Rapid Detection of the Fish-Pathogenic Bacterium *Pasteurella piscicida* by Polymerase Chain Reaction Targetting Nucleotide Sequences of the Species-Specific Plasmid pZP1.

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(Received October 24, 1996)

A species-specific plasmid (pZP1) has been isolated from the fish-pathogenic bacterium *Pasteurella piscicida*. Two DNA fragments of PZP1 (PZP1-1 with 964 bp and PZP1-4 with 477 bp) were cloned from the plasmid pZP1. Two 20-mer primer sets, PZP1-1a and PZP1-1b, and PZP1-4a and PZP1-4b, were constructed according to the nucleotide sequences of fragments PZP1-1 and PZP1-4. A 484-bp DNA fragment was amplified from template DNA from 40 strains of *P. piscicida* by PCR using the PZP1-1a/1b primer set. The strains were isolated at different times of the year at different places in Japan and the USA. A 321-bp PCR product was amplified from all of the above strains of *P. piscicida* except strain ATCC17911 using the PZP1-4a/4b primer set. No such PCR products were obtained from template DNAs of *Beneckeia proteolytica*, *Photobacterium damsela*, *Ph. histaminum*, *Ph. leiognathi*, 15 standard strains of *Vibrio* spp. or four fish pathogenic bacteria (*Aeromonas hydrophila*, *A. salmonicida*, *Edwardsiella tarda* and *Enterococcus seriolicida*). The PCR products were amplified with the PZP1-1a/1b primer set from DNA from the kidney of yellowtail (*Seriola quinqueradiata*) infected naturally with *P. piscicida*, but not from DNA from healthy yellowtail.

Key words: *Pasteurella piscicida*, PCR, diagnosis, plasmid

Control of bacterial infection in the intensive fish-culture industry requires a rapid, sensitive and specific method for identifying and detecting fish-pathogenic bacteria. Standard culture techniques are time-consuming, and immunological methods are not sufficiently sensitive and specific for detecting fish pathogens. In addition, these methods cannot easily detect fish-pathogenic bacteria from the early stage of infection.

The polymerase chain reaction (PCR) (Saiki *et al.*, 1985; Mullis and Faloona, 1987) is an *in vitro* method of nucleic acid synthesis by which a particular segment of DNA can be specifically replicated. Species-specific primer sets for the identification and detection of fish-pathogenic bacteria by PCR have been developed for many pathogens, including *Aeromonas salmonicida* (Miyata *et al.*, 1995), *Edwardsiella tarda* (Aoki and

Hirono, 1995), *Cytophaga psychrophila* (Toyama *et al.*, 1994), *Renibacterium salmoninarum* (Brown *et al.*, 1994), *Vibrio anguillarum* (Hirono *et al.*, 1996), and *Vibrio trachuri* (Iwamoto *et al.*, 1995).

Pasteurella piscicida is the causative agent of pseudotuberculosis in yellowtail, *Seriola quinqueradiata* (Kimura and Kitao, 1971). Pseudotuberculosis is one of the most important causes of mortality in yellowtail. We developed a primer set for the amplification of a species-specific 629-bp DNA fragment (Pp1) from the chromosome of *P. piscicida* (Aoki *et al.*, 1995). However, the same size PCR product was detected in some species of the genus *Vibrio* by PCR using this primer set under low-stringent PCR conditions.

Previously, we also found a species-specific plasmid, pZP1, of *P. piscicida* (Zhao and Aoki, 1992). In the present study, two primers were selected from the region of the species-specific fragment of pZP1. We demonstrate their use for the rapid identification and detection of *P. piscicida* by PCR.

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Materials and Methods

Bacterial strains

The bacterial strains used in this study are listed in Tables 1 and 2. *Escherichia coli* JM109 were cultured in 2× YT broth (1.6% tryptone, 1.0% yeast extract, 0.5% NaCl) at 37°C for 24 h. All other strains were cultured in brain-heart-infusion (BHI) broth and BHI agar (Difco Laboratories, USA) containing 2% NaCl at 25°C for 24 h.

