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EXPERIMENTAL ARTICLES

Characterization of *Vibrio gigantis* and *Vibrio pomeroyi* Isolated from Invertebrates of Peter the Great Bay, Sea of Japan

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Abstract—Previously unknown associations of *Vibrio gigantis* with the trepang *Apostichopus japonicus* and of *V. pomeroyi* with the sea urchin *Strongylocentrotus nudus* were described in Vostok Bay of Peter the Great Bay, Sea of Japan. Bacterial isolates were identified based on their morphological, cultural and biochemical characteristics, as well as by 16S rRNA gene sequencing. Strain 915 (*V. gigantis*) was found to possess amylase, gelatinase, chitinase, pustulanase, glucosidase, galactosidase, and alginase, while strain 929 (*V. pomeroyi*) possessed amylase, gelatinase, chitinase, and fucoidanase. *S. nudus* and *A. japonicus* probably provide favorable niches for *V. gigantis* and *V. pomeroyi* and act as natural reservoirs for these bacteria in Peter the Great Bay. The broad spectrum of enzymes in associated vibrios suggests their role in food digestion of the above marine invertebrates.

Key words: Vibrio gigantis, V. pomeroyi, associations, enzymes.

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Vibrios are widespread in marine environments, both in the water column and in hydrobionts, from zooplankton to vertebrates; they are highly important for the ecology of these organisms [1]. Over 60 validly described species of these microorganisms are presently known, including both saprophytic or symbiotic species and those pathogenic to humans and animals. The number of known marine vibrios is ever increasing. The recently isolated species include, among others, *V. pacini* from maricultured fish and shrimp [2]; *V. ponticus* from seawater, bivalves, and diseased fish [3]; and *V. comitans, V. rarus*, and *V. inusitatus* from sea-ear (*Haliotis*) [4]. *V. gigantis* was originally isolated from the hemolymph of infected Mediterranean cultured oysters [5], while *V. pomeroyi* was obtained from the larvae of the *Nodopecten nodosus* mollusk in Brazil [6]. *V. pomeroyi* was subsequently revealed in intestinal microflora of coastal Japanese fish [7] and of the polychaetes *Paralvinella sulfincola* and *P. piscesae* from the deep-sea hydrothermal vents of the eastern Pacific [8]. However, the nature of the relationships between marine invertebrates and associated microflora is still poorly known.

The goal of the present work was a comprehensive physiological and ecological characterization of two vibrio strains, *V. gigantis* and *V. pomeroyi*, originally isolated from the trepang *Apostichopus japonicus* and the sea urchin *Strongylocentrotus nudus*, respectively, in Peter the Great Bay, Sea of Japan.

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MATERIALS AND METHODS

Strains 915 (*Vibrio gigantis*) and 929 (*V. pomeroyi*) were isolated from the intestinal content of the trepang *Apostichopus japonica* and the sea urchin *Strongylocentrotus nudus*, respectively, of Peter the Great Bay, Sea of Japan, in August 2006. The animals were collected by divers at a 6-m depth, placed into sterile plastic bags, and transported live to the laboratory within 1 h after capture.

Phenotypic characterization of the isolates. In the laboratory, the invertebrates were washed three times with sterile seawater and dissected under aseptic conditions. The intestines with their contents were homogenized, and 10- and 100-fold dilutions of the homogenate (0.1 ml) were plated on TCBS agar. All the procedures were carried out aseptically. The plates were incubated at room temperature for 2 days. The colonies were purified on TCBS agar, grown on tryptose soy agar (TSA, Oxoid) with 2% NaCl, and stored at –80°C in Marine broth (Difco) with 30% glycerol (vol/vol). The morphology of the colonies was determined on TSA under an MBS-10 stereo microscope. Bacterial motility was determined under dark field illumination. Cell morphology and cell-wall type were determined on gram-stained preparations. The presence of cytochrome oxidase, catalase, lysine and ornithine decarboxylases, arginine dihydrolase (by Moller's method), oxidation and fermentation of glucose (Hugh and Leifson's test), citrate utilization on the Simmons medium, nitrate reduction, and indole pro-

