Archives of Environmental Protection
Vol. 48 no. 4 pp. 95–101

PL ISSN 2083-4772
DOI 10.24425/aep.2022.143712

Composition of norfloxacin-resistant bacteria and isolation of norfloxacin-degrading bacteria in subtropical aquaculture ponds in China

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Keywords: high-throughput sequencing, norfloxacin, antibiotic resistant bacterium, pond culture

Abstract: To analyze the composition of norfloxacin-resistant bacteria and norfloxacin-degrading bacteria in pond water and sediment in subtropical China, the composition of antibiotic resistant bacteria in pond water and sediment enriched with norfloxacin-containing medium was analyzed by high-throughput sequencing. Sediment and water samples were collected from 3 fish ponds in subtropical China, and domesticated with norfloxacin, subsequently norfloxacin-resistant bacteria through high-throughput sequencing of 16S rDNA, and isolated norfloxacin--degrading bacteria. Our results showed that the pond sediment and water contain a variety of norfloxacin-resistant bacteria, mainly from Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes, and Chloroflexi. Moreover, we isolated two norfloxacin-degrading bacteria (NorXu-2 and NorXu-3). The norfloxacin-degrading rate by NorXu-2 and NorXu-3 in the culture mediums with 200 μg/mL was the highest, which was up to 49.71% and 35.79%, respectively. When the norfloxacin concentration was 200 μg/mL, NorXu-2 and NorXu-3 had the best norfloxacin--degrading effect at pH of 6, and the degradation rates were 53.64% and 45.54%, respectively. Moreover, NorXu-3 exhibited a good tolerance to high NaCl concentration. These results not only provided basic data for the follow-up study of the molecular mechanism of antimicrobial microbial degradation, but also provided potential norfloxacin degrading bacteria for norfloxacin removal and bioremediation in aquaculture environment.

Introduction

The majority of aquatic products are obtained from aquaculture owing to a decrease in wild fishery resources and development of high-density and intensive culture (Gong et al. 2021). For instance, global fish production was estimated to have reached approximately 179 million tons in 2018, of which aquaculture accounted for 46% and 52% of the total production and human consumption, respectively (FAO 2020). China has remained a major fish producer, accounting for 35% of the global fish production in 2018 (FAO 2020). Pond high-density and intensive culture is the main form of aquaculture in China, accounting for 37.22% of the total aquaculture area and 48.84% of the total output of aquatic products in China (Zhang et al. 2020). Although the pond high-density and intensive culture greatly increases the productivity and efficiency of the unite aquaculture area, this culture method has increased the pollution of aquaculture water and tine incidence of fish diseases.

To prevent the disease of cultured fish, antibiotics are widely used in pond high-density and intensive culture, which leads to a large number of antibiotics remain in the pond water and sediment (Mao et al. 2019). For instance, Wang et al. (2011) reported that fish only absorb about 1/5 of antibiotics in feed, and most of the antibiotics enter the water and sediment. Extensive use of antibiotics has caused a serious threat of antibiotic resistance (Laxminarayan et al. 2013). Although the usage of antibiotics in agricultural production is strictly restricted presently, residues of antibiotics in environment and illegal use of antibiotics still cause prevalence of antibiotic resistance, especially in lakes and fish ponds (Hao et al. 2017, Liu and Lu 2018, Mao et al. 2019, Lemańska et al., 2021). Liu and Lu (2018) reported that Baiyangdian Lake, Taihu Lake, Chaohu Lake, Wulungu Lake, Besiteng Lake, Daliao River were polluted by a variety of antibiotics. The norfloxacin concentration was 4.3–214 ng/L, and the concentration in Daliao River was the most serious.