DNA isolation

Bacterial total DNA was isolated by a modification of the method described by Ausubel *et al.* (1987). Plasmid DNA was prepared as described by Sambrook *et al.* (1989). Concentration of the purified DNA was determined using the Gene Quant system (Pharmacia, Sweden). Total DNAs from *P. piscicida* were extracted by a boiling method (Aoki and Hirono, 1995).

Subcloning of pZPI

The *P. piscicida*-specific plasmid pZPI (Fig. 1) (Zhao and Aoki, 1992) was cloned into plasmid vector pUC19.

The cloned pZPI DNA was digested with *Hinc*II or with a combination of *Hinc*II and *Eco*RI. The DNA fragment digested with *Hinc*II was self-ligated by using a DNA Ligation Kit Ver. 2 (Takara, Japan). The DNA fragment digested with *Hinc*II and *Eco*RI was ligated with pUC119.

Colony hybridization

Horseradish peroxidase-labelled single-strand DNA probes were prepared using ECL direct nucleic acid labelling and detection systems (Amersham, UK) according to the manufacturer's instructions.

Colony hybridization was carried out by a modification of the method described by Grunstein and Hogness (1975). Bacterial colonies on BHI agar were picked up with a sterilized toothpick and fixed on a nylon membrane (Hybond N⁺, Amersham, UK). Duplex membranes were prepared and hybridized with the peroxidase-labeled DNA probe. Hybridization and washing conditions were as described in the ECL direct nucleic acid labelling and detection systems.

Table 1. Bacterial strains used in thi study

Strains		Source and references
<i>Pasteurella piscicida</i>	see table 2	
<i>Beneckea proteolytica</i>	NCBC1326	Aoki <i>et al.</i> , 1989
<i>Photobacterium damsela</i>	ATCC33539	Smith <i>et al.</i> , 1991
<i>Ph. histaminum</i>	JCM8968 T	Okuzumi <i>et al.</i> , 1994
<i>Ph. leiognathi</i>	ATCC25521	Aoki <i>et al.</i> , 1989
<i>Vibrio alginolyticus</i>	NCMB1903	"
<i>V. anguillarum</i>	ATCC19264	"
<i>V. campbellii</i>	ATCC25920	"
<i>V. costicola</i>	NCMB701	"
<i>V. diazotrophicus</i>	ATCC33466	"
<i>V. fischeri</i>	ATCC7744	"
<i>V. gazogenes</i>	ATCC29988	"
<i>V. harveyi</i>	ATCC14126	"
<i>V. hollisae</i>	JCM01283	"
<i>V. natriegens</i>	CCM2575	"
<i>V. nereis</i>	ATCC25917	"
<i>V. ordalii</i>	ATCC33509	"
<i>V. parahaemolyticus</i>	ATCC17802	"
<i>V. pelagius</i>	ATCC25916	"
<i>V. vulnificus</i>	ATCC27562	"
<i>Aeromonas hydrophila</i>	ATCC7966	Popoff M., 1984
<i>A. salmonicida</i>	ATCC14174	Smith, I. W., 1963
<i>Edwardsiella tarda</i>	MZ8901	in this study.
<i>Enterococcus seriolocida</i>	NG8206	in this study.
<i>Escherichia coli</i>	Mos Blue carrying pMos Blue	Amersham, USA
<i>E. coli</i>	JM109 carrying pUC19	Sambrook <i>et al.</i> , 1989
<i>E. coli</i>	JM109 carrying pUC119	Sambrook <i>et al.</i> , 1989

Table 2. Amplification of species-specific DNA fragment from various strains of *Pasteurella piscicida* by PCR with primer sets of PZP1-1a/1b and PZP1-4a/4b

