Taiwania, **44**(3): 404-412, 1999

Detection of *Vibrio anguillarum* and *Vibrio alginolyticus* by Randomly Cloned DNA Fragments

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(Manuscript received 30 July, 1999; accepted 8 September, 1999)

ABSTRACT: *Vibrio anguillarum* and *Vibrio alginolyticus* were used as tested organisms. Restriction endonuclease *Hin*dIII and *Eco*RI digested DNA fragments from the above two bacteria were randomly selected and inserted into vector pUC19. Among 0.5-2.0 kb DNA fragments were recovered from agarose gel to prepare non-radioactive DIG-labeled probes. One out of 102 cloned fragments could hybridize only to *V. alginolyticus*. Three out of 94 cloned fragments could hybridize only to *V. anguillarum* and one fragment is specific for serotype C.

KEY WORDS: ***Vibrio anguillarum*, *Vibrio alginolyticus*, Colony hybridization, Randomly cloned fragments of DNA, Serotype.**

INTRODUCTION

Vibrio anguillarum and *V. alginolyticus* are the major pathogens for both marine (Brunn and Heiberg, 1932; Nybelin, 1935; Rucker *et al.*, 1953; Smith, 1961; Anderson and Conroy. 1970; Sindermann, 1970) and fresh water fishes (Ross *et al.*, 1968; Kou *et al.*, 1976; Huang, 1977; Tung *et al.*, 1985) as well as shellfishes (Bowser *et al.*, 1980). The detection and identification of these pathogens are important aspects of both diagnostic and therapy (Aoki *et al.*, 1990). In the diagnosis of etiological agents, pathogenic microorganisms required selective media or special cultural conditions for growth. Identification of pathogens has used combinations of biochemical and serological tests after growth on agar media. These processes are time consuming and costly. DNA probe hybridization technology, widely used now for the direct detection and identification of microorganisms, provides rapid and accurate diagnosis.

This report describs probes isolated from the random fragments of chromosomal DNA are specific for *V. anguillarum* and *V. alginolyticus.*

MATERIALS AND METHODS

Bacteria strains and vectors

Vibrio species used in this study are listed in Table 1. Transformation recipient *Escherichia coli* RR1 was a gift from Molecular Biology Laboratory, and *E. coli* TG1 and vector pUC19 were from Genetic Laboratory, Department of Botany, National Taiwan University. *V. anguillarum* NIE 275 and *V. alginolyticus* ATCC 17749 were selected for the preparation of detection probes.

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Table 1. Bacterial strains used in this study.

Strain no.	Species	No. of strains	Source or strains designation(s)*
	Vibrio		
1 - 6	*V. alginolyticus*	6	ATCC 17749, NPUST
7 - 15	*V. anguillarum*	9	ATCC 19264, NTU-Z, T. Aoki
16 - 17	*V. campbellii*	2	ATCC 25920, NTU-AC
18 - 19	*V. carchariae*	2	NTU-Z
20 - 25	*V. damsela*	6	ATCC 33539, NTU-Z, NPUST
26	*V. fischeri*	1	FIRDI
27	*V. fluvialis*	1	NTU-AC
28 - 29	*V. harveyi*	2	ATCC-14126, NPUST
30 - 32	*V. mediterarranei*	3	NPUST, NTU-Z
33	*V. minicus*	1	NTU-AC
34	*V. natriegens*	1	NTU-Z
35	*V. ordallii*	1	NPUST
36 - 38	*V. parahaemolyticus*	3	ATCC-27519, ATCC-27969, NPUST
39	*V. percolans*	1	NTU-AC
40	*V. Proteolyticus*	1	NPUST
41	*V. salmonicida*	1	ATCC 43839
42	*V. splendidus*	1	ATCC 33125
43 - 44	*V. vulnificus*	2	NPUST
45 - 61	*V. spp.*	17	NTU-Z, NTU-AC, NPUST,
	Other genera		
62	*Bacillus subtilus*	1	NTU-B
63 - 65	*Escherichia coli*	3	NTU-B
66	*Klebsiella pneumonia*	1	NTU-AC
67	*Micrococcus lutea*	1	NTU-AC
68	*Salmonella typhi*	1	NTU-AC
69	*S. typhimurium*	1	NTU-AC
70	*Staphylococcus aureus*	1	NTU-AC

*: FIRDI: Culture Collection and Research Center (CCRC), the Food Industry Research and Development Institute, Institute, Taiwan, R.O.C.; NTU-B: Department of Botany, National Taiwan University; NTU-Z: Department of Zoology, National Taiwan University, Taipei, Taiwan, R.O.C.; NTU-AC: Department of Agricultural Chemistry, National Taiwan University, Taipei, Taiwan, R.O.C.; NPUST: Department of Veterinary Medicine, National Pingtung University of Science and Technology, Pingtung, Taiwan, R.O.C.; ATCC: American Type Culture Collection; T. Aoki: Department of Biological Resources, Miyazaki University, Miyazaki, Japan.

