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Genotyping of *Flavobacterium psychrophilum* using PCR-RFLP analysis

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ABSTRACT: Genetic variability among 242 strains of *Flavobacterium psychrophilum* was characterized using polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) analysis. Universal Primers GYR-1 and GYR-1R, which were designed to amplify the gyrase subunit B gene (*gyrB*), yielded a 1178 bp PCR product encoding *gyrB* and a 290 bp PCR product of anonymous DNA from all *F. psychrophilum* strains tested. In the RFLP analysis of the anonymous 290 bp DNA marker, the restriction enzyme *Hin*fI generated 2 cleavage patterns (Genotypes A and B). Genotype A was found only in isolates from ayu (n = 109), while Genotype B was found in isolates from coho salmon (n = 11), ayu (n = 35), rainbow trout (n = 43) and other fishes (n = 44). In the second experiment, Primers PSY-G1F and PSY-G1R specific for *F. psychrophilum*, were used to amplify *gyrB*. The specific primer pair amplified the expected size (1017 bp) PCR product from all *F. psychrophilum* strains. In the RFLP analysis of the *gyrB*, the restriction enzyme *Rsa*I produced 2 genotypes, R and S. Genotype R was found in isolates from coho salmon (n = 6), ayu (n = 27), rainbow trout (n = 39) and other fishes (n = 4). Genotype S was found in isolates from coho salmon (n = 5), ayu (n = 117), rainbow trout (n = 4) and other fishes (n = 40).

KEY WORDS: Genotyping · *Flavobacterium psychrophilum* · PCR-RFLP · Bacterial cold-water disease · *gyrB* gene

INTRODUCTION

Flavobacterium psychrophilum (formerly *Cytophaga psychrophila* and *Flexibacter psychrophilus*) is the causative agent of bacterial cold-water disease (BCWD) and rainbow trout fry syndrome (RTFS). This pathogen was originally isolated from juvenile coho salmon *Oncorhynchus kisutch* in the USA in 1948 (Borg 1960). Over the last decade, BCWD or RTFS have been reported in the USA, Europe (Bernardet et al. 1988), Japan (Wakabayashi et al. 1991), Australia (Schmidtke & Carson 1995), Chile (Bustos et al. 1995) and Korea (Lee & Heo 1998) in many species, e.g. the eel *Anguilla anguilla*, the common carp *Cyprinus carpio*, the crucian carp *Carassius carassius*, the tench *Tinca tinca* and the oikawa *Zacco platypus* (Lehmann et al. 1991, Iida & Mizokami 1996). In Japan, since the first isolation of *F. psychrophilum* was reported in a local ayu *Plecoglossus altivelis* farm in 1987, this bacterium has widely spread over many host species, such as coho salmon, rainbow trout *Oncorhynchus mykiss* and oikawa in various local areas. Because of the serious losses in rivers as well as in fish farms, BCWD caused by *F. psychrophilum* is

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currently the most economically important fish disease in Japan.

Some typing methods have been used to classify *Flavobacterium psychrophilum* strains for the epizootiological analysis of BCWD or RTFS. Serologic analysis (Wakabayashi et al. 1994, Lorenzen & Olesen 1997, Izumi & Wakabayashi 1999) and electrophoretic pattern of proteases (Bertolini et al. 1994), as well as genotyping using randomly amplified polymorphic DNA (RAPD) (Chakroun et al. 1997), ribotyping (Chakroun et al. 1998, Madsen & Dalsgaard 2000) and plasmid profiling (Lorenzen et al. 1997, Chakroun et al. 1998, Madsen & Dalsgaard 2000) have been reported. In these genotyping studies of *F. psychrophilum*, however, the numbers of Japanese isolates tested were very small and do not seem to reflect the epizootiological situation of BCWD in Japan. The present study aimed to develop a new genotyping method for *F. psychrophilum* by restriction fragment length polymorphism based on PCR amplification (PCR-RFLP) of the gyrase subunit B gene (*gyrB*) and anonymous products, and its suitability for classifying 242 *F. psychrophilum* strains including 225 Japanese isolates from various host-fish species.