Norfloxacin belongs to the third generation of fluoroquinolones. It has the characteristics of strong broad--spectrum antibacterial activity, insolubility in water, and easy adsorption by soil minerals and organisms (Yang et al. 2012, Jałowiecki et al. 2019). It has become one of the widely used antibiotics (Hao et al. 2017). Guo et al. (2016) reported that fluoroquinolones dominated by norfloxacin have been detected in sewage inflow tanks in cities such as Guangdong and Beijing, which affects human health and safety. Liang

et al. (2013) reported that antibiotics such as norfloxacin accumulated with the accumulation of aquaculture time.

Fluoroquinolone antibiotics such as norfloxacin, which remain in the natural environment, are difficult to transform and degrade by natural self-purification ability (Yang et al. 2020). The ecotoxicity of antibiotics has also received extensive attention (Yang et al. 2020, Gamoń et al. 2022). Zhao et al. (2016) reported that norfloxacin has high ecological risk. Therefore, the residue of norfloxacin in aquaculture brings potential safety hazards to human, animals or environment. Therefore, to explore the removal technology of antibiotics in the environment has become an important topic in the field of environmental remediation (Wu et al. 2019).

Although there are physicochemical methods (such as activated carbon adsorption, low-temperature plasma technology, soil infiltration system, and ultrasonic degradation methods) and microbial degradation method for degrading antibiotics (Yang et al. 2012, Wu et al. 2019, Zhang et al. 2019), considering the limitations of treatment conditions and cost, physicochemical methods cannot be well used for the removal of antibiotics in water environment, and that is why microbial degradation became the main way to degrade environmental antibiotics (Wu et al. 2019). Analyzing the composition of antibiotic resistant bacteria in pond environment and isolating antibiotic degrading bacteria are the basis for further study on the molecular mechanism of antibiotic microbial degradation. To analyze the composition of norfloxacin resistant bacteria and norfloxacin degrading bacteria in pond water and sediment in subtropical China, the composition of antibiotic resistant bacteria in pond water and sediment enriched with norfloxacin containing medium was analyzed by high-throughput sequencing, and norfloxacin degrading bacteria were isolated and cultured by culture method. Our results not only provided basic data for the follow-up study of the molecular mechanism of antimicrobial microbial degradation, but also provided potential norfloxacin degrading bacteria for norfloxacin removal and bioremediation in aquaculture environment.

Materials and Methods

Sample collection

Sediment (SedM) and water (WatB) samples were collected for analyzing norfloxacin resistant microbiota from 3 Cyprinidae fish mixed ponds located at Yuanzhou Town (113°57′ E, 23°07′ N) in Huizhou, a subtropical city in southern China on January 1, 2016. Moreover, a sediment sample was collected from one pond for directional domestication test using different concentrations of norfloxacin.

Directional domestication, DNA extraction and high-throughput sequencing analysis of microbiota

Each 1 L basic medium contains 0.0023 g $FeCl_3 \cdot 6H_2O$, 0.025 g $CaCl_2 \cdot 6H_2O$, 1.6 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.4 g K_2HPO_4, 0.5 g NH_4NO_3, and 0.2 g yeast extract powder. Three samples of sediment (SedM) and water (WatW) were cultured using medium with 50 μg/mL norfloxacin (Sinopharm Shantou Jinshi Pharmaceutical Co., Ltd., Shantou, China). Four sediment samples (NorC) were cultured using medium with 0, 10, 50, and 100 μg/mL norfloxacin respectively to study the effect of norfloxacin concentration on the sediment microbiota. In the above experiments, 1 g sediment or 1 ml water was added to every 100 ml of culture medium and mixed evenly. After incubation in a shaking table with 150 r/min at 28°C for 24 h, each 2 mL bacterial solution was transferred into a 2 ml centrifuge tube and centrifuged at 1200 rpm for 10 min, then the bacteria were collected for DNA extraction.

Microbiota DNA was extracted by revised CTAB method (Ni et al. 2017). The DNA was purified with DNA purification kit (Beijign Dingguo, Beijing, China). The DNA quality and concentration were detected by NanoDrop 2000 micro--spectrophotometer (Thermo, USA).

