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Note

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Phenotypic Diversity of *Vibrio ichthyoenteri* Isolated from the Gastrointestinal Tract of Larval Olive Flounder *Paralichthys olivaceus*

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Abstract

Vibrio ichthyoenteri is a facultatively anaerobic gram-negative bacterium with straight or slightly curved rod morphology. The bacterium is an etiological agent of bacterial enteritis of Olive flounder *Paralichthys olivaceus*. Only a handful of studies, using a limited number of isolates, have investigated the phenotypic and genetic characteristics of *V. ichthyoenteri.* We isolated 40 *V. ichthyoenteri* strains, identified based on the 16S rRNA gene sequence, from the diseased flounder larvae and investigated the API 20E and ZYM profiles. The isolates exhibited highly divergent phenotypic characteristics regardless of sampling time point and location, and fish age. Essential enzymes produced by *V. ichthyoenteri* seemed to be alkaline phosphatase, leucine arylamidase, and N-acetyl-β-glucosaminidase. This study reveals a much greater enzymatic and biochemical phenotype diversity than has been evident to date. These results suggest that a given population of *V. ichthyoenteri* could be heterogeneous in terms of its phenotypic characteristics.

Key words: *Vibrio ichthyoenteri*, *Paralichthys olivaceus* Olive flounder larvae, API 20E, API ZYM, 16S rRNA gene

Introduction

The etiological bacterium *Vibrio ichthyoenteri* is a facultatively anaerobic gram-negative bacterium, which has straight or slightly curved rod morphology that is motile by means of a single, polar flagellum. The bacterium causes bacterial enteritis, which is often characterized by opaque intestine (intestinal necrosis) and high larval susceptibility (Muroga et al., 1990; Kim et al., 2004). The *V. ichthyoenteri* strains isolated from flounder do not produce arginine dihydrolase, chitinase, gelatinase, and lipase, and do not utilize D-cellobiose and citrate (Ishimaru et al., 1996). However, few studies, using a limited number of isolates, have investigated the phenotypic and genotypic characteristics of *V. ichthyoenteri* (Ishimaru et al., 1996; Kim et al., 2004).

The aim of this study was to obtain fundamental data for identification of *V. ichthyoenteri* isolated from olive flounder larvae. We isolated 40 *V. ichthyoenteri* strains from diseased olive flounder from 2007 to 2009 in South Korea and investigated their characteristics in terms of the API20E profile, API-ZYM profile, and 16S rRNA gene sequence.

Materials and Methods

Bacterial isolation

Strains of *V. ichthyoenteri* were isolated from the gastroin-

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Table 1. Phenotypic profiles of the *Vibrio ichthyoenteri* isolated from diseased Olive flounder as determined by the API 20E system

