A specific DNA hybridization probe for detection of *Pasteurella piscicida*

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ABSTRACT: A 692 base pair (bp) DNA fragment from the bacterial fish pathogen *Pasteurella piscicida* was cloned into pUC9 and used as a hybridization probe for the identification of *P. piscicida*. The radiolabeled probe hybridized with itself, but did not hybridize in colony hybridization with other fish pathogens (including *Aeromonas hydrophila*, *A. salmonicida*, *Edwardsiella tarda*, *Pseudomonas anguilliseptica*, *Vibrio anguillarum* and *Yersinia ruckeri*), or with other related organisms (*Haemophilus influenzae*, *Pasteurella haemolytica*, and *P. multocida*). Photobiotin-labeled probe hybridization detected *P. piscicida* chromosomal DNA by Southern and dot blot hybridizations. The radiolabeled probe directly detected *P. piscicida* on nitrocellulose filters smeared with infected kidney and spleen tissue of yellowtail *Seriola quinqueradiata*. The ³²P-labeled DNA probe was capable of detecting 3.9 ng of purified DNA and 10⁵ cells of *P. piscicida*. The cloned specific DNA probe can be used for rapid detection and identification of *P. piscicida*.

INTRODUCTION

Pasteurella piscicida is the causative agent of pseudotuberculosis in yellowtail *Seriola quinqueradiata* (Kimura & Kitao 1971), one of the most important causes of mortality in cultured yellowtail. The identification of *P. piscicida* has been based on its morphological and biochemical characteristics. However, classification using these features is cumbersome and time-consuming. The different morphological and biochemical characteristics of the species of bacteria are prescribed by their genes. Recently, randomly cloned chromosomal DNA fragments and cloned genes encoding species-specific characteristics have been used as specific hybridization probes for rapid identification of organisms (Salys et al. 1983, Nishibuchi et al. 1985, Carter et al. 1987, Mifflin et al. 1987). By using a species-specific DNA probe to a specific nucleotide sequence of chromosomal DNA from *Vibrio anguillarum*, we easily and rapidly identified *V. anguillarum* using colony hybridization (Aoki et al. 1989).

In the present study, we describe a species-specific DNA fragment of chromosomal DNA from *Pasteurella piscicida* which, by direct hybridization, specifically detects the causative agent in diseased marine fish.

MATERIALS AND METHODS

Bacterial strains and plasmid. The bacterial strains used are listed in Table 1. Twenty-nine strains of *Pasteurella piscicida* were collected from diseased yellowtail in various areas of Japan and 1 strain from white perch, *Morone americana*, in the USA (Janssen & Surgalla 1968). One *P. piscicida* strain (KG8601), isolated in 1986 from Kagoshima in Japan, was used for the cloning of its chromosomal DNA. Other fish pathogens (4 strains of *Aeromonas hydrophila*, 4 of *A. salmonicida*, 4 of *Edwardsiella tarda*, 2 of *Pseudomonas anguilliseptica*, 4 of *Vibrio anguillarum*, and 2 of *Yersinia ruckeri*), were used in testing the specificity of the probe, as were 1 strain each of *Haemophilus influenzae*, *Pasteurella haemolytica* and *Pasteurella molitocida*. *Escherichia coli* Strain JM103 (Maniatis et al. 1982) was used as the host for propagating the plasmid. The plasmid, pUC9 (Maniatis et al. 1982), was used for the cloning of *P. piscicida* chromosomal DNA.

Media and growth conditions. *Pasteurella piscicida* strains were cultured at 25 °C in nutrient (N) broth (beef extract: 15 g; polypeptone: 10 g; NaCl: 5 g; distilled water: 1 l; pH: 7.2) supplemented with 0.3 % yeast extract and 1.5 % NaCl. The other *Pasteurella* species and *Haemophilus influenzae* were grown at 37 °C in heart infusion broth (HI, Nissui Co. Ltd.) containing 5 %

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Table 1. Bacterial strains used in this study

