Identification of *Cytophaga psychrophila* by PCR Targeted 16S Ribosomal RNA

Takashi Toyama^{*1}, Kumiko Kita-Tsukamoto^{*2}
and Hisatsugu Wakabayashi^{*1}

^{*1} Department of Fisheries, Faculty of Agriculture, University of Tokyo,
Yayoi 1-1-1, Bunkyo-ku, Tokyo 113, Japan

^{*2} Ocean Research Institute, University of Tokyo,
Minamidai 1-15-1, Nakano-ku, Tokyo 164, Japan

(Received August 31, 1994)

On the basis of the 16S rRNA sequence data analysis among the closely related species, the specific primers for *Cytophaga psychrophila* were constructed. The specificity in amplifying the 16S rRNA of *C. psychrophila* was confirmed by using some selected strains of the related species. In addition, it was revealed that these specific primers distinguished *C. psychrophila* from other principal fish pathogens. The present PCR technique is expected to be a powerful tool for the diagnosis of cold-water disease.

Cytophaga psychrophila, the causative agent of cold-water disease, was originally isolated from coho salmon (*Oncorhynchus kisutch*) in Washington State, USA, in 1948 (Borg 1960). Outside of the North America, Bernardet and Kerouault (1989) reported *Flexibacter psychrophilus* (syn. *C. psychrophila*) in diseased rainbow trout (*Oncorhynchus mykiss*) in France. In Japan, it was isolated from coho salmon, rainbow trout and ayu (*Plecoglossus altivelis*) (Wakabayashi *et al.*, 1991, 1994). The epizootic has been spreading over Japan, especially among ayu in both farms and rivers.

So far, identification of *C. psychrophila* has been generally conducted on the basis of its phenotypical and/or serological characteristics (Bernardet *et al.*, 1989; Wakabayashi *et al.*, 1994). Recently, many reports have been published on the 16S rRNA sequences of various bacteria and the phylogenetic relationships deduced from analyses of these sequences, and some particular regions of the 16S rRNA sequences are known to be species or genus specific (Nishimura *et al.*, 1992). When we use the specific primers for *C. psychrophila*, they enable us a rapid and accurate identification, and provide a useful tool in the epidemiological investigations.

The aim of the present study is to construct the specific primers for *C. psychrophila* based on the 16S rRNA sequences, and to use them for identification of *C. psychrophila* by means of PCR.

Materials and Methods

Construction of the specific primers for *C. psychrophila*

The species selected for construction of the specific primers are listed in Table 1. These bacteria were selected according to Nakagawa's Ph. D dissertation (1993), in which, on the basis of 16S rRNA sequences, they were classified from cluster 9 through 12 phylogenetically. The 16S rRNA sequence data of all bacteria from cluster 9 through 12 were taken by accessing Nucleotide Sequence Database (GenBank). The selected data were dealt with multiple alignment analysis (CLUSTAL V) (Higgins *et al.*, 1992), and the specific regions for *C. psychrophila* found out and confirmed the specificity by FASTA (Lipman *et al.*, 1985). The specific primers were synthesized by Sawady Technology Co., Tokyo, Japan according to our profile. These primers, designated PSY1 (5'-CGATCCTACTTGCGTAG-3') and PSY2 (5'-GTTGGCATCAACACACT-3'), were located at positions corresponding to positions 190 to 206 and 1278 to 1262, respectively, on the *Escherichia coli* 16S rRNA numbers. And universal primers conserved in all 16S rDNAs, designated 20F (5'-AGAGTTTGATCATGGCTCAG-3') and 1500R (5'-GGTTACCTTGTTACGACTT-3'), were also employed (Weisburg *et al.*, 1991).

Table 1. Species selected for construction of the specific primers for *C. psychrophila* and their sequences corresponding to the positions of the primers

