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Characteristics of *Cytophaga columnaris* isolated from rainbow trout (*Oncorhynchus mykiss*), goldfish (*Carassius auratus*), and ayu (*Plecoglossus altivelis*) in Korea

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국내에서 양식되는 무지개송어(*Oncorhynchus mykiss*), 금붕어(*Carassius auratus*), 은어(*Plecoglossus altivelis*)로부터 분리된 *Cytophaga columnaris*의 특성

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초 록 : 국내의 무지개송어, 금붕어, 은어양식장에서 아가미와 지느러미의 부식과 괴사를 나타내는 물고기로부터 균주를 분리하여 형태학적, 생화학적, 항원적시험 그리고 전기영동을 통하여 분리주와 미국과 일본에서 분리된 표준주(NCMB 2248^T, EK 28)의 성상을 비교하였다. 즉, 형태학적으로 cytophaga agar에서 강한 점착성, rhizoid 그리고 노랗고 편평한 집락을 나타내었으며 flexirubin type pigment를 산생하고 동종과 이종에 대한 항원적 시험에서도 응집역가는 1024, 512를 나타냈다. 비록 분리주는 각기 다른 어종에서 분리되었음에도 불구하고 형태학적, 생화학적 그리고 혈청학적인 특성에 있어서 미국과 일본에서 분리된 표준주와 유사하였다. SDS-PAGE에 의한 세포외막단백질(outer membrane proteins, OMPs)의 분석에서도 표준주와 gel pattern이 일치하는 형태를 나타내었다. 이상의 결과를 미루어볼 때 국내 무지개송어, 금붕어 및 은어양식장에서 분리된 균주들은 *Cytophaga columnaris* 로 분리 동정할 수 있었다.

Key words : *Cytophaga columnaris* , rainbow trout, goldfish, ayu.

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Introduction

Columnaris disease, caused by *Cytophaga columnaris*, is a disease of many freshwater fishes and worldwide distribution[1,2]. This bacterium has been described in many freshwater fishes, and can be of economical importance in intensive farming. The importance of the disease has led to a significant volume of publications, which have been adequately reviewed by various authors[3,4]. However, taxonomy of the bacterium has not been completely resolved. Classification of the organism from the first proposed name, *Bacillus columnaris*[5] was changed to *Condrococcus columnaris*[6] and then to *Flexibacter columnaris*[7,8]. Now, the bacterium was named to *C columnaris*[9,10].

Although the taxonomy of *C columnaris* is still in a state of flux[11], the morphological, physiological, and biochemical properties of the bacterium is reasonably clear. According to Bernardet and Grimont[8], characteristics of *C columnaris* that distinguish it from other, nonpathogenic, gliding bacteria of fish include the follwoings; strongly adherent and rhizoid colonies on cytophaga agar; adsorption of Congo red dye; production of flexirubin-type pigments; reduction of nitrate; and intense hydrolysis of lecitin. The absence of a reaction on any carbohydrate and no growth in media containing more than 0.5% NaCl was also characteristics of the bacterium.

A gram-negative, yellow-pigmented bacteria (YPB) resembling *C columnaris* was recently isolated from rainbow trout (*Oncorhynchus mykiss*), goldfish (*Carassius auratus*), and ayu (*Plecoglossus altivelis*) affected with columnaris disease in Korea.

This study was done to compare the morphological, phenotypical, antigenic, and OMPs profiles of these isolates with those of reference strains of *C columnaris* (NCMB 2248^T and EK 28).

Materials and Methods

Bacterial strains : Bacteria isolated from February to July in 1997. Five strains of gram-negative YPB were isolated (Table 1). Media used in this experiment were cytophaga medium containing 0.9% agar (0.9% CA). The isolates were maintained and subcultures on 0.9% CA at 25℃ or stored in cytophaga broth (CB, pH 7.0) containing 15% glycerol at -70℃ until use. Two reference strains (Table 1) of *Cytophaga columnaris* NCMB 2248^T and EK 28 were also used for comparative purposes.

Table 1. Korean isolates and reference strains of *C columnaris* used in this study

Strain	Year of isolation	Host species
Korean isolates		
KRT 1	1997	Rainbow trout (*Oncorhynchus mykiss*)
KRT 2	1997	Rainbow trout
KGF 1	1997	Goldfish (*Carassius auratus*)
KGF 2	1997	Goldfish
KAU 1	1997	Ayu (*Plecoglossus altivelis*)
Reference strains		
NCMB 2248^T	?	Salmonid (USA)
EK 28	1967	Japanese eel (*Anguilla japonica*) (Japan)

^{T} Type strain : morphological variant of strain NCMB 1038 (salmonid, USA).