MATERIALS AND METHODS

Bacterial strains and growth conditions. We examined 242 strains of *Flavobacterium psychrophilum*, comprising 11 strains from coho salmon, 144 strains from ayu, 43 strains from rainbow trout, and 44 strains from other species (amago *Oncorhynchus rhodurus*, yamame *O. masou*, iwana *Salvelinus pluvins*, grayling *Thymallus thymallus*, oikawa, common carp, ginbuna *Carassius auratus langsdorfii*, ugui *Tribolodon hakonensis*, tench, Japanese eel *Anguilla japonica*, and other unidentified salmonids). Strains from coho salmon were isolated in Japan and the USA, those from rainbow trout and carp were isolated in Japan and Europe, those from ayu, amago, yamame, iwana, oikawa, eel and ugui were isolated in Japan, and those from grayling, tench and other salmonids were isolated in Europe. Isolates from ayu were collected from 19 different Japanese prefectures. All strains were stored at −80°C and grown in tryptone and yeast extract (TYE) agar (0.4% tryptone, 0.05% yeast extract, 0.05% $CaCl_2 \cdot 7H_2O$, 0.05% $MgSO_4 \cdot 7H_2O$, 1.5% agar, distilled water, pH 7.2) at 18°C.

DNA extraction. Total genomic DNA was prepared from individual strains (Walsh et al. 1991, Izumi & Wakabayashi 1997); 1 loop of bacterial pellet was mixed with 300 µl of 5% Chelex100 (Sigma) and incubated for 30 min at 55°C. Following vortex mixing at high speed for 5 to 10 s, the mixture was boiled for 20 min and then centrifuged for 10 min at 10 000 × *g*. Without further purification, an aliquot of the supernatant containing DNA was used as a template for PCR amplification.

PCR amplification. We used 2 pairs of PCR primers. GYR-1 and GYR-1R are a degenerate universal primer pair amplifying 1178 bp of the partial *gyrB* and also amplifying approximately 300 bp of anonymous DNA from *Flavobacterium psychrophilum*. PSY-G1F and PSY-G1R are specific primers for *gyrB* of *F. psychrophilum*, amplifying a 1017 bp PCR product. The sequences of GYR-1, GYR-1R, PSY-G1F and PSY-G1R are 5′-CAYGCNGGNGGNAARTTYGA-3′, 5′-CCRTCNACRTCNGCRTCNGT-3′, 5′-TGCAGGAAATCTTACACTCG-3′ and 5′-GTTGCAATTACAATGTTGT-3′, respectively (Izumi & Wakabayashi 2000).

PCR amplification was performed in a total reaction volume of 20 µl with a GeneAmp PCR System 2400 (Perkin-Elmer). The reaction mixture contained 4 µl of template DNA, 0.2 nmol of each dNTP, 20 pmol of each primer and 1 U of *Taq* DNA polymerase (Takara). The following temperature profile was used for the amplification: preheating at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 90 s and a final extension at 72°C for 5 min.

Sequencing of PCR products. Approximately 300 bp PCR products from 15 *Flavobacterium psychrophilum* strains, obtained using the universal primer pair (GYR-1 and GYR-1R), were sequenced. The *gyrB* sequence of the 15 strains had been determined in a previous study (Izumi & Wakabayashi 2000). The 300 bp PCR products were purified and cloned into pT7Blue T-Vector (Novagen) using a DNA Ligation Kit ver.2 (Takara), and used to transform competent *Escherichia coli* strain JM109. Sequencing was performed with a 'dye terminator cycle sequencing FS ready reaction kit' (Perkin-Elmer) in an ABI Prism 377 DNA sequencer (Perkin-Elmer).