duction (with Kovac's reagent) were determined. The capacity for utilization of lactose, sucrose, mannose, arabinose, mannitol, and inositol with formation of acid was studied on the Giss medium. Production of fucoidanase, chitinase, pustulanase, glucosidase, galactosidase, and alginase was determined according to Nelson [9]. Bacteria were grown in the Yoshimizu–Kimura medium on a rotor shaker at 24–26°C for 1 day. Sensitivity to O129 (10 and 150 μg per disk), ampicillin (10 μg), chloramphenicol (30 μg), polymyxin B (300 U), streptomycin (30 μg), and tetracycline (30 μg) was determined by the diffusion method with Oxoid disks. The requirement for Na^+ was determined on TSA agar with NaCl content from 0 to 10%. The temperature range for growth was determined on TSA agar with 3% NaCl (wt/vol) after 14-day incubation at 4, 20, 25, 30, 35, and 40°C.

DNA isolation, amplification, sequencing, and analysis. DNA was isolated according to Marmur [10]. The DNA G+C content was determined from temperature denaturation curves [11].

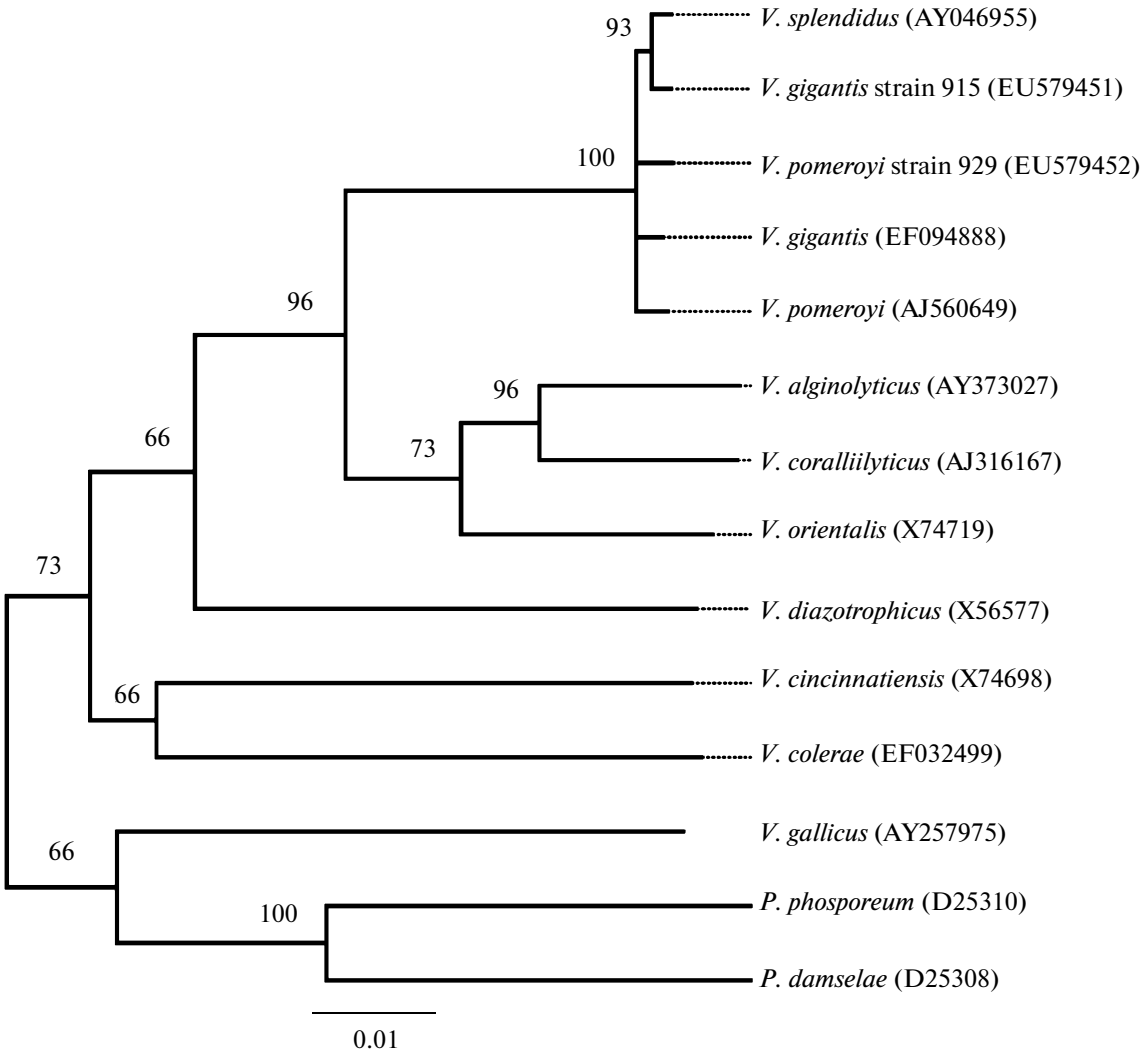
Total DNA was extracted from 1 ml of the bacterial culture by lysis in a solution containing SDS and proteinase K and subsequent deproteinization with phenol and chloroform [12]. The 16S rRNA gene fragment was amplified in 25 μl of the reaction mixture containing 2.5 μl 10 × PCR buffer, 2 μl 10 mM dNTP mixture (2.5 mM each), and 2.5 μl of each primer (2.5 μM, 10 ng DNA, and 1 U *Taq* DNA polymerase). The primers used were 5'-GTTTGATCMTGGCTCAG-3' and 5'-TACGGYTACCTTGTTACGACTT-3' [13]. The conditions for PCR amplification (Perkin-Elmer GeneAmp PCR System 9600) included initial denaturation for 3 min at 95°C; 30 cycles of 1 min at 94°C, 1 min at 52°C, and 1 min at 72°C; and the final cycle of 5 min at 72°C. The purity and size of amplification products were determined in 1% agarose gel. The purified amplification products were used as templates for