Species	Cluster ^{*1}	Sequence	
		190–206 ^{*2}	1278–1262 ^{*2}
<i>C. psychrophila</i>	12	5'-GTTGGCATCAACACACT-3'	5'-CGATCCTACTTGCGTAG-3'
<i>F. branchiophilum</i>	12	* ⁴ XX·C· · · · GGTTTTTCXC	· · C· · · · · CA· · · · ·
<i>C. columnaris</i>	12	TA·X· · · C· · · TTTG·T·	· · C· · · · · · · · · ·
<i>F. aquatile</i>	12	AA· · · · · · · TTTT·TA	· · C· · · · · GC· · · · · C· ·
<i>C. aquatilis</i>	12	CA· · · · · · GC·AC· · GC	· · · · · · · GG·C· · CC· ·
<i>C. flevensis</i>	12	TG·T· · · · GGXXXAGXG	· · · · · · · GG·CA·CC· ·
<i>Fx. maritimus-1</i> ^{*3}	10	TACTA·C·XGTACT·T·	G·CAT·CTG· · A·CAG·
<i>Fx. maritimus-2</i> ^{*3}	10	AA· · · · · · GTTTT·AA	· · C· · TCTG· · CAGA
<i>F. indologenes</i>	9	· · ATAGGGAT·A· · CTG	T·CAT·ACA·C· · TGT·
<i>F. breve</i>	9	AA· · · · · · TTTA·T·	· · CAT· · TG· · · · CA· ·
^{*5} -----			
<i>Fx. aurantiacus</i>	12	AG· · · · · · CTTT·TA	· · C· · · · · GG·CA·CC· ·
<i>C. johnsonae</i>	12	AGX· · · · · XCTTT·TA	· · · · · · · GG·CA·CC· ·
<i>C. pectinovora</i>	12	XXXC· · · · G· · TCXG· ·	· · · · · · · GG·C· · CC· ·
<i>C. saccharophila</i>	12	AA· · · · · · TTTTGT A	· · · · · · · GG·C·ACC· ·
<i>C. succinicans</i>	12	AA· · X· · · · TTTTCAC	· · · · · · · GG·CA·CC· ·
<i>C. sp.</i>	12	AG· · · · · · CTTA·TA	· · · · · · · GG·X· · CC· ·
<i>F. odoratum</i>	11	AA· · · · · · XGTTTXTA·	· · · AT·C·G· · · · C·G·
<i>C. uliginosa</i>	10	TAC· · · · · · GTATTTA·	· · C· · TGCG· · · · CGCA
<i>C. latercula</i>	10	· A· · · · · · TTTA·TC	· · X· · · · T· · A·XAA· ·
<i>C. marinoflava</i>	10	· A· · · · · · TC·TTTTAA	· · X· · · · TA·C· · TA· ·
<i>C. lytica</i>	10	TA· · · · · · TTTA·A·	· · C· · · · TA·C· · TA· ·
<i>F. meningosepticum</i>	9	AC· · · · · · GTTA·TA	T·CAT·CTA·C· · TAG·
<i>F. balustinum</i>	9	XXX· · · · · · GTXXXXXA	· · CAT·C·G·C· · C·G·
<i>F. indoltheticum</i>	9	AG· · · · · · CTTATTA	· · CAT·CTA·C· · TAG·
<i>F. gleum</i>	9	AG· · · · · · CTTA·TA	T·CAT·ACT·C· · AGT·

^{*1} Clusters based on 16S rRNA sequences according to Nakagawa's Ph. D. dissertation (1993).

^{*2} *Escherichia coli* numbering.

^{*3} Two different data can be accessed.

^{*4} X in the sequence indicates uncertainty in the determination.

^{*5} The species above this line were used for the PCR, and the species below this line not.

Bacterial strains used for the PCR

The bacterial strains used for the PCR are listed in Table 2. A total of 33 strains was consisted of 23 strains of *Flavobacterium-Cytophaga* complex from cluster 9 through 12 and 10 strains of fish pathogens belonging to the other genera. The strains are mostly part of our fish pathogen collection (FPC). All strains belonging to the genus *Cytophaga* or *Flavobacterium* were grown in tryptone and yeast extract (TYE) broth (0.4% tryptone, 0.05% yeast extract, 0.02% CaCl₂·7H₂O, 0.05% MgSO₄·7H₂O, distilled water, pH 7.2). The strain of *Flexibacter maritimus* was cultured in Marine TYE broth containing aged sea water instead of distilled water. And the strains belonging to the other genera were grown in brain heart infusion broth. The temperature for cultiva-

tion was 25°C except for *C. psychrophila*, which was grown at 18°C. Cells were harvested at approximately early stationary phase by centrifugation, washed with sterile distilled water, suspended in distilled water to give a cell concentration of 30 mg of wet weight per ml, and stored at -20°C.