Strains	Source and reference	amplification with primer		
		PZP1-1a/1b	PZP1-4a/4b	Pp1 primers* ¹
ATCC 17911	Snieszko <i>et al.</i> (1964)	+* ²	—	+
KP9016	Kagoshima (1990)	+	+	+
KP9038	Kagoshima (1990)	+	+	+
KP9406	Kagoshima (1994)	+	+	+
KP9409	Kagoshima (1994)	+	+	+
KP9411	Kagoshima (1994)	+	+	+
KP9422	Kagoshima (1994)	+	+	+
KP9432	Kagoshima (1994)	+	+	+
KP9445	Kagoshima (1994)	+	+	+
KP9464	Kagoshima (1994)	+	+	+
KP9435	Kagoshima (1994)	+	+	+
KP9433	Kagoshima (1994)	+	+	+
KP9483	Kagoshima (1994)	+	+	+
KP9511	Kagoshima (1995)	+	+	+
KP9550	Kagoshima (1995)	+	+	+
KP9578	Kagoshima (1995)	+	+	+
KP9579	Kagoshima (1995)	+	+	+
KP9580	Kagoshima (1995)	+	+	+
SP90-078	Ehime (1990)	+	+	+
SP90-043	Ehime (1990)	+	+	+
SP91-142	Ehime (1991)	+	+	+
SP91-041	Ehime (1991)	+	+	+
SP91-149	Ehime (1991)	+	+	+
SP91-278	Ehime (1991)	+	+	+
SP93-052	Ehime (1993)	+	+	+
SP93-041	Ehime (1993)	+	+	+
SP95-096	Ehime (1995)	+	+	+
SP95-098	Ehime (1995)	+	+	+
SP95-102	Ehime (1995)	+	+	+
SP95-103	Ehime (1995)	+	+	+
SP95-109	Ehime (1995)	+	+	+
NA90-09	Nagasaki (1990)	+	+	+
NA90-25	Nagasaki (1990)	+	+	+
NA90-29	Nagasaki (1990)	+	+	+
NA90-30	Nagasaki (1990)	+	+	+
NP91-10	Nagasaki (1991)	+	+	+
NP91-45	Nagasaki (1991)	+	+	+
91-278	USA (1991)	+	+	+
94-110	Supplied from Dr. P. Hawke USA (1994)	+	+	+
94-165	Supplied from Dr. P. Hawke USA (1994)	+	+	+
	Supplied from Dr. P. Hawke			

+: amplified, -: not amplified.

*¹ Aoki *et al.*, (1995)*² The molecular size of PCR product differed from that of other tested strains of *P. piscicida*.

DNA Sequencing

Dideoxy sequencing (Sanger *et al.*, 1977) of the cloned DNA fragments was carried out using the Thermo Sequenase fluorescent-labelled primer cycle sequencing

kit with 7-deaza-dGTP (Amersham, UK). Electrophoresis and data collection was performed on a DNA sequencer, Model 4000L (LI-COR, USA).

Fig. 1. Restriction map of the common plasmid pZP1 isolated from *Pasteurella piscicida*.

Black bar region (PZP1-1) was ligated into pUC19.

White bar region (PZP1-4) was ligated into pUC119.

Identification test using the synthesized primer sets

Total DNAs from four strains of *P. piscicida* (ATCC 17911, KP 9038, SP 91-142 and 91-278), the above-mentioned single strain of *B. proteolytica*, 3 strains of *Photobacterium* spp., 15 strains of *Vibrio* spp., and four species of fish-pathogenic bacteria (*A. hydrophila*, *A. salmonicida*, *E. tarda* and *E. seriolocida*) were prepared as template DNAs to examine the species specificity of our designed primer sets. PCR was carried out using 1 ng of template DNA, 0.2 mM of dNTP mixture, 0.5 μ M of each primer, 0.5 unit of Ex Taq polymerase (Takara, Japan) and 2 μ l of Ex Taq buffer, in a total volume of 20 μ l. Following initial denaturation at 95°C for 30 to 120 s, 30 thermal cycles consisting of 30 s at 95°C to denature, 30 s at 58°C to anneal and 30 s at 72°C to extend were performed. The samples were post-heated at 72°C for 5 min after thermal cycling. The PCR products were mixed with 0.2 volumes of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol) and electrophoresed on a 1.0% agarose gel. The gel was stained with ethidium bromide and visualized under UV light.

