RFLP and sequence analysis of 16S-23S rDNA intergenic spacer region of strains of *Flavobacterium columnare* from diseased fishes

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Abstract

The restriction length fragment polymorphism (RFLP) and sequence analysis of 16S-23S rDNA intergenic spacer region of 23 strains of *Flavobacterium columnare* were undertaken. The 16S-23S rDNA was amplified by PCR using universal primers. The RFLP analysis was conducted by digesting the amplified 16S-23S rDNA with restriction enzymes *Hha*I, *Hinf*I, *Rsa*I and *Msp*I. The characteristic restriction profiles allowed us to differentiate 23 strains of *Fl. columnare*. The strains of genomovars 1, 2, and 3 were separated in the different phylogenetic branch. Futhermore, the strains of genomovar 1 were subdivided into RFLP-type Ia, Ib, and Ic. Amplified 16S-23S rDNA of representative strains were cloned to *Escherichia coli* JM 109 and sequenced directly. The sequence analysis demonstrated that homology values ranged from 80.92% to 99.60%.

Keywords: Flavobacterium columnare – *RFLP – 16S-23S rDNA – sequencing*

Introduction

Flavobacterium columnare (Bernardet *et al.*, 1996), formerly *Flexibacter columnaris*, is one of important bacterial pathogens in freshwater fishes. The bacterium has been a subject of taxonomic confusion since its first description by Davis in 1922 as *Bacillus columnaris*. Several molecular studies have been done in order to elucidate taxonomic position of the bacterium (Bader and Shotts, 1998; Bernardet and Grimont, 1989; Bernardet *et al.*, 1996; Nakagawa and Yamasato, 1993). Our recent study based on 16S rDNA analysis and DNA-DNA hybridization revealed that there was an intra-specific variation among strains of *Fl. columnare* and we proposed to divide the strains into three genomovars (Triyanto and Wakabayashi, 1999).

It has been reported that 16S-23S rRNA gene represented a potential target within bacterial genom to find suitable site for probes and from which to derive additional phylogenetic information. Gurtler and Stanisich (1996) stated that 16S-23S rRNA gene were more variable than the adjacent genes and often showed species-specific sequence traits useful for designing molecular markers. Roth *et al.* (1998) found

that the 16S-23S rRNA gene sequence of genus *Mycobacterium* represented a supplement to 16S rRNA gene sequence for differentiation of closely related species.

This study was conducted in order to investigate the intra-specific variation among strains of *Fl. columnare* on the basis of 16S-23S rDNA analysis. We also discuss the possibility of 16S-23S rDNA analysis for identification and taxonomy of *Fl. columnare*.

Materials and Methods

Bacterial strains, cultivation and DNA extraction

The 23 strains of *Fl. columnare* isolated from different host species at various localities were used in this study (Table 1). All strains were cultured on tryptone yeast extract salt (TYES) broth medium containing 0.4% tryptone, 0.04% yeast extract, 0.05% $MgSO_4 7H_2O$, 0.05% $CaCl_2 10H_2O$, pH 7.0-7.2. One ml of bacterial cell cultures was harvested

Table 1. *Flavobacterium columnare* strains used in this study

Strain	Date isolation	Host species	Locality
LP8	1966	Loach	Tokyo, Japan
EK 28 (=IAM 14820)	1967	Japanese eel	Shizuoka, Japan
FPC 77	1973	Goldfish	Tokyo, Japan
FK 401	1978	Japanese eel	Shizuoka, Japan
FPC 492	1980	Carp	Indonesia
FPC 666	1987	Loach	Tokyo, Japan
FPC 667	1987	Goldfish	Tokyo, Japan
ATCC 49512 (=JIP 44/87)	1987	Brown trout	France
ATCC 49513 (=JIP 39/87)	1987	Black bullhead	France
LVDL 3414/89	1989	European eel	France
LVDL 2027/89	1989	Sturgeon	France
JIP-P06/90	1990	Black bullhead	France
LVDI 39/I	1992	?	France
JIP 08/94	1994	Rainbow trout	France
JIP-P11/91	1994	Rainbow trout	France
CH 97022	1997	Carp	Hiroshima,Japan
CH 97027	1997	Carp	Hiroshima,Japan
PH 97028 (=IAM 14821)	1997	Ayu	Hiroshima,Japan
CH 97030	1997	Goldfish	Hiroshima,Japan
IBR-98	1998	Carp	Ibaraki, Japan
FK-1	?	Ayukake	Fukui, Japan
IAM 14301^T(=NCMB 2248^T)	?	Salmonid fish	USA
G4	?	Grasscarp	China

