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Short Paper

Identification of *Vibrio halioticoli* using 16S rDNA PCR/RFLP (restriction fragment length polymorphism) analysis

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Vibrio halioticoli, identified as a new member of the *Vibrio* genus,[1] has been isolated from the gut of abalone *Haliotis discus hannai* as an alginolytic, non-motile fermentative marine bacterium.[2] Abalones secrete polymannuronate lyase but no polyguluronate lyase as a digestive enzyme.[3,4] As the bacterium dominated the microflora in the gut of abalone *H. discus hannai*,[2] and most of the strains produced polyguluronate lyase rather than polymannuronate lyase,[2] a contribution of the bacterium in the digestion of ingested seaweed was expected. Although a new symbiotic relationship in marine invertebrate-gut microorganism may be found, the distribution of *V. halioticoli* in the marine environment and the developmental process of the *V. halioticoli* in the abalone gut is not well understood. Prior to ecological investigation of *V. halioticoli*, the development of a rapid and accurate identification system on species level is needed. It is possible to identify *V. halioticoli* and to distinguish it from other vibrios by phenotypic characterization, but it is a time-consuming method. Instead of the quantitative DNA–DNA hybridization for the identification of bacterial species, some useful molecular-based tools (e.g. fingerprinting techniques), were recently developed for vibrios and related bacterial species.[5,6] We developed a simple and rapid fingerprinting coupling 16S rDNA amplification with restriction fragment length polymorphism (16S rDNA/RFLP), and demonstrated the applicability in the present paper. Twenty-seven species belonging to the genera *Vibrio* and *Photobacterium* were used for the PCR/RFLP analysis (Table 1). Fresh over-night cultures in ZoBell 2216E broth were harvested and the DNA was extracted from each culture using a genomic DNA purification kit (Promega, Madison, USA). A 100 ng DNA template was applied to amplify the small-subunit rRNA (16S rRNA) gene using an initial denaturation step at 94°C for 180 s, 30 cycles of thermal-cycle step consisted of denaturation at 94°C for 60 s, annealing at 55°C for 60 s and extension at 72°C for 90 s. Two DNA primers corresponded to the following position in the *Escherichia coli* sequence: primer 24F, positions 8–24, 5′-AGAGTTTGATCATGGCT-3′; primer 1540R, positions 1541–1522, 5′-AAGGAGGTGATCCAACCGCA-3′, were used in this amplification. The polymerase chain reaction (PCR) products were analyzed on a 1.5% agarose gel with a pHY molecular standard marker (Takara, Tokyo, Japan). Among 27 species, only a single 1.5 kb band was observed for 11 species (14 strains, Table 1); *V. halioticoli* IAM 14596^T, IAM 14597, IAM 14598, IAM 14599, '*V. algosus*' LMG 7905, *V. fischeri* ATCC 7744^T, *V. hollisae* LMG 17719^T, *V. mediterranei* LMG 11258^T, *V. nigripulchritudo* LMG 3896^T, *V. orientalis* LMG 7897^T, *V. pelagius* ATCC 25916^T, *V. splendidus* ATCC 33125^T, *V. tubiashii*, LMG 10936^T and *Photobacterium damselae* subsp. *damselae* LMG 7892^T. No PCR product was observed from the other 16 species using PCR condition at 45°C for annealing (data not shown). Using the 1509R universal primer[7] instead of the 1540R primer, a 1.5 kb single PCR product from all strains tested in the present study was produced (data not shown).

Polymerase chain reaction products that pro-

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Table 1 Bacterial strains and the estimated and experimented fragments number of the 16S rDNA PCR/RFLP analysis

