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NOTE

The Diversity of Multi-drug Resistance Profiles in Tetracycline-Resistant *Vibrio* Species Isolated from Coastal Sediments and Seawater

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In this study we examined the multi-drug resistance profiles of the tetracycline (TC) resistant genus *Vibrio* to determine its susceptibility to two β-lactams, ampicillin (ABPC), and mecillinam (MPC), as well as to macrolide, erythromycin (EM). The results showed various patterns of resistance among strains that were isolated from very close geographical areas during the same year, suggesting diverse patterns of drug resistance in environmental bacteria from this area. In addition, the cross-resistance patterns suggested that the resistance determinants among *Vibrio* spp. are acquired differently within the sediment and seawater environments.

Keywords: drug resistance, tetracycline, *Vibrio*, sediment, seawater

Antimicrobial agents have been extensively applied in aquaculture to prevent and control disease. Tetracycline (TC) is one of the most popular antibiotics that is used in fish culture because it is effective over a broad spectrum of pathogens and has a low cost. The release of antibiotics into the environment, however, presents selective pressures for not only fish pathogens, but also for environmental bacteria. Samuelsen *et al.* (1992) reported that antibiotic-resistant bacteria persisted in fish farm sediments for at least 18 months after antibiotic administration. Fish farmers generally know that TC-resistant bacteria easily occur when TC is administered; however, the resistant bacteria disappear after drug administration has stopped. On the other hand, bacteria resistant to ampicillin (ABPC) and erythromycin (EM) tend to be continuously present on the farm, even after months of no treatment.

Drug resistance arises by the acquisition of drug resistance genes, although spontaneous resistance is also known to occur (Walsh, 2003). There are two predominate mechanisms for TC resistance: efflux and ribosomal protection (Roberts, 2005), which move among the bacterial community. However, recent reports revealed that some determinants of TC resistance have been detected among a variety of species, and that some distribute in specific species (Kim *et al.*, 2003, 2004), suggesting the presence of non-transferable *tet* gene(s). The resistance profiles for various drugs are a result of a combination of different resistance genes that are transferred among the community. In this study, we examined the cross-resistance of TC-resistant *Vibrio* species to other β-lactams and macrolide. The *Vibrio* strains were isolated from sediment and seawater in areas where TC had been administered.

Ninety-three TC-resistant strains were isolated from coastal aquaculture sites in Seto Inland Sea during April to December, 2004. The locations of the sampling sites are

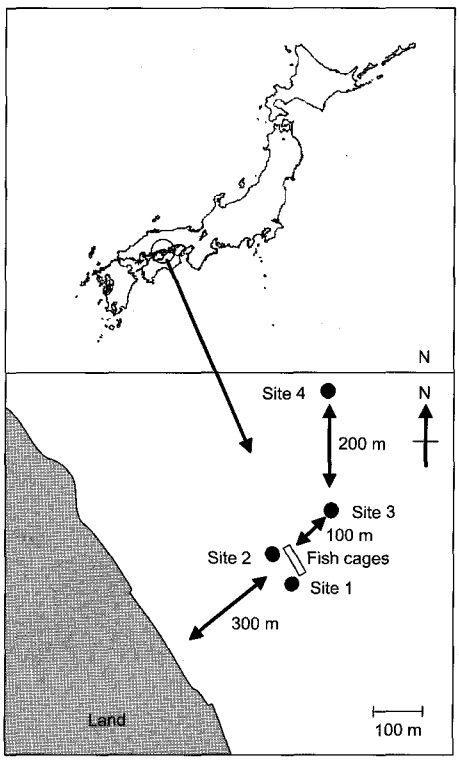

Fig. 1. Sampling sites in Seto Inland Sea

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shown in Fig. 1. The seawater samples were taken using a plastic bucket from the water surface at all sites. The sediment cores were collected by a KK-core sampler under or beside the fish cage. After removing the water phase, the sediment samples were horizontally sectioned at 3 cm. The cut samples were stored in sterile plastic bags and transported to the laboratory on ice.