vested using centrifugation and used for DNA extraction using Qia-Amp Kit (Qiagen, Germany) according to protocol recommended by the manufacturer.

Amplification of 16S-23S rDNA

The universal primers number 4 and 5 were used to amplify 16S-23S rDNA intergenic spacer region and their sequences are 5'TGCGGCTGGATCACCTCCTT-3' (position at 1525-1545 based on *Escherichia coli* numbering) and 5'-CTAGGCATCCCCC-ATAC-3' (position at 35-19 *E. coli* numbering), respectively (Navarro *et al.*, 1992). PCR was performed in 50 µl containing 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.2 mM of each dNTP (the nucleotide triphosphate of adenine, guanine, cytosine and thymine), 50 pM of each primer, 1.25 units of *Taq* DNA polymerase and 5 ng of purified DNA. Sample was subjected to 30 cycles of amplification in a DNA thermal cycler (Gene Amp PCR System 2400, Perkin-Elmer). A preheating cycle at 94°C for 3 min was included. The amplification cycles were as follows: denaturation at 94°C for 1 min, annealing to the template at 55°C for 1 min, and primer extension at 72°C for 1 min.

Table 2. 16S-23S rDNA restriction patterns of *Flavobacterium columnare* strains

Number of strain	Restriction pattern				RFLP type	Genomovar
	*Hha*I	*Hinf*I	*Rsa*I	*Msp*I		
15[1]	H1	F1	R1	M1	Ia	1
4[2]	H1	F3	R1	M1	Ib	1
1[3]	H4	F4	R4	M4	Ic	1
2[4]	H2	F2	R2	M2	II	2
1[5]	H3	F3	R3	M3	III	3

[1]FK 401, FPC 77, G4, FPC 492, FPC 666, FPC 667, LVDL3414/89, JIP-P06/90, JIP-P11/91, CH 97022, CH 97027, CH 97030, FK-1, ATCC 49512, LVDI 39/I and LVDL 2027/89
[2]FPC 666, ATCC 49513, IAM 14301^T and IBR-98
[3]JIP 08/94
[4]EK 28 and LP 8
[5]PH 97028

RFLP analysis

PCR products of 16S-23S rDNA were cut with restriction endonucleases, *Hha*I, *Msp*I, *Hinf*I and *Rsa*I. Digested fragments were separated using Mupid minigel electrophoresis aparatus with 4% NuSieve agarose (FMC BioProducts, USA). The results were detected by staining with 0.3-0.5% ethidium bromide for 30 min and visualized at 320 nm UV transillumination.

Cloning and transformation

PCR-amplified 16S-23S rDNA of representative strains (IAM 14301^T, FK 401, JIP 08/94, ATCC 49513, EK 28, LP 8 and PH 97028) were purified using Microcon-100 microconcentrator (Amicon). Sixty nanogram of purified 16S-23S rDNA were inserted to plasmid pT7Blue T-vector (Novagen) in ligation mix solution (Takara, Tokyo, Japan) at 16°C for 2-3 h and used directly for transformation to *E. coli* strain JM 109. Transformation of plasmid carrying 16S-23S rDNA into *E. coli* JM 109 was performed according to Sambrook *et al.* (1989). Plasmidic colonies (white colonies) were checked the presence of 16S-23S rDNA using primers of SQ M 13-RV (5'CAGGAAACAGCTATGACCAT-3') and SQ M 13-47 (5'CGCCAGGGTTTTCCCAGTCACGAC-3') which amplify the region of *Lac*-Z gene. The PCR condition to amplify this region was conducted as described on 16S-23S rDNA amplification. Positive colonies indicated by amplification of sufficient size of DNA region were subcultured on Luria-Bertani (LB) agar medium for plasmid purification using Mini-plasmid Kit (Qiagen, Germany) according to protocol recommended by the manufacturer.