Species	Strain	Amplification[a,b]	Restriction patterns digested with:					
			*Eco57*I		*Acc*I		*Sma*I	
			Estimated[b]	Experimented	Estimated[b]	Experimented	Estimated[b]	Experimented
Vibrio halioticoli	IAM 14596^T	+	3	3	4	3	3	3
Vibrio halioticoli	IAM 14597	+	3	3	4	3	3	3
Vibrio halioticoli	IAM 14598	+	3	3	4	3	3	3
Vibrio halioticoli	IAM 14599	+	3	3	4	3	3	3
Vibrio aestuarianus	LMG 7909^T	–						
Vibrio alginolyticus	LMG 4409^T	–						
'Vibrio algosus'	LMG 7905	+	2	2	3	2	3	3
Vibrio campbellii	LMG 11216^T	–						
Vibrio cincinnatiensis	LMG 7891^T	–						
Vibrio diazotrophicus	LMG 7893^T	–						
Vibrio fischeri	ATCC 7744^T	+	2	2	3	2	3	3
Vibrio fluvialis	LMG 7894^T	–						
Vibrio harveyi	LMG 4044^T	–						
Vibrio hollisae	LMG 17719^T	+	2	2	3	2	3	3
Vibrio mediterranei	LMG 11258^T	+	2	2	3	2	3	3
Vibrio metschnikovii	LMG11664^T	–						
Vibrio mytili	LMG 16865^T	–						
Vibrio natriegens	LMG 10935^T	–						
Vibrio navarrensis	LMG 15976^T	–						
Vibrio nereis	LMG 3895^T	–						
Vibrio nigripulchritudo	LMG 3896^T	+	2	2	3	2	3	3
Vibrio ordalii	LMG 13544^T	–						
Vibrio orientalis	LMG 7897^T	+	2	2	3	2	3	3
Vibrio parahaemolyticus	LMG 2850^T	–						
Vibrio pelagius	ATCC 25916^T	+	2	2	3	2	3	3
Vibrio proteolyticus	LMG 3772^T	–						
Vibrio splendidus	ATCC 33125^T	+	2	2	3	2	3	3
Vibrio tubiashii	LMG 10936^T	+	2	2	3	2	3	3
Vibrio vulnificus	LMG 13545^T	–						
Photobacterium damselae subsp. *damselae*	LMG 7892^T	+	2	2	3	2	3	3

LMG, Laboratory for Microbiology University of Gent. ATCC, American Type Culture Collection. IAM, IAM Culture Collection.

[a] PCR amplification of 16S rRNA gene was done using 24F and 1540R primer set. +, the amplification working; –, indicates no amplification.

[b] The number of estimated fragments was calculated using Web Cutter program, and the sequence data were obtained from EMBL/GeneBank/DDBJ database.

duced a single band on agarose gels were purified using the Wizard™ PCR preps DNA purification system (Promega) according to the instruction manual provided from the supplier. After quantification of the purified PCR product, 10 ng of PCR product was digested with *Eco57*I (Fermentas, Vilnius, Lithuania), *Acc*I (Promega) at 37°C and *Sma*I (Promega) at 25°C for 2 h. *Eco*57I and *Acc*I were selected to generate specific fragment pattern only from *V. halioticoli* strains. The estimated digesting site was predicted by the Web Cutter program (http://www.medkem.gu.se/cutter/). Digested DNA were analyzed by horizontal electrophoresis using a 1.5% agarose gel. Electrophoresis was carried out at 50 V with a Mupid mini-gel electrophoresis apparatus (Advance, Tokyo, Japan) in TBE buffer (0.5x). The gel was stained with ethidium bromide and exposed to a Polaroid film.

The estimated and experimented fragments of 11 species (14 strains) digested by *Eco*57I, *Acc*I and *Sma*I are shown in Table 1. *Eco*57I recognized two digestion sites on the 16S rRNA gene of *V. halioticoli* strains and generated three fragments: 220 bp, 480 bp and 830 bp (Fig. 1a). *Eco*57I, however, recognized only one digestion site and generated two fragments from 10 other *Vibrio* strains. *Acc*I produced four fragments (50 bp, 180 bp, 640 bp, and 660 bp) for *V. halioticoli* strains, but the 50 bp fragment could not be observed (Fig. 1b). The 180 bp fragment generated by *Acc*I digestion was specifically detected from *V. halioticoli* strains and was never observed from the 10 other tested *Vibrio-Photobacterium* species. *Sma*I was used as a control cutter, and produced three fragments for all strains (Fig. 1c).

A good and confidential PCR/RFLP method for differentiating *V. halioticoli* strains from other *Vibrio* and *Photobacterium* spp. was developed. The method was combined with a selected PCR amplification of the 16S rRNA gene using the 24F and 1540R primer set, and *Eco*57I and *Acc*I digestion of the amplified 1.5 kb fragment. However, the method has a limitation for the actual identification of *V. halioticoli* strains isolated from environmental samples. The method could not be applied directly for environmental isolates without phenotypic characterization due to a related homology of the 16S rRNA gene sequences of bacterial strains, because the same PCR/RFLP pattern may be observed. Therefore, prior to the PCR/RFLP analysis, a phenotypic characterization, or at least a tentative identification for vibrios, is necessary. Furthermore, some known *Vibrio* species and brand-new vibrios could not be tested in the