Bacteria	Strain	Host	Source
<i>Aeromonas hydrophila</i>	ATCC15467		Schubert (1974)
	A-10	Eel	Wakabayashi et al. (1981)
	A-17		
	A-54		
<i>Aeromonas salmonicida</i>	ATCC14174	Brooktrout	Nomura & Aoki (1985)
	N8004		Nomura & Aoki (1985)
	OB8101	Amago	Kitao et al. (1985)
	HR8101		
<i>Edwardsiella tarda</i>	SA8318	Flounder	This study
	NG8407		
	MZ8401	Eel	
	E-11-2		
<i>Haemophilus influenzae</i>	NCTC8143		Kilian & Biberstein (1984)
<i>Pasteurella haemolytica</i>	NCTC9380		Carter (1984)
<i>Pasteurella multocida</i>	NCTC10322		Carter (1984)
<i>Pasteurella piscicida</i>	EH8218	Yellowtail	This study
	KG8206		
	KG8232		
	MEP8215		
	EH8306		
	EH8310		
	MZ8303		
	MZ8309		
	NG8304		
	EH8405		
	EH8411		
	EHP84133		
	EHP84135		
	SA8406		
	SA8407		
	KG84305		
	EHP85468		
	EHP85477		
	WKY8575		
	MZ8574		
	MZ8575		
	SA8514		
	SA8516		
	KG8601		
	EH8601		
	EH8606		
	EH8610		
	NG8609		
	MZ8615		
	Snieszko	White perch	Janssen & Surgalla (1968)
<i>Pseudomonas anguilliseptica</i>	NCMB1949	Eel	Nakajima et al. (1983)
	ET-2		
<i>Vibrio anguillarum</i>	ATCC19264		Nakajima et al. (1983)
	PT8341	Ayu	Baumann et al. (1984)
	PT-24		Aoki et al. (1989)
	ET-1	Eel	Kitao et al. (1983)
	11-4		Kitao et al. (1983)
<i>Yersinia ruckeri</i>	11-29	Rainbow trout	Stave et al. (1987)
			Stave et al. (1987)

fildes extract (Oxoid Ltd.). *Vibrio anguillarum* strains were cultured at 25 °C in HI broth containing 1 % NaCl. *Pseudomonas anguilliseptica* and *Aeromonas salmonicida* were cultured at 20 °C in HI and N broth, respectively. *A. hydrophila*, *Edwardsiella tarda*, and *Yersinia*

ruckeri were cultured at 25 °C in N broth. *Escherichia coli* was grown at 37 °C in Luria-Bertani broth (LB) (polypeptone: 10 g; yeast extract: 5 g; NaCl: 5 g; distilled water: 1 l; pH 7.0) (Maniatis et al. 1982). Each medium also contained 1.5 % agar to isolate organisms.

Isolation of DNA. The chromosomal DNA of *Pasteurella piscicida*, Strain KG8601, and the fish-pathogens *Aeromonas hydrophila* A-10, *A. salmonicida* ATCC14174, and *Vibrio anguillarum* PT8341 were isolated by previously reported methods (Silhavy 1984). Plasmid DNA was prepared by the alkaline lysis method of Birnboim & Doly (1979). Both isolated chromosomal DNA and plasmid DNA were purified by centrifugation to equilibrium in cesium chloride-ethidium bromide gradients.

Molecular cloning of DNA probe. The chromosomal DNA of *Pasteurella piscicida* was digested with *Hind* III. The vector plasmid, pUC9, was also digested with the same enzyme and then dephosphorylated with bacterial phosphatase (Takara Shuzo Co. Ltd, Kyoto, Japan). The digested DNA was mixed together and ligated with T4 ligase (Nippon Gene Co. Ltd, Osaka, Japan). The recombinant plasmids were transformed to *Escherichia coli* JM103, and transformants selected on L-agar (Maniatis et al. 1982) supplemented with 50 $\mu\text{g ml}^{-1}$ ampicillin and X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside).

Labeling of DNA probe. A cloned DNA fragment with a molecular weight of about 0.7 kilobase (kb) was selected, at random, for use as the probe, and was isotopically labeled with (α - ^{32}P)dCTP (New England Nuclear Corp., Boston, USA) by nick translation (Rigby et al. 1977).

The DNA probe was labeled with photobiotin (Biotechnology Research Enterprises S.A. Pty Ltd), and the Blu GENE nonradioactive DNA detection system (Bethesda Research Laboratories, USA) was used. Probe DNA was mixed with equal volumes of photobiotin acetate in an Eppendorf tube, irradiated for 20 min with a 400W lamp from a distance of 10 cm, and diluted to 100 μl with TE buffer (10 mM Tris HCl, 1mM EDTA, pH 8.0) followed by 100 μl 2-butanol. The mixture was centrifuged and the 2-butanol phase discarded. Photobiotin-labeled DNA was purified by 3 successive ethanol precipitations, and finally resuspended in TE buffer and stored at -20°C .

Southern and dot blot hybridization. For Southern blot hybridization, chromosomal DNAs of *Pasteurella piscicida*, *Aeromonas hydrophila*, *A. salmonicida*, and *Vibrio anguillarum* were cleaved by *Hind* III, and electrophoresed in 0.8% agarose gel at 80 mA for ca 3 h. Electrophoresed gels containing digested DNAs were transferred to nitrocellulose filters (0.45 μm , Schleicher & Schuell Inc., FRG) using a transfer pyramid for Southern blotting (Southern 1975).