Restriction endonuclease digestion and agarose gel electrophoresis. An aliquot (8 µl) of PCR amplicon was checked by electrophoresis using a 1% agarose gel. Without further purification, the remaining PCR product was digested with 3 U of the restriction endonucleases *Rsa*I or *Hin*fI in a final volume of 20 µl for 2 to 3 h at 37°C. Electrophoresis was carried out in horizontal slab gel on a 4% NuSieve (FMC) agarose gel in TAE (Tris acetate EDTA) buffer at 4°C. The low temperature was necessary because of the prolonged electrophoresis required to achieve separation of restriction fragments in the 4% gel. The cleavage patterns were predicted by the 'Webcutter 2.0' program available on the internet (www.firstmarket.com/cutter/cut2.html) from the DNA sequences determined in this and a previous study (Izumi & Wakabayashi 2000).

RESULTS

PCR amplification and sequencing of PCR products

The Universal Primers GYR-1 and GYR-1R yielded 1178 bp and approximately 300 bp PCR amplification products from all *Flavobacterium psychrophilum* strains tested. The product of approximately 3000 bp found in a previous study using these primers (Izumi & Wakabayashi 2000) was not found using the PCR conditions described above. The Specific Primers PSY-G1F and PSY-G1R yielded a single PCR product of 1017 bp. The nucleotide sequences of the approximately 300 bp PCR product amplified with universal primers from 15 *F. psychrophilum* strains were determined and deposited in DDBJ, EMBL and GenBank nucleotide-sequence databases under the accession numbers in Table 1. The sequence of this product was not identical in all strains examined. The length of this product was 290 bp (Fig. 1), and no homologous sequences were found in GenBank. This anonymous DNA seems to be from chromosomal DNA of *F. psychrophilum* and not mobile DNA such as plasmid or transposon, since the 290 bp PCR product was obtained from all the 242 *F. psychrophilum* strains used in this study.

PCR-RFLP

For the RFLP analysis, DNA fragments shorter than 150 bp were not taken into consideration because they were near the DNA detection threshold using agarose gel electrophoresis and ethidium bromide staining. With the restriction enzyme *Hin*fI and PCR products amplified with the universal primer pair GYR-1 and GYR-1R, 2 cleavage patterns were expected from the sequences of the *Flavobacterium psychrophilum gyrB* and anonymous DNA. We obtained 2 clear cleavage patterns of Genotypes A and B in the RFLP analysis. The cleavage pattern of Genotype A was estimated to comprise 429, 217 and 161 bp DNA fragments. The cleavage pattern of Genotype B was estimated to comprise 429, 217 and 186 bp DNA fragments (Fig. 2). The difference between Genotypes A and B results from mutation at the 268th nucleotide of the anonymous DNA marker that affects a *Hin*fI cleavage site (Fig. 1). When RFLP analysis was performed on the PCR products amplified with the specific primer pair PSY-G1F and PSY-G1R, the restriction enzyme *Rsa*I generated 2 cleavage patterns of Genotypes R and S, as expected from the *gyrB* sequences of *F. psychrophilum*. Genotypes R and S were estimated to be composed of 429 and 327 bp DNA fragments and 429 and 367 bp DNA fragments, respectively (Fig. 3).

Relationship between *Flavobacterium psychrophilum* genotypes and host-fish species or isolation locality

Although Genotype A was found only in isolates from ayu, Genotype B was found in all fish species examined (Table 2). Genotype S was found in all fish species examined, while Genotype R was not found in cyprinids and eels (Table 2). A relationship between genotypes and the host fish species is possible.

In this study, 40 *Flavobacterium psychrophilum* strains from Okayama Prefecture, Japan, were examined: 25 strains from ayu, 4 strains from rainbow trout, 6 strains from amago and 5 strains from oikawa. The

Table 1. *Flavobacterium psychrophilum* strains used for sequence determination of 290 bp PCR product. Acc. No: sequence accession number. ^{T}: type strain