Sampling location (year)	Isolate code	Day post-hatch	API reactions						API20E resulting no.	Growth on TCBS
			ONPG	ADH	GLU	MAN	SAC	MEL		
Korea, Jeju (2007)	Vi079-1	9	-	-	+	-	+	-	0004024	Y
	Vi079-2	9	-	-	+	-	+	-	0004024	Y
	Vi0711-1	11	-	-	+	-	+	-	0004024	Y
	Vi0711-2	11	-	-	+	-	+	-	0004024	Y
	Vi0711-3	11	-	-	+	-	+	-	0004024	Y
	Vi0711-4	11	-	-	+	-	+	-	0004024	Y
	Vi0714-1	14	-	-	+	+	-	-	0004104	G
	Vi0717-1	17	-	-	-	-	+	-	0004004	Y
	Vi0717-2	17	-	-	-	-	+	-	0000024	Y
	Vi0717-3	17	-	-	+	+	-	-	0004124	G
	Vi0717-4	17	-	-	+	-	+	-	0004024	Y
	Vi0717-5	17	-	-	+	-	+	+	0004044	Y
Korea, Jeju (2008)	Vi0830-1	30	+	+	+	-	+	+	3004064	Y
	Vi0830-2	30	+	+	+	-	+	+	3004064	Y
	Vi0830-3	30	-	+	+	-	-	-	2004004	G
	Vi0830-4	30	+	-	+	-	+	-	1004024	Y
	Vi0830-5	30	-	-	+	+	+	-	0004124	Y
	Vi0830-6	30	-	+	+	-	+	+	2004064	Y
	Vi0830-7	30	-	-	+	+	+	-	0004124	Y
Korea, Pohang (2008)	Vi0855-1	55	+		+	-	+	-	1004020	Y
	Vi0855-2	55	-	+	+	+	+	-	2004124	Y
	Vi0855-3	55	-	-	+	+	+	-	0004124	Y
	Vi0855-4	55	-	+	+	-	+	-	2004024	-
	Vi0855-5	55	-	+	+	-	+	-	2004024	-
Korea, Jeju (2009)	Vi099-7	9	-	+	+	-	-	-	2004004	G
	Vi099-8	9	-	+	+	-	-	-	2004004	G
	Vi099-9	9	-		-	-	-	-	0000004	G
	Vi099-11	9	-	+	+	-	-	-	2004004	G
	Vi0914-1	14	-	-	-	-	-	-	0200004	G
	Vi0914-3	14	-	-	-	-	-	-	0000004	G
	Vi0914-8	14	-	-	-	-	-	-	0000004	G
	Vi0917-1	17	-	-	-	-	-	-	0000004	G
	Vi0917-2	17	-	+	+	-	-	-	2004004	G
	Vi0921-3	21	-	+	+	-	-	-	2004004	G
	Vi0921-4	21	-	+	+	-	-	-	2004004	G
	Vi0921-6	21	+	+	+	-	-	-	3004004	G
	Vi0921-11	21	-	+	+	-	-	-	2004004	G
	Vi0921-12	21	-	+	+	-	-	-	2004004	G
	Vi0921-15	21	-	+	+	-	-	-	2004004	G
	Vi0921-16	21	-	-	-	-	-	-	0200004	G
Strains positive (%)		N/A	13	45	80	18	50	13	N/A	N/A
Japan (1986)	IFO-15876*	N/A	-	-	+	+	-	+	ND	ND
Japan (1988)	IFO-15847*	N/A	-	-	+	+	-	+	ND	ND
Korea, Wando (2003)	Vic-CS1†	N/A	ND	-	+	+	+	-	ND	Y

ONPG, beta-galactosidase; ADH, arginine dihydrolase; GLU, utilization glucose; MAN, utilization mannitol; SAC, utilization sucrose; MEL, utilization melibiose; TCBS, thiosulfate citrate bile salt sucrose; **-**, no growth; Y, yellow colony formation; G, green colony formation; N/A, not applicable; ND, not done.
*Masumura et al. (1989), †Li et al. (2006).

testinal tracts of 9-30 days post-hatching (dph) Olive flounder larvae (approximately 30 at each sampling point) with symptoms of bacterial enteritis in the Jeju hatchery from 2007 to 2009. To acquire more isolates of *V. ichthyoenteri*, 55-dph Olive flounder larvae (n = 30) obtained from the Pohang hatchery in 2008 were also used for bacterial isolation.

Fish were initially disinfected with 0.1% (w/v) benzalkonium chloride before being rinsed with sterile distilled water. The intestine was then aseptically removed and homogenized in 10 mL of sterile 0.85% (w/v) saline. Volumes (0.1 mL) of 10-fold dilutions of the homogenate were individually spread onto tryptic soy agar (Difco, Sparks, MD, USA) containing 1% (w/v) sodium chloride (TNA). The plates were incubated at 25°C for 48 h, after which representative colonies, normally in pure culture, were selected and streaked for isolation. Forty isolates of *V. ichthyoenteri* were isolated for 3 y (Table 1).

Biochemical characterization

All isolates were tested for biochemical characteristics using the API20E system (bioMérieux, Marcy I'Etoile, France). Enzymatic activities were detected using APIZYM commercial kits (bioMérieux) according to the manufacturer's protocol. API20E and APIZYM tests were conducted three times for each isolate. Growth was observed on TNA at 35°C after 48 h and on thiosulfate citrate bile salt sucrose (TCBS) 25°C for 48 h.

16S rRNA gene analysis

Bacterial genomic DNA was isolated using a Genomic DNA extraction kit (Bioneer, Daejeon, Korea) and following the manufacturer's protocol. The DNA templates were amplified using PCR in a Gene Amp PCR system 2400 (Perkin Elmer, Wellesley, MA, USA) using universal primers amplifying a 1,343-bp region of the 16S rRNA gene (63F: 5-CAGGCCTAACACATGCAAGTC-3′; 1406R: 5′-ACGGGCGGTGTGTRC-3′) obtained from Bioneer. The DNA templates were amplified by initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1.5 min, and a final extension at 72°C for 5 min. Direct sequencing of the amplified DNA fragment was performed using a 3730xl DNA automatic sequencer (Applied Biosystems, Foster City, CA, USA). All sequences were submitted for similarity searches with BLAST (Altschul et al., 1990).

