

Diverse Tetracycline Resistant Bacteria and Resistance Genes from Coastal Waters of Jiaozhou Bay

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Abstract Environmental microbiology investigation was carried out in Jiaozhou Bay to determine the source and distribution of tetracycline-resistant bacteria and their resistance mechanisms. At least 25 species or the equivalent molecular phylogenetic taxa in 16 genera of resistant bacteria could be identified based on 16S ribosomal deoxyribonucleic acid sequence analysis. *Enterobacteriaceae*, *Pseudomonadaceae*, and *Vibrionaceae* constituted the majority of the typical resistant isolates. Indigenous estuarine and marine *Halomonadaceae*, *Pseudoalteromonadaceae*, *Rhodobacteraceae*, and *Shewanellaceae* bacteria also harbored tetracycline resistance. All the six resistance determinants screened, *tet(A)*–(*E*) and *tet(G)*, could be detected, and the predominant genes were *tet(A)*, *tet(B)*, and *tet(G)*. Both

anthropogenic activity-related and indigenous estuarine or coastal bacteria might contribute to the *tet* gene reservoir, and resistant bacteria and their molecular determinants may serve as bioindicators of coastal environmental quality. Our work probably is the first identification of *tet(E)* in *Proteus*, *tet(G)* in *Acinetobacter*, *tet(C)* and *tet(D)* in *Halomonas*, *tet(D)* and *tet(G)* in *Shewanella*, and *tet(B)*, *tet(C)*, *tet(E)*, and *tet(G)* in *Roseobacter*.

Introduction

Extensive use and misuse of antibiotics in medication, veterinary, agriculture, and aquaculture have caused antibiotic-resistant bacteria to be widespread in the environment [29, 30]. Elevated number of resistant bacteria, especially clinical strains, could be found near civic or hospital sewage discharging locations [8, 22]. High incidences of resistant bacteria in response to antibiotic usage have also been reported in coastal mariculture areas [25]. Aquatic ecosystems have been proposed as reservoirs of antibiotic resistance [5], and resistant bacteria in coastal environments represent a serious biotic contamination and a means for the spread and evolution of resistance genes and their vectors [46]. Although all these aspects raised the concern about the potential threat of wide-spread antibiotic resistance to public health and mariculture industry, resistance mechanisms in natural marine environments were seldom studied [2, 33].

Tetracycline class antibiotics have been widely used for microbial disease treatment and prevention [9]. Oxytetracycline used to be a common medicine in China and is still being intensively and intensely used as a therapeutic drug in veterinary and mariculture. Among the 39 resistance determinants discovered so far [1, 36], genes encoding

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active efflux pumps, such as *tet(A)*–(*E*) and *tet(G)*, occurred frequently among marine Gram-negative bacteria [27]. The *tet* genes isolated from marine environments might have the same origins as those from clinical strains [22, 34]. However, most of the studies focused on antibiotic-resistant pathogens. Appraisals of the source, abundance, diversity, distribution, dynamics, and mechanisms of bacterial resistance in natural marine environments are largely untackled globally.

Jiaozhou Bay, located on the western coast of the Yellow Sea, is a semi-enclosed waterbody with a total area of 367 km² and an average water depth of 7 m on the eastern part of the Shandong Peninsula of China. Environmental quality of Jiaozhou Bay has deteriorated dramatically in the past three decades because of rapid development in agriculture, industry, urbanization, and mariculture in the surrounding areas. More than 30% of the bay area has been lost over the past 80 years because of coastal land usage, and more than 60% of the remaining water body has been polluted or contaminated to some extent. Red tides and species loss are serious environmental and ecological issues in Jiaozhou Bay [38]. Surrounding small rivers used to form the major sources of discharged sewage, and improperly processed hospital effluent and civic wastewater could be a source of antibiotic-resistant bacteria. Excessive usage of antibiotics, such as oxytetracycline, in mariculture might also stimulate the propagation of resistant bacteria in Jiaozhou Bay.

To gain an understanding of antibiotic resistance contamination in a typical coastal environment that has been dramatically influenced by human activity and to define the source, diversity, distribution, and dynamics of the corresponding resistance determinants, cultivation and

molecular techniques were employed to determine the phylogenies of typical tetracycline-resistant bacteria and their resistance genes in Jiaozhou Bay.

Materials and Methods

Sample Collection and Processing

Surface seawater from ten stations (Fig. 1) of Jiaozhou Bay was collected aseptically on the 12th and 13th of September and the 11th and 12th of October of 2004. Water samples were kept on ice before being transferred to the laboratory, where samples for total microbial counts were fixed with formaldehyde (2% final concentration) and stored at 4°C in the dark, and samples for bacterial cultivations were processed immediately. A multifunction water quality monitoring system (model U-2001, Horiba, Japan) was used for *in situ* environmental factor measurements, except for seawater depth and diaphaneity. The environmental conditions seemed to be in normal ranges during the sampling season, except dramatic drops of seawater pH have been recorded in all the sampling stations in October (Table 1).