The prokaryotic V4–V5 hypervariable region was amplified using primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 909R (5'-CCCCGYCAATTCMTTTRAGT-3') (Xiang et al. 2018). PCR products were purified using a GeneJET gel recovery kit (Thermo Scientific, USA). Then all amplicons were pooled together with an equal molar amount from each sample and sequenced using an Illumina MiSeq system at Guangdong Meilikang Bio-Science, Ltd., China.

The raw reads were merged using FLASH 1.2.8 (Magoc and Salzberg 2011) and processed using QIIME 1.9.0 (Caporaso et al. 2010) as previously described (Xiang et al. 2018). In brief, the low-quality sequences and chimera sequences were identified and removed before further analysis. Then the sequences were clustered into operational taxonomic units (OTUs) at 97% identity using UPARSE (Edgar 2013). Subsequently, all samples were randomly resampled to obtain the same number of sequences. Taxonomic assignments of each OTU were determined using the RDP classifier.

Isolation of norfloxacin-degrading bacteria and determination of degradation rate

Independent colonies from the high-concentration norfloxacin plates of sediment bacteria were selected. Then, the colonies were further purification until their descendent colonies with consistent morphology. Norfloxacin-resistant bacteria were isolated and numbered according to the colony morphology.

The purified norfloxacin-resistant bacteria were inoculated into liquid inorganic salt mediums containing different concentrations of norfloxacin, and cultured in shaking table with 150 r/min at 30°C for 24 h. The liquid inorganic salt mediums without bacteria were used as the blank control and cultured using the same culture conditions. All mediums were filtered using 0.22 μm filter membranes to remove macromolecular particulate matter and bacteria. Then 1 ml of filtrate was taken and diluted to 100 mL with mobile phase, and the norfloxacin content was analyzed by high performance liquid chromatography (HPLC).

Morphological observation and 16S rDNA sequencing of norfloxacin-degrading strains

A single colony of each norfloxacin-degrading strain was drawn on the plate of solid inorganic salt medium and cultured upside down at 30°C for 3 d. The bacterial micro-morphologies were observed by microscope with Gram staining.

A single colony of each norfloxacin-degrading strain was inoculated into liquid inorganic salt medium and cultured in shaking table with 150 r/min at 30°C for 24 h. Each 2 mL bacterial solution was transferred into a 2 ml centrifuge tube and

centrifuged at 1200 rpm for 10 min, and then the bacteria were collected and DNA extracted using the revised CTAB method (Ni et al. 2017). The DNA was purified with DNA purification kit (Beijign Dingguo, Beijing, China). Subsequently, the 16S rDNA was amplified using bacterial universal primers 9bfm and 1512uR as previously described (Mühling et al. 2018). PCR products were sequenced at BGI (Shenzhen, China). Afterwards, low-quality bases were removed by BioEdit software, and similar sequences were blasted online using blastn in GenBank database. The phylogenetic tree was constructed using Clustal W and MEGA7 software.

Optimization of norfloxacin-degrading conditions by norfloxacin-degrading bacteria

To analyze the optimal degradation conditions of norfloxacin-degrading bacteria, the norfloxacin concentration of from 100 to 500 μg/ml, pH of from 4 to 9, and NaCl concentration of from 0 to 4.0% were set separately, then 1 mL of the norfloxacin-degrading bacteria suspensions (OD value was 1.0) was inoculated into 100 mL inorganic salt medium for shake flask degradation experiment. Three parallel samples were set in each group, and the blank control (CK) was set without bacterial inoculation. Then the mediums were cultured in shaking table with 150 r/min at 30°C for 24 h. The norfloxacin concentrations were detected using HPLC and the degradation rates of norfloxacin were calculated according to the standard curve.

Data Availability Statement

The merged sequences were submitted to NCBI sequence read archive with accession number PRJNA794364. The 16S rDNA sequences of NorXu-2 and NorXu-3 were submitted to NCBI GenBank database with accession number OM149364 and OM149365, respectively.