Strain	Isolation year	Host fish	Locality	Acc. No.
NCIMB1947^{T}	Unknown	*Oncorhynchus kisutch*	Oregon, USA	AB097199
FPC828	1990	*Oncorhynchus kisutch*	Miyagi, Japan	AB097200
FPC817	1991	*Oncorhynchus kisutch*	Miyagi, Japan	AB097201
Ch8-80	1980	*Oncorhynchus kisutch*	Oregon, USA	AB097202
TG-P01/88	1988	*Oncorhynchus mykiss*	Brittany, France	AB097203
OKR9801	1998	*Oncorhynchus mykiss*	Okayama, Japan	AB097204
OKM9801	1998	*Oncorhynchus rhodurus*	Okayama, Japan	AB097205
YMY9520	1996	*Oncorhynchus masou*	Yamanashi, Japan	AB097206
YNU9803	1998	*Anguilla japonica*	Yamanashi, Japan	AB097207
FPC945	1993	*Zacco platypus*	Hiroshima, Japan	AB097208
FPC840	1987	*Plecoglossus altivelis*	Tokushima, Japan	AB097209
FPC924	1992	*Plecoglossus altivelis*	Wakayama, Japan	AB097210
FPC956	1994	*Plecoglossus altivelis*	Shiga, Japan	AB097211
OKA9805	1998	*Plecoglossus altivelis*	Okayama, Japan	AB097212
YNA9801	1998	*Plecoglossus altivelis*	Yamanashi, Japan	AB097213