The CFUs were measured using the plate spread method. Sediment samples of 0.5 g were suspended in 4.5 ml of phosphate buffered saline (PBS) and mixed vigorously by vortex for 1 min to detach the bacteria. Seawater samples of 0.5 ml were mixed in 4.5 ml of PBS and serial 10 fold dilutions were prepared. The dilutions were spread on Marine Broth 2216 (BD, USA) plus 1.5% Bacto Agar (BD, USA) with the appropriate concentration of TC (Sigma). The plates were incubated at 25°C for 4 days. The viable bacterial

Table 1. Drug resistance and plasmid possessing profiles of strains isolated from coastal aquaculture site

Strain ID	Genus	Site	Origin	Plasmid	TC	ABPC	MPC	EM
04Ya033	*Vibrio*	Site 2	Sediment	−	128	>256	0.25	48
04Ya042	*Vibrio*	Site 3	Sediment	−	32	>256	0.032	>256
04Ya044	*Vibrio*	Site 3	Sediment	−	64	>256	0.19	32
04Ya047	*Vibrio*	Site 3	Sediment	+	64	>256	0.38	48
04Ya051	*Vibrio*	Site 3	Sediment	+	32	>256	0.19	48
04Ya083	*Vibrio*	Site 1	Sediment	−	64	>256	0.38	64
04Ya100	*Vibrio*	Site 1	Sediment	−	64	>256	0.38	>256
04Ya101	*Vibrio*	Site 1	Sediment	−	32	>256	0.75	32
04Ya102	*Vibrio*	Site 1	Sediment	−	64	>256	0.75	>256
04Ya108	*Vibrio*	Site 1	Sediment	−	32	>256	1	64
04Ya153	*Vibrio*	Site 3	Sediment	−	64	>256	0.5	64
04Ya154	*Vibrio*	Site 3	Sediment	+	128	>256	2	>256
04Ya208	*Vibrio*	Site 3	Sediment	−	128	>256	0.38	64
04Ya228	*Vibrio*	Site 1	Sediment	+	128	>256	2	>256
04Ya234	*Vibrio*	Site 1	Sediment	+	128	>256	4	48
04Ya246	*Vibrio*	Site 2	Sediment	+	128	>256	1.5	>256
04Ya248	*Vibrio*	Site 2	Sediment	−	64	>256	1.5	>256
04Ya249	*Vibrio*	Site 2	Sediment	−	128	>256	1.5	64
04Ya045	*Vibrio*	Site 3	Sediment	+	64	>256	0.19	32
04Ya155	*Vibrio*	Site 3	Sediment	−	128	>256	1.5	64
04Ya244	*Vibrio*	Site 2	Sediment	+	128	>256	0.75	48
04Ya255	*Vibrio*	Site 2	Sediment	−	128	>256	0.25	>256
04Ya261	*Vibrio*	Site 3	Sediment	−	128	>256	1.5	96
04Ya266	*Vibrio*	Site 4	Sediment	−	128	>256	1	96
04Ya267	*Vibrio*	Site 4	Sediment	−	128	>256	0.25	48
04Ya268	*Vibrio*	Site 4	Sediment	−	64	>256	0.38	64
04Ya269	*Vibrio*	Site 4	Sediment	−	128	>256	0.5	48
04Ya230	*Vibrio*	Site 1	Sediment	−	64	>256	>256	16
04Ya245	*Vibrio*	Site 2	Sediment	−	64	>246	>256	24
04Ya061	*Vibrio*	Site 3	Sediment	−	128	0.064	0.047	48
04Ya104	*Vibrio*	Site 1	Sediment	−	64	0.19	0.38	32
04Ya186	*Vibrio*	Site 1	Sediment	−	64	0.38	0.032	96
04Ya231	*Vibrio*	Site 1	Sediment	+	64	0.19	>256	6
04Ya233	*Vibrio*	Site 1	Sediment	+	64	0.19	>256	4
04Ya239	*Vibrio*	Site 1	Sediment	+	64	>256	0.125	12
04Ya258	*Vibrio*	Site 3	Sediment	−	128	>256	2	3
04Ya048	*Vibrio*	Site 3	Sediment	+	128	>256	0.125	16
04Ya004	*Vibrio*	Site 1	Sediment	−	64	>256	0.125	2
04Ya046	*Vibrio*	Site 3	Sediment	+	32	>256	0.094	4
04Ya049	*Vibrio*	Site 3	Sediment	−	128	>256	0.125	6
04Ya247	*Vibrio*	Site 2	Sediment	+	64	32	0.064	4
04Ya274	*Vibrio*	Site 4	Sediment	+	128	>256	0.75	12