Morphological and phenotypic characterization : The criteria of Reichenbach[9] were used for identification of the YPB from affected fishes.

Columnar formation was determined on wet mounts under phase contrast microscopy. Gliding movement was determined as described by Perry[13]. Colonial spreading was determined by point inoculation on a freshly poured 0.9% CA plate and incubation in a humid chamber. Morphology of the cells was determined by observation of Gram-stained cells under a light microscopy.

Anaerobic growth was tested by steel wool method in anaerobic jar[14]. Growth temperature was determined by incubating the cultures aerobically at 15, 25, 30, and 40℃ for 5 days. The tolerance to NaCl was determined by inoculating to CB containing 0, 0.5, and 1.0% NaCl and recording the amount of growth for more than 5 days.

Standard methods[15] were employed by using CA or CB

Table 2. Morphological and physiological characteristics of Korean isolates and *C columnaris* reference strains

Characteristic	KRT 1	KRT 2	KGF 1	KGF 2	KAU 1	NCMB 2248^T	EK 28
Gram reaction	-	-	-	-	-	-	-
Rhizodiodal edges	+	+	+	+	+	+	+
Gliding movement	+	+	+	+	+	+	+
Anaerobic growth	-	-	-	-	-	-	-
Growth at							
15℃	+	+	+	+	+	+	+
25℃	+	+	+	+	+	+	+
30℃	+	+	+	+	+	+	+
40℃	-	-	-	-	-	-	-
Growth in NaCl							
0%	+	+	+	+	+	+	+
0.5%	+	+	+	+	+	+	+
1.0%	-	-	-	-	-	-	-
Growth at							
pH 5.0	-	-	-	-	-	-	-
pH 6.0	-	-	-	-	-	-	-
pH 7.0	+	+	+	+	+	+	+
pH 8.0	+	+	+	+	+	+	+
pH 9.0	+	+	+	+	+	+	+
Growth in TSB	-	-	-	-	-	-	-
Sensitivity to O/129	+	+	+	+	+	+	+

+ : positive or present, - : negative or absent.

cultures for biochemical characterization of the strains unless otherwise stated. Additional criteria and methods of Bernardet[8] were used to compare the biochemical characteristics of the Korean isolates with *C columnaris* isolated in USA and Japan. The presence of cell-wall-associated flexirubin-like pigment was determined by flooding 0.9% CA colonies with 20% KOH (w/v). Hydrolysis of gelatin was detected by mercuric chloride procedure of Frazier[15]. Production of an extracellular galactosamine glycan was revealed by adsorption of Congo red[16]. To examine aerobic carbohydrate utilization, the low peptone medium described by Wakabayashi *et al*[17] was used.

Antigenic characterization : Rabbit antisera were raised against each formalin-killed cells of Korean isolates and reference strains. The agglutination and immunodiffusion tests were used to examine the serological relationship among the strains. For agglutination tests by microtiter technique, whole cell antigens were washed twice in PBS and adjusted to an optical density of 1.0 (550nm) with PBS prior to use. For immunodiffusion tests, soluble antigens were prepared by sonication (six times at 200W for 30 sec) of formalin-killed bacteria. The double immunodiffusion test was performed with the use of 1% agarose gel. Soluble antigens were added to the duplicate outer peripheral wells and the undiluted antiserum was added to the central well (approximate volume per well, 25μl). Gels were incubated for 2 days at 20℃ before examination.

Electrophoresis :

1) Outer membrane proteins (OMPs) preparation : The method of Thomson *et al*[18] was followed for the preparation of OMPs of Korean isolates and *C columnaris* NCMB 2248^T with some modifications. The whole-cell suspension was sonicated (three times at 200W for 10min) in 1mM EDTA-20mM Tris HCl (pH 7.8). After centrifugation at 10,000×g for 20min, 1% Sarkosyl (N-lauroylsarcosine ; Sigma) was added to the supernatant, and the mixture was incubated at 4℃ overnight. After centrifugation, the pellet was resuspended in distilled water and was stored at -20℃. The protein concentration was determined by a modified Lowry technique[19].

2) Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) : The OMPs were electrophoresed on polyacrylamide gels (12% separating, 4% stacking) according to the method of Laemmli[20]. Electrophoresis was performed at 90V for 4h.