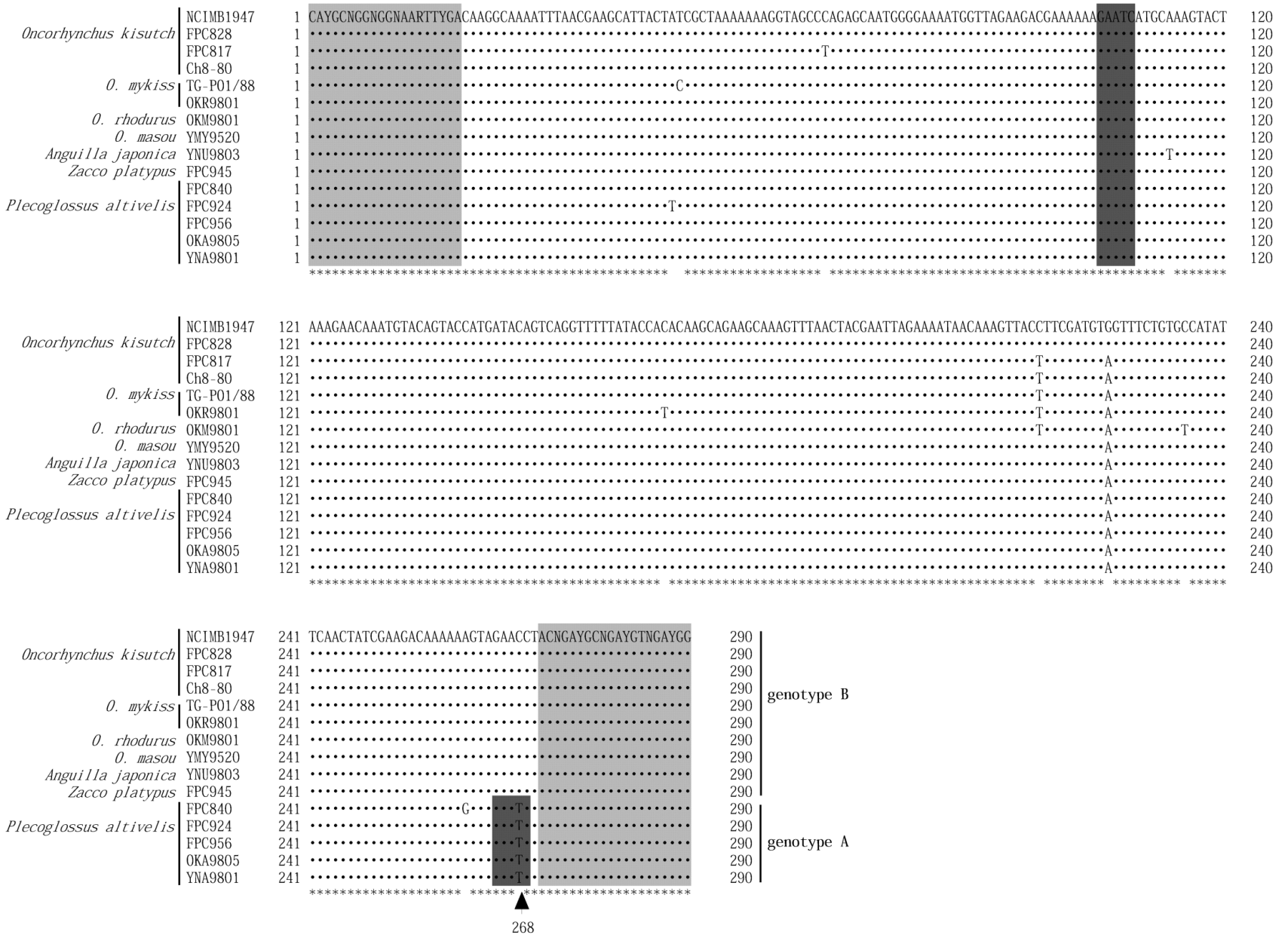

Fig. 1. *Flavobacterium psychrophilum*. DNA sequence alignment of 290 bp PCR product from 15 *F. psychrophilum* strains. Host fish are indicated to left of strain designation. A mutation at the 268th nucleotide (arrowed) differentiated Genotypes A and B. ▬: *Hin*fI recognition site, ┈┈: universal primer annealing site

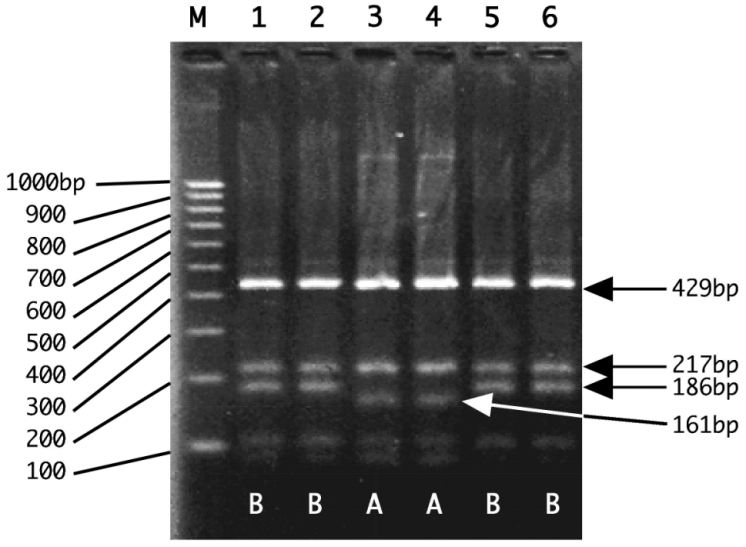

Fig. 2. *Flavobacterium psychrophilum.* Agarose gel electrophoresis of PCR products amplified with *gyrB* universal primer pair, GYR-1 and GYR-1R, digested with *Hin*fI and separated on 4% agarose gel. Lane M: molecular mass standard of 100 bp; Lanes 1 and 2: PCR products of *F. psychrophilum* isolates from coho salmon; Lanes 3 and 4: PCR products of *F. psychrophilum* isolates from ayu; Lanes 5 and 6: PCR products of *F. psychrophilum* isolates from rainbow trout. Arrows indicate digested fragments of 429, 217, 186 and 161 bp. A, B: Genotypes A and B, respectively

genotypes of these 40 strains varied, and seemed to be related to the host fish species even in a geographically limited area (Table 3). *F. psychrophilum* strains of ayu tested were isolated in 19 different prefectures in Japan. *F. psychrophilum* strains belonging to Genotype A, which was specific and dominant for the isolates from ayu, were found in 18 of 19 prefectures. Strains belonging to Genotype S, which was dominant for the isolates from ayu, were found in 19 prefectures (Table 4). There was no significant relationship between genotypes of ayu isolates and locality of isolation. From coho salmon, 11 strains were examined: 3 strains from the USA and 8 strains from Japan. Genotypes of B-R and B-S were found in both the USA and Japanese isolates (Table 5).