Results

Bacterial identification

A total of 40 representative isolates of *V. ichthyoenteri* at each sampling time point were used in this study. Various ages of sampled larval fish with opaque intestine were expressed as days post-hatch as indicated in Table 1. A pure culture containing only one type of bacterium was generally grown on TNA. According to the 16S rRNA gene sequences of the isolates, all isolates except for Vi0855-1 (id% = 99.7) (GenBank accession no. AB518046) had identical nucleotide sequences (GenBank accession no. AB518045) compared to that of *V. ichthyoenteri* FK3 (GenBank accession no. AM181658) and were all identified as *V. ichthyoenteri* with 100% identities, including the Vi0855-1 isolate.

Biochemical characterization

All isolates were negative for lysine decarboxylase, ornithine decarboxylase, citrate utilization, H_2S production, urease, tryptophan deaminase, indole production, acetoin production, gelatinase, fermentation/oxidation inositol, fermentation/oxidation sorbitol, fermentation/oxidation rhamnose, fermentation/oxidation amygdalin, and fermentation/oxidation arabinose (data not shown). Overall, β-galactosidase (ONPG), arginine dihydrolase, citrate utilization, and/or acid production in four different carbohydrates, including glucose, mannitol, sucrose, and/or melibiose were observed in various isolates (Table 1). No strain grew at 35°C. Almost half of the isolates (19 of 40) produced yellow colonies on TCBS (Table 1).

Intensities of enzymatic activities were established using the APIZYM system. Strong alkaline phosphatase, leucine arylamidase, and N-acetyl-β-glucosaminidase activities were induced by *V. ichthyoenteri* cells. The enzymatic activities of esterase, esterase-lipase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, and β-glucosidase varied depending on the isolates. Only enzymatic activities that exhibited a positive reaction in at least one isolate and the percentage of positive isolates are shown in Table 2.

Discussion

Presently, all isolates derived from the intestine of diseased olive flounder larvae were identified as *V. ichthyoenteri* based on 16S rRNA gene sequences. The isolates displayed an abundant diversity of phenotypic traits. Since the first introduction of this species (Ishimaru et al., 1996), few studies have focused on this pathogen and its morphological and biochemical characteristics (Kim et al., 2004; Li et al., 2006). Li et al. (2006) used only nine isolates from larvae and juvenile olive flounder, and characterized their biochemical activity. They did not assess enzymatic activities.

Biochemical characterization is still used for identification of bacteria such as *Vibrio parahaemolyticus* and *Vibrio cholera*, although molecular analyses are needed to confirm phenotypic characterizations in some cases (Choopun et al., 2002; Fabbro et al., 2010). Phenotypic characteristics of the

isolates obtained in this study varied regardless of sampling time points, sampling locations, and larval ages. Many strains isolated from larvae of the same age and at the same sampling time exhibited diverse biochemical and enzymatic phenotypes. Some isolates in this study showed arginine dihydrolase activity, ONPG hydrolysis, and acid production from melibi-

Table 2. Enzymatic profiles of the *Vibrio ichthyoenteri* isolated from diseased Olive flounder as determined by the API ZYM system