Microbial Number Counts

For total microbial and cultivable bacteria counts, 4',6-diamidino-2-phenylindole staining epifluorescence microscopy and 2,216 marine agar plating techniques were used as described previously [16, 17]. For cultivable tetracycline-resistant bacteria counts, a membrane filter technique was used to concentrate aquatic bacteria [37]. Water

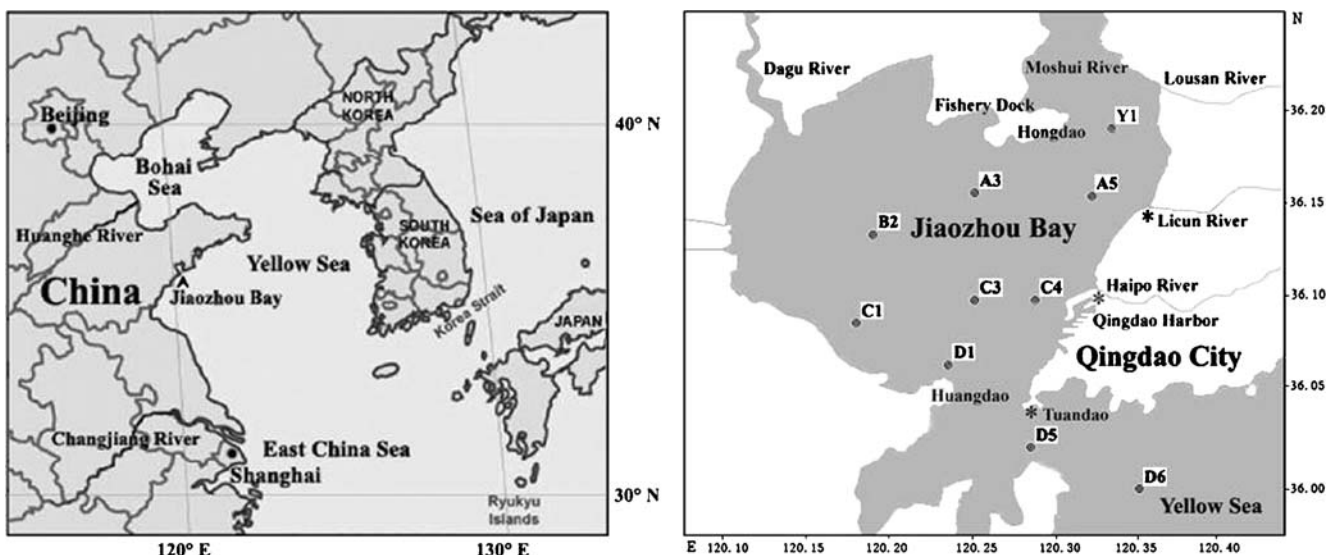


Figure 1 Maps show the Yellow Sea (*left*) and the sampling stations of Jiaozhou Bay (*right*). The locations of sewage processing plants are indicated with the *asterisk symbols*

Table 1 Measurements of *in situ* environmental factors of Jiaozhou Bay in September (Sep) and October (Oct) of 2004

Station	Water depth (m)		Water temperature (°C)		Salinity (‰)		pH		DO (dissolved O ₂) (mg/L)		Diaphaneity (m)	
	Sep	Oct	Sep	Oct	Sep	Oct	Sep	Oct	Sep	Oct	Sep	Oct
A3	3.7	5.1	24.0	20.4	31.0	31.7	8.0	5.7	4.54	3.84	1.2	1.0
A5	9.0	8.0	24.4	21.0	31.0	31.8	7.9	5.7	4.04	3.53	1.0	1.5
B2	2.2	3.8	24.1	21.1	30.0	32.4	8.0	5.6	4.45	3.61	0.6	3.0
C1	3.7	4.6	24.5	20.5	31.0	31.7	8.0	5.8	4.40	4.55	1.1	2.8
C3	16.0	14.6	25.0	21.3	32.0	32.6	8.1	5.6	4.49	3.35	2.0	2.0
C4	6.9	4.6	24.9	20.8	32.0	32.1	8.2	5.6	4.56	3.32	1.5	1.0
Y1	3.5	3.6	24.1	20.7	30.0	31.2	8.1	5.7	4.10	3.86	1.0	1.0
D1	16.0	10.0	25.3	20.8	32.0	32.3	6.5	5.6	3.87	3.37	1.0	2.1
D5	38.0	39.0	25.0	22.1	32.0	32.7	8.1	7.0	4.24	3.49	1.8	5.0
D6	28.0	29.0	24.9	22.1	32.0	31.9	8.1	6.9	3.94	3.54	1.3	4.4

samples from each station were diluted into a tenfold series in sterilized artificial seawater, and 3-mL portions were filtered onto sterilized 0.22- μ m-pore-size polycarbonate membrane filters in triplicates. The filters were placed onto tryptic soy agar (Difco formula) plates supplemented with 3% (w/v) NaCl and 10 μ g/mL oxytetracycline (OTC₁₀). OTC₁₀-resistant colonies on the positively growing highest or the second highest dilution (if there were too few colonies on the highest) plates were further screened with 30 μ g/mL oxytetracycline (OTC₃₀) based on the method described for antibiotic susceptibility tests [27]. Only OTC₃₀-resistant isolates were counted as tetracycline-resistant bacteria, which were further assayed for higher resistance to 100 μ g/mL oxytetracycline (OTC₁₀₀).