DISCUSSION

In recent years, investigators have revealed that *Flavobacterium psychrophilum* possesses a remarkable degree of genetic diversity closely related to its epizootiological and pathological characteristics. In these studies various genotyping methods have been proposed, including PCR-based RAPD fingerprinting, ribotyping and plasmid profiling (Chakroun et al. 1997, 1998, Lorenzen et al. 1997, Madsen & Dalsgaard 2000). Some of these reports (Chakroun et al. 1997, 1998) suggested that Japanese isolates of *F. psychrophilum* had different genotypes than the European and USA isolates. However, the Japanese isolates used in these studies were mainly from ayu or rainbow trout and the numbers examined were relatively small. It is, therefore, difficult to determine whether the genotypes of these Japanese isolates were related to the geographical features of Japan or specific characteristics of host-fish species, as ayu is a geographically limited species.

In a preliminary study on PCR-RFLP genotypings, we also examined PCR amplification of the small-subunit rRNA (16S rRNA) gene and internal transcribed spacer (ITS) region. For the 16S rRNA gene amplification, Universal Primers 20F and 1500R were used. For ITS amplification, Universal Primers ITS16F and ITS23R were used. Sequences of 20F, 1500R, ITS16F nd ITS23R are 5′-AGAGTTTGATCMTGGCTCAG-3′, 5′-GGTTACCTTGTTACGACTT-3′, 5′-TGCGGCTGGATCACCTCCTT-3′ and 5′-TGGATGCCTTGGCACCAGGAGCCGAT-3′, respectively. The restriction endonucleases *Alu*I, *Dde*I, *Hae*III, *Hha*I, *Hin*fI, *Mbo*I, *Msp*I, *Pst*I, *Rsa*I and *Sau*3AI were used for digestion. Unfortunately, no useful cleavage patterns to differentiate *Flavobacterium psychrophilum* strains were found (data not shown). We thus selected the *gyrB* that encodes the B

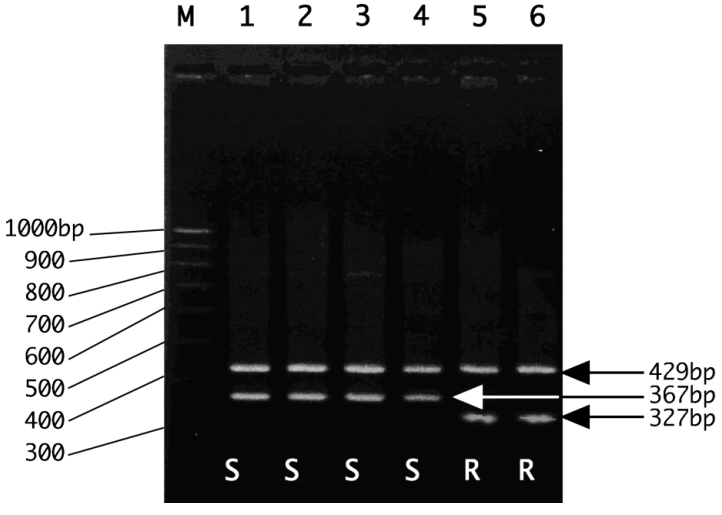

Fig. 3. *Flavobacterium psychrophilum.* Agarose gel electrophoresis of PCR products amplified with primer pair specific for *gyrB* of *F. psychrophilum*, PSY-G1F and PSY-G1R, digested with *Rsa*I and separated on 4% agarose gel. Lanes labelled as in Fig. 2. Arrows indicate digested fragments of 429, 367 and 327 bp. S, R: Genotypes S and R, respectively