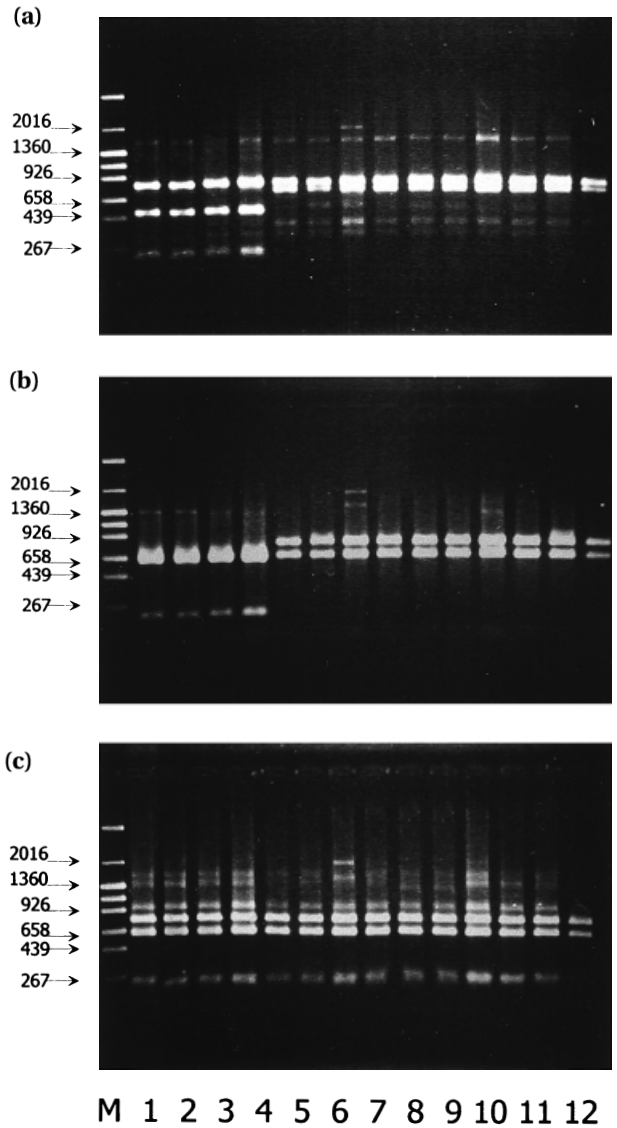

Fig. 1 Restriction patterns of 16S rRNA genes digested with *Eco*57I (a), *Acc*I (b) and *Sma*I (c). Lanes: 1, *Vibrio halioticoli* IAM 14596^T; 2, *V. halioticoli* IAM 14597; 3, *V. halioticoli* IAM 14598; 4, *V. halioticoli* IAM 14599; 5, '*V. algosus*' LMG 7905; 6, *V. fischeri* ATCC 7744^T; 7, *V. hollisae* LMG 17719^T; 8, *V. mediterranei* LMG 11258^T; 9, *V. nigripulchritudo* LMG 3896^T; 10, *V. orientalis* LMG 7897^T; 11, *V. pelagius* ATCC 25916^T; 12, *V. splendidus* ATCC 33125^T; 13, *V. tubiashii* LMG 10936^T; 14, *Photobacterium damselae* subsp. *damselae* LMG 7892^T; Lane M, size marker (pHY marker; Takara, Tokyo, Japan).

present study. Although the comparative analysis did not cover all the *Vibrio* and *Photobacterium* species, a restriction site prediction of the 16S rRNA gene of seven *Vibrio* species (*V. cholerae, V. mimicus, V. furnissii, V. anguillarum, V. gazogenes, V. salmonicida,* and *V. logei*) and four *Photobacterium* species (*P. phosphoreum, P. leiognathi, P. angustum,* and *P. histaminum*) using the WebCutter program, showed that their RFLP patterns were not the same as the *V. halioticoli* patterns. To avoid misidentification, two more characteristics (i.e. motility on the ZoBell 2216E medium containing sodium alginate and alginate degradation), could increase the confidence for the *V. halioticoli* identification with the PCR/RFLP method. We applied the PCR/RFLP method to identify environmental isolates, isolated from the seawater supply of an abalone aquaculture station. The strains were identified as *V. halioticoli* using the PCR/RFLP faster than using the conventional quantitative DNA-DNA hybridization methods (Tanaka R., unpubl. data, 1999). Of course, a molecular phylogenetic method[8] without isolation and culturing bacteria would be a powerful and fast method. The PCR/RFLP method could be very helpful for decreasing background noise from a cloned 16S rRNA gene library for a future study for the detection of *V. halioticoli* in the gut of abalone.

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