Phylogenetic Analysis of Typical Tetracycline-resistant Isolates

OTC₁₀₀-resistant bacteria were randomly selected from each station, and some colonies were also picked based on their unique colony morphology and color features. As all of these isolates were originally screened from the positively growing highest or the second highest dilution OTC₁₀ plates, they represented the predominant tetracycline-resistant bacteria in Jiaozhou Bay. Eventually, 89 isolates (about eight to ten isolates from each station) were selected for subsequent amplified ribosomal deoxyribonucleic acid (rDNA) restriction analysis (ARDRA) genotyping, 16S rDNA sequencing, and *tet* gene screening.

A simple boiling method was used for rapid bacterial genomic DNA extraction [18]. The primers 27F and 785R were used for bacterial 16S rDNA amplification as described previously [16, 17]. Polymerase chain reaction (PCR) amplicons were digested in separate reactions with *Msp*I and *Taq*I (Fermentas, USA) according to the manufacturer's instructions. Restriction fragments were resolved by electrophoresis on 4% (w/v) agarose gels and

digitally photographed with an ImageMaster VDS imaging system (Pharmacia Biotech, USA). The band patterns were compared to identify unique sequence types. Bacteria with identical ARDRA pattern using both enzymes were assumed to be of the same genotype and phylogenetic affiliation in this study.

One isolate from each genotype was selected for 16S rDNA sequencing and phylogenetic determination. Partial 16S rDNA was PCR amplified to serve as the template, and primer 27F was used as the sequencing primer. The DNA fragments sequenced were usually about 600–700 bp long, covering at least the V1 to V3 hypervariable regions of the bacterial 16S rDNA.

Bioinformatic determination of sequence affiliations followed standard methods described previously [13]. Phylogenetic trees were constructed by using the DNADIST and NEIGHBOR programs within the PHYLIP package (version 3.6) [19].

Multiplex PCR Detection of Efflux *tet* Genes

The above selected 89 OTC₁₀₀-resistant isolates were screened for the *tet*(A)–(E) and *tet*(G) genes via a multiplex PCR method developed by Jun *et al.* [27]. The original primers (SI_Table 1), experimental procedure, and PCR amplification condition were used, only with a minor modification for total DNA (including genomic and plasmid DNA) extraction [18]. Representative PCR products from each of the six *tet* gene types identified were sequenced to confirm their molecular identities as the typical efflux types of tetracycline resistance genes.

Nucleotide Sequence Accession Numbers

The 16S rDNA sequences determined have been deposited in the National Center for Biotechnology Information

GenBank database under accession nos. DQ316101 to DQ316136 and the six representative *tet* gene sequences determined under accession nos. DQ316137 to DQ316142.

Results

Microbial Abundance and Distribution

During the sampling period, the aquatic microbial density ranged from 2.7×10^6 to 3.8×10^7 cells/mL, the cultivable bacterial abundance ranged from 1.9×10^2 to 5.2×10^5 cells/mL, and the OTC₃₀-resistant bacteria ranged from 1 to 4.2×10^2 cells/mL in the surface seawater samples of Jiaozhou Bay (Table 2). In general, the OTC₃₀-resistant bacteria accounted for less than 0.6% of the total cultivable bacteria. However, their spatial distribution was not homogeneous. The highest OTC₃₀-resistant bacteria abundance occurred at station A5 of the northeastern part of Jiaozhou Bay, around the estuary of river Licun (Fig. 1).

Decrease of total microbial densities occurred in October compared to those in September (Table 2). However, the temporal changes of culturable and OTC₃₀-resistant bacteria abundance did not have clear trends, and the percentages of resistant bacteria to culturable bacteria were not consistent for any sampling stations between the two different sampling periods. Currently, it is not evident about the relationship of the changing environment conditions, such as the dramatic drop of seawater temperature and pH (Table 1), with the temporal change of the microbial abundance in Jiaozhou Bay.

Phylogenetic Affiliations of the Typical Resistant Isolates

A total of 704 OTC₃₀-resistant bacteria (402 from September and 302 from October) were isolated from surface seawater samples of Jiaozhou Bay, and 270 isolates (117

from September and 153 from October) showed resistance to at least 100 µg/mL oxytetracycline. A total of 89 typical resistant isolates (52 from September and 37 from October) were selected for further molecular analyses.

