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'Flexibacter columnaris': first description in France and comparison with bacterial strains from other origins

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ABSTRACT: Three strains of gliding bacteria resembling a known fish pathogen, *Flexibacter columnaris*, were recently isolated from diseased freshwater fishes in France. Morphological, physiological, and biochemical characteristics were very similar to those of 6 *F. columnaris* strains previously identified in other countries and used here for reference. This is the first unequivocal identification of this fish pathogen in France. Characteristics of this organism that distinguish it from other, apparently non-pathogenic, gliding bacteria of fish origin include its strongly adherent and rhizoid, yellow, flat colonies on solid media; its absorption of Congo red dye, and its production of flexirubin-type pigments. Other useful distinguishing characteristics are the production of H_2S and the reduction of nitrates, the absence of action on any carbohydrates, the rapid and intense hydrolysis of lecithin, and the absence of growth in media containing more than 0.5% NaCl.

INTRODUCTION

'Columnaris' disease, caused by the bacterial pathogen *'Flexibacter columnaris'* (Leadbetter 1974), is a disease of many freshwater fishes and has a worldwide distribution. This bacterium has not been listed in the 'Approved Lists of Bacterial Names' (Skerman et al. 1980, Moore et al. 1985), nor has it been validated in the International Journal of Systematic Bacteriology. Thus, its name must be cited in quotation marks.

'Columnaris' disease has been described in many freshwater fishes (Bullock et al. 1971, Mc Carthy 1975), and can be of economical importance in intensive fish farming (Amend 1982). Low virulence bacterial strains become pathogenic for salmonids at water temperatures exceeding 20°C, while high virulence strains may be pathogenic at temperatures above 15°C. Mortality rates range from ca 10 to 100% depending on water temperature (Holt et al. 1975). The clinical signs and lesions are usually restricted to the body surface and take the form of skin erosions and gill necrosis. Characteristic 'saddleback' lesions can be found in advanced cases in catfish. A systemic infection may occur in severe cases, depending on the virulence of the strains (Snieszko & Bullock 1976, Amend 1982).

In France, gliding bacteria are commonly isolated from external lesions of diseased fish. The current classification of this group of organisms makes accurate identification difficult, at best. Additionally, *'Flexibacter columnaris'* has never been definitively identified in France and most isolates probably belong to non-pathogenic members of the order Cytophagales.

Recent investigations carried out on gliding bacteria isolated from several species of freshwater fish in France resulted in the identification of 3 bacterial isolates as *'F. columnaris'*. This study was done to compare the morphological, physiological, and biochemical characteristics of these isolates with those of 6 *'F. columnaris'* strains originating from the USA, Japan, and Hungary.

MATERIALS AND METHODS

Origin of the strains. The host and geographical origin of each isolate is given in Table 1.

Strain 39/87 originated from an outbreak of heavy mortality occurring among a black bullhead *Ictalurus melas* population in shallow waters of a gravel-pit. Diseased fish had extensive yellow-edged skin ero-

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Table 1. Origins of the 9 strains of '*Flexibacter columnaris*' NCMB: National Collection of Marine Bacteria, Aberdeen, Scotland, UK. Strain NCMB 1038 was isolated by E. J. Ordal. American strains were kindly provided by R. A. Holt (Department of Microbiology, Oregon State University, Corvallis, Oregon); the Japanese strain by H. Wakabayashi (Department of Fisheries, Faculty of Agriculture, University of Tokyo); and the Hungarian strain by J. Farkas (Fisheries Research Institute, Szarvas)

Strains	Fish species	Isolated from	Origin	Year
Reference strains				
NCMB 1038	Salmonid	?	USA	?
NCMB 2248	Salmonid	Subculture of NCMB 1038	USA	?
DD3	Adult chinook salmon *Oncorhynchus tshawytscha*	Gill lesions	Dexter Dam, Oregon, USA	1969
IC8	Young catfish *Ictalurus* sp.	Kidney	Idaho, USA	1969
EK 28	Japanese eel *Anguilla japonica*	Gill lesions	Japan	1967
H82/7	Carp *Cyprinus carpio*	Skin ulcer	Hungary	1982
New strains				
39/87	Adult black bullhead *Ictalurus melas*	Skin ulcer	France	1987
44/87	Brook trout fry *Salmo trutta*	Skin lesions	France	1987
P03/87	Young sheatfish *Silurus glanis*	Kidney	France	1987

sions on the head, from which *Citrobacter freundii* and *Aeromonas hydrophila* were also isolated. Strain 39/87 was also abundant in the spleen and the kidney. Bacterial strain 44/87 was isolated from a brown trout *Salmo trutta* fry on a fish-farm where chronic mortalities were being experienced following a rise in the water temperature to 16°C. Skin around the dorsal fin was blanched but showed no real ulceration. Strain P03/87 was isolated from the kidney of a young sheatfish *Silurus glanis* kept at 20°C in our laboratory. The affected specimen was one of a number originating from a fish-farm in Belgium but had earlier been imported from Hungary as young fry. The skin was locally blanched and ulcerated, and mortality in the affected stock reached 3% per day. The bacterium was also abundant in the skin lesions.