Data analysis

Data were showed as mean ± standard error. Principal coordinate analysis (PCoA) was conducted using the QIIME 1.9.0 (Caporaso et al. 2010). Student's t-test and Kruskal-Wallis test were conducted using R 4.0.3. Boxplots, heatmap, and curve diagrams were drawn using ggpubr, pheatmap, and ggplot2 packages of R, respectively.

Results

Composition of pond and sediment norfloxacin-enriched microbiota

A total of 324,711 (32471.10 ± 7741.95) high-quality sequences were obtained from 10 norfloxacin-enriched microbiota. To eliminate the interference of sequencing depth difference on the analysis results, 11,000 high-quality sequences were randomly resampled from each sample for subsequent analysis. The OTU number and Shannon index of norfloxacin-enriched microbiota from pond sediment were significantly higher than those from pond water (Figure 1A and 1B), which caused that the Goods' coverage of the sequences to norfloxacin-enriched microbiota from pond sediment was significantly lower than that from pond water (Figure 1D). However, Chao1 index of the microbiota was no significantly different (Figure 1C). PCoA also showed that the norfloxacin-enriched microbiota from pond sediment and water could be clearly distinguished (Figure 1E). These results implied that the number of norfloxacin-resistant bacteria in sediment microbiota was significantly higher than that in pond water microbiota.

Taxonomic assignments of the norfloxacin-enriched microbiota showed that except for a few sequences (accounting for 0.10±0.05% of all the analyzed high-quality sequences), other sequences were divided into 38 phyla, in which Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes, and Chloroflexi dominated the norfloxacin-enriched microbiota (Figure 1F). The norfloxacin-enriched bacteria mainly belonged to *Delftia*, *Pseudomonas*, *Mycoplana*, *Chyseobacterium*, *Wautersiella*, *Pedobacter*, *Deefgea*, *Cloacibacterium*, *Stenotrophomonas*, *Comamonas*, *Acinetobacter*, *Sinomonas*, *Arthrobacter*, *Ralstonia*, *Citrobacter*, *Salinispora*, *Burkholderia*, *Erwinia*, *Pandoraea*, *Dyella*, *Lactococcus*, *Methanolinea*, *Klebsiella*, *Anaerolinea*, and a lot of unidentified genera (Figure 1G).

Different norfloxacin concentrations were used to screen the norfloxacin-resistant bacteria. When the concentration of norfloxacin was less than 100 μg/mL, Proteobacteria was the phylum with the highest relative abundance. The relative abundances of Chloroflexi, Planctomycetes, and Euryarchaeota were increased together with increased norfloxacin, especially when the concentration of norfloxacin was 100 μg/mL, Euryarchaeota and Chloroflexi were evidently enriched (Figure 2A). When the concentration of norfloxacin was 10 μg/mL, *Alicyclobacillus*, *Citrobacter*, and some unidentified genera were significantly enriched. When the concentration of norfloxacin was 50 μg/mL, *Acinetobacter*, *Burkholderia*, *Streptomyces*, and some unidentified genera were significantly enriched. When the concentration of norfloxacin was 100 μg/mL, Candidatus Methanoregula, *Chryseobacterium*, and some unidentified genera were enriched (Figure 2B).

Isolation and identification of norfloxacin-degrading bacteria

Two bacterial strains, NorXu-2 and NorXu-3, were isolated and purified from norfloxacin-enriched bacteria. The colony of NorXu-2 was milky white, round, opaque, medium-sized, flat surface, irregular edge, and no hyphae (Figure 3A). It was small, rod-shaped and Gram-positive bacterium (Figure 3C). The colony of NorXu-3 was milky white, round, opaque, small, flat surface, irregular edge, and no hyphae (Figure 3B). It was small, rod-shaped and Gram-positive bacterium (Figure 3D). The phylogenetic tree constructed based on 16S rDNA sequences showed that both of them were clustered into one branch with Bacillus (Figure 3E). Therefore, NorXu-2 and NorXu-3 were preliminarily identified as *Bacillus*.