Strain ID	Genus	Site	Origin	Plasmid	TC	ABPC	MPC	EM
04Ya001	*Vibrio*	Site 1	Sediment	−	32	0.094	0.047	3
04Ya151	*Vibrio*	Site 3	Sediment	−	64	0.38	0.125	6
04Ya016	*Vibrio*	Site 1	Sediment	−	64	0.19	0.19	3
04Ya094	*Vibrio*	Site 1	Sediment	−	128	0.19	0.047	6
04Ya103	*Vibrio*	Site 1	Sediment	−	64	0.19	0.125	3
04Ya131	*Vibrio*	Site 2	Sediment	+	64	0.047	0.094	3
04Ya133	*Vibrio*	Site 2	Sediment	+	128	0.023	0.25	1
04Ya142	*Vibrio*	Site 2	Sediment	+	64	0.047	0.19	1.5
04Ya139	*Vibrio*	Site 2	Sediment	−	64	0.19	0.19	3
04Ya090	*Vibrio*	Site 1	Sediment	−	32	0.19	0.064	6
04Ya117	*Vibrio*	Site 2	Sediment	−	32	0.75	0.023	8
04Ya091	*Vibrio*	Site 1	Sediment	−	64	1	<.016	3
04Ya129	*Vibrio*	Site 2	Sediment	−	64	0.19	<.016	0.75
04Ya150	*Vibrio*	Site 3	Sediment	−	64	0.19	0.032	16
04Ya146	*Vibrio*	Site 3	Sediment	−	64	0.064	0.047	12
04Ya147	*Vibrio*	Site 3	Sediment	−	64	0.125	0.047	6
04Ya148	*Vibrio*	Site 3	Sediment	−	128	0.125	0.023	6
04Ya002	*Vibrio*	Site 1	Sediment	−	32	0.064	0.016	1.5
04Ya121	*Vibrio*	Site 2	Sediment	−	64	0.094	0.064	3
04Ya141	*Vibrio*	Site 2	Sediment	−	64	0.25	<.016	0.064
04Ya143	*Vibrio*	Site 2	Sediment	+	64	0.023	0.19	0.75
04Ya262	*Vibrio*	Site 3	Sediment	−	64	0.032	0.064	4
04Ya111	*Vibrio*	Site 2	Sediment	−	64	0.5	0.016	8
04Ya293	*Vibrio*	Site 2	Seawater	+	64	>256	>256	>256
04Ya304	*Vibrio*	Site 3	Seawater	+	64	>256	>256	>256
04Ya305	*Vibrio*	Site 3	Seawater	−	64	>256	>256	>256
04Ya311	*Vibrio*	Site 4	Seawater	−	128	>256	>256	>256
04Ya313	*Vibrio*	Site4	Seawater	−	128	>256	>256	>256
04Ya280	*Vibrio*	Site 1	Seawater	+	128	>256	1	>256
04Ya312	*Vibrio*	Site 4	Seawater	−	32	2	>256	>256
04Ya297	*Vibrio*	Site 2	Seawater	+	64	128	>256	24
04Ya300	*Vibrio*	Site 3	Seawater	−	64	>256	>256	6
04Ya303	*Vibrio*	Site 3	Seawater	−	64	>256	>256	16
04Ya279	*Vibrio*	Site 1	Seawater	+	64	>256	>256	24
04Ya290	*Vibrio*	Site 1	Seawater	+	32	>256	>256	16
04Ya296	*Vibrio*	Site 2	Seawater	−	64	>256	>256	12
04Ya292	*Vibrio*	Site 2	Seawater	−	32	6	>256	16
04Ya295	*Vibrio*	Site 2	Seawater	+	64	4	>256	8
04Ya281	*Vibrio*	Site 1	Seawater	−	64	1	>256	12
04Ya283	*Vibrio*	Site 1	Seawater	−	64	2	>256	12
04Ya284	*Vibrio*	Site 1	Seawater	−	64	0.19	>256	6
04Ya285	*Vibrio*	Site 1	Seawater	−	32	1.5	>256	12
04Ya301	*Vibrio*	Site 3	Seawater	−	32	>256	0.064	6
04Ya288	*Vibrio*	Site 1	Seawater	−	64	12	0.023	16
04Ya287	*Vibrio*	Site 1	Seawater	−	64	0.19	0.023	6
04Ya302	*Vibrio*	Site 3	Seawater	−	32	0.125	<.016	0.75
04Ya007	*Shewanella*	Site 1	Sediment	−	64	>256	8	12
04Ya112	*Shewanella*	Site 2	Sediment	−	64	0.25	1.5	24
04Ya181	*Shewanella*	Site 1	Sediment	+	64	3	24	8
04Ya082	*Alteromonas*	Site 1	Sediment	−	64	>256	0.094	16
04Ya080	*Alteromonas*	Site 3	Seawater	−	64	0.032	0.047	0.125