Table 2. *Flavobacterium psychrophilum.* Relationship between Genotypes A, B, R, S and host-fish species. Genotypes A and B were generated using *gyrB*-universal primer and *Hin*fI, and Genotypes R and S using *gyrB*-specific primer and *Rsa*I

Host fish	Genotypes using: *gyrB* univ + *Hin*fI		*gyrB* specif + *Rsa*I		Total
	A	B	R	S	
Plecoglossidae					
Plecoglossus altivelis	109	35	27	117	144
Salmonidae					
Oncorhynchus kisutch	0	11	6	5	11
Oncorhynchus mykiss	0	43	39	4	43
Thymallus thymallus	0	2	1	1	2
Oncorhynchus rhodurus	0	13	2	11	13
Salvelinus pluvins	0	1	0	1	1
Oncorhynchus masou	0	5	0	5	5
Other salmonids	0	3	1	2	3
Cyprinidae					
Tribolodon hakonensis	0	1	0	1	1
Zacco platypus	0	12	0	12	12
Carassius auratus	0	2	0	2	2
Cyprinus carpio	0	2	0	2	2
Tinca tinca	0	2	0	2	2
Anguillidae					
Anguilla japonica	0	1	0	1	1
Total	109	133	76	166	242

Table 3. *Flavobacterium psychrophilum.* Genotypes of strains isolated in Okayama, Japan

Host fish	Genotypes using: *gyrB*-univ + *Hin*fI		*gyrB*-specif + *Rsa*I		Total
	A	B	R	S	
Plecoglossidae					
Plecoglossus altivelis	20	5	6	19	25
Oncorhynchus mykiss	0	4	3	1	4
Salmonidae					
Oncorhynchus rhodurus	0	6	1	5	6
Cyprinidae					
Zacco platypus	0	5	0	5	5
Total	20	20	10	30	40

subunit protein of DNA gyrase for further PCR-RFLP analysis. Although it has been commonly used for the detection and phylogenetic differentiation of related bacterial species, 16S rRNA is highly conservative and seems to be less appropriate for differentiation among bacterial strains belonging to the same species than protein-encoding genes such as *gyrB* (Yamamoto & Harayama 1995).

We successfully classified the *Flavobacterium psychrophilum* isolates into 4 genotypes by RFLP analysis of PCR products amplified with primers targeting *gyrB*. The PCR-RFLP genotypings and host-fish species are suggested to have a close relationship. The relationship between our PCR-RFLP genotypings and host-fish species was statistically significant (χ^2 test, $p > 0.05$). Genotype A was found only in *F. psychrophilum* isolates from ayu, and there is no geographical relationship with genotypes among the isolates from ayu. These results suggest that the *F. psychrophilum* isolates exhibiting Genotype A are specifically infectious to the ayu as a host fish and barely infectious to other fish species, even those inhabiting the same water system. Earlier serologic analysis had indicated this hypothesis of specific characteristics of the *F. psychrophilum* isolates from ayu (Wakabayashi et al. 1994, Izumi & Wakabayashi 1999). Also, in other *F. psychrophilum* genotyping studies with RAPD, ribotyping and plasmid profiling, Japanese isolates from ayu exhibited very characteristic profiles (Chakroun et al. 1997, 1998). Furthermore, all the 4 ayu isolates used in those studies exhibited the same RFLP genotype, corresponding to Senotypes A and S in the present study. This suggests that the other *F. psychrophilum* isolates that exhibited Genotypes A and S could possess the same characteristic profiles of RAPD, ribotyping and plasmid profiling observed by Chakroun et al. (1997, 1998). Further studies on ayu isolates exhibiting RFLP genotypes other than A and S are needed for detailed epizootiological analysis of BCWD in ayu farming. The coho salmon isolates from Japan and the USA shared common RFLP genotypes, i.e. Genotypes B and R or Genotypes B and S (Table 5). This confirms previous data using RAPD and ribotyping (Chakroun et al. 1997, 1998). It seems likely that some *F. psychrophilum* strains were introduced into Japan with infected eggs from the USA, as mentioned in a previous study on PCR detection of *F. psychrophilum* from imported coho salmon eggs (Izumi & Wakabayashi 1997). In the rainbow trout isolates, 39 Japanese and European isolates exhibited Genotypes B and R, and 4 Japanese isolates exhibited Genotypes B and S together with the majority of the other salmonid isolates (Table 2). The discovery of cyprinid and eel isolates from Japan is of great concern, since they were isolated from fishes inhabiting natural water systems. All isolates used in this