Optimization of degrading conditions of norfloxacin-degrading bacteria

The degradation rates by NorXu-2 and NorXu-3 in the culture mediums with 100–300 μg/mL of norfloxacin were higher than in those with 400–500 μg/mL of norfloxacin. The norfloxacin-degrading rate by NorXu-2 and NorXu-3 in the culture mediums with 200 μg/mL was the highest, which was up to 49.71% and 35.79%, respectively. However, when the norfloxacin concentration increased to 400 μg/mL, the degradation rates of the two strains were reduced

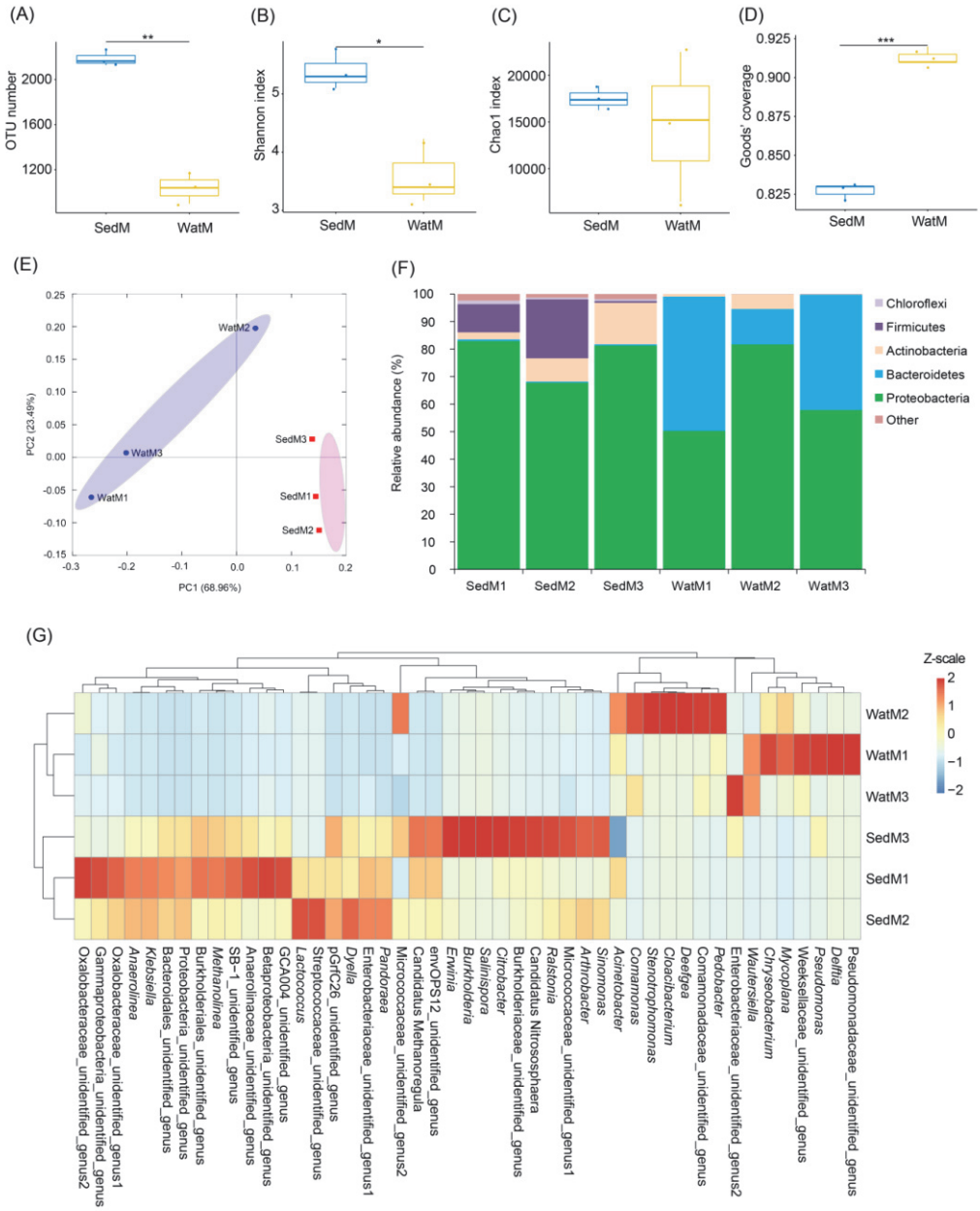

Fig. 1. Norfloxacin-enriched microbiota characteristics of pond water and sediment. (A), OTU number; (B), Shannon index; (C), Chao1 index; (D), Goods' coverage; (E), PCoA profile; (F), Relative abundances of dominant phyla; (G), Heatmap profile of dominant genera. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

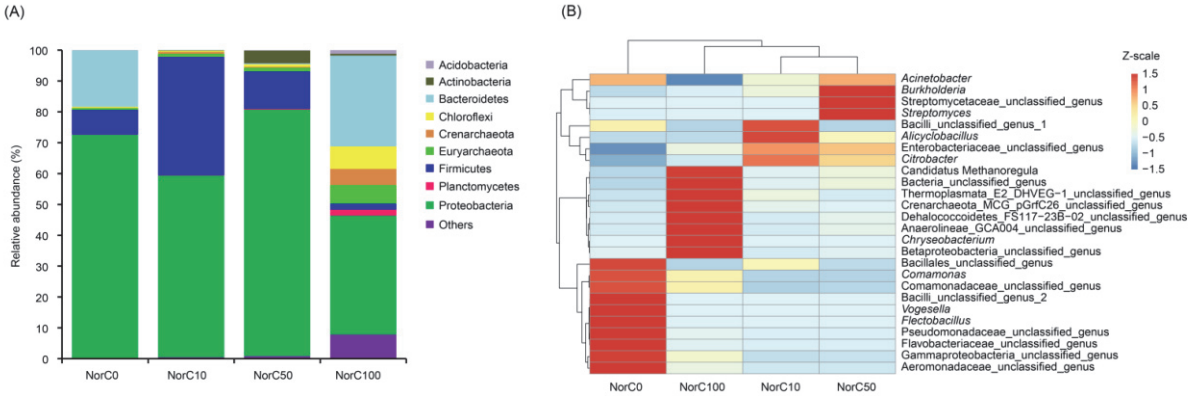

Fig. 2. Norfloxacin-enriched bacteria using different concentrations of norfloxacin. (A), Relative abundances of dominant phyla; (B), Heatmap profile of dominant genera.

evidently, and when the norfloxacin concentration increased to 500 μg/mL, the degradation rates of the two strains were reduced to 14.02% and 12.75% respectively (Figure 4A).

When the norfloxacin concentration was 200 μg/mL, NorXu-2 and NorXu-3 had the best norfloxacin-degrading effect at pH of 6, and the degradation rates were 53.64% and 45.54%, respectively. The degradation effect at lower and higher pH was reduced (Figure 4B). These results implied that in the neutral pH environment, the norfloxacin-degrading potential was the highest. Commonly, the pH of aquaculture water was ranged from 6 to 9. The above two strains showed high degradation rate at pH 6, which was consistent with the original habitat conditions isolated from the sediment of aquaculture farm.

The NaCl content in seawater in subtropical China is about 3.5%. When the norfloxacin concentration was 200 μg/mL, and pH was 6, the degradation ability of NorXu-2 was stable when NaCl concentration was lower than 2.50%, and decreased significantly when NaCl concentration was higher than 3% (Figure 3C), suggesting that the strain was not suitable for the bioremediation of norfloxacin residues in mariculture water. NorXu-3 exhibited a good tolerance to high NaCl concentration. Although the degradation rate decreased slightly, the degradation effect remained stable (Figure 3C). It was further domesticated and expected to be applied to the bioremediation of norfloxacin residues in freshwater and mariculture environment.