Based on ARDRA analyses, 32 unique bacterial genotypes could be identified for the 89 OTC₁₀₀-resistant isolates. They were further analyzed by 16S rDNA sequence determination. All the representative 32 isolates had 97% or greater 16S rDNA sequence similarity to their best-match sequences retrieved from the GenBank database (SI Table 2). The phylogenetic tree constructed further verified their phylogenetic affiliations (Fig. 2). The OTC₁₀₀-resistant bacteria were quite diverse; at least 25 species or the equivalent molecular phylogenetic taxa in 16 genera could be identified. For the two strains that were closely related to *Pseudomonas stutzeri*, two genomovars could be distinguished from each other [39]. Most of the isolates belonged to the γ -Proteobacteria subdivision, and some might be potential human or marine animal pathogens, such as *Acinetobacter*, *Citrobacter*, *Escherichia*, *Klebsiella*, *Proteus*, *Photobacterium*, and *Vibrio*. The remaining isolates belonged to either the α -Proteobacteria subdivision or the phylum Bacteroidetes.

Bacteria affiliated to *Enterobacteriaceae* contribute to 37.5% (12 strains) of the 32 sequenced OTC₁₀₀-resistant isolates, including species closely related to *Citrobacter freundii*, *Escherichia coli*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Kluyvera intermedia*, *Proteus mirabilis* and *Raoultella ornithinolytica*. Most of these species occurred at stations A5, B2, C4, D6 and Y1 of September. The *Acinetobacter* strains occurred exclusively at station A5 of September. The other OTC₁₀₀-resistant isolates may be indigenous estuarine or coastal marine bacteria, such as *Halomonas*, *Photobacterium*, *Pseudoalteromonas*, *Pseudomonas*, *Psychrobacter*, *Roseobacter*, *Shewanella*, and *Vibrio*.

Bacteria affiliated to *Enterobacteriaceae*, *Pseudomonadaceae*, and *Vibrionaceae* constituted the majority of the

Table 2 Abundance of total microbes (TM), cultivable bacteria (CB) and OTC₃₀-resistant bacteria (ORB) and their percentages in the cultivable microflora (ORP) in Jiaozhou Bay of September (Sep) and October (Oct) of 2004

Station	TM (cells/mL)		CB (cells/mL)		ORB (cells/mL)		ORP (%)	
	Sep	Oct	Sep	Oct	Sep	Oct	Sep	Oct
A3	1.5×10^7	2.7×10^6	4033	3833	14	5	0.35	0.13
A5	3.8×10^7	8.2×10^6	516000	24333	417	30	0.08	0.12
B2	2.0×10^7	8.0×10^6	2033	777	1	1	0.05	0.13
C1	1.4×10^7	5.3×10^6	360	1433	1	2	0.28	0.14
C3	5.4×10^6	5.4×10^6	187	1833	1	1	0.53	0.05
C4	9.5×10^6	4.4×10^6	7133	9233	7	3	0.1	0.03
Y1	3.0×10^7	2.1×10^7	35667	19000	5	11	0.01	0.06
D1	9.2×10^6	5.4×10^6	9933	3333	16	3	0.16	0.09
D5	2.2×10^7	3.8×10^6	4933	5767	2	6	0.04	0.1
D6	2.1×10^7	4.7×10^6	5467	7200	9	3	0.16	0.04