sequencing. The reaction was carried out with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The purified sequencing products were treated by electrophoresis in an ABI Prism 3130 genetic analyzer on an 80-cm capillary mount. The direct and reverse sequences for each species were aligned using the SeqScape v2.5 software package (Applied Biosystems). The fragments of 16S rRNA gene sequences were deposited in the NCBI/GenBank database (EU579451 and EU579452). The consensus sequences were compared to the homologous 16S rDNA sequences of vibrios from the database (AJ316167, AJ560649, AY046955, AY257975, AY373027, EF032499, EF094888, D25308, D25310, X56577, X74698, and X74719). The sequences were aligned using the Geneious-Alignment algorithm. The optimal model of nucleotide replacement was determined with the PAUP 4.0 b 10 [14] and Modeltest 3.7 [15] software packages. The phylogenetic tree was constructed with the MrBayes v. 3.1.2 software package [16] using the GTR + gamma (K = 4) model of nucleotide replacement and the MCMC parameters of 1 100 000 generations and digitization every 200 generations.

RESULTS

According to 16S rRNA gene sequencing, strains 915 and 929 were assigned to the genus *Vibrio*. The sequences of strains 915 and 929 were compared to the sequences of known *Vibrio* species. The results are presented in the figure. Strains 915 and 929 fall into the *Splendidus* group. The phylogenetic tree, however, did not suggest unequivocal species identification of the strains. Even the GenBank sequences of 16S rRNA genes of *V. gigantis* (EF094888) and *V. pomeroyi* (AJ560649) form a single cluster. However, the nucleotide sequence of 16S rRNA genes of strain 915 was closest to that of *V. gigantis* (EF094888): 1306 matching nucleotides out of 1308 (99.8% similarity). In the case of strain 929, the 16S rRNA gene sequence was closest to that of *V. pomeroyi* (AJ560649) (99.1% similarity).