Discussion

Antibiotic residues in aquaculture water and sediment have become an important problem affecting the quality of cultured fish (Monteiro et al. 2016). Understanding the composition of antibiotic resistant bacteria in aquaculture water and sediment, and developing antibiotic removal technology are not only of great value to aquaculture, but also of great ecological

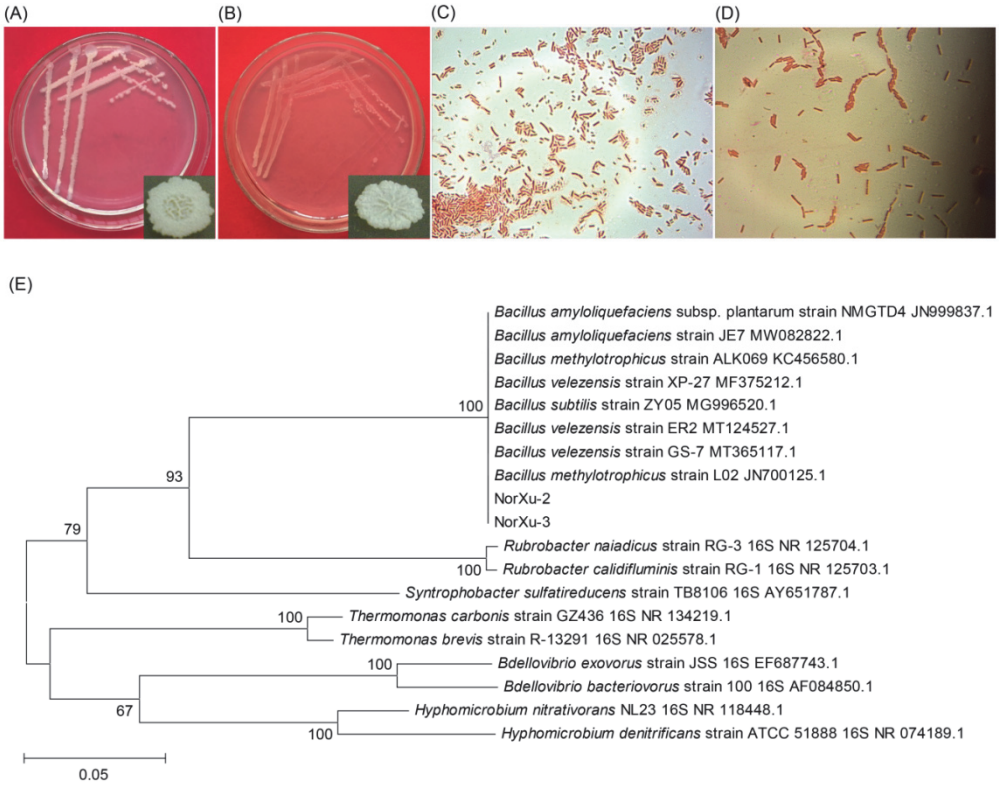

Fig. 3. Morphology and phylogeny of norfloxacin-degrading bacteria. (A), NorXu-2 strain morphology; (B), NorXu-3 strain morphology; (C), microphology of NorXu-2; (D), micromorphology of NorXu-3; (E), a phylogenetic tree was constructed based on 16S rDNA sequences of NorXu-2, NorXu-3, and reference bacteria.

