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A Possible Mechanism of Quinolone Resistance in *Vibrio anguillarum*

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ABSTRACT—To study a possible quinolone resistance mechanism in *Vibrio anguillarum*, we examined *gyrA* and *parC* mutations among the oxolinic acid-resistant strains and laboratory-derived mutants. One point mutation only in the quinolone resistance-determining region (QRDR) of the *gyrA* gene was detected in one strain isolated from ayu *Plecoglossus altivelis* and laboratory-derived mutants with low-level resistance. On the other hand, point mutations in both the *gyrA* and *parC* QRDRs were detected in one strain isolated from ayu and a laboratory-derived mutant with high-level resistance. These results suggest that as in other Gram-negative bacteria GyrA and ParC are the primary and secondary targets, respectively, of quinolone in *V. anguillarum*.

Key words: *Vibrio anguillarum*, quinolone resistance, *gyrA*, *parC*, oxolinic acid

Vibrio anguillarum, the causative bacterium of vibriosis, is classified in the genus *Listonella* on the basis of analysis of the 5S rRNA sequence[1)], but it is still described as *V. anguillarum* in many reports. Vibriosis caused by *V. anguillarum* has been particularly a serious problem in the marine and freshwater aquaculture[2)]. In Japan, *V. anguillarum* was first reported as the causative agent of vibriosis in ayu *Plecoglossus altivelis* in 1967, then the disease has caused the most serious damage in ayu culture industry until around 1990[3)]. Among drugs licensed for fisheries use in Japan, oxolinic acid (OXA), which is a synthetic quinolone, is one of the antimicrobial agents allowed for wide use in aquaculture, and has been used for the treatment of various bacterial infections in cultured fish including vibriosis in ayu[4)]. As the amount of use of OXA is increased, appearance of OXA-resistant *V. anguillarum* has also been increased[5, 6)], but the mechanism of resistance to OXA in this bacterium has never been reported. To study the quinolone resistance mechanism in *V. anguillarum*, we used quinolone-resistant strains isolated from ayu and laboratory-derived quinolone-resistant mutants. Mutations in *gyrA* (the gene encoding DNA gyrase GyrA subunit) and *parC* (the gene encoding topoisomerase IV ParC subunit), which were known as determinants of quinolone resistance in other bacteria[7)], were compared among these strains.

Materials and Methods

Thirty-three strains of *V. anguillarum* were isolated from healthy and diseased fish, ayu, Japanese eel *Anguilla japonica*, yellowtail *Seriola quinqueradiata* and purplish amberjack *Seriola dumerili*, and stocked in our laboratories. The OXA susceptibility patterns of these *V. anguillarum* strains were examined and then obtained four OXA-resistant strains were subjected for comparison of *gyrA* and *parC* mutations.

V. anguillarum PT-24, which had been isolated from diseased ayu at Tokushima Prefecture in 1973, was used for laboratory-derived quinolone-resistant mutants and cloning of the *gyrA* and *parC* sequences. *In vitro* selection of OXA-resistant mutants of PT-24 was performed by plating the test strain onto Trypto-Soya Agar (TSA, Nissui) containing OXA. A mutant resistant to OXA at 0.05 μg/mL was selected first, and mutants resistant to higher concentrations of OXA were obtained from the mutant in a step by step manner. To determine the quinolone resistance-determining region (QRDR) sequence of the *gyrA* gene, the sequence was amplified by PCR using primers that were designed from the *gyrA* sequence reported in *V. parahaemolyticus*[8)]. The QRDR sequence of the *gyrA* gene spanning from amino acids position 25 to 124 was amplified (300 bp) with VP-gyr73 (5'-GCGATGTCAGTTATCGTGGGT-3') and VP-gyr372 (5'-TTCGGTGTAACGCATTGCCGC-3') primers. PCR amplification was performed in a total reaction volume of 20 μL containing 50 ng of template DNA, 10 pmol of each primer, and 0.5 U of ExTaq polymerase (Takara). The following temperature profile was used for the amplification: preheating at 95°C for 1 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 1 min and extension at 72°C for 90 s; and a final extension at 72°C for 5 min. After PCR product was ligated with the pGEM-T easy vector (Promega), the nucleotide sequence was determined. To determine the QRDR sequence of the *parC* gene, the sequence was amplified using the same strategy that was reported in the previous paper to amplify the *parC* QRDR of *V.*

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parahaemolyticus[8]. The first PCR was performed using VGYR-1 and -4 primers[8], then the second PCR was carried out using VGYR-3 and -4 primers[8]. This resulted in an amplicon of 149 bp. After this amplicon was ligated with the pGEM-T easy vector, the nucleotide sequence was determined. To amplify the *gyrA* and *parC* QRDRs from quinolone-resistant strains and laboratory-derived mutants, the VP-gyr73 and VP-gyr372 primers for the *gyrA* QRDR and VA-par1 (5'-AAGTACCACCCGCACGGT-3') and VA-par149 (5'-TCGGTGTAACTCATGGCCG), primers for the *parC* QRDR were used.

Results and Discussion

A 300-bp PCR product for the *gyrA* QRDR and a 149-bp PCR product for the *parC* QRDR were amplified from *V. anguillarum* PT-24. The amplified fragments were sequenced and the deduced amino acid sequences were compared with GyrA and ParC of other bacterial species reported so far. The deduced amino acid sequences of the 300-bp and 149-bp fragments matched perfectly with the corresponding sequences of GyrA (accession no. AB023569) and ParC (accession no. AB023570) of *V. parahaemolyticus* AQ3815[8], respectively. The nucleotide sequences of the *gyrA* and *parC* sequences of *V. anguillarum* PT-24 will appear in the GenBank nucleotide sequence database under accession no. DQ343844 and DQ343845, respectively.