Results

Morphological and phenotypic characterization : The bacteria showed a slow gliding motility and somtimes gathered into columns (Fig 1).

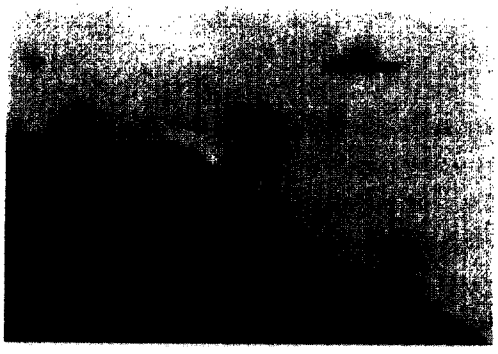

Fig 1. Columnar formations of Korean isolate KGF 1 along the margin of a piece of the skin. Bar = 50μm.

All test isolates of *Cytophaga columnaris* and the reference strains exhibited gram-negative slender rods. Actively growing cells were approximately 0.5μm in diameter and 4~12μm long. Generally, the organisms tended to become shorter and thicker as the cultures aged. Their colonial morphology are gliding, strongly adherent, rhizoid, yellow and flat colonies on CA (Fig 2).

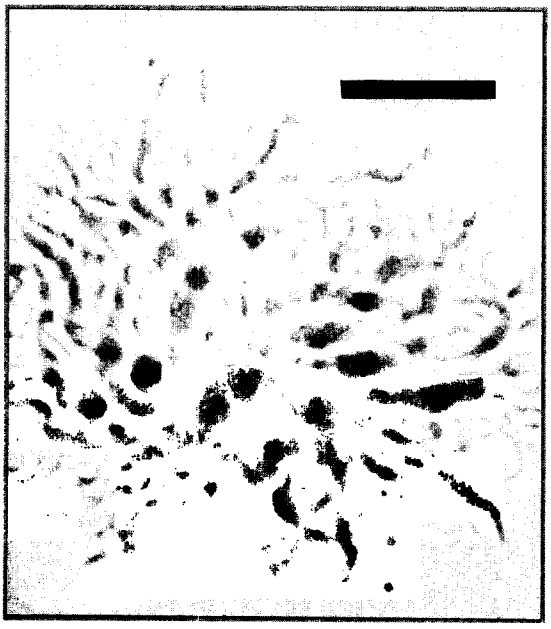

Fig 2. Typical rhizoid colony of Korean isolate KRT 1 on CA (48h, 25℃, Phase contrast microscopy). Bar = 0.5mm.

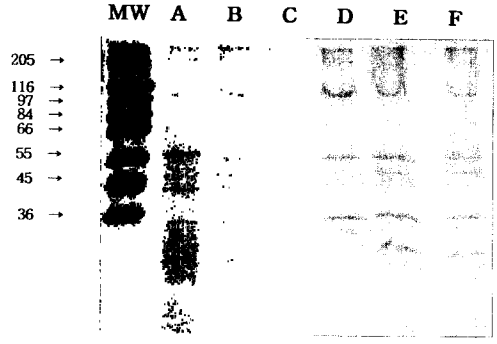

Fig 3. SDS-PAGE analysi of OMPs preparations of Korean isolates and *C columnaris* NCMB 2248^T. MW : molecular weight standards (values in kDa). Lane A : KRT 1, lane B : KRT 2, lane C : KGF 1, lane D : KGF 2, lane E : KAU 1, lane F : NCMB 2248^T.

As shown in Table 2, all isolates and reference strains were able to grow with NaCl concentration below 1.0%, at pH values above 6.0, and at 15, 25 and 30℃. None of the

Table 3. Biochemical characteristics of Korean isolates and *C columnaris* reference strains

Characteristic	KRT 1	KRT 2	KGF 1	KGF 2	KAU 1	NCMB 2248^T	EK 28
Production of							
Flexirubin	+	+	+	+	+	+	+
Cytochrome-*c* oxidase	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+
Hydrogen sulfide	+	+	+	+	+	+	+
Ornithine decarboxylase	-	-	-	-	-	-	-
Degradation of							
starch	-	-	-	-	-	-	-
Gelatin	+	+	+	+	+	+	+
Casein	+	+	+	+	+	+	+
Congo red test	+	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	+	+
Lecitin hydrolysis	+	+	+	+	+	+	+

+ : postive or present, - : negative or absent.