For dot blot hybridization, chromosomal DNA from *Pasteurella piscicida* was denatured by heating at 90°C for 5 min and diluted from 125 μg to 1.95 $\mu\text{g ml}^{-1}$ with $2\times\text{SSC}$ ($1\times\text{SSC} = 0.15\text{ M NaCl} + 0.015\text{ M sodium citrate}$, pH 7.0). Samples of 250, 125, 62.5, 31.3, 15.7,

7.8, and 3.9 ng of DNA were blotted on nitrocellulose filters.

Hybridization with ^{32}P labeled DNA probe was carried out on filters at 65°C for 36 h in $4\times\text{SSC}$ containing $10\times\text{Denhardt's solution}$ (1% Ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin), 0.5% sodium dodecyl sulfate (SDS), and 1.5 mg ml^{-1} heat-denatured salmon-sperm DNA. Filters were washed with $2\times\text{SSC} + 0.1\%$ SDS, dried, and subjected to autoradiography. Hybridization with the photobiotin-labeled probe was carried out at 42°C for 12 to 18 h in a hybridization solution containing 45% formamide, $5\times\text{SSC}$, $1\times\text{Denhardt's solution}$, 25 mM sodium phosphate (pH 6.5), 5% dextran sulfate, and 0.2 mg ml^{-1} heat-denatured salmon-sperm. After hybridization, the probe-target DNA hybrid was detected with conjugate dyes of the Blu GENE (Bethesda Research Laboratories) streptavidin-alkaline phosphatase.

Colony hybridization. The organisms were inoculated onto autoclaved, gridded nitrocellulose filters (Toyo Roshi Co. Ltd, Japan) overlayed on an agar plate. After incubation, the filter was removed from the plate and treated with NaOH, neutralized, baked, and hybridized with probe DNA. Hybridization reactions were carried out by the method of Grunstein & Hogness (1975).

The minimum number of *Pasteurella piscicida* (Strain KG8601) cells detected was determined by hybridization with the same probe. Bacterial cells diluted 10-fold from 10^2 to 10^8 were spotted on a nitrocellulose filter. DNA hybridization was then performed as described by Grunstein & Hogness (1975).

Detection of *Pasteurella piscicida* in fish. Nineteen diseased and 3 healthy *Seriola quinqueradiata* were obtained from an experimental fishery station. These fish were dissected and examined to determine whether nodules of kidney and spleen, characteristic of the pseudotuberculosis, were present in the kidney and spleen of fish. Kidney and spleen tissue from each fish was sampled using a platinum loop and smeared onto nitrocellulose filters. Hybridization was then carried out with the same probe and by the same method as used in the colony hybridization procedure. At the same time, the causative agent was isolated on agar media and identified.

Restriction sites and nucleotide sequence of the probe. A cloned DNA fragment of *Pasteurella piscicida*, which was chosen at random and used as the probe, was digested by various endonucleases: *Av*II, *B*amHI, *E*coRI, *H*apII, *H*incII, *H*indIII, *H*infI, *H*paI, *R*saI, *S*alI, *S*au3AI, *S*maI, *T*aqI, and *X*hoI (Takara Shuzo Co. Ltd, Nippon Gene Co. Ltd, Japan). Nucleotide sequences of this cloned DNA fragment were determined using pUC9 by the dideoxy method of Hattori et al. (1985).

Fig. 1. Southern blot hybridization of ^{32}P -labeled cloned DNA fragment from *Pasteurella piscicida* with chromosomal DNAs of various fish pathogens. Panel 1: agarose gel electropherogram of chromosomal DNAs digested by *Hind*III. Panel 2: autoradiogram of a nitrocellulose filter blotted with DNA from Panel 1 and probed with ^{32}P labeled cloned DNA of *P. piscicida*. Lane A: standard molecular size of λ DNA digested by *Hind*III; Lane B: chromosomal DNA of *P. piscicida*; Lane C: chromosomal DNA of *Vibrio anguillarum*; Lane D: chromosomal DNA of *Aeromonas salmonicida*; Lane E: chromosomal DNA of *A. hydrophila*.

RESULTS

Screening of DNA probe

The molecular weights of the cloned DNA fragment obtained from 14 recombinant plasmids ranged from 0.4 to 4.0 kb. A cloned DNA fragment with a molecular size of about 0.7 kb was chosen as the probe for experiments.