The PCR conditions for the primer set Pp1 were the same as the above conditions except for the annealing temperature, which was 62°C. The primer set Pp1 is

described by Aoki *et al.* (1995).

Determination of PCR sensitivity

The minimum amounts of *P. piscicida* DNA or *P. piscicida* cells that are needed to produce a PCR product using the PZP1-1a/1b and PZP1-4a/4b primer sets were determined. Extracted total DNA of *P. piscicida* SP91-142 was diluted 10 times (from 1 ng/ μ l to 100 ag/ μ l) and used for PCR. *Pasteurella piscicida* SP91-142 cells were diluted 10^6 times (from 1×10^7 cells/ml to 1×10 cells/ml) and 1 ml of the diluted cell suspension was boiled for 5 min. Aliquots of the supernatants (1 μ l) were used for PCR templates. Secondary PCR was carried out using 1 μ l of the first PCR product as a template.

Detection of *P. piscicida* from naturally infected fish

Five yellowtail, with a mean body weight of 61 g and whose head kidneys and spleens had white spot tubers, were collected from fish farms in Ehime in 1996. Another five fish without white spot tubers were used as a negative control. Template DNAs were extracted from the kidney homogenates of these fish. At the same time, the bacterial isolation from the kidneys of both groups was attempted using BHI agar containing 2% NaCl.

Results

A HincII-digested DNA fragment of pZP1 (designated PZP1-1) hybridized with tested 14 strains of *P. piscicida* (ATCC 17911, KP 9038, SP 91-142, 91-278, KP 9511, KP 9550, KP 9578, KP 9579, KP 9580, SP 95-096, SP 95-098, SP 95-102, SP 95-103 and SP 95-109) but did not hybridize with the single strain of *B. proteolytica*, 3 strains of *Photobacterium* or 15 strains of *Vibrio* spp.

A HincII- and EcoRI- digested DNA fragment (designated PZP1-4) hybridized with all of the tested strains of *P. piscicida* except ATCC17911, but did not hybridize with the single strain of *B. proteolytica*, 3 strains of *Photobacterium* or 15 strains of *Vibrio* spp.

The nucleotide sequences of PZP1-1 (947 bp) and PZP1-4 (477 bp) are shown in Figs. 2 and 3, respectively. The forward primer (PZP1-1a) and the reverse primer (PZP1-1b) were the 20-mers 5'-GCCCCATTCCAGT-CACACA-3' and 5'-TCCCTAAGCACACCGACAGG-3', respectively, both constructed from the nucleotide sequences of PZP1-1 (Fig. 2). The other primers were based on the nucleotide sequence of PZP1-4. The forward primer (PZP1-4a) and the reverse primer (PZP1-

