FISHERIES SCIENCE 2002; **68**: 227–229

Short Paper

Identification of *Vibrio halioticoli* by colony hybridization with non-radioisotope labeled genomic DNA probe

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KEY WORDS: **colony hybridization, identification, non-radioisotope labeling, *Vibrio halioticoli*.**

Vibrio halioticoli was isolated from the gut of abalone *Haliotis discus hannai* and identified as a new member of the genus *Vibrio*.[1] *Vibrio halioticoli* was first identified as a non-motile fermenter (NMF) because of its facultative anaerobic, alginolytic, and non-motile traits.[2] Most of the gut isolates from abalone *H. discus hannai* were identified as NMF using phenotypic characterization, and *V. halioticoli* strains were found to be dominant in the gut.[2] From an ecophysiological view point, NMF strains have an alginate-degrading ability, which probably degrades the polyguluronate block, and might assist in the breakdown of the alginate-rich microalgae ingested by abalone.[2] In other words, a symbiotic relationship similar to that of ruminants may be found between NMF and abalone.

We have developed a species-specific detection and identification method for *V. halioticoli* that is able to differentiate precisely and rapidly *V. halioticoli* strains from other NMF isolates. An applicable fingerprinting technique based on 16S rDNA polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP) has been reported previously by Tanaka *et al.*[3] *V. halioticoli* strains were differentiated from others using RFLP; however, as the PCR/RFLP technique is suitable only for phenotypically characterized isolates, cost and time restricts its application to huge amounts of isolates.

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Received 18 September 2000. Accepted 5 February 2001.

In the present study, we developed a colony hybridization technique for the species-specific detection of *V. halioticoli* using alkaline phosphatase-labeled genomic DNA probes instead of conventional radioisotope probes.[4] This method allows the rapid identification and enumeration of *V. halioticoli* strains grown on agar plates without phenotypic characterization and isolation.

Vibrio halioticoli IAM14596^T genomic DNA was purified according to Marmur,[5] and the DNA concentration was adjusted 20 ng/μL in a 1.5 mL microcentrifuge tube. The DNA was denatured at 100°C for 5 min, then transferred immediately on ice and kept for 1 h. The denatured DNA solution was randomly broken by 30 times sonication at 50 W for 5 min. The denatured and sonicated DNA fragments were labeled using the Alkphos direct labeling system™ (Amersham Pharmacia Biotech, Chicago, IL, USA) according to the instruction manual. The labeled mixture, containing heat-stable alkaline phosphatase provided from the labeling system, was added to 10 μL of the above-prepared DNA solution, and incubated at 37°C for 30 min The *V. halioticoli* genomic DNA probes (Vh probes) labeled with alkaline phosphatase were stored at 4°C before hybridization.

The nylon membrane (Hybond™-N$^+$, 82 mm diameter, Amersham Pharmacia Biotech) spotted template DNA or transferred colonies were placed onto Whatman 3MM paper that had been immersed previously in a denaturation buffer (0.5 M NaOH–1.5 M NaCl) for 5 min at room temperature, hence causing lysis of cells and the denaturation of genomic DNA. The membrane was placed briefly onto Whatman paper to remove any

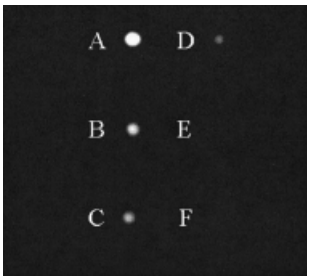

Fig. 1 Determination of Vh-probe sensitivity on dot blot hybridization. (a) 800 ng/μL; (b) 400 ng/μL; (c) 200 ng/μL; (d) 100 ng/μL; (e) 50 ng/μL; and (f) 25 ng/μL of *Vibrio halioticoli* IAM 14596^{T} genomic DNA were fixed on nylon membrane.