Culture condition

Tryptic soy agar (TSA) supplemented with 2% NaCl was used to culture all the *Vibrio* spp. at 30℃. Other bacterial cultures were grown on Luria-Bertani (LB) medium at 37℃.

Preparation of DNA

After organisms were grown overnight, the extraction and purification of bacterial chromosomal DNA were performed according to the method of Ausubel *et al.* (1995). Fragments of *Vibrio* DNA were generated by digestion of appropriate amount of chromosomal DNA with 2-3 units of *Hin*dIII or *Eco*RI for every one μg DNA at 37℃ overnight and separated by agarose gel electrophoresis (Ausubel *et al.*, 1995). Vector DNA was purified and similarly digested with the same restriction enzyme and then ligated with the *Vibrio* fragments using T4 DNA ligase (Ausubel *et al.*, 1995).

E. coli competent cell was prepared following the methods of Ausubel *et al.* (1995), and transformed with recombinant plasmids in the ligation mixture.

Construction of the DNA probe specific for *V. anguillarum* and *V. alginolyticus*

Random fragments of chromosomal DNA from *V. anguillarum* NIE 275 and *V. alginolyticus* ATCC 17749 were cloned in pUC19. Recombinant plasmids were digested with *Eco*R1 and *Hin*dIII, and DNA fragments with a size between 0.5 and 2 kb were purified and recovered by electroelution onto DEAE membrane (Ausubel *et al.*, 1995). The probes were labeled with digoxigenen (DIG) using random oligonucleotide primers (Sambrook *et al.*, 1989) according to the specifications of the manufacturer (DIG labeling kit, Boehringer Mannheim).

Colony hybridization

Tested organisms listed in Table 1 were grown on TSA supplemented with 2% NaCl and transfered to Nitroplus membrane (Sumbrook *et al.*, 1989). Colonies on Nitroplus membranes were lysed and detected by DIG-labeled probe with DIG detection kit (Boehringer Mannheim).

RESULTS

Screening of randomly cloned fragments specific for *V. alginolyticus*

V. alginolyticus ATCC 17749 was used as type organism for the preparation of probes. Recombinant plasmids containing DNA fragments from this strain were constructed after digestion of the bacteria and vector pUC19 DNA with *Eco*R1 and *Hin*dIII. Recombinant plasmids containing *V. alginolyticus* DNA fragments with a size between 0.5 and 2.0 kb were selected to hybridize *Vibrio* species DNA. The fragments hybridized only with type organism but not with other *Vibrio* species were selected. Nine out of 102 fragments purified from recombinant plasmids were collected (Fig. 1). Hybridization of these selected fragments with the collected *V. alginolyticus* strains were shown in Table 2. Probe 8 could hybridize with all the strains tested. All the probes could hybridize with strain numbers 1, 5 and 6.

Screening of randomly cloned fragments specific for *V. anguillarum*

V. anguillarum NIE275 native strain (serotype C) was selected as type organism. Recombinant plasmids containing DNA fragments from this strain were constructed after digestion of the bacteria and vector pUC19 DNA with *Eco*R1 and *Hin*dIII. Recombinant plasmids containing *V. anguillarum* DNA fragments with a size between 0.5 and 2.0 kb were selected to hybridize *Vibrio* species DNA. The fragments hybridized only with *V. anguillarum* but not with other *Vibrio* species were selected. Fourteen out of 94 fragments purified from recombinant plasmids were collected (Fig. 2). Hybridization of these selected fragments with the collected *V. anguillarum* strains were shown in Table 3. Probes 13, 17 and 19 could hybridize with all the tested *V. anguillarum.* Probe 23 could only hybridize with serotype C (strain numbers 9 and 10), but not with other serotypes. Probes 14 and 18 could hybridize with serotype C (strain numbers 9 and 10) and D (strain number 11). Probes 10 and 16 could hybridize with all the tested organisms except serotype B (strain number 8). Probes 12 and 22 could hybridize with all the strains but not with serotype F and H respectively. Probe 24, originally selected for serotype A by Dr. Aoki, could only hybridize with strain number 7.