Specificity of DNA probe

The specificity of the 0.7 kb fragment was verified by Southern blot hybridization with chromosome DNAs of various fish pathogens. The radio- and photobiotin-labeled probes of the 0.7 kb fragment only hybridized with themselves. Probe DNA did not hybridize with chromosomal DNA from other fish pathogens tested (Fig. 1).

Further colony hybridization experiments were carried out to determine the specificity of the 0.7 kb fragment. Four colonies of different strains of *Pasteurella piscicida* and 1 of *Escherichia coli* (carrying the recombinant plasmid contained the 0.7 kb fragment from *P. piscicida*) hybridized with the ^{32}P -labeled probe (Fig. 2). Others, i.e., *Aeromonas hydrophila*, *A. salmonicida*, *Edwardsiella tarda*, *Pseudomonas anguilliseptica*, *Vibrio anguillarum*, and *Yersinia ruckeri* did not hybridize (Fig. 2). No cross hybridization was seen between the probe and closely related *Haemophilus influenzae*, *Pasteurella haemolytica*, and *P. multocida* (Fig. 3).

Fig. 4 shows that the probe hybridized, not only with 29 strains of *Pasteurella piscicida* isolated from *Seriola quinqueradiata* cultured in various districts of Japan, but also with the 1 strain of *P. piscicida* isolated from white perch, *Morone americana*, in the USA. (Janssen & Surgalla 1968). Photobiotin-labeled probe DNA used for colony hybridization was unsuccessful due to the high background it produced.

Fig. 2. Colony hybridization of ^{32}P -labeled cloned DNA fragment from *Pasteurella piscicida* with chromosomal DNA of various fish pathogens. DNA samples of bacterial strains located above numbers. 1 to 4: *Vibrio anguillarum*; 5 to 8: *Aeromonas hydrophila*; 9 to 12: *A. salmonicida*; 13 to 16: *Edwardsiella tarda*; 17 to 20: *Pasteurella piscicida*; 21 to 22: *Yersinia ruckeri*; 23 to 24: *Pseudomonas anguilliseptica*; 25: *Escherichia coli* JM103; 26: *E. coli* JM103 carrying recombinant pUC9-0.7 kb fragment from *P. piscicida*

Quantitation of DNA and bacteria containing probe

The sensitivity of the probe technique was determined by dot blot hybridization with radio- and photobiotin-labeled probe DNA. The ^{32}P labeled DNA probe detected 3.9 to 250 ng of purified DNA in *Pasteurella piscicida*. The same amounts of DNA were detected by photobiotin-labeled probe DNA. Again, there was no hybridization with chromosomal DNAs of the other fish pathogens, *Aeromonas hydrophila*, *A. salmonicida* and *Vibrio anguillarum* (Fig. 5).

The number of *Pasteurella piscicida* cells detectable by the probe was also determined by colony hybridization using radiolabeled probe DNA and was from 10^5 to 10^8 cells (Fig. 6).

Fig. 3. Colony hybridization of ^{32}P -labeled cloned DNA fragment from *Pasteurella piscicida* with closely related pathogens. 1: *Pasteurella multocida* NCTC10322; 2: *P. haemolytica* NCTC9380; 3: *Haemophilus influenzae* NCTC8143; 4: *Pasteurella piscicida* KG8601; 5: *P. piscicida* Snieszko

Fig. 4. Colony hybridization of ^{32}P -labeled cloned DNA fragment from *Pasteurella piscicida* with different strains of *P. piscicida* isolated from various areas. 1 to 30 (except 24): different strains of *P. piscicida* isolated from diseased *Seriola quinqueradiata* from various locations in Japan; 24: strain of *P. piscicida* isolated from white perch, *Morone americana*, in the USA; 31: *Escherichia coli* JM103; 32: *E. coli* JM103 carrying recombinant pUC9-0.7 kb fragment from *P. piscicida*

Fig. 5. Dot blot hybridization of radio- and photobiotin-labeled probe with chromosomal DNAs of various fish pathogens. Panel 1: hybridization of ^{32}P -labeled cloned fragment DNA from *Pasteurella piscicida*. Panel 2: hybridization of photobiotin-labeled fragment DNA from *P. piscicida*. Lane A: chromosomal DNA of *Vibrio anguillarum*; Lane B: chromosomal DNA of *P. piscicida*; Lane C: chromosomal DNA of *Aeromonas hydrophila*; Lane D: chromosomal DNA of *A. salmonicida*

Fig. 6. Minimum number of of *Pasteurella piscicida* cells detectable using ^{32}P -labeled DNA fragment

Analysis of DNA Probe

Fig. 7 illustrates the endonuclease restriction sites and the nucleotide sequences of cloned DNA fragment of *Pasteurella piscicida*, (i.e., the probe). The cloned DNA fragment had single *Hap*II, *Hind*III, and *Taq*I sites, and double sites for *Rsa*I; there were no restriction sites for the other endonucleases, i.e., *Ava*II, *Bam*HI, *Eco*RI, *Hinc*II, *Hin*FI, *Hpa*I, *Pst*II, *Sal*II, *Sau*3AI, *Sma*I, and *Xho*I. The fragment contained 692

base pairs (bp) and the guanine + cytosine (G + C) content was 39.2 %.