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gtcgacATGA CTTTACACAC AATCGCCTAA TTAGAATGAG ATCGCAGAAA GATACGCTCG 60
Hinc II site
CGCTCAATAA GATGTTTATA TGATTTTGAA ATAGAAAAGC CGAGCAAAAA GGAAGTCGCC 120
AAAGGTCAGC TTGATTGCCT TTCGGCTGCG CCCCCCTACC CAATTTTCAGC TAAATTTTAG 180
CGAAATCGAG TATCTTTTGT GTCGCATCAG ATCGGGGTTT GTCTCTTACC CTTTCGGGCG 240
TTCCTGCGCT GTGTGTAAAG GCTTTCGCCC CCATTCCAGT CACACAATCT TCGAGGTTAT 300
                                     PZP1-1a
TATCATTAAG TCGCAGCCCC AACCTGAACG CTATAGTTGG GCGACACATT ACGTCATTTT 360
CCCGAAGACC TTTACCTCCA CCTATGGAAT ATCCCTTGAT CGGTGGTGAT GCGCATCGCT 420
CTATCCAAAA CATCTTGATC CAAATAGAGT GCAGGAGTAC TTGAAGTGCT TCTATGAATT 480
GTCTATCATG AAAGGACTTA ACGAGCTTCG AAAATCGTCT TAAGTATTC GAATAAACCA 540
GCACGCCAAT GCTGGTTTTT TCACATCTGG ACAAAGATAC TACTCTGCCA CTCAAATATA 600
CTCAAGCACA ACCTCTCACC CATCAATCAA ACCGCAAAAA CAGAATAATC TAAGTAAAC 660
ATAACCTTAT TATACTTTTT TCTTTAATAT ATCATGCTAT CTCGTAGAGA TTTTAGGGTT 720
TTAGGGAAAA TCCCTAAGCA CACCGACAGG GCACGACGAT TAAACAAACG AGGAACGAGT 780
                                     PZP1-1b
TTGTATACAA TCGGTAGCGT GCCTGTATAC GTCGGTAGCG TGCCTATATT AATTAAGCTC 840
TAAAGTGTGA CCGCCCCCAC AGATTCACCA CAAATCCCC AAAAGTTCAC CTAGAAATCA 900
CCACACATTC CCCATTTCAA TAGTTGATAT TGCAAAAGT TCCCCATAAA ATCACTACga 960
                                     EcoR I site
attc 964

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Fig. 2. The nucleotide sequence of PZP1-1 cloned from pZP1 of *Pasteurella piscicida*. The nucleotide sequences of the forward and reverse primers are underlined.

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gaattcACCA TAAATCCCC ATGAGTTCAC CAAGGACAA CAAATGAACG AAGAAGAGTT 60
EcoR I site
AAAAACCTTA TTATCACCTT TTATTAGAGA CATTATGGT GGTGTTTTAA GTAATTTAGC 120
CTGTATCAAA ACACATGCTG AATTACTGTT ATCACTACGT GCAAAAGGTG TCCCTTTAAG 180
                                     PZP1-4a
GCTGATTGTT GAATTATCAG GAACTAGTTA TAGCAATAAA ACCTTTAGTA AAAAGCTAAG 240
TGGCTTTAAA AAAAGCCTAG CTATTAAACC AAAAGAAATG CCAACAGCAG AAAAAGGGAC 300
TTTTTCAAAA GCGGCTTTGA GTTCTTCTAA ACCAGAACT AAACAGGAAC CGATTAAGCC 360
TGACAACAAA GAAGAATCGG ACGAATTAGA ATATAGCTTA GATGATTGGA TTAGTAACAC 420
TCGCCTAAGT AAAGGAAACA TTCGCCTCTA TCGACAAGGA GAGAAAGACG Ggttgac 477
                                     PZP1-4b Hinc II site

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Fig. 3. The nucleotide sequence of PZP1-4 cloned from pZP1 of *Pasteurella piscicida*. The nucleotide sequences of the forward and reverse primers are underlined.

4b) were the 20-mers 5'-CTACGTGCAAAAGGTG-TTCC-3' and 5'-AAGGAGAGAAAGACGGGTTG-3', respectively (Fig. 3).

A specific DNA fragment having a length of 484 bp was amplified by PCR with the PZP1-1a/1b primer pair from 4 strains of *P. piscicida*, although the amplified DNA from ATCC17911 was larger than that of the other strains of *P. piscicida* (Fig. 4). A 484-bp PCR product was subsequently obtained from boiled-extracted DNA from 36 strains of *P. piscicida* using the PZP1-1a/1b primer pair (Table 2). No fragment was amplified from the above-mentioned 15 strains of *Vibrio* spp., 3 strains

of *Photobacterium*, 1 strain of *B. proteolytica* or 4 strains of fish-pathogenic bacteria (Fig. 4).