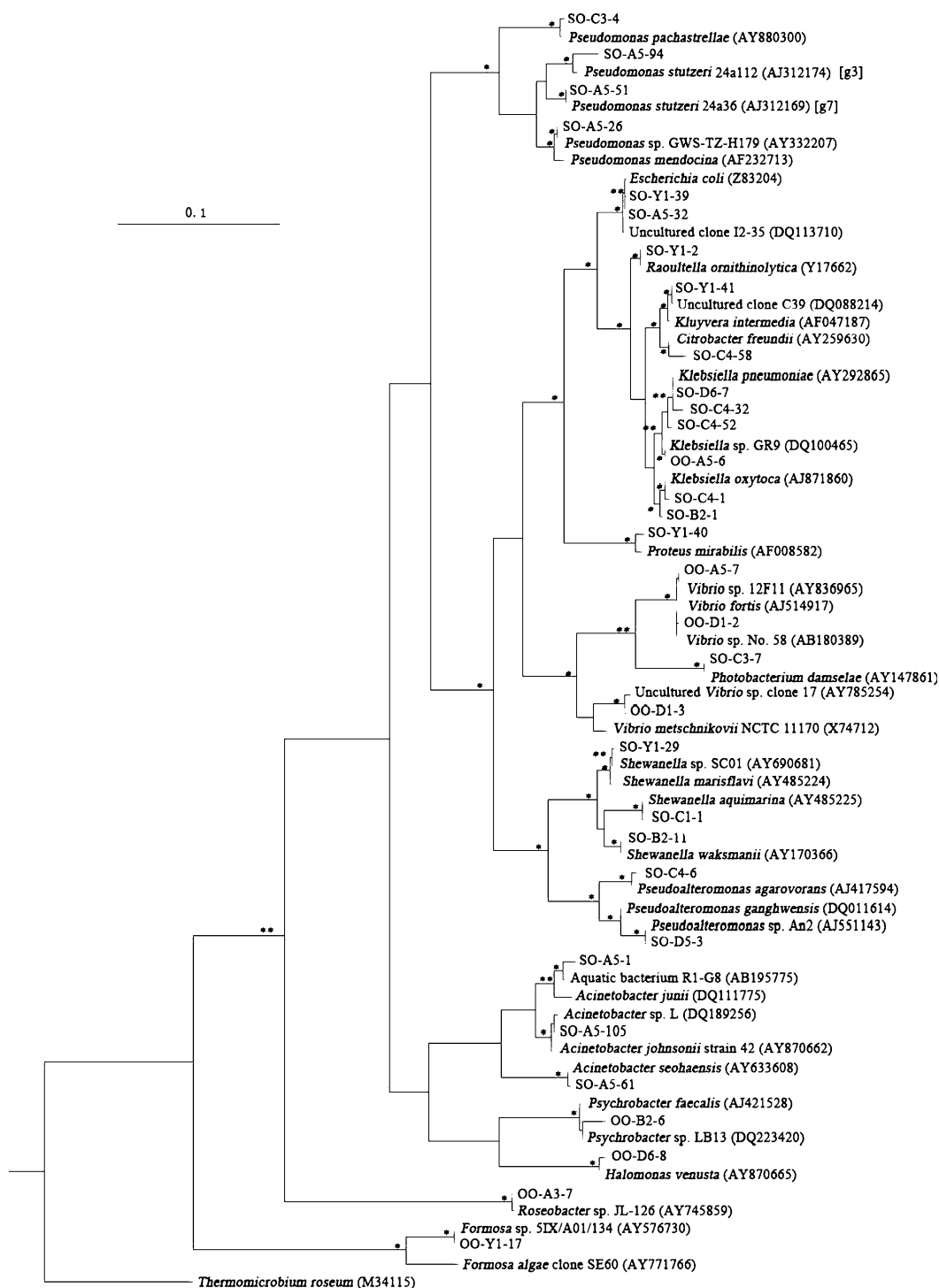


Figure 2 Phylogenetic tree of the 32 typical OTC₁₀₀-resistant strains isolated from Jiaozhou Bay constructed based on partial 16S rDNA sequences in a 620-bp frame length using the neighbor-joining method with the Kimura two-parameter model for nucleotide change. The tree branch distances represent nucleotide substitution rate, and the scale bar

represents the expected number of changes per homologous nucleotide position. Bootstrap values greater than 70% of 100 resamplings are shown near nodes as double asterisk, and those greater than 90% are shown as asterisk

selected typical OTC₁₀₀-resistant isolates. The combination of ARDRA and 16S rDNA sequence analyses indicated that 31.5% (28 out of 89) of the resistant isolates were affiliated to *Enterobacteriaceae*, including 15 isolates of

Klebsiella, four isolates of *Citrobacter*, four isolates of *Escherichia*, two isolates of *Kluyvera*, two isolates of *Raoultella*, and one isolate of *Proteus* (SI_Table 2). Most of these bacteria (82.1%) were isolated from stations A5,

Y1, and C4, which are located on the eastern coast of Jiaozhou Bay (Fig. 1). *Pseudomonadaceae* bacteria contributed to 21.3% of the 89 analyzed resistant isolates, including 16 *Pseudomonas* isolates and three *Acinetobacter* isolates. *Vibrionaceae* bacteria contributed to 19.1% of the 89 analyzed resistant isolates, including 16 *Vibrio* isolates and one *Photobacterium* isolate.

Diversity of Efflux *tet* Genes

All the six *tet* genes screened, *tet*(A)–(E) and *tet*(G), were detected. Of the 49 *tet*-positive isolates (*tet* gene carrying strains), 16 carried only *tet*(A), ten carried only *tet*(B), two carried only *tet*(C), two carried only *tet*(D), one carried only *tet*(E), 13 carried only *tet*(G), one carried both *tet*(B) and *tet*(C), one carried both *tet*(C) and *tet*(D), and three carried both *tet*(E) and *tet*(G) (SI_Fig. 1, SI_Table 2). The molecular mechanism encoding for tetracycline resistance of the remaining 40 strains could not be identified by the current multiplex PCR method. The predominant tetracycline resistance genes in Jiaozhou Bay during our sampling period were *tet*(A), *tet*(B), and *tet*(G). Most of the *tet* genes were found in bacteria isolated from stations A3, A5, B2, C1, C4, D6, and Y1, whereas quite a little or even none was detected in C3, D1, or D5, which are located near the mouth of Jiaozhou Bay (Table 3, Fig. 1). The resistance gene composition was highly dynamic; the same type of the screened *tet* genes was seldom shared by the same station of both sampling months (Table 3). The bacterial hosts of the detected *tet* genes were quite diverse, including both

anthropogenic activity-related strains and indigenous estuarine or marine strains (Table 3, SI_Table 2).