Analysis of 16S rDNA sequences is not always sufficient for differentiation between closely related species. Traditional bacteriological techniques are required for the purpose. Identification of strain 915 as *V. gigantis* and of strain 929 as *V. pomeroyi* on the basis of 16S rRNA gene sequence analysis was confirmed by analysis of their phenotypic characteristics (table). On TCBS agar, strain 915 formed green convex colonies 3–4 mm in diameter after 2 days of incubation at room temperature. Under the same cultivation conditions, the colonies on tryptose soy agar (Difco) were white-beige, opaque, convex, and 0.2–0.3 cm in diameter. Na^+ ions were required for growth. The cells grew at 3, 6, and 8% NaCl at 4°C and at room temperature; no growth occurred at 30–42°C. The cells were motile and rod-shaped with a gram-negative cell wall. They were facultative anaerobes, possesses cytochrome oxidase, catalase, DNase, nitrate reductase, arginine dihydrolase, amylase, gelatinase, chitinase, fucoidanase, pustulanase, glucosidase, galactosidase, and alginase. Bacteria produced indole, formed acid from mannose and mannitol, and were sensitive to the vibriostatic agent at 10 and 150 μg per disk.

Strain 929 exhibited insignificant differences from strain 915 in its morphological and biochemical characteristics. It formed smaller (0.1–0.2 cm), white-beige, semitransparent, convex, and round colonies on TSA. The strain was resistant to ampicillin and did not grow at 8% NaCl. It possessed a smaller range of hydrolytic enzymes, namely, amylase, gelatinase, chitinase, and fucoidanase.



Phylogenetic position of *V. gigantis* 915 and *V. pomeroyi* 929 within the genus *Vibrio* determined from 16S rRNA gene sequences. *Photobacterium phosphoreum* and *P. damselae* were taken as an outgroup. Numbers indicate posterior probability values from Bayesian analysis.

DISCUSSION

The phenotypic traits of new species have much in common with those of known species, thus hindering phenotype-based identification. Molecular genetic techniques, including 16S rRNA gene sequencing, make it possible to establish the species position of an isolate, but provide no information concerning its physiological characteristics. A polyphasic approach combining the standard bacteriological methods and molecular identification techniques makes it possible both to assign the isolates to specific taxa and to gain insight to their physiological traits, biotechnological potential, and role in marine ecosystems. Molecular genetic techniques revealed close relationships between the newly described *Vibrio* species and monophyletic taxa of known vibrios [17]. For example, *V. rotiferanus* exhibits high similarity to *V. parahaemolyticus, V. harveyi, V. alginolyticus*, and *V. campbellii* [18] of the *Harveyi* group [4]. *V. gigantis, V. pomeroyi, V. lentus, V. pelagius*, and a number of other species fall into the *Splendidus* group. Sawabe et al. [4] maintain that the species within a group have closely related genomes and a high degree of phenotypic similarity and may occupy identical ecological niches. This notion is supported by comparative analysis of the genotypic and phenotypic characteristics of isolates 915 and 929 belonging to the *Splendidus* group.

In general, the phenotypic characteristics of strains 915 and 929 are very similar to those of the well-known species *V. splendidus*. Similarly to *V. splendidus*, they are psychrophilic and halotolerant. Alginase was found in strain 915. Production of extracellular alginases is a known trait of *V. splendidus* [19], while such