On the other hand, a 321-bp PCR product with primers PZP1-4a/4b was obtained from 3 strains of *P. piscicida* but not from strain ATCC17911 (Fig. 5). The PZP1-4a/4b primer pair also amplified products from all other strains of *P. piscicida* (Table 2). A similar sized DNA fragment was amplified from *B. proteolytica*, *Ph. leiognathi*, *V. anguillarum*, *V. diazotrophicus*, and *V. hollisae* (Fig. 5). The amounts of PCR products from *B. proteolytica*, *Ph. leiognathi*, *V. anguillarum*, *V. diazotrophicus*, and *V. hollisae* were lower than that from

Fig. 4. Agarose gel electrophoresis of PCR products using the *P. piscicida*-specific primer set, PZP1-1a and 1b. Lanes: M, ϕ X 174-DNA digested with *Hae*III as a molecular size marker; 1, *Pasteurella piscicida* ATCC17911; 2, *P. piscicida* KP9038; 3, *P. piscicida* SP91-142; 4, *P. piscicida* SP91-278; 5, *Photobacterium damsela* ATCC33539; 6, *Ph. histaminum* JCM8968; 7, *Ph. leiognathi* ATCC25521; 8, *Beneckeia proteolytica* NCMB1903; 9, *Vibrio alginolyticus* NCMB1903; 10, *V. anguillarum* ATCC19264; 11, *V. campbellii* ATCC25920; 12, *V. costicola* NCMB701; 13, *V. diazotrophicus* ATCC33466; 14, *V. fischeri* ATCC7744; 15, *V. gazogenes* ATCC29988; 16, *V. harveyi* ATCC14126; 17, *V. hollisae* JCM01283; 18, *V. natriegens* CCM2575; 19, *V. nereis* ATCC25917; 20, *V. ordalii* ATCC33509; 21, *V. parahaemolyticus* ATCC17802; 22, *V. plagius* ATCC25916; 23, *V. vulnificus* ATCC27562; 24, *Aeromonas hydrophila* ATCC7976; 25, *A. salmonicida* ATCC14174; 26, *Edwardsiella tarda* MZ8901; 27, *Enterococcus seriolicida* NG8206.

Fig. 5. Agarose gel electrophoresis of PCR products using the *P. piscicida*-specific primer set, PZP1-4a and 4b. Lanes: M, ϕ X 174-DNA digested with *Hae*III as a molecular size marker; numbers in lanes 1–27 refer to strains shown in Fig. 4.

P. piscicida (Fig. 5).

A 629-bp PCR product was detected from 4 strains of *P. piscicida* and *Ph. damsela* when using the primer set Pp1 (Fig. 6). In addition, when PCR was performed with an annealing temperature of 58°C, the PCR product was detected in 4 strains of *P. piscicida*, the single strain of *B. proteolytica*, 3 strains of *Photobacterium* spp., and 15 strains of *Vibrio* spp. (data not shown).

The minimum amount of DNA detectable in the first PCR using the PZP1-1a/1b and PZP1-4a/4b primer sets was 100 fg. However, using the secondary PCR method, the minimum amount of DNA was 10 fg (data not shown). The products obtained from the PZP1-1a/1b and PZP1-4a/4b primer pairs required a minimum of

10^3 bacterial cells/ml in the first PCR, but only 10^2 cells/ml in the second PCR (data not shown).

Two DNA fragments having lengths of 484 and 530 bp were obtained from DNA extracted from kidney homogenates of all 5 yellowtail in which *P. piscicida* was detected by a standard culture method (Fig. 7, lanes 3 to 7). On the other hand, these specific fragments were not detected in five apparently healthy yellowtail in which *P. piscicida* was not detected by a standard culture method (Fig. 7, lanes 8 to 12).

Fig. 6. Agarose gel electrophoresis of PCR products using the Pp1 primers set.

Lanes: M, ϕ X 174-DNA digested with *Hae*III as a molecular size marker; numbers in lanes 1–27 refer to strains shown in Fig. 4.

Fig. 7. Detection of *Pasteurella piscicida* from naturally infected yellowtail and apparently healthy yellowtail by PCR with the PZP1-1a/1b primer set.

