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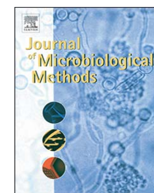

Development of a simple and practical method of discrimination between *Vibrio furnissii* and *V. fluvialis* based on single-nucleotide polymorphisms of 16S rRNA genes observed in *V. furnissii* but not in *V. fluvialis*

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ABSTRACT

Vibrio furnissii and *V. fluvialis* are closely related, the discrimination of which by conventional biochemical assay remains a challenge. Investigation of the sequence of the 16S rRNA genes in a clinical isolate of *V. furnissii* by visual inspection of a sequencing electropherogram revealed two sites of single-nucleotide polymorphisms (SNPs; positions 460 A/G and 1261 A/G) in these genes. A test of 12 strains each of *V. fluvialis* and *V. furnissii* revealed these SNPs to be common in *V. furnissii* but not in *V. fluvialis*. Divergence of SNP frequency was observed among the strains of *V. furnissii* tested. Because the SNPs described in *V. furnissii* produce a difference in the target sequence of restriction enzymes, a combination of polymerase chain reaction (PCR) of the 16S rRNA genes using conventional primers and restriction fragment length polymorphism analysis using *Eco* RV and *Eae* I was shown to discriminate between *V. fluvialis* and *V. furnissii*. This method is simple and alleviates the need for expensive equipment or primer sets specific to these bacteria. Therefore, we believe that this method can be useful, alongside specific PCR and mass spectrometry, when there is a need to discriminate between *V. fluvialis* and *V. furnissii*.

1. Introduction

Originally classified into the same species, *Vibrio furnissii* and *V. fluvialis* are closely related although the former produces gas while the latter does not (Lee et al., 1981; Igbinosa and Okoh, 2008; Brenner et al., 1983). Both of these halophilic bacteria are found in estuarine waters as well as in the seafood and animals living in and around them (Brenner et al., 1983; Austin, 2010). Diarrhea caused by both of these bacteria has been reported in humans (Igbinosa and Okoh, 2008; Hickman-Brenner et al., 1984; Morris and Black, 1985). A case of bacteremia and skin lesions after the ingestion of seafood has also been reported (Derber et al., 2011). Bacterial characterization is important in clinical and epidemiological studies. Moreover, Lesmana et al. reported a difference in sensitivities to antibiotics between *V. furnissii* and *V. fluvialis* (Lesmana et al., 2002). In selecting the appropriate antibiotic for treatment, discrimination between *V. furnissii* and *V. fluvialis* is useful, but achieving this by conventional biochemical methods remains a challenge. To overcome this issue, determination of the occurrence of gas production, molecular biological methods such as specific polymerase chain reaction (PCR), and mass spectrometry have been reported to be useful (Chakraborty et al., 2006; Schirmeister et al., 2014).

As for the use of PCR to identify bacteria in general, analysis of the 16S rRNA gene, which is encoded in the ribosomal RNA operon, has frequently been employed because this gene has a consensus sequence that is conserved in the majority of bacteria (Drancourt et al., 2000). Bacterial cells have been reported to harbor 1–15 rRNA operons, many of which have been found to have single-nucleotide polymorphisms (SNPs) (Moreno et al., 2002; Acinas et al., 2004; Pei et al., 2010).

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Investigation of the 16S rRNA gene sequence in a clinical isolate of *V. furnissii* revealed SNPs at two sites, which we did not observe in *V. fluvialis* by visual inspection of sequencing electropherograms. In the current study, therefore, we tested a number of strains of *V. furnissii* and *V. fluvialis* to determine whether the above-described SNPs are specific features of *V. furnissii*. Furthermore, we developed a simple and practical method of discriminating between *V. furnissii* and *V. fluvialis* using restriction fragment length polymorphism (RFLP) analysis of these SNPs.

2. Materials and methods

2.1. Bacterial strains and preparation of the template for PCR

Reference strains of *V. fluvialis* and *V. furnissii* were obtained from Microbe Division, Japan Collection of Microorganisms RIKEN BioResource Center (RIKEN BRC) and Research Institute for Microbial Diseases (RIMD), Osaka University. Five strains of *V. fluvialis* and two strains of *V. furnissii* were obtained from Nagasaki Prefectural Institute of Environment and Public Health (NPIEPH). Two strains of *V. fluvialis* and one strain of *V. furnissii* (proband strain, MSF-1) were clinical isolates cultured at University of Miyazaki Hospital (Table 1). Accordingly, 12 strains each of *V. fluvialis* and *V. furnissii* were available for the analysis. They were cultured on Sheep Blood Agar (T) (Nippon Becton Dickinson, Tokyo, Japan) at 35 °C for 18 h. Part of the colony was suspended in purified water, and turbidity was adjusted to McFarland 0.5 (estimated microbial count: 1.5×10^8 CFU/ml). Fifty microliters of the bacterial suspension was added to an equal volume of 50 mM NaOH and heated at 98 °C for 5 min. To neutralize the solution, 100 μl of 100 mM Tris-HCl (pH 7.0) was added. The neutralized solution was used as a template for PCR. The nucleotide position number of 16S rRNA was according to DDBJ/EMBL/GenBank accession No. X76336 (*V. furnissii*).