Cultivation. The bacterial strains were grown in the medium of Anacker & Ordal (1955). The medium was used as a broth (AOB) or as an agar (AOA). The composition of the medium was as follows: tryptone 0.05%; yeast extract 0.05%; sodium acetate 0.02%; beef extract 0.02%. To obtain AOA, 0.9% agar was added. The pH of the medium was adjusted to between 7.2 and 7.4. All cultures were incubated at 22°C.

Morphological studies. Wet mounts of infected tissue from external lesions (French isolates only) were checked under the microscope (×100 magnification) for the presence of gliding bacteria.

On AOA, the morphology of 2- to 5-d-old colonies was studied using magnifications of ×20 and ×100 when necessary.

Broth cultures were checked at 48 h for the following features: (1) appearance of the growth in an AOB flask culture grown on an orbital shaker; (2) gliding motility of the bacteria using a hanging drop and phase contrast microscopy (×1000 magnification); (3) morphology of Gram-stained bacteria, observed under the microscope (×1000 magnification).

Biochemical tests. The following tests were performed on the 9 strains:

Presence of flexirubin-type pigments was determined using the KOH method of Reichenbach et al. (1974).

Production of an extracellular galactosamine glycan was revealed by absorption of Congo red (Johnson & Chilton 1966, McCurdy 1969).

Respiratory metabolism: Anaerobic growth was tested in deep AOA tubes inoculated before solidification; the presence of a cytochrome oxidase was determined using the appropriate filter paper reagent disks (Diagnostics Pasteur, Marnes-la-Coquette, France); the presence of a catalase was tested for with hydrogen peroxide; the ability to reduce nitrates was investigated in 0.1% potassium nitrate AOB tubes (Bullock 1972); and the production of hydrogen sulfide was detected using a strip of filter paper impregnated with

lead acetate and suspended above inoculated AOB (Pacha 1968).

Carbohydrate metabolism: Presence of a β-galactosidase was tested for with ONPG (*o*-nitrophenyl-β-D-galactopyranoside) filter paper disks (Diagnostics Pasteur).

Ability to degrade polysaccharides was investigated in the following media: sterile filter paper (cellulose) on Dubos agar (Reichenbach & Dworkin 1981); 3 % carboxymethylcellulose AOB (modified from Lewin & Lounsbery 1969); a thin top layer of chitin agar containing 20 % chitin above a thick layer of the same medium lacking chitin (Reichenbach & Dworkin 1981); 0.2 % soluble starch AOA (Bullock 1972); aesculin agar (Diagnostics Pasteur); and classical AOA to test for agar digestion.

Ability to use other carbohydrates was tested by 2 methods: firstly, on AOA slants containing 0.2 % bromothymol blue and 1 % carbohydrate (fructose, galactose, glucose, mannose, glycerol); secondly, using a microtechnique with 49 different carbohydrates (API 50 CH strips, API System SA, La Balme-les-Grottes, France) inoculated with a suspension of bacteria in Ammonium Salt Sugars broth (medium composition modified from Cowan & Steel 1974). In both methods, oxidation of the carbohydrate was demonstrated by a change in colour, from blue to green and yellow.

Protein metabolism: Gelatin hydrolysis was tested for by the film method of Le Minor & Piechaud (1963); casein digestion was determined using 5 % skim milk AOA (modified from Pacha 1968); tyrosine hydrolysis and/or pigment production was investigated using 0.5 % L-tyrosine AOA (Bullock 1972); and presence of decarboxylases and dihydrolase was determined using liquid media containing 1 % L-lysine, L-ornithine, or L-arginine hydrochloride (Diagnostics Pasteur).

Lipid metabolism: Lipase production was tested for on agar supplemented with 1 % tributyrin (glycerol tributyrate) emulsified with 0.1 % polyvinylic alcohol (Mourey & Kilbertus 1976); lecithinase production was investigated using 5 % sterile egg yolk AOA (modified from Cowan & Steel 1974); and esterase production was determined on agar containing 1 % Tween 20 or Tween 80 (modified from Sierra 1957).

Enzyme production: Production of 19 different enzymes was tested for using a special microtechnique (API ZYM strips, API System SA, La Balme-les-Grottes, France); results are read after 12 h incubation at 22 °C.

Antibiotic sensitivity: Sensitivity to the following drugs was tested for on AOA by the diffusion method: ampicillin, cefalotin, streptomycin, gentamycin, neomycin, kanamycin, tetracycline, chloramphenicol, erythromycin, polymyxin B, novobiocin, sulphonamides, trimethoprim, nalidixic acid, furans (all disks by Diagnostics Pasteur), and actinomycin D (disks prepared at the laboratory). Sensitivity to the vibriostatic compound O/129 (Diagnostics Pasteur) was tested for on a separate AOA plate. Inhibition zone diameters were recorded after 48 h incubation at 22 °C.

Physiological studies. The tolerance of the strains to temperature, NaCl concentration, and pH was studied as follows: AOB and TSB (trypticase soy broth) tubes were incubated at 15, 22, 25, and 37 °C; tubes of AOB containing 0.5, 1, 2, and 3 % NaCl were incubated at 22 °C; tubes of AOB, adjusted to various pH values (4 to 11), were incubated at 22 °C. Bacterial growth at the different temperatures, NaCl concentrations