Table 4. Carbohydrate reactions of Korean isolates and *C columnaris* reference strains

Characteristic	KRT 1	KRT 2	KGF 1	KGF 2	KAU 1	NCMB 2248^T	EK 28
Sucrose	-	-	-	-	-	-	-
Glucose	-	-	-	-	-	-	-
Inuline	-	-	-	-	-	-	-
Xylose	-	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-
Trehalose	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-

Acid production from carbohydrate ; - : negative or absent.

strains grew anaerobically, at 40℃, nor at 1.0% NaCl. All of the strains failed to grow at pH values below 7.0. No growth occurred in TSB. All strains used were sensitive to O/129.

Cytochrome-c oxidase, hydrogen sulfide and catalase were positive in all strains. Non diffusible flexirubin type pigments were present. All strains failed to produce ornithine decarboxylase. All of the strains tested hydrolyzed gelatin, lecitin and casein, but not starch. Congo red was absorbed by colonies. Nitrate was reduced to nitrite (Table 3).

Table 5. Agglutinin titers of six rabbit anti-*C columnaris* sera against homologous and heterologous antigens

Agglutinogen	Agglutinin titers antisera to					
	KRT 1	KRT 2	KGF 1	KGF 2	KAU 1	NCMB 2248^T
KRT 1	**1024**	512	512	512	512	512
KRT 2	512	**1024**	512	512	512	512
KGF 1	512	512	**4096**	1024	512	1024
KGF 2	512	512	1024	**2048**	512	512
KAU 1	512	512	512	512	**2048**	512
NCMB 2248^T	512	512	1024	512	1024	**2048**

All strains tested did not produced acid from any carbohydrate after 7 days of incubation (Table 4).

Antigenic characterization : Agglutinin titers of antisera for homologous formalin-killed cells ranged from 1024 to 4096 and those for heterologous formalin-killed cells ranged from 512 to 1024 (Table 5). The Korean isolates shared varying degrees of antigenic similarity with *C columnaris* NCMB 2248^T.

The Korean isolates and reference strain (NCMB 2248^T) shared many lines of precipitation in double immunodiffusion gels using formalin-killed antigens.

Electrophoretic characterization : SDS-PAGE analysis showed that the OMPs of all isolates and reference strain NCMB 2248^T almost had similar gel patterens, regardless of whether the isolates were derived from different fishes (Fig 3).

Discussion

Morpological, pysiological, and biochemical characteristics showed that all isolates were very similar to the two tested reference strains. Also, our results with respect to *Cytophaga columnaris* agree quite well those of other authors for this organism[21~24].

Serologically, the isolates shared many lines of precipitation in double immunodiffusion gels and high agglutinin titer with the reference strain. Korean isolates and refrence strain (NCMB 2248^T) are serologically related. The antisera greatly facilitated the identification of *C columnaris* and permited serological comparisons among the isolates.

Electrophoretic analysis of Korean isolates revealed similar pattern in profiles of OMPs when compared with reference strain. Therefore, it appears that there is no variation in *C columnaris* strains on the OMPs level. Only minor differences were observed in the intensity of staining reactions.

It was reported that the base compositions of *C columnaris* NCMB 2248^T were 32mol% G+C[8]. To enhance the abilities to recognize *C columnaris*, further studies of DNA homology between the Korean isolates and other reference strains would help establish their genetic relationship.

Columnaris disease has been known to occur in many species of cultured freshwater fishes and has caused economic loss in Korea. Yoo[25] and Chun[26] reported that *Flexibacter columnaris* was isolated from catfish and tilapia (*Tilapia* sp.) but the etiological agent was not isolated and identified except this report before. This is the first description that the Korean isolates from columnaris disease outbreaks of these three species were confirmed to be *C columnaris*.

In conclusion, the morphologic, phenotypic, antigenic, and electrophoretic characteristics of our five test isolates clearly identified them as *C columnaris* regardless of the species of origin.

Summary

Five strains of gram-negative and yellow-pigmented bacteria were recently isolated from diseased freshwater fishes in Korea. All isolates were confirmed as a known fish pathogen of columnaris disease, *Cytophaga columnaris* based on

their colonial and cellular morphology, and on physiological, biochemical and antigenic characteristics. Although the isolates were from different fish species, their characteristics of them were very similar to those of the reference strains of *C columnaris* (NCMB 2248^T and EK 28). Also, profiles of OMPs of Korean isolates were similar to those of the reference strain, *C columnaris* NCMB 2248^T when analyzed by SDS-PAGE.

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