The MICs of 29 OXA-susceptible *V. anguillarum* strains (16 strains isolated from healthy and diseased ayu, one strain from diseased Japanese eel, two strains from diseased yellowtail, 10 strains from purplish amberjack) ranged from 0.025 to 0.1 μg/mL. The three strains (PB-26, PT-8001, PT-82018) isolated from ayu showed low-level resistance with MIC of 0.2 or 0.39 μg/mL, but one point mutation only in the *gyrA* QRDR was detected only in PT-8001, i.e., one base change in the *gyrA* sequence responsible for a Ser-to-Ile change at residue position 83 was detected (Table 1). On the other hand, point mutations in both the *gyrA* and *parC* QRDRs were detected in one strain (PT-87089) from ayu with high-level resistance (6.25 μg/mL). One base change in the *gyrA* sequence responsible for a Ser-to-Ile change at residue position 83 and one base change in the *parC* gene causing a Glu-to-Gly change at residue position 89 was detected (Table 1).

Six laboratory-derived mutants of PT-24 (VA-M1 ~

Table 1. Comparison of the *gyrA* and *parC* mutations among four oxolinic acid (OXA)-resistant *V. anguillarum* strains

Strain No.	Host fish	Isolation year	Isolation locality	MIC (μg/mL) OXA	*gyrA* mutation			*parC* mutation		
					Amino acid residue		Base change	Amino acid residue		Base change
					Position[a]	Mutation		Position[a]	Mutation	
PB-26	healthy ayu[b]	1967	Shizuoka	0.20		–[c]			–	
PT-8001	healthy ayu	1980	Tokushima	0.39	83	Ser→Ile	AGT→ATT		–	
PT-82018	diseased ayu	1982	Tokushima	0.39		–			–	
PT-87089	diseased ayu	1987	Tokushima	6.25	83	Ser→Ile	AGT→ATT	89	Glu→Gly	GAA→GGA

[a] Position corresponds to that of the sequences of GyrA and ParC of *V. parahaemolyticus* AQ3815 (accession no. AB023569 and AB023570)
[b] *Plecoglossus altivelis*
[c] –, no change.

Table 2. Isolation of oxolinic acid (OXA)-resistant mutants of *V. anguillarum* PT-24 and mutations detected in the *gyrA* and *parC* sequences of the mutants

Mutant No.	MIC of OXA (μg/mL)	Mutation in:					
		gyrA			*parC*		
		Amino acid residue		Base change	Amino acid residue		Base change
		Position[a]	Mutation		Position[a]	Mutation	
PT-24	0.025						
VA-M1	0.10		–[b]			–	
VA-M2	0.39	87	Asp→Tyr	GAT→TAT		–	
VA-M3	1.56	87	Asp→Tyr	GAT→TAT		–	
VA-M4	3.13	87	Asp→Tyr	GAT→TAT		–	
VA-M5	6.25	87	Asp→Tyr	GAT→TAT		–	
VA-M6	25	83	Ser→Ile	AGT→ATT	89	Glu→Gly	GAA→GGA

[a] Position corresponds to that of the sequences of GyrA and ParC of *V. parahaemolyticus* AQ3815 (accession no. AB023569 and AB023570)
[b] –, no change.

M6) were compared for the *gyrA* and *parC* mutations (Table 2). One point mutation in the *gyrA* QRDR responsible for a Asp-to-Tyr change at residue position 87 was observed among VA-M2, VA-M3, VA-M4, and VA–M5 mutants with low- to high-level resistance. However, in VA-M6 mutant with the highest-level resistance (25 μg/mL), one point mutation in the *gyrA* QRDR responsible for a Ser-to-Ile change at residue position 83 was detected. Furthermore, a point mutation in the *parC* QRDR causing a Glu-to-Gly change at residue position 89 was also detected in this mutant. Although VA-M6 mutant was isolated by plating VA-M5 mutant onto TSA containing OXA at 12.5 mg/mL, the *gyrA* mutation responsible for a Asp-to-Tyr change at residue position 87 detected in VA-M5 disappeared in VA-M6, and the *gyrA* mutation responsible for a Ser-to-Ile change at residue position 83 newly appeared in VA-M6. The same point mutations in both the *gyrA* and *parC* QRDRs were also observed in one strain (PT-87089) with high-level resistance (6.25 μg/mL) (Table 1). From these results, it is suspected that combination of point mutations in both the *gyrA* QRDR responsible for a Asp-to-Tyr change at residue position 87 and the *parC* QRDR responsible for a Glu-to-Gly change at residue position 89 is lethal. Therefore, non-lethal point mutations in both the *gyrA* QRDR responsible for a Ser-to-Ile change at residue position 83 and the *parC* QRDR responsible for a Glu-to-Gly change at residue position 89 could be selected to result in high-level resistant strains such as PT-87089 and VA-M6.

The present results suggest that as in other Gram-negative bacteria GyrA and ParC are the primary and secondary targets, respectively, of quinolone in *V. anguillarum*. However, since no mutations other than one point mutation in the *gyrA* QRDR responsible for a Asp-to-Tyr change at residue position 87 was found in the *gyrA* and *parC* QRDRs of the VA-M2, VA-M3, VA-M4, and VA-M5 (Table 2), increases in the level of resistance from VA-M2 to VA-M5 may be explained by a mutation(s) in non-QRDRs. Mutations in the *gyrB* gene or those that can result in decreased drug permeability or increased drug efflux are possible explanations[7]. These possibilities have to be investigated in future study.

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