16S-23S rDNA sequencing

Purified-inserted plasmid was used as template for amplification of the 16S-23S rDNA region using primers of SQ M 13-RV and SQ M 1347. Amplicon was purified using microcon-100 microconcentrator (Amicon) and used as template in cycle sequence amplification. PCR products were purified by Centri-Sep column and sequenced directly with ABI Prism 377 DNA sequencer (Perkin-Elmer). Sequences data were compiled with Genetix-Mac (Software Development Co., Tokyo, Japan) and compared with those of *E. coli* and *B. subtilis* as outgroup references obtained from Genbank using Clustal-W program (Thompson *et al.*, 1994).

Nucleotide sequence accession number

The 16S-23S rDNA sequences in the present study have been deposited in DDJB, EMBL and GenBank nucleotide sequence databases under accession numbers AB030748, AB031216, AB031219, AB031220, AB031217, AB031221, and AB031218 for IAM 14301^T, FK 401, ATCC 49513, JIP 08/94, EK 28, LP 8, and PH 97028, respectively.

Results and Discussion

RFLP analysis of 16S-23S rDNA

Amplification of 16S-23S rDNA of *Fl. columnare* strains yielded approximately 600 bp PCR product. All strains of *Fl. columnare* had a single band of 16S-23S rDNA in gel electrophoresis and there was no variation in terms of size with exception of strain JIP 08/94 which had a shorter size than the other strains. RFLP analysis of 16S-23S rDNA was performed using 4 restriction enzymes, *Hha*I, *Hinf*I, *Rsa*I and *Msp*I. Independent cleavage by these enzymes gave 4 different restriction patterns (Fig. 1). The characteristic restriction profiles generated by digestion with these enzymes differentiated 23 strains of *Fl. columnare* (Table 2). Two strains of genomovar 2 (EK 28 and LP 8) produced the same

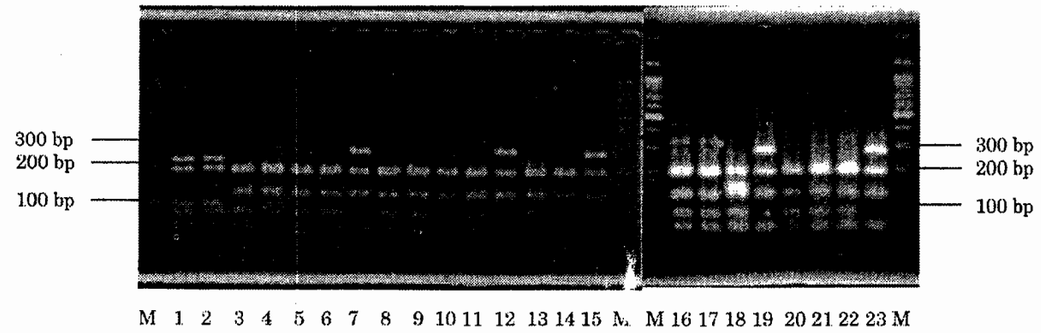

Fig. 1. Restriction profiles of 16S-23S rDNA intergenic spacer region from 23 strains digested with *Hinf*I. Lanes M, 100-bp ladder; lane 1, EK 28; lane 2, LP 8; lane 3, FK 401; lane 4, FPC 77; lane 5 G4; lane 6, FPC 492; lane 7, FPC 666; lane 8, FPC 667; lane 9, LVDL 3414/89; lane 10, JIP-P06/91 lane 11, JIP-P11/91; lane 12, IAM 14301^T; lane 13, CH 97022; lane 14, CH 97027; lane 15, PH 97028; lane 16, CH 97030; lane 17, FK-1; lane 18, JIP 08/94; lane 19, ATCC 49513; lane 20, ATCC 49512; lane 21, LVDI 39/I; lane 22, LVDL 2027/89 and lane 23, IBR-98.