Table 4. *Flavobacterium psychrophilum.* Relationship between genotypes and isolation locality for *Flavobacterium psychrophilum* of ayu *Plecoglossus altivelis* in Japan

Prefecture	Genotypes using: *gyrB*-univ + *Hin*fI		*gyrB*-specific + *Rsa*I		Total
	A	B	R	S	
Iwate	4	0	2	2	4
Miyagi	2	1	1	2	3
Yamagata	1	0	0	1	1
Tochigi	1	0	0	1	1
Gunma	5	1	1	5	6
Kanagawa	7	4	0	11	11
Nagano	2	1	2	1	3
Yamanashi	2	2	3	1	4
Gifu	5	3	1	7	8
Aichi	0	2	0	2	2
Kyoto	1	0	0	1	1
Shiga	15	4	1	18	19
Wakayama	11	4	4	11	15
Hyogo	3	1	0	4	4
Okayama	20	5	6	19	25
Hiroshima	6	0	1	5	6
Yamaguchi	1	1	1	1	2
Tokushima	22	4	4	22	26
Kumamoto	1	2	0	3	3
Total	109	35	27	117	144

Table 5. *Flavobacterium psychrophilum.* Genotypes B, R and S of strains isolated from coho salmon *Oncorhynchus kisutch*

Strain	Locality	Isolation year	Genotype
Ch8-80	Oregon, USA	1980	B, R
SH3-81	Oregon, USA	1981	B, S
NCIMB1947	Oregon, USA	Unknown	B, S
FPC829	Miyagi, Japan	1990	B, S
FPC828	Miyagi, Japan	1990	B, S
FPC830	Miyagi, Japan	1990	B, S
FPC818	Miyagi, Japan	1991	B, R
FPC819	Miyagi, Japan	1991	B, R
FPC820	Miyagi, Japan	1991	B, R
FPC817	Miyagi, Japan	1991	B, R
FPC831	Miyagi, Japan	Unknown	B, R

study exhibited Genotypes B and S. The results indicate that most *F. psychrophilum* isolates, which are pathogenic for rainbow trout and ayu, may not be infectious to natural populations of eel and cyprinids.

The anonymous 290 bp DNA fragment that differentiated Genotypes A and B was amplified with degenerate universal primers for *gyrB*. As shown by the sequencing analysis of this anonymous PCR product, it was not derived from *gyrB.* Genotype A is thus independent of mutation in *gyrB* of *Flavobacterium psychrophilum* isolates. On the other hand, the difference between Genotypes R and S was clearly dependent on the mutation of *gyrB* of *F. psychrophilum* isolates. However, this mutation, resulting in the difference between Genotypes R and S, does not alter the amino acid sequences of DNA gyrase subunit B. Therefore, the difference between Genotypes R and S may not correlate with the susceptibility to quinolone antibiotics, which sometimes depends on mutation of DNA gyrase.

PCR-RFLP analysis has been widely developed as an effective tool for the genotyping and differentiation of many bacteria at the species and strain levels. In this study, we determined that PCR-RFLP is a powerful tool for characterizing genetic variability among strains of *Flavobacterium psychrophilum*. Furthermore, the analysis procedure is easy and fast compared to other genotyping methods for *F. psychrophilum* such as ribotyping, RAPD, and plasmid typing. PCR-RFLP analysis of *F. psychrophilum* is, therefore, a potential analysis method for fish-disease control in the laboratories of fish farms which lack sophisticated gene analysis equipment.

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