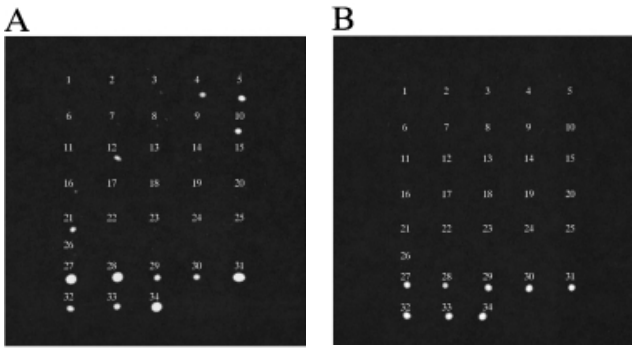

Fig. 2 Effect of hybridization temperature on specific detection of *V. halioticoli*. Hybridization temperature was set at (a) 55°C for 4 h and (b) at 60°C for 4 h. Code nos. 1–26 are reference strains of *Vibrio* and *Photobacterium* species, and 27–34 are *V. halioticoli* strains.

Fig. 3 Colony hybridization using Vh-probes against *Vibrio-Photobacterium* species. Hybridization was conducted at 60°C for 4 h. All bacterial strains were cultured for 5 days. Code nos. 1–26 are reference strains of *Vibrio* and *Photobacterium*, and 27–34 are *V. halioticoli* strains.

excess solution. Then, the membrane was transferred to a paper soaked with a neutralization buffer (1.0 M Tris–1.0 M HCl, pH 7.5) at room temperature for 3 min. The membrane was washed twice in 1× standard saline citrate (SSC), and air-dried and baked at 80°C for 2 h in a hybridization oven.

The baked membrane was prehybridized with 25 mL of hybridization buffer and attached to the labeling system for 15 min. Approximately 200 ng/membrane of Vh probe was added to the hybridization buffer, and then hybridized. The membrane was washed twice with a primary wash buffer, and again washed twice at room temperature in a secondary wash buffer. The membrane was then washed three times at room temperature in 0.15 M NaCl–0.015 M sodium citrate (1× SSC, pH 7.0). Alkaline phosphatase activity was assayed with CDP star^{TM} detection reagent (Amersham Pharmacia Biotech) as the substrate. One milliliter of detection reagent was spread onto the membrane, which was then wrapped in clear plastic film (saran wrap; Asahi kasei, Japan), and incubated for 5 min. The X-ray film (Fuji photo film; Fuji, Tokyo, Japan) was kept in contact for 5 min with the side on which the substrate was spread, and chemical luminescence signals derived from the positive hybridization reaction were exposed to the film. The film was developed according to the instruction manual.

Prior to colony hybridization, to determine the optimum template DNA concentration required by the Vh probe to give a satisfactory signal, 1 μL of diluted genomic DNA varying between 800 ng and 25 ng of *V. halioticoli* IAM14596^{T}, was blotted onto a membrane. Then, hybridization was conducted at 55°C for 4 h with 200 ng of Vh probe. The Vh probes were able to detect 100 ng of *V. halioticoli* template DNA, although the signal was weak. No signal was detected with either the 50 ng or 25 ng of template DNA (Fig. 1).

Twenty-six species of *Vibrio*, including *V. halioticoli* and one species of *Photobacterium*, were used to carry out the dot blot hybridization with the Vh probe in order to investigate the specificity of the probe (Table 1). For its technical development, eight strains of *V. halioticoli* were used: the four strains IAM14596^{T}, IAM14597, IAM14598, and IAM14599 have been identified previously by Sawabe *et al.*;[1] and another four strains, 2Y223, 25Y110, KN1Y29, and KL1Y210, which were isolated from the gut of abalone, were identified by DNA–DNA hybridization experiments showing 82.1%, 88.0%, 97.0%, and 101.5% DNA homology against IAM14596^{T}, respectively. All genomic DNA were blotted onto the nylon membrane. The membrane was washed, air-dried, and baked for 2 h at 80°C, and then used for hybridization. Hybridization was conducted at 55°C or 60°C for 4 h using the 200 ng Vh probe. At a hybridization temperature of 55°C, in addition to the *V. halioticoli* strains, strong signals were observed for five other *Vibrio* strains: *V. campbelli*, *V. cincinnatiensis*, *V. hollisae*, *V. metschnikovii*, and *V. pelagius* (Fig. 2a, spot 4, 5, 10, 12, and 21, respectively). Faint signals were observed for eight strains (Fig. 2a, spot 3, 7, 8, 14, 15, 16, 20, 23, and 26; see Table 1 for names of species). Increasing the hybridization temperature