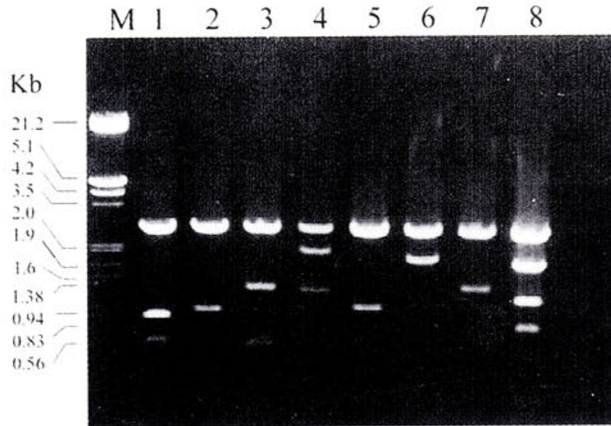

Fig. 1. Agarose gel electrophoresis of *Hin*dIII and *Eco*RI-digested DNA from pUC19 carrying *V. alginolyticus* fragment. M: λ digested by *Hin*dIII and *Eco*R1. Probe 1: second fragment of lane 1, about 0.83 kb; Probe 2: second fragment of lane 2; about 0.93 kb, Probe 3: second fragment of lane 3, about 1.38 kb; Probe 4: third fragment of lane 4, about 1.38 kb; Probe 5: second fragment of lane 5, about 1.0 kb; Probe 6: second fragment of lane 6, about 1.9 kb; Probe 7: second fragment of lane 7, about 1.38 kb; Probe 8: third fragment of lane 8, about 1.38 kb; Probe 9: forth fragment of lane 8, about 0.83 kb.

Table 2. Detection of *V. alginolyticus* by colony hybridization with randomly cloned DNA fragment.

Bacterial strain number	Probe number[1]								
	1	2	3	4	5	6	7	8	9
1	+	+	+	+	+	+	+	+	+
2	-	-	-	-	-	-	-	+	-
3	-	-	-	-	-	-	-	+	-
4	+	-	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+

[1] The molecular weight of the probes is indicated in Fig. 1.

DISCUSSION

A probe (Macario and De Macario, 1990), used for the identification of microorganisms, can be a whole cell chromosomal DNA (Morotomi *et al.*, 1988 ; Moncla *et al.*, 1988 ; Roberts *et al.*, 1987), specific genes (Moseley and Falkow, 1980; Moseley *et al.*, 1982 ; Morris *et al.*, 1987 ; Datta *et al.*, 1987), ribosomal RNA (DeLong, *et al.*, 1989; Romaniuk and Trust, 1987, Juha *et al.*, 1994), plasmids (Totten *et al.*, 1983 ; Horn *et al.*, 1986), or oligonucleotides (Datta *et al.*, 1988 ; Karch and Meyer, 1989 ; Miliotis *et al.*, 1989; Lee *et al.*, 1992). Even the randomly cloned fragments of chromosomal DNA were also used as the probe for the identification of *Pasteurella piscida, V. anguillarum* (Aoki *et al.*, 1990), *Campelobacter spp.* (Totten *et al.*, 1985), *Leptospira interrogans* (van Eys *et al.*, 1988), *Salmonella* spp. (Fitts *et al.*, 1983; Tompkins *et al.*, 1986) and *Bacteroides thetaitamicron* (Salyers *et al.*, 1986).

In this study, probe 8 was found to be specific for *V. alginolyticus* and probes 13, 17 as well as 19 for *V. anguillarum*. These probes did not hybridized with any other *Vibrio* species or with any other non-vibrio bacteria tested. Probe 23 was specific only for serotype C of *V. anguillarum*. During cross hybridization tests of the selected probes (data not shown), only probes 4 and 8 could hybridize with each other and showed similar hybridization patterns. The rest of the probes did not cross hybridize with any other probes selected. Since some of the tested organisms were gifts from other laboratories. In order to confirm our results, we had reidentified the tested organisms with Nissui ID test EB-20 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). The identification results do match hybridization results (Hsieh, 1993). Thus, these optimally selected randomly cloned DNA fragments can be used as probes to identify those organisms whose specific gene probe is not available.