TC, tetracycline; ABPC, ampicillin; MPC, mecillinam; EM, erythromycin

counts were 3.5×10^3 to 1.0×10^6 CFU/g in the sediment and 4.3×10^3 to 3.6×10^4/ml in the seawater throughout all the sites. The TC resistant bacteria percentages in the sediment under the fish cages (sites 1 and 2) were 2.7 to 60.7% and 11.1 to 34.5% in site 3. The TC resistant bacteria percentages in the seawater were 4.7 to 64.8% in sites 1 and 2, whereas site 3 showed 7.0 to 35.0%. Site 4 was a 300 m distance from the culture cage where we isolated some TC resistant bacteria in September only, but detailed data about the rate of occurrence were not obtained. Strains that were resistant to 32 μg/ml of TC were defined as "resistant strains." This criterion (32 μg/ml) has been commonly used (Walsh, 2003). The strains consisted of 69 sediment strains and 24 seawater strains, and were classified to the genus level by 16S rDNA sequencing, targeting the V3 region (194 bp). The primer sequences for PCR were reported by Muyzer *et al.* (1993). A total of 88 strains were identified to be the genus *Vibrio*, 2 strains were *Alteromonas,* and 3 strains were *Shewanella*. Therefore, the genus *Vibrio* was the dominant strain in this area. Details on the classification of the TC-resistant bacteria in this area will be reported elsewhere. Culturing of the strains was performed on Marine Broth 2216 contained in 1.5% bacto agar at 25°C.

The minimum inhibitory concentrations (MIC) were determined using Etest strips (AB BIODISK, Sweden) for ampicillin (ABPC), mecillinam (MPC), and erythromycin (EM). A bacterial cell suspension was prepared in PBS and the cell density was adjusted to Macfarland No. 1.0. The suspension was spread on a Muellar Hinton agar (BD, USA) plate supplemented with 2% NaCl. Etest strips were then placed on the agar plate and incubated at 25°C for 24 h. The MIC values were determined according to the MIC scale of the strip. In the case of TC, the Etest would not give a reliable result. Therefore, an agar dilution method (Nonaka *et al.*, 2000) was employed. Two μl of bacterial cell suspension with a density of MacFarland No. 1.0 was spotted on Mueller Hinton agar medium supplemented with 2% NaCl containing an appropriate concentration of TC. Resistance to an antibiotic was defined as a MIC value greater than 32 μg/ml. The significance of the percent occurrence was evaluated using Fisher's Exact test at $P<0.05$.

Plasmid extraction was performed according to the plasmid miniprep procedure (Sambrook *et al.*, 2001). The extracted plasmids were visualized by agarose gel electrophoresis.

The Shannon-Weaver index (H') (Shannon and Weaver, 1949) was used to estimate the diversity of the cross-resistance patterns. H' was calculated with the following equation:

$$H' = -\sum Pi \log Pi$$

where $Pi=ni/N$ (ni=number of strains having each resistance pattern, N=total strain number).

The susceptibility of TC-resistant strains against other antibiotics is shown in Table 1. Four patterns of resistance were found among the strains, even though all the strains were isolated from very close geographical areas. This suggests that the drug resistance patterns of the environmental bacteria from this area are diverse. The *Vibrio* strains were separated into sediment and seawater origins (Table 1). The *Shewanella* and *Alteromonas* strains were resistant to TC only or to TC-ABPC. However, more strain numbers are needed to effectively determine the resistance properties in these genera.