Table 1 Bacterial strains used in the study

Species	Strain	Code no.
Vibrio aestuarianus	LMG 7909^T	1
Vibrio alginolyticus	LMG 4409^T	2
Vibrio algosus	LMG 7905	3
Vibrio campbellii	LMG 11216^T	4
Vibrio cincinnatiensis	LMG 7891^T	5
Vibrio diazotrophicus	LMG 7893^T	6
Vibrio fischeri	ATCC 7744^T	7
Vibrio fluvialis	LMG 7894^T	8
Vibrio harveyi	LMG 4044^T	9
Vibrio hollisae	LMG 17719^T	10
Vibrio mediterranei	LMG 11258^T	11
Vibrio metschnikovii	LMG11664^T	12
Vibrio mytili	LMG 16865^T	13
Vibrio natriegens	LMG 10935^T	14
Vibrio navarrensis	LMG 15976^T	15
Vibrio nereis	LMG 3895^T	16
Vibrio nigripulchritudo	LMG 3896^T	17
Vibrio ordalii	LMG 13544^T	18
Vibrio orientalis	LMG 7897^T	19
Vibrio parahaemolyticus	LMG 2850^T	20
Vibrio pelagius	ATCC 25916^T	21
Vibrio proteolyticus	LMG 3772^T	22
Vibrio splendidus	ATCC 33125	23
Vibrio tubiashii	LMG 10936^T	24
Vibrio vulnificus	LMG 13545^T	25
Photobacterium damselae Subsp. *damselae*	LMG 7892^T	26
Vibrio halioticoli	IAM 14596^T	27
Vibrio halioticoli	IAM 14597	28
Vibrio halioticoli	IAM 14598	29
Vibrio halioticoli	IAM 14599	30
Vibrio halioticoli	2Y223	31
Vibrio halioticoli	25Y110	32
Vibrio halioticoli	KN1Y29	33
Vibrio halioticoli	KL1Y210	34

to 60°C reduced the non-specific signal for the species-specific detection of *V. halioticoli* strains, and positive signals were observed only for *V. halioticoli* strains (Fig. 2b, spots 27–34). A longer hybridization period did not improve specificity (data not shown).

Using the optimum conditions (60°C for 4 h), colony hybridization was applied. Thirty-four strains, including eight strains of *V. halioticoli* (Table 1), were inoculated on a ZoBell 2216E agar plate and cultured for 5 days at 20°C. Prior to the lifting of colonies, the plate was incubated at 4°C for 5 min. The nylon membrane was applied for 1 min to the colonies growing on the plate, and colonies were lifted onto the membrane. Denaturation and hybridization were conducted as described earlier. The colony hybridization worked well in detecting only *V. halioticoli* strains grown on the agar plate; strong Vh probe-positive signals were observed (Fig. 3, spots 27–34). The Vh probes detected *V. halioticoli* strains grown from 2–6-day-old cultures, but the positive signals of cultures older than 7 days seemed to decrease (data not shown).

A rapid and accurate method to enumerate *V. halioticoli* strains amongst a mixed population such as environmental samples is necessary for studying *V. halioticoli* in the gut of abalone *H. discus hannai* and for studying the possibility of a symbiotic relationship between *V. halioticoli* and abalone.[2]

We now prefer to use the colony hybridization system to environmental samples. However, some difficulties may still remain. To overcome the divergence of bacteria, we decided to test Vh probes derived from the genomic DNA of *V. halioticoli* with only 14% homology with other *Vibrio* species under optimal conditions.[1] Hybridization using the Vh probe at 55°C showed non-specific signals amongst *Vibrio* and *Photobacterium* species, which are closely related to *V. halioticoli* (Fig. 2a) but, at 60°C, non-specific signals were reduced (Fig. 2b). Also, the incubation time required by bacterial strains on agar plates may be problematic. In fact, the strongest signals were observed only in samples growing 2–6-day-old *V. halioticoli* cells. Younger and older cells gave weak signals and were observed as faint and donut-shaped signals, which may cause a false negative result. It is recommended that 5–6-day-old plate cultures be used for the colony hybridization experiments.

This work was supported by a Research Fellowship of the Japan Society for the Promotion of Young Scientists.

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