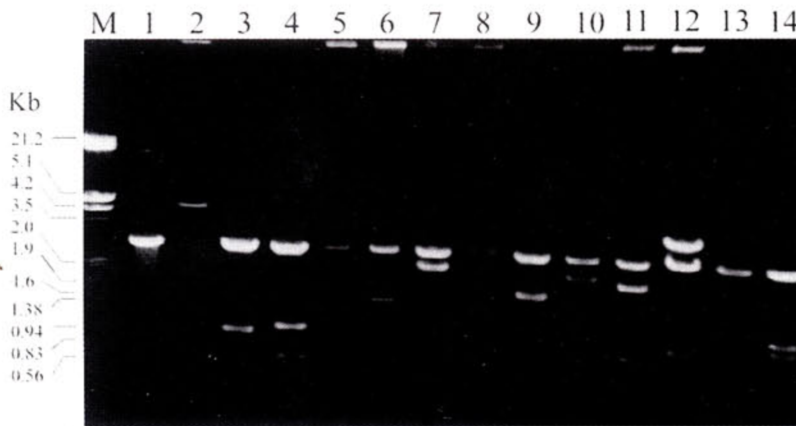

Fig. 2. Agarose gel electrophoresis of *Hin*dIII and *Eco*R1-digested DNA from pUC19 carrying *V. anguillarum* fragment. M: λ digested by *Hin*dIII and *Eco*R1. Probe 10: second fragment of lane 1, about 0.56 kb; Probe 11: second fragment of lane 2, about 0.56 kb; Probe 12: second fragment of lane 3, about 0.83 kb; Probe 13: second fragment of lane 4, about 0.94 kb; Probe 14: third fragment of lane 5, about 1.2 kb; Probe 15: second fragment of lane 6, about 1.38 kb; Probe 16: second fragment of lane 7, about 2.0 kb; Probe 17: second fragment of lane 8, about 1.38 kb; Probe 18: second fragment of lane 9, about 1.6 kb; Probe 19: second fragment of lane 10, about 2.0 kb; Probe 20: second fragment of lane 11, about 2.0 kb; Probe 21: second fragment of lane 12, about 0.94 kb; Probe 22: second fragment of lane 13, about 1.38 kb; Probe 23: second fragment of lane 14, about 1.2 kb.

Table 3. Detection of *V. anguillarum* by colony hybridization with randomly cloned DNA fragment.

Bacterial strain no.	Serotype	Probe number[1]														
		10	11	12	13	14	15	16	17	18	19	20	21	22	23	24[2]
7	A	+	+	+	+	-	+	+	+	-	+	-	-	+	-	+
8	B	-	-	+	+	-	-	-	+	-	+	+	-	+	-	-
9	C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
10	C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
11	D	+	-	+	+	+	-	+	+	+	+	+	+	+	-	-
12	E	+	-	+	+	-	+	+	+	-	+	+	+	+	-	-
13	F	+	+	-	+	-	+	+	+	-	+	+	-	+	-	-
14	H	+	-	+	+	-	+	+	+	-	+	+	+	-	-	-
15	I	+	-	+	+	-	+	+	+	-	+	+	+	+	-	-

[1] The molecular weight of the probes is indicated in Fig. 2.
[2] probe number 24: 562 bp DNA fragment, gift from Dr. T. Aoki (Aoki *et al.*, 1990).

ACKNOWLEDGMENT

This study was supported by a grant from the Council for Agricultural Planning and Development.

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應用隨機基因探針偵測 *Vibrio anguillarum* 和 *Vibrio alginolyticus*

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(收稿日期：1999 年 7 月 30 日；接受日期：1999 年 9 月 8 日)

摘　　要

以 *Vibrio anguillarum* 和 *Vibrio alginolyticus* 作為測試菌株。利用限制酶 *Hin*dIII 和 *Eco*RI 將細菌的基因體 DNA 切割後，選出介於 0.5-2.0 kb 之片段選殖到質體 pUC19 上。利用非放射性探針標定篩選的片段，由 102 選殖的片段中找到一段只能與 *V. alginolyticus* 雜交;由 94 選殖的片段中找到三段只能與 *V. anguillarum* 雜交的片段，並找到一段只能與 *V. anguillarum* 血清型 C 作用的片段。

關鍵詞： *Vibrio anguillarum*，*Vibrio alginolyticus*，菌落雜交， 隨機選殖 DNA 片段，血清型。

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