PCR identification

For each strain, 40 µl portions of the above sample was mixed with 10 µl portions of proteinase K (1 mg/ml) and 50 µl portions of 2×K buffer (40 mM Tris buffer, 0.2% nonidet p-40, 0.2 mM EDTA, 1% Tween 20, distilled water, pH 8.0). The mixture was heat-treated at 60°C for 20 min, followed at 100°C for 5 min, then cooled rapidly on ice and centrifuged by 8,000 rpm/min for 5 min and stored at -20°C

Table 2. Bacterial strains used for the PCR

No.* ¹	Species* ²	Strain	Source
1	<i>C. psychrophila</i>	NCIMB1947	Coho salmon, Washington, USA
2	<i>C. psychrophila</i>	FPC840	Ayu, Tokushima, Japan
3	<i>C. psychrophila</i>	FPC828	Coho salmon, Miyagi, Japan
4	<i>C. psychrophila</i>	FPC813	Rainbow trout, Tokyo, Japan
5	<i>C. psychrophila</i>	FPC808	Rainbow trout, Nagano, Japan
6	<i>C. psychrophila</i>	FPC924	Ayu, Wakayama, Japan
7	<i>C. psychrophila</i>	FPC928	Ayu, Kyoto, Japan
8	<i>C. psychrophila</i>	FPC931	Ayu, Hiroshima, Japan
9	<i>C. psychrophila</i>	FPC938	Rainbow trout, Yamagata, Japan
10	<i>C. psychrophila</i>	SH3-81* ³	Coho salmon, Oregon, USA
11	<i>C. psychrophila</i>	TG-P01/88* ⁴	Rainbow trout, France
12	<i>C. psychrophila</i>	FPC955	Ayu, Shiga, Japan
13	<i>C. psychrophila</i>	FPC956	Ayu, Shiga, Japan
14	<i>C. psychrophila</i>	FPC957	Ayu, Shiga, Japan
15	<i>F. branchiophilum</i>	ATCC35036	Chinook salmon, Oregon, USA
16	<i>F. branchiophilum</i>	ATCC35035	Yamame, Gunma, Japan
17	<i>C. columnaris</i>	FPC53	Eel, Shizuoka, Japan
18	<i>F. aquatilis</i>	IAM12216	
19	<i>C. aquatilis</i>	IAM12365	
20	<i>C. flevenensis</i>	IAM12375	
21	<i>Fx. maritimus</i>	NCIMB2154	Red sea bream, Hiroshima, Japan
22	<i>F. indologenes</i>	FPC954	Ayu, Shiga, Japan
23	<i>F. breve</i>	ATCC14234	
24	<i>E. coli</i>	K-12	
25	<i>Ed. tarda</i>	FPC801	Eel pond, Shizuoka, Japan
26	<i>A. hydrophila</i>	FPC868	Eel, Japan
27	<i>A. salmonicida</i>	NCIMB2020	
28	<i>P. piscicida</i>	FPC851	Yellowtail, Japan
29	<i>Y. ruckeri</i>	FPC442	USA
30	<i>Ps. anguilliseptica</i>	NCIMB1950	Eel, Tokushima, Japan
31	<i>Ps. fluorescens</i>	FPC348	Japanese Char, Japan
32	<i>V. anguillarum</i>	FPC346	Ayu, Shiga, Japan
33	<i>V. anguillarum</i>	NCIMB6	

*¹ Numbers used to label lanes in Fig. 1 and Fig. 2.

*² Abbreviations: *C.*, *Cytophaga*; *F.*, *Flavobacterium*; *Fx.*, *Flexibacter*; *E.*, *Escherichia*; *Ed.*, *Edwardsiella*; *A.*, *Aeromonas*; *P.*, *Pasteurella*; *Y.*, *Yersinia*; *Ps.*, *Pseudomonas*; *V.*, *Vibrio*.

*³ Supplied by Dr. R. A. Holt.

*⁴ Supplied by Dr. J. F. Bernardet.

until used for the PCR. Five-microliter portions of these heat-treated cell extracts and 10 pmol primers were used in the PCR. Samples were subjected to 30 cycles of amplification in a DNA thermal cycler (MiniCycler TM; MJ RESEARCH, INC., Massachusetts, USA). A preheating cycle at 94°C for 2 min was included. The amplification cycles used for denaturation, primer annealing to the template, and primer extension were as follows: 94°C for 2 min, 45°C for 1.5 min, and 72°C for 2 min for the 20F-1500 R and PSY1-PSY2 primer pairs. After 30 cycles of amplification, 10 µl portions of the PCR products

were electrophoresed in a 1.0% agarose gel, stained with ethidium bromide, and photographed.