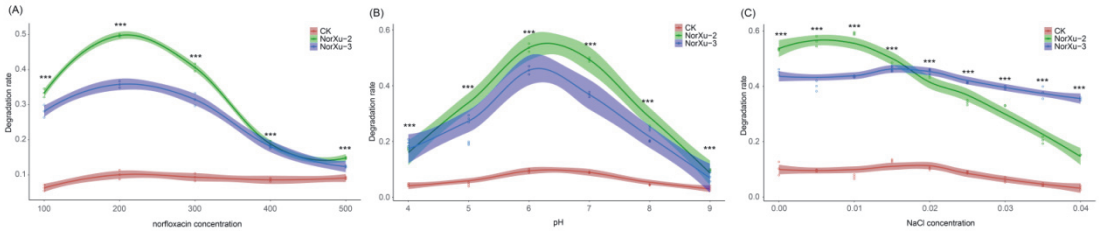

Fig. 4. Degradation efficiency of two norfloxacin-degrading bacteria under different norfloxacin concentrations (A), pH (B), and NaCl concentration (C). CK, blank control. ***, $p < 0.001$.

value to deal with the generation of antibiotic resistance and protect the health of humans and other animals (Wu et al. 2019). Additionally, effect of the application of fishmeal on the antibiotic resistance genes in the sediment of mariculture sediment has also attracted attention (Han et al. 2017). Our results showed that the water and sediment of fish culture ponds in subtropical China contained a variety of norfloxacin-resistant bacteria. Whether these bacteria enter the cultured fish and spread to other regions and environments with the processing and transportation of the fish still needs further investigation and evaluation.

Due to the advantages of low cost, easy operation and wide application range, microbial degradation is considered to be an important way to remove antibiotic residues in various environments (Yang et al. 2012, Wu et al. 2019, Jałowiecki et al. 2019). A large number of bacteria with antibiotic degradation ability have been isolated, such as *Labrys* sp. SMX-W11, *Ochrobactrum* sp. SMX-PM1-SA1, and *Gordonia* sp. SMX-W2-SCD14, which can degrade sulfamethoxazole (Mulla et al. 2018), *Bacillus cereus* J2 degrading sulfadimidine (Zhang et al. 2019), *Ochrobactrum* sp. KSS10 (Shao et al. 2018) degrading oxytetracycline, and *Pseudomonas* sp. (Lin et al. 2015), and *Shewanella* sp. (Liu et al. 2016), which can degrade cefalexin. However, only a few norfloxacin-degrading bacteria have been reported, such as *Staphylococcus caprae* NOR-36 (Fu et al. 2017). In this study, we isolated two strains of Bacillus and proved that they had strong norfloxacin-degradation ability. It provided candidate strains for the subsequent removal of norfloxacin in ponds. Moreover, we also found that NorXu-3 had the potential to remove norfloxacin residues in mariculture water and sediment, as it still had high norfloxacin removal efficiency when the NaCl concentration was 4%. However, the removing effect in the real aquaculture environment needs to be further verified by the remediation experiment on the filed aquaculture farm.

Conclusions

The water and sediment of fish culture ponds in subtropical China contained a variety of norfloxacin-resistant bacteria, mainly from Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes, and Chloroflexi. Moreover, we isolated two norfloxacin-degrading bacteria (NorXu-2 and NorXu-3). The norfloxacin-degrading rate by NorXu-2 and NorXu-3 in the culture mediums with 200 μg/mL was the highest, which was up to 49.71% and 35.79%, respectively. When the norfloxacin concentration was 200 μg/mL, NorXu-2 and NorXu-3 had the best norfloxacin-degrading effect at pH of 6, and the degradation rates were 53.64% and 45.54%, respectively. Moreover, NorXu-3 exhibited a good tolerance to high NaCl concentration.

Acknowledgments

This research was funded by the Rural Science and Technology Special Fund for Rural Revitalization Strategy of Guangdong Province, grant number 170 [2019]; Guangdong Science and Technology Project "Overseas Master" Project, grant number 2020A1414010091; Innovation and Entrepreneurship Training Program for College Students, grant number 202110577007. We would like to thank Dr. Jiajia Ni from Guangdong Meilikang Bio-Science Ltd., China for his help with data analysis.

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