2.2. Biochemical testing and mass spectrometry

The above-described strains of *V. fluvialis* and *V. furnissii* were examined biochemically using VITEK 2™ GNID card by VITEK 2™ (Sysmex-biomerieux, Tokyo, Japan). Gas production from glucose was detected by TSI Agar (BD) including 0.5% NaCl after 24-h incubation. These strains were also tested by mass spectrometry using MALDI Biotyper™ (Bruker Daltonik, Bremen, Germany), in accordance with the manufacturer's instructions. In brief, bacteria were cultured on Sheep Blood Agar (T) at 35 °C for 18 h. Colonies were applied to Micro Scout Plate 48 target polished steel Barcode (Bruker Daltonik). One drop of matrix (α-cyano-4-hydroxycinnamic acid) including 2-cyan-3 (4-hydroxyphenyl) acrylic acid (Bruker Daltonik), was added to the area to which bacteria had been applied. After drying, MALDI-TOF MS analysis was performed using Bruker Biotyper 3.1 software, in accordance with the instruction manual.

2.3. Direct sequencing of 16S rRNA genes

To amplify the 16S rRNA gene with high fidelity, PrimeSTAR[TR] GXL DNA polymerase (Takara-Bio, Otsu, Japan) and the following primers were used: forward primer (1F: 5′-AGAGTTTGATCMTGGCTCAG-3′ positions 1–20) and reverse primer (1517R: 5′-TACGGTTACCTTGTTACGAC-3′ positions 1517–1498), based on the method described by Masaki et al. (2006). PCR was performed using 2 μl of bacterial solution samples prepared as described above under the following conditions: one cycle of 98 °C for 20 s and then 25 cycles of denaturing at 98 °C for 10 s, annealing at 55 °C for 20 s, and extension at 72 °C for 30 s. PCR products were purified by ethanol precipitation. The DNA sequence of the amplified 16S rRNA gene was analyzed by Applied Biosystem 3500 Genetic Analyzer (Life Technology Japan, Tokyo, Japan) using the Big Dye™ Terminator V.3.1 Cycle Sequencing Kit (Life Technology). To confirm the species of strains, the amplicon sequences were assembled and then compared to reference sequences and other entries in the DDBJ (http://www.ddbj.nig.ac.jp/index-j.html) and EZBioCloud (http://www.ezbiocloud.net/identify).

2.4. Cloning and sequencing of PCR products of 16S rRNA genes

We found SNPs in two regions (positions 459/460 and 1261) of the amplified 16S rRNA genes of *V. furnissii*, as described below. To

Table 1
Bacterial strains used in this study.

Species	Strain				16S rRNA gene
	No	ID	Derived from		Accession no
V. fluvialis	1	JCM 1281	Type strain	RIKEN BRC	LC271220
	2	MFV-1	Bile	Clinical isolate	LC271221
	3	MFV-2	Pus	Clinical isolate	LC271222
	4	RIMD2220083	Reference strain	RIMD	LC271223
	5	RIMD2220085	Reference strain	RIMD	LC271224
	6	RIMD2220098	Reference strain	RIMD	LC271225
	7	FP12019	*Turban shell*	NPIEPH	LC271226
	8	FP130066	*Rapana venosa*	NPIEPH	LC271227
	9	RIMD2220099	Reference strain	RIMD	LC316971
	10	FP12018	*Rapana venosa*	NPIEPH	LC316972
	11	FP130068	*Rapana venosa*	NPIEPH	LC316973
	12	FP130072	*Rapana venosa*	NPIEPH	LC316974
V. furnissii	13	JCM1282	Reference strain	RIKEN BRC	LC275181
	14	MFS-1	Tissue	Clinical isolate	LC275182
	15	RIMD2223001	Reference strain	RIMD	LC275183
	16	RIMD2223004	Reference strain	RIMD	LC275184
	17	RIMD2223006	Reference strain	RIMD	LC275185
	18	RIMD2223007	Reference strain	RIMD	LC275186
	19	OB-0121	–	NPIEPH	LC275187
	20	FP130069	*Rapana venosa*	NPIEPH	LC275188
	21	RIMD2223008	Reference strain	RIMD	LC316975
	22	RIMD2223010	Reference strain	RIMD	LC316976
	23	RIMD2220016	Reference strain	RIMD	LC316977
	24	RIMD2223005	Reference strain	RIMD	LC316978

RIKEN BRC, Microbe Division/Japan Collection of Microorganisms RIKEN BioResource Center; RIMD, Research Institute for Microbial Diseases, Osaka University; NPIEPH, Nagasaki Prefectural Institute of Environment and Public Health.

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