The combination of ARDRA, 16S rDNA sequence, and *tet* gene analyses indicated that ten *Klebsiella* strains out of the 89 analyzed bacteria carried a *tet* gene. It is interesting to note that all the *tet*-positive *K. oxytoca*-related strains carried *tet*(B), and all the *tet*-positive *K. pneumoniae*-related strains carried *tet*(A), except for strain OO-A5-6, which carried *tet*(D) and was only remotely related to the other *tet*-positive *K. pneumoniae* strains found in Jiaozhou Bay (see Fig. 2). Of the other *Enterobacteriaceae* bacteria, the *C. freundii*-like isolates carried either *tet*(A) or *tet*(B), and the *P. mirabilis*-like isolate carried *tet*(E).

The *Acinetobacter* isolates mainly carried *tet*(B) or *tet*(G). Most of the *Pseudomonas* isolates carried *tet*(A) or *tet*(G) and occasionally *tet*(C). The *Vibrio* strains carried mainly *tet*(B) and occasionally *tet*(A) or *tet*(G). Five isolates carried two different types of *tet* genes simultaneously. They belonged to the genus *Roseobacter* or *Halomonas*; both are typical coastal marine bacteria.

The identities of the representative *tet* genes detected via the multiplex PCR method were further verified by DNA sequencing. One representative strain from each group of the bacterial isolates related to a single type of the six *tet* genes screened was randomly selected for *tet* gene sequencing (Table 4). All the representative sequences of the screened *tet* gene types had 99% similarity to their best-match known genes retrieved from the GenBank database, and they also had 99% or higher similarity to the best-match known efflux-type tetracycline resistance protein sequences after

Table 3 The *tet* gene distribution in Jiaozhou Bay of September (Sep) and October (Oct) of 2004

<i>tet</i> gene	Station		Resistance species	
	Sep	Oct	Sep	Oct
<i>tet</i> (A)	A5, C4, D6, Y1	A3, C1, C3, C4, Y1	<i>Citrobacter</i> <i>Klebsiella</i> <i>Pseudomonas</i> <i>Vibrio</i>	<i>Citrobacter</i> <i>Klebsiella</i> <i>Pseudomonas</i>
<i>tet</i> (B)	A5, C4	A5, B2, D1, Y1	<i>Acinetobacter</i> <i>Klebsiella</i> <i>Vibrio</i>	<i>Citrobacter</i> <i>Roseobacter</i> <i>Vibrio</i>
<i>tet</i> (C)	A5	B2, D6	<i>Pseudomonas</i>	<i>Halomonas</i> <i>Roseobacter</i>
<i>tet</i> (D)		A5, C4, D6		<i>Halomonas</i> <i>Klebsiella</i> <i>Shewanella</i>
<i>tet</i> (E)	Y1	A3	<i>Proteus</i>	<i>Roseobacter</i>
<i>tet</i> (G)	A5, Y1	A3, B2, C1, C4, D6	<i>Acinetobacter</i> <i>Pseudomonas</i> <i>Shewanella</i>	<i>Pseudomonas</i> <i>Roseobacter</i> <i>Shewanella</i> <i>Vibrio</i>

Table 4 Representative *tet* gene sequences determined in the current study

Isolate	Closest sequence match	Sequence similarity	Accession number
SO-A5-51 ^a	<i>Salmonella typhimurium</i> plasmid pU302L <i>tet</i> (A)	99%	AY333434
SO-C4-1	<i>Pasteurella aerogenes</i> plasmid pPAT2 <i>tet</i> (B)	99%	AJ278685
SO-A5-26	<i>Escherichia coli</i> <i>tet</i> (C)	99%	Y19114
OO-A5-6	<i>Salmonella</i> sp. TC67 <i>tet</i> (D)	99%	AB089602
SO-Y1-40	<i>Vibrio salmonicida</i> plasmid pRVS1 <i>tet</i> (E)	99%	AJ289103
SO-Y1-29	<i>Pseudomonas</i> sp. Plasmid pPSTG2 <i>tet</i> (G)	99%	AF133140

^a SO stands for September oxytetracycline-resistant isolates and OO for October oxytetracycline-resistant isolates

they were translated into conceptual amino acid sequences. The multiplex PCR method adopted [27] provides a rapid and reliable approach for typical *tet* gene detection and differentiation in the marine environment studied.