restriction profiles, H2, F2, M2 and R2. The single strain of genomovar 3 isolated from Ayu produced the H3, F3, M3 and R3 profiles for *Hha*I, *Hinf*I, *Rsa*I and *Msp*I enzymes, respectively. Interestingly, the genomovar 1 was subdivided into RFLP-type Ia, Ib and Ic. The RFLP-type Ia consisted of 15 strains and type Ib comprised 4 strains including the type strain (IAM 14301^T). Strain JIP 08/94 isolated from rainbow trout in France gave different restriction profiles with the other strains of genomovar 1 all restriction enzymes. Therefore, the strain was assigned as RFLP-type Ic.

Sequences analysis of 16S-23S rDNA

The sequence analysis revealed that among strains of *Fl. columnare* on the basis of 16S-23S rDNA sequences had a wide range in homology of 80.92-99.60%. The sequence homology within strains of genomovar 1 was 85.34-99.60%. The strains of genomovar 2 (EK 28 and LP 8) exhibited 99.60% sequence homology to each other and 83.94-96.18% with the other strains. The single strain of genomovar 3, has a low sequence homology (80.92-86.55%) with other strains of *Fl. columnare*. The phylogenetic tree showed that strain of PH 97028 formed a different branch and distantly from the other strains of *Fl. columnare*. The strains of genomovar 2 (EK 28 and LP 8) were placed in the same cluster with the sequence homology of 99.60%. However, the strains of genomovar 1 were separated into two clusters (Fig. 2).

The 16S-23S rDNA has been reported to be useful for discrimination of bacteria, since the considerable variations occurred in this region (Gurtler and Stanisich, 1996). The variation was particularly caused by the copy number of rRNA operon existed in the bacteria. Some reports revealed that the multiple copies of rRNA operon occurred in *E. coli* (Morgan *et al.*, 1977), *B. subtilis* (Loughney *et al.*, 1982), *Streptomyces griseus* (Kim *et al.*, 1993), and *Mycoplasma* (Amikam *et al.*, 1984). The copy number ranged from 2 in *Frankia* strain ORS020606 (Normand *et al.*, 1992) to 10 in *B. subtilis* (Loughney *et al.*, 1982). The multiple copies of rRNA operon could be recognized by gel electrophoresis of PCR product (Riffard *et al.*, 1998). Gurtler and Stanisich (1996) stated that there are two reasons for the recovery of only a single PCR product: a) the use of primer number 5