From the resistance tests, 23/65 (35%) of the sediment strains showed single resistance against TC, whereas only 3/23 (13%) of the seawater strains expressed resistance. This difference between sediment and seawater resistance was statistically significant (Fisher's Exact test, $P<0.05$). However, multi-drug resistance (MDR) showing 3- or 4-drug resistance was found in 29/65 (45%) of the sediment strains, and 13/23 (57%) of the seawater strains; this difference was not statistically significant. There are two possible hypotheses for the mechanism of MDR. The first is that independent genes are located in various loci on the chromosome or plasmid and independently expressed. The second is that an MDR gene cluster is present on the same locus and MDR occurs at the same time (George, 1996). We found many MDR strains without plasmids suggesting that MDR-related genes were present on the chromosomes of *Vibrio*. In marine bacteria, drugs belonging to different categories may be pumped out by such a mechanism. For example, in *Pseudomonas aeruginosa* a multiple efflux operon is encoded in the chromosome, which causes resistance to TC and other β-lactams (Nikaido, 1994). However, so far such operons have not been reported in *Vibrio.*

The cross-resistance patterns of the *Vibrio* strains to each drug are summarized in Table 2. We found four types of resistance patterns in our study. The sediment strains showed two types while the seawater strains showed four types. The Shannon-Weaver index in the sediment strains was 0.35, whereas in the seawater strains it was 0.57, indicating that the seawater strains had more diverse profiles of drug resistance than the sediment bacteria. ABPC-EM-resistance was a primary combination for the sediment strains. However, in the seawater strains the occurrence rates of ABPC-EM-resistance were low, but β-lactam (ABPC and MPC) resistance was high. This difference in patterns suggests that the resistance determinant for ABPC is different from that of MPC in the sediment strains. In the seawater strains there were more patterns in common, although ABPC and MPC are in different β-lactam categories. A single drug can lead to the cross-resistance of other unrelated drugs (George, 1996). Since the sampling sites in this study had TC administration, it is likely TC would have been a selective pressure for obtaining not only TC resistance, but also resistance to other drugs. The rates of occurrences for resistance to ABPC and MPC in the seawater strains were almost the same with 13/23 and 18/23, respectively. However, we would have expected higher resistance to ABPC because ABPC

Table 2. Occurrence rates of cross-resistance patterns in *Vibrio* species

Resistance to	Sediment strain N=65	Seawater strain N=23
TC-ABPC-EM	42%[1*]	4%[1]
TC-ABPC-MPC	3%[2]	26%[2]
TC-EM-MPC	0%	4%
TC-ABPC-EM-MPC	0%[3]	22%[3]

*, same number indicates a significant difference ($P<0.05$)

had been applied in areas near the sampling sites, whereas MPC was never used. Since the plasmid possession rate was not high, the reason for the high occurrence of β-lactam resistance in the seawater strains is of particular interest and should be examined further.

A recent study (Sayah *et al.*, 2005) reported that TC resistance was found in a high percentage of multi-drug resistant *E. coli* isolates from various domestic animal species and farm environments, which suggests that having resistance to various antibiotics can relate specifically to TC-resistance. Therefore, TC-resistance might increase the risk for becoming resistant to additional drugs. Similarly, our results suggest that there may be a linkage between TC-resistance and resistance to other drugs.

We know that resistance genes are transferred within a population in the environment (Schmidt *et al.*, 2000). According to the Shannon index, our results from the *Vibrio* strains show that drug resistance patterns were higher in the seawater strains than in the sediment strains. With regard to gene transfer between different environments among marine *Vibrio*, plasmid transfer occurs in seawater and sediment microcosms and no difference between the two environments has been indicated (Paul *et al.*, 1991). Furthermore, resistance genes can be transferred from marine *Vibrio* to human enteric bacteria by plasmids (Furushita *et al.*, 2003). This evidence indicates that drug resistance determinants, and their transfer, are complicated among environments. In this study, the presence or absence of plasmids was not related to the drug sensitivity profiles. This seems to imply that TC resistance and MDR occur from genes on the chromosome, in which transfer is performed not only by plasmids, but also by chromosomal gene-transformation. Chromosomal gene transfer rates may be different between sediment and seawater environments despite a former report suggesting otherwise (Paul *et al.*, 1991). Our earlier research showed that a chromosomal TC resistance determinant, *tet* (34), was detected from only one *Vibrio* species (Kim *et al.*, 2003), which suggests the presence of species-specific drug resistance genes. Mazel *et al.* (1998) reported that environmental non-pathogenic *Vibrio* species often possess an integron-like cassette sequence, which is thought to have originated from the pre-antibiotic era. Here, our results suggest that drug resistance in *Vibrio* occurs through very complex mechanisms that are not random, as was indicated by the comparisons between strains isolated from sediment and seawater. We should consider drug resistance from two sides: the exogenous transfer among species and the endogenous reconstruction within a genome without transfer. Both of which could occur in a variety of environments and result in diverse drug resistance patterns.

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