner et al. reported that the sulfur-modified strain is less tolerant to HOCl than the non-sulfur modified strain (Kellner et al., 2017).

Therefore, the sulfur modification system in the Δ*dndB* strain may be able to increase the sensitivity of the cells to chlorine in the environment, but this does not rule out the fact that *dndB* protein exerts a protective effect on the chlorine tolerance of the cells. Hence, further studies should be conducted to explore this phenomenon.

In this study, chloramine was used as a disinfection agent in the DWTPs. An increase in the relative abundance of *dndB* in the effluent and the strong correlation of *dndB* with chloramine indicate that *dndB* protects ARB and helps them to survive the multiple barriers in the WSSs, specifically after chloramine disinfection in the water treatment process (Fig. 2 and Table 1). Furthermore, in the water treatment processes, chlorine dosage is usually below 3 mg/L; hence, the WT strain is resistant to chlorine at concentrations ranging from 0.5 to 3 mg/L, thereby indicating that *dndB* protects the ARB against chloramine disinfection during the water treatment process and helps in the spread of ARB in the WSS.

4.3. Bdellovibrio: a core genus carrying MCR, NDM, and dndB

Bdellovibrio possess an obligatory predatory life cycle and are characterized as gram-negative, motile, and uniflagellated bacteria. They are mainly found in wet and aerobic environment and were first isolated from soil (Stolp and Starr, 1963). Additionally, they can also be found in fresh and brackish water, sewage, water reservoirs, and seawater (Kelley et al., 1997; Schoeffield and Williams, 1990). Biofilms are another environmental niche where bdellovibrios reside, due to high prey density which is necessary for their survival (Kelley and Williams, 1992; Williams et al., 1995).

The treatment process used at DWTPs consists of several steps, and most microbes are removed during coagulation, biological sand filtration, ozonation, and chlorination. However, the presence of ARGs (*MCR* and *NDM*) and *dndB* in ARB provides resistance to last-resort antibiotics, thereby facilitating their survival through the water treatment process and growth in the biofilms in the distribution system. Further research is warranted to compare this new mechanism with other well-known mechanisms (i.e., HGT and gene mutation), with regard to their contributions to the prevalence of ARGs in municipal drinking water systems.

5. Conclusion

It has been demonstrated that *dndB* protects ARB from chloramine disinfection during water treatment. The significant positive correlation of *dndB* with ARGs (*MCR* and *NDM*) in the water supply system is worrisome. A core genus of *Bdellovibrio* was found carrying *MCR*, *NDM*, and *dndB* in the water supply system. The role of *dndB* in the spread of ARGs opens new avenues for exploration and elucidation of the underlying mechanisms through which ARGs can disseminate from untreated water sources to treated drinking water supply systems. Prospective studies should focus on highlighting the in-depth mechanism by which *dnd* genes help bacteria to survive multiple barriers in the water treatment process. In addition to chlorination, the effect of other water treatment processes, such as UV disinfection and ozonization, on *dndB* needs to be explored. Furthermore, apart from the role of *dndB* in facilitating the dissemination of ARGs, the role of the other *dnd* genes (*dndACDE*) should also be investigated. This will help to control the dissemination of ARGs and improve the performance of DWTPs.

Authorship statement

Hira. Khan: Data curation, Formal analysis, Investigation, Writing - original draft preparation. Mingkun. Liu: Data curation. Masood ur Rehman Kayani: Data curation. Shakeel. Ahmad: Writing- Review and Editing, Visualization. Jingdan Liang: supply model strain and related experiment training, Xiaohui. Bai: Conceptualization, Methodology, Validation, Supervision, Writing-Review and Editing, funding support.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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