Sampling location (year)	Isolate code	Enzymatic activities									
		2	3	4	6	11	12	13	14	15	18
Korea, Jeju (2007)	Vi079-1	++++	++	+	+++	-	++	-	-	-	++
	Vi079-2	++++	+	+	+++	-	++	-	-	-	+
	Vi0711-1	++++	+	+	+++	-	+	-	-	-	++
	Vi0711-2	++++	+	+	+++	-	+	-	-	-	+++
	Vi0711-3	++++	+	+	+++	-	++	-	-	-	++
	Vi0711-4	++++	++	+	+++	-	++	-	-	-	++
	Vi0714-1	++++	+	+	++++	-	+	-	-	-	++++
	Vi0717-1	++++	+	+	++++	-	+	-	-	-	++++
	Vi0717-2	++++	+	+	++	-	+	-	-	-	+++
	Vi0717-3	++++	+	+	++++	-	+	-	-	-	++++
	Vi0717-4	+++	+	+	++++	+	+	-	-	-	+++
	Vi0717-5	+++	+	-	-	+	-	-	-	-	+
Korea, Jeju (2008)	Vi0830-1	++++	+	+	++++	-	+	-	-	-	+++
	Vi0830-2	++++	+	+	+++	-	+	-	-	-	+++
	Vi0830-3	++++	+	-	++++	-	-	-	-	-	++
	Vi0830-4	++++	+	-	++++	-	-	+	+	-	++
	Vi0830-5	++++	+	+	+++	+++	+	-	-	-	+++
	Vi0830-6	++++	+	+	++++	-	+	-	-	-	+++
	Vi0830-7	++++	+	-	++	+++	-	-	-	-	++
Korea, Pohang (2008)	Vi0855-1	++++	+	+	++++	-	-	-	-	-	++++
	Vi0855-2	++++	+	+	++++	-	-	-	-	-	++++
	Vi0855-3	++++	+	+	++++	-	-	-	-	-	++++
	Vi0855-4	+++	+	+	++	-	+	-	-	++++	+++
	Vi0855-5	++++	+	-	+	-	-	-	-	++++	+++
Korea, Jeju (2009)	Vi099-7	+++	+	+	++	-	+	-	-	-	+
	Vi099-8	+++	+	-	++++	-	+	-	-	-	++
	Vi099-9	+++	+	-	+++	-	+	-	-	-	++
	Vi099-11	++++	+	-	++++	-	+	-	-	-	++++
	Vi0914-1	++++	+	+	+++	-	+	-	-	-	+++
	Vi0914-3	+++	+	-	+++	-	+	-	-	-	++
	Vi0914-8	+++	+	-	++	-	+	-	-	-	++
	Vi0917-1	+++	++	-	++	-	+	-	-	-	++
	Vi0917-2	++++	+	+	+++	-	+	-	-	-	+++
	Vi0921-3	++++	+	-	++++	-	+	-	-	-	++++
	Vi0921-4	++++	+	-	+++	-	+	-	-	-	+++
	Vi0921-6	++	+	-	+++	-	+	-	-	-	++
	Vi0921-11	++++	+	-	++++	-	+	-	-	-	+++
	Vi0921-12	++++	+	-	+++	-	+	-	-	-	++
	Vi0921-15	++++	+	-	++++	-	+	-	-	-	+++
	Vi0921-16	++++	+	-	++	-	+	-	-	-	++
Strains positive (%)		100	100	55	98	10	83	3	3	5	100

2, alkaline phosphatase; 3, esterase; 4, esterase-lipase; 6, leucine arylamidase; 11, acid phosphatase; 12, naphthol-AS-BI-phosphohydrolase; 13, α-galactosidase; 14, β-galactosidase; 15, β-glucosidase; 18, N-Acetyl-β-glucosaminidase; - (no activity) to ++++ (strong activity) indicate the degree of activity.

ose and utilization of citrate, all of which were not detected in the six strains of *V. ichthyoenteri* (including type strain F-2) isolated previously (Ishimaru et al., 1996). Also, all nine isolates used in a previous study (Li et al., 2006) were negative for dehydrolase production of arginine, ONPG reaction, and mannitol and melibiose utilization, and were positive for glucose utilization. This indicates that *V. ichthyoenteri* exists as highly divergent phenotypes. Although the API 20E system is widely used in routine identification of bacteria, it is difficult to apply to fish bacterial pathogens (Santos et al., 1993). This is probably because of the relative paucity of API 20E data for bacterial fish pathogens; results to date in other studies should be collected and analyzed. The most important enzymes produced by *V. ichthyoenteri* appear to be alkaline phosphatase, leucine arylamidase, and N-acetyl-β-glucosaminidase; these might be related to pathogenesis. General enzymatic activities of *V. ichthyoenteri* were similar to those reported previously for various species within the family.

In conclusion, our data suggest that most isolates of the same identity as determined by the 16S rRNA gene sequence exhibited diverse enzymatic and biochemical phenotypes. These results suggest that pathogenic *V. ichthyoenteri* isolated in South Korea exhibits heterogeneous phenotypic characteristics.

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