Discussion

Environmental bacteria may play an important role as reservoirs of antibiotic resistance, and resistance genes are exchanged by bacteria from different ecosystems [35]. A basic understanding of the source, abundance, diversity, distribution, and dynamics of antibiotic-resistant bacteria and their resistance determinants is necessary for effective prevention and control of antibiotic resistance and its dissemination. Modern molecular techniques have been proven to be suitable for studying resistant bacteria and their resistance mechanisms [2, 27]. The employment of molecular methods in the current project helps us uncover diverse tetracycline-resistant bacteria and their resistance genes. However, higher resistant bacteria abundance and diversity should be expected in Jiaozhou Bay if samples were collected from the middle or bottom of the seawater, as the surface seawater might have much reduced bacterial populations because of the effect of UV sunlight irradiation during our sampling period.

Diverse Antibiotic-resistant Bacteria from Coast Marine Environments

Two sources of OTC₁₀₀-resistant bacteria could be identified in Jiaozhou Bay, the indigenous estuarine or coastal marine bacteria and the terrestrial bacteria related to anthropogenic activities. *Enterobacteriaceae* bacteria were detected mainly along the densely populated eastern coast of Jiaozhou Bay. Civic and hospital wastewater discharges via rivers or sewage processing plants on the seashore might be a major

source of *Enterobacteriaceae* in the seawater. Most of the *Enterobacteriaceae* strains were isolated in September, suggesting a seasonal succession likely introduced by the change of environmental factors (Table 1) or the variation of the terrestrial bacterial source. Some bacteria might also enter the viable but nonculturable state with the drop of seawater temperature [10], contributing to the drop of culturable *Enterobacteriaceae* abundance.

The distribution of OTC₁₀₀-resistant bacteria was quite heterogeneous. Stations on the eastern coast of Jiaozhou Bay, such as A5, C4, and Y1, were highly contaminated with *Enterobacteriaceae*. Station A5 is around the river mouth of Licun and the Licun River sewage processing plant (Fig. 1), which discharges about 50,000 tons of processed sewage into the bay daily. Station Y1 is located at the river mouths of Loushan and Moshui near the Hongdao aquacultural area. Station C4 is located near the river mouth of Haipo and the Haipo River sewage processing plant, which discharges about 80,000 tons of processed sewage into the bay daily. Our current study indicates that the spatial distribution of antibiotic resistant microbes might be closely related to geographical characteristics and anthropogenic activities and that sewage contamination might be a serious environmental problem of Jiaozhou Bay, at least in our sampling period.

The Bulletin of Marine Environmental Quality of Qingdao City of the year 2004 (<http://www.coi.gov.cn/dfhygb/2004/qingdao/index.html>) shows that the eastern coast of Jiaozhou Bay was heavily polluted by inorganic nitrogen, phosphate, heavy metal, oil, and other organic pollutants, indicating that anthropogenic activity has an important impact on the coastal environment. Although the seawater of Jiaozhou Bay is highly dynamic and water mixing happens frequently because of currents, tides, and other hydrological factors, confined distribution of resistant bacteria, especially the terrestrial strains, still could be identified along the eastern coast, indicating a strong terrestrial or anthropogenic impact.

Diverse Efflux *tet* Genes from Coastal Marine Environments

Molecular characterization of *tet* genes helped decode partially the tetracycline-resistance mechanisms in Jiaozhou Bay. Other or even novel mechanisms might also exist. All the *tet(A)*–*tet(E)* and *tet(G)* genes could be detected, and *tet(A)*, *tet(B)*, and *tet(G)* were the most predominant determinants in Jiaozhou Bay, in contrary to *tet(E)* that was found to be dominant in marine sediments of Norway and Denmark a decade ago [2]. The *tet* determinants could vary with sampling origin, suggesting ecosystem-specific reservoirs for certain antibiotic resistance [24].

Basically, two sources of *tet* genes could be identified, one from the terrestrial bacteria coming from anthropogenic activities of the surrounding environments and one from the indigenous estuarine or coastal marine bacteria (Table 3). Transfer of resistance determinants between different environmental compartments has been demonstrated [34], indicating that a common gene pool might be shared between environmental microorganisms and clinical strains. Terrestrial bacteria entering into seawater with antibiotic-resistant plasmids were proposed to be partially responsible for the prevalence of resistance genes in marine environments [4, 7]. The resistance gene flow between the indigenous environmental bacterial assemblage and the human activity-related bacterial assemblage in Jiaozhou Bay will be determined in the future.