Lanes: M, ϕ X 174-DNA digested with *Hae*III as a molecular size marker; 1, strain ATCC17911; 2, strain KP9038; 3 to 7, naturally infected yellowtail; 8 to 12, apparently healthy yellowtail which were not infected with *P. piscicida* cells.

Discussion

Gauthier *et al.* (1995) proposed reclassifying *Pasteurella piscicida* as a new species, *Photobacterium damsela* subspecies *piscicida*, based on its 16S rRNA sequences. Previously, we reported a primer set for the amplification of a 629-bp DNA fragment (Pp1) from the chromosome of *P. piscicida* (Aoki *et al.*, 1995). In the present study, however, this primer set amplified the same size PCR product from *Ph. damsela* even under the high-stringent PCR condition (Fig. 6). In addition, under the low-stringent PCR condition, the same size PCR product was amplified from several species of the genus *Vibrio*. In this study, we tried to amplify products by PCR using our PZP1-1a/1b and PZP1-4a/4b primer sets and template DNAs from *B. proteolytica*, *Photobacterium* spp. and *Vibrio* spp. The primers PZP1-1a and 1b amplified a 484-bp sequence from only *P. piscicida* and did not amplify a DNA fragment of any size from *B. proteolytica*, *Photobacterium* spp., or *Vibrio* spp. This sequence therefore appears to be specific for *P. piscicida* and the primers PZP1-1a and 1b can be used for identification of *P. piscicida*. On the other hand, the primers PZP1-4a and 4b amplified a 321-bp DNA fragment from each of the tested strains of *P. piscicida* except ATCC17911, and also amplified a similar sized DNA fragment from *B. proteolytica*, *Ph. leiognathi*, *V. anguillarum*, *V. diazotrophicus*, and *V. hollisae* (Fig. 5). This result suggests that *B. proteolytica*, *Ph. leiognathi*, *V. anguillarum*, *V. diazotrophicus*, and *V. hollisae* possess a sequence homologous to the primers PZP1-4a and 4b.

Zhao and Aoki (1992) reported that all Japanese isolates of *P. piscicida* examined carried pZP1. We have found that strain ATCC17911 does not carry this plasmid (Zhao and Aoki, 1992). When using the PZP1-1a/1b primer set, the amplified DNA from ATCC17911 was larger in size than that from other strains of *P. piscicida* including 3 strains isolated in the USA (Fig. 4, Table 2). We cloned the PCR products from ATCC17911 to the pMOSBlue T-vector kit (Amersham, USA) and determined its nucleotide sequence. The length of the larger sequence from ATCC17911 was 530 bp. Strain ATCC17911 was isolated from white perch in the 1963 in the U. S. A. (Snieszko *et al.*, 1964). It is thought that a partial region of pZP1 might have become integrated into the chromosomal DNA of *P. piscicida* ATCC17911. In an experiment to see if *P. piscicida* could be detected in naturally infected yellowtail, we obtained two PCR products from *P. piscicida*-infected

fish using the PZP1-1a/1b primer set. One was 484 bp and the other was 530 bp in length (Fig. 7). The 484-bp sequence was recognized as part of PZP1 and the 530-bp sequence was recognized as part of the chromosomal DNA. Further work is needed to explain this result.

In the experiment to determine the minimum number of bacterial cells for a PCR product, a PCR product was obtained from the 2nd PCR using 0.1 cell in the reaction. This indicates that the copy number of pZP1 is greater than 10 copies per one cell and suggests that the copy number of the PCR target region is important for the sensitive detection of fish infected with bacteria.

PCR amplification by *P. piscicida*-specific primers was able to detect diseased fish infected with *P. piscicida*. The specific *P. piscicida* primers PZP1-1a and 1b could be used widely for detection of etiological agents from diseased fish and water at hatcheries and farms.

Acknowledgements

This research was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan. We are grateful to Kagoshima Prefectural Fisheries Experimental Station, Nagasaki Prefectural Institute of Fisheries, and Ehime Prefectural Fish Disease Control Center for kindly providing strains of *Pasteurella piscicida*.

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