Detection of *Pasteurella piscicida* in clinical specimens

All filters from diseased yellowtail proved positive, both by radiolabeled probe and by culture for *Pasteurella piscicida*. Some hybridizations results are

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Hind III
AAGCTTGTAGCTCTTGTGGAGTAATGCTGACAGAAGAACAATAGCCAGCCTTTGCGTGCCAGA
TATCTCAACCTGAAATAGAAAAGTGGGCCCTAGAGCTCACTTTTAGCCCCATGCTCGCTAA
TTTAGCGGGCAATTTTTATTTATATCACTACAATACATATTGTTGGCCATATCTATGAGT
AAACACAGCTAGAAAAAGAAGAAGAAACCACAAAGAAATGGCGTTCGGTTCAGCTTCAGTGC
TTTGTAGCTATGAGCAATATGAACACATGATATGTCATCAGTGTTAAATGCCAATTGATAC
TGCACCACCAAAGATCAGATGAAGAAATAACACCAATTTATCAATATCTTATGATAAGTTTG
GTGACACATCGACTACTGCATTCAAATTACATGAAGTATTAATCTCGCTTGCTCATTAGCAGA
GCGAGATGGTACATGACATACTAACAGTGATATGCATGTGTCCTAGAGCCACCAAACTCATCT
TTAATAAAGATGAGCTTGAAAAATATCTCAGCACTGTGGCGATAACATCACCACCAATCAA
ATAGAGTGATAGAAAAATGCTTCGCTTGCGGTACAGCAAAGTGGGCAGTAAGCACTACGA
ATGTGAAAACCCTTATTGTGAGCACCAGCAAAGTCATCTATAACTCTTGTAAGAGTAAAGCTT

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Fig. 7. Restriction sites and nucleotide sequence in the 692 base pair fragment of DNA from *Pasteurella piscicida* (the probe)

Fig. 8. Autoradiogram of ^{32}P -labeled cloned DNA fragment from *Pasteurella piscicida* hybridized on nitrocellulose filter with smeared kidney and spleen tissues from diseased (i.e., 1 to 6) and healthy (7 to 9) *Seriola quinqueradiata*. For each yellowtail Smears 1 to 5 represent kidney tissue, Smear 6 to 9 spleen tissue

shown in Fig. 8. The hybridization signal strength appeared to be a function of pathogen concentration in tissues. Healthy fish proved negative both by the probe and culture techniques.

DISCUSSION

A 692 bp fragment of chromosomal DNA from *Pasteurella piscicida* was cloned in the pUC9 plasmid and used as a probe for detecting *P. piscicida*. The probe only hybridized with itself. There was no hybridization with any other tested fish pathogens or with closely related organisms. The 692 bp nucleotide sequence differed from any of the sequences in the nucleotide sequence library of GENAS (Kuhara et al. 1984).

The probe was successfully used to detect *Pasteurella piscicida* on filters smeared with kidney and spleen tissues from diseased *Seriola quinqueradiata*.

All smears from diseased yellowtail yielded positive reactions, the strength of reaction apparently being related to the number of *P. piscicida* cells in the smear. We did not determine the detecting limit of the filter hybridization procedure for the pathogen, but we think that it is lower than the 10^5 cells per sample detected by the direct hybridization method in this study. This method allowed us to rapidly detect and identify *P. piscicida* in infected tissues and to rapidly identify colonies of the pathogen on culture plates.

The photobiotin-labeled probe used in these experiment was successful in detecting the chromosomal DNA of *Pasteurella piscicida* by dot blot hybridization. However, the photobiotin-labeled probe could not be used to detect *P. piscicida* by colony hybridization. In colony hybridization, some component of the bacterial cells on the nitrocellulose filter appeared to bind, non-specifically, with the coloring reagents of the detection system, thus resulting in unacceptably high background signals.

The colony hybridization technique is more easily and quickly applied than the dot blot hybridization method. However, radiolabeled probes are generally unacceptable for routine clinical use and further development of detection procedures based on non-isotope labeling is, therefore, still required.

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