Results

First of all, we confirmed that all preparations contained DNA which could be amplified. The universal 20F-1500R primer pair yielded a fragment of the expected size (Fig. 1). The PSY1-PSY2 primer pair specifically amplified the 16S rDNA of all 14 *C. psychrophila* strains but none of 19 strains belonging to the other species (Fig. 2). The PSY1-

Fig. 1. Results of PCR-assisted amplification by the universal 20F-1500R primer pair complementary to conserved regions in all eubacterial 16S rRNA sequences. See Table 2 for an explanation of the bacterial strain numbers above the lanes. The chromosomal DNA from bacteria was subjected to 30 cycles of amplifications including the primer annealing, at 45°C. The resulting PCR product was then electrophoresed in an agarose gel, stained with ethidium bromide, and photographed.

Fig. 2. Results of PCR-assisted amplification by *C. psychrophila*-specific PSY1-PSY2 primer pair. See Table 2 for an explanation of the bacterial strain numbers above the lanes. The chromosomal DNA from bacteria was subjected to 30 cycles of amplification including the primer annealing, at 45°C. The resulting PCR product was then electrophoresed in an agarose gel, stained with ethidium bromide, and photographed.

PSY2 primer pair was assessed for the usefulness for discriminating *C. psychrophila* from other bacteria.

Discussion

The present results suggested that the PSY1-PSY2 primer pair could differentiate *C. psychrophila* from the other species of *Flaobacterium-Cytophaga* complex as well as other fish pathogens. The validity of this primer pair for the strains of *Flaobacterium-Cytophaga* complex from cluster 9 through 12 not used for the PCR was assessed by comparing the sequence patterns of the primer positions (Table 1). According to this comparison, the sequence patterns of these regions were significantly different between *C. psychrophila* and other species belonging to the cluster 9 through 12. This comparison confirmed that the differences of the sequence patterns considerably had an influence on amplifying the 16S rDNA. So it is supposed that the PSY1-PSY2 primer pair will not amplify the 16S rDNA of the other species within these ranges. It is expected that the PSY1-PSY2 primer pair will be a useful tool to identify and detect *C. psychrophila* in the epidemiological investigations.

Acknowledgments

We greatly thank Dr. Y. Nakagawa for generously supplying the 16S rRNA sequence data of his Ph. D dissertation. We are also grateful to Dr. K. Ohwada, Dr. K. Kogure, Dr. M. Nishimura, and the members in Marine Microbiology Division, Ocean Research Institute, University of Tokyo, for their advices and encouragements.

References

- Bernardet, J. F. and B. Kerouault (1989): Phenotypic and genomic studies of *Cytophaga psychrophila* isolated from diseased rainbow trout (*Oncorhynchus mykiss*) in France. *Appl. Environ. Microbiol.*, **55**, 1796–1800.
- Borg, A. F. (1960): Studies on myxobacteria associated with diseases in salmonid fishes. *Wildl. Dis.*, **8**, 1–85, 2 microcards.
- Higgins, D. G., A. J. Bleasby and R. Fuchs (1992): CLUSTAL V; improved software for multiple sequence alignment. *Comput. Appl. Biosci.*, **8**, 189–191.
- Lipman, D. J. and W. R. Pearson (1985): Rapid and sensitive protein similarity searches. *Science*, **227**, 1435–1441.
- Nakagawa, Y. (1993): Phylogenetic study of *Flaobacterium-Cytophaga* complex. Ph. D dissertation. The university of Tokyo.
- Nakagawa, Y. and K. Yamasato (1993): Phylogenetic diversity of the genus *Cytophaga* revealed by 16S rRNA sequencing and menaquinone analysis. *J. Gen. Microbiol.*, **139**, 1155–1161.
- Nishimura, M., K. Kita-Tsukamoto, K. Kogure and K. Ohwada (1992): Enumerations of *Vibrio cholerae* in aquatic environments by MPN-16S rRNA hybridization method. *Bull. Jpn. Soc. Microbiol. Ecol.*, **7**, 43–46.
- Wakabayashi, H., M. Horiuchi, T. Bunya and G. Hoshiai (1991): Outbreaks of cold-water disease in coho salmon in Japan. *Fish Pathol.*, **26**, 211–212.
- Wakabayashi, H., T. Toyama and T. Iida (1994): A study on serotyping of *Cytophaga psychrophila* isolated from fishes in Japan. *Fish Pathol.*, **29**, 101–104.
- Weisburg, W. G., S. M. Barns, D. A. Pelletier and D. J. Lane (1991): 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.*, **173**, 697–703.