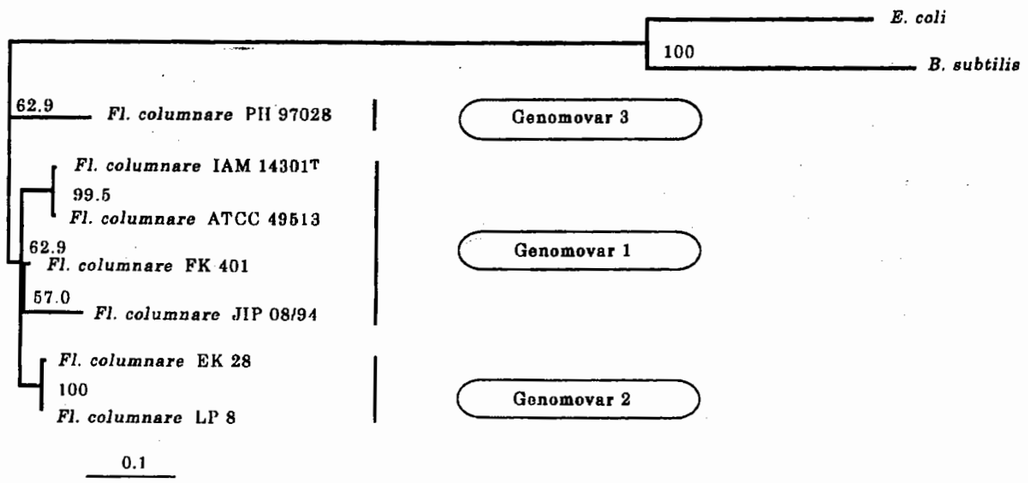

Fig 2. A phylogenetic tree of *Flavobacterium columnare* on the basis of 16S-23S rDNA intergenic spacer region sequences. The numbers on the branches are confidence limits (expressed as percentages) estimated by bootstrap analysis with 1,000 replicates. E; *Escherichia*, B.; *Bacillus*, Fl; *Flavobacterium*.

located at 2138 (23S rDNA, *E. coli* numbering system), and b) only one copy of 16S-23S rDNA is actually present per genom. In the present study, the single PCR product was not caused by the use of primer number 5, because the single PCR product was also found when the primers of number 2 located at 1390-1407 (16S rDNA, *E. coli* numbering system) and number 10 located at 456-474 (23S rDNA, *E. coli* numbering system) were used as recommended by Gurtler and Stanisich (1996). These results indicated that the size variation and copy number of 16S-23S rDNA could not be used for discriminating the existence of genomovars in *Fl. columnare* strains. This is in accordance with those of *Legionella dumoffii*, *L. gormanii*, and *L. quinlivanii* (Riffard *et al.*, 1998).

To obtain the discriminative difference from a single PCR product of 16S-23S rDNA, some methods have been developed. These are, for example, RFLP analysis (Liveris *et al.*, 1995; Matar *et al.*, 1992; de Wit and Klatser, 1994), denaturing gradient gel electrophoresis (Sheffield *et al.*, 1989), temperature-gradient gel electrophoresis (Wartell *et al.*, 1990) and single-strand conformation polymorphism (de Wit and Klatser, 1994). In our study, the RFLP analysis was applied to differentiate the 16S-23S rDNA of *Fl. columnare* strains. The results revealed that the RFLP analysis of 16S-23S rDNA could separate the 23 strains of *Fl. columnare*. However, comparing to the RFLP analysis of 16S rDNA, this results is different where the strains of genomovar 1 are subdivided into RFLP type Ia, Ib and Ic. It is suggested that intergenic spacer region is more variable than the adjacent genes.

Leblond-Bouget *et al.* (1996) compared the 16S and 16S-23S rDNA sequence analysis of *Bifidobacterium* and found that the two trees were similar. Similar finding was also demonstrated in *Mycobacterium* (Roth *et al.*, 1998). These results suggested that the two type of the molecules provide the same kind of phylogenetic information. Our results concerning 16S-23S rDNA sequence analysis of *Fl. columnare* strains revealed that it supported the phylogenetic information of

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16S rDNA. On the basis of 16S rDNA sequence analysis, strains of *Fl. columnare* were clearly separated into three clusters which consists of genomovar 1, 2 and 3 (Triyanto and Wakabayashi, 1999). In the present study, the strains of genomovar 1, 2 and 3 were separated in the different phylogenetic branch. However, the strains of genomovar 1 were subdivided into two clusters with the sequence homology of 85.34-99.60%. The differences might be caused by the fact that evolutionary rate of 16S-23S rDNA is 10 times greater than that of 16S rDNA (Leblond-Bouget *et al.*, 1996). Moreover, in this region, the insertion-deletion, substitution and mutation events have occurred at higher frequencies than those in the adjacent genes. Therefore, it could prevent accurate sequence alignment and make phylogenetic similarity difficult to assess. Similar finding was reported in other bacteria (Yoon *et al.*, 1998). We conclude that the 16S-23S rDNA analysis is an appropriate data as an additional taxonomic information particularly for separating genomovars of *Fl. columnare*.

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