Members of *Enterobacteriaceae* are usually found in the gastrointestinal tract of humans and animals, and certain strains are primary or opportunistic pathogens [31]. Tetracycline-resistant *Enterobacteriaceae* strains seem to be prevalent globally [21, 28]. Multidrug-resistant *K. pneumoniae* isolated from Nigeria harbored *tet(A)* [40]. *Klebsiella* strains isolated from Europe carried *tet(A)*, *tet(B)*, or *tet(D)* [20]. The *tet(A)* gene was also detected in most of the *K. pneumoniae* strains isolated from Jiaozhou Bay, indicating a possible global distribution of the common *Klebsiella* tetracycline-resistance determinants. Multidrug-resistant *Citrobacter* strains isolated from fish farms in southern Japan harbored *tet(D)* [22], whereas the *Citrobacter* strain isolated from Jiaozhou Bay carried *tet(A)* or *tet(B)*. This difference seems to suggest regional or ecosystem-specific reservoirs of tetracycline resistance determinants for this genus. Our work probably is the first identification of *tet(E)* in *Proteus*.

Acinetobacter strains are usually commensal, but in the past few decades, they have emerged as important opportunistic nosocomial pathogens [44]. Most of the clinical strains isolated from Europe carried either *tet(A)* or *tet(B)* [23, 26], whereas the aquatic strains isolated from sewers receiving waste effluent from a hospital and a pharmaceutical plant in Denmark carried only *tet(B)* [23]. In Jiaozhou Bay, some of the *Acinetobacter* isolates

harbored either *tet(B)* or *tet(G)*. The *tet(G)* gene was probably the first identification in *Acinetobacter*, suggesting regional or ecosystem-specific reservoirs of this resistance determinant in *Acinetobacter*.

Marine vibrios have long been recognized as important reservoirs and vehicles of antibiotic resistance because of their importance as potential human and/or marine animal pathogens [41], their abundance and diversity in coastal waters [42], their ability to readily develop and acquire antibiotic resistance in response to selective pressure, and their ability to spread resistance by horizontal genetic material exchange [3]. The major vibrio tetracycline-resistance gene in Jiaozhou Bay was *tet(B)* and occasionally *tet(A)* or *tet(G)*. The *tet* genes carried by vibrios isolated recently from a mariculture farm in northern China were *tet(A)*, *tet(B)*, and *tet(D)* [16, 17]. The *tet* gene carried on R plasmids from fish-pathogenic *V. anguillarum* isolated from 1973 to 1977 in Japan was *tet(B)*, from 1980 to 1983 was *tet(G)*, and from 1989 to 1991 was *tet(D)* [3]. The *tet* gene carried on R plasmids from fish-pathogenic *Edwardsiella tarda* in Korea was mainly *tet(A)* and *tet(D)*, and *tet(B)* and *tet(G)* were occasionally detected on nonmobile nucleic acids [27]. The prevalence of similar *tet* genes in Korea and China at the present time to the originally isolated *tet* genes from Japan decades ago might aid in the speculation about the origin and spread of certain antibiotic resistance factors among the three neighboring countries [17], although local strains might also contribute to resistance gene transfer because of anthropogenic activities in coastal marine environments.

Besides *Enterobacteriaceae*, *Pseudomonadaceae*, and *Vibrionaceae*, many typical indigenous estuarine and coastal marine bacteria also harbored *tet* genes in Jiaozhou Bay. It is probably the first identification of *tet(B)*, *tet(C)*, *tet(E)*, and *tet(G)* in *Roseobacter*, *tet(C)* and *tet(D)* in *Halomonas*, and *tet(D)* and *tet(G)* in *Shewanella* (SI_Table 2). The *Roseobacter* clade is one of the major marine groups of coastal bacterioplankton communities [6, 14, 15, 45]. *Roseobacter* isolates occurred abundantly and widely in Jiaozhou Bay (SI_Table 2). All the *Roseobacter* isolates are *tet* positive, and more than half of them actually harbored two different *tet* genes simultaneously. This unique phenomenon further emphasizes the ecological and environmental importance of this clade of marine bacteria.

Deterioration of coastal environmental quality is becoming a global issue because of pollution and eutrophication. Oxygen-starved areas in bays and coastal waters have been expanding since the 1960s, and the number of known dead zones around the world has doubled since 1990 [43]. The occurrence and persistence of diverse antibiotic-resistant bacteria and their resistance genes makes the situation even worse and more complicated. The study of tetracycline-resistant bacteria isolated from marine sediments indicated that the resistant species were different between the

polluted and the unpolluted sites [2]. Our current study also indicates the possibility that antibiotic-resistant bacteria and their molecular resistance determinants may serve as bioindicators of coastal environmental quality consistently influenced by anthropogenic activities.

Epidemic diseases caused by antibiotic-resistant bacteria have been reported all over the world. More than 10,000 deaths caused by cholera were reported to the World Health Organization in 1998, and antibiotic-resistant *Vibrio cholera* strains have been reported frequently [11, 12]. Epidemics of pathogens in coastal marine environments may spread in large scale and at extremely rapid speed because of their potential for long-term survival outside the host and the lack of barriers to dispersal [32]. Antibiotic-resistant microbes may pose a particular threat in coastal areas. Uncovering the genetic mechanisms behind resistance gene transfer will be an important research orientation for public health and coastal environment management.

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