Phenotypic characterization of *V. gigantis* and *V. pomeroyi*

Characteristics/test	915 (*V. gigantis*)	929 (*V. pomeroyi*)	F. Le Roux et al., 2005 (*V. gigantis*)	F.L. Thompson et al., 2003 (*V. pomeroyi*)
Oxidation/fermentation	+/+	+/+	+/+	+/+
Arginine dihydrolase	+	+	+	+
Lysine decarboxylase	–	–	–	–
Ornithine decarboxylase	–	–	–	–
Oxidase	+	+	+	+
Catalase	+	+	+	+
Indole	+	+	ND	+
Simmons citrate	–	–	ND	ND
Nitrate reduction	+	+	+	+
Urease	–	–	–	–
Gelatinase	+	+	+	+
Amylase	+	+	+	+
DNase	+	+	ND	ND
Chitinase	+	+	ND	ND
Growth at:				
0% NaCl	–	–	–	–
6% NaCl	+	+	V	+
8% NaCl	+	–	–	+
10% NaCl	–	–	ND	–
4°C	+	+	+	+
30°C	–	–	ND	ND
35°C	–	–	ND	–
40°C	–	–	ND	–
Acid from: mannose	+	+	ND	ND
lactose	–	–	ND	ND
sucrose	–	–	+	+
arabinose	–	–	ND	ND
mannitol	+	+	ND	ND
inositol	–	–	ND	ND
Sensitivity to: 0129 (10/150 μg)	+/+	+/+	+/+	+/+
Polymyxin B	+(15)*	+(15)	ND	+
Ampicillin	+(24)	–	ND	–
Tetracycline	+(20)	+(24)	ND	ND
Streptomycin	+(15)	+(25)	ND	ND
Chloramphenicol	+(20)	+(22)	ND	ND
G+C content, mol %	46.7	44.6	ND	44.1

Notes: "+," positive reaction; "–," negative reaction; "V," variable characteristics. "ND," no data.
* Diameter of growth inhibition zones, mm.

characteristics as acid production from sucrose, the presence of arginine dihydrolase, and growth at 8% NaCl vary within the species [20].

The physiological characteristics of strains 915 and 929 generally correlate with those of *V. gigantis* [5] and *V. pomeroyi* [6], respectively (table). Together with other characteristics, resistance of strain 929 to ampicillin confirms its classification as *V. pomeroyi*. Unlike type strains, the isolates 915 and 929 do not produce acid from sucrose and have different growth characteristics on the medium with 8% NaCl. These intraspecific variations characterize the ecological traits of individual strains and probably reflect the effect of the environment. Unfortunately, since a limited number of *V. gigantis* and *V. pomeroyi* strains are presently available to researchers, comparative analysis of their characteristics is difficult, limiting our understanding of the phenotypic and ecological traits of these species.

We discovered associations between *V. pomeroyi, V. gigantis*, and the echinoderms *Apostichopus japonicus* and *Strongylocentrotus nudus*, respectively, which are widespread in the Sea of Japan. Both strains were isolated from the digestive tract of the echinoderms. Associations between *V. gigantis* and *A. japonicus, V. pomeroyi* and *S. nudus* are of ecological importance. Vibrios are known to possess high metabolic plasticity and can degrade biopolymers (carbohydrates, complex polysaccharides, and proteins) [1]. These microorganisms are well adapted to anaerobic microniches in internal cavities of hydrobionts [19]. For the first time, members of the species *V. gigantis* and *V. pomeroyi* were analyzed for a broad range of hydrolytic enzymes. The presence of hydrolytic enzymes (chitinase, gelatinase, amylase, DNase, fucoidanase, pustulanase, glucosidase, and galactosidase, as well as alginase, which belongs to lyases) (table) in animal-associated strains 915 and 929, suggests their involvement in echinoderm digestion.

Our analysis confirmed classification of the isolates as *V. gigantis* and *V. pomeroyi*. We believe that marine invertebrates *S. nudus* and *A. japonicus* are a favorable niche for proliferation and spreading of *V. gigantis, V. pomeroyi*, and other vibrios in Peter the Great Bay. Detection of vibrios in winter in associated microflora of these hydrobionts, when they were not retrieved from the water samples confirms this notion [21]. *S. nudus* and *A. japonicus* probably act as a natural reservoir of these bacteria in Peter the Great Bay. The discovery of associations between *V. gigantis* and *A. japonicus, V. pomeroyi* and *S. nudus* is an important contribution to our understanding of ecology of these bacteria. The broad spectra of enzymes in associated vibrios are an indirect indication of their role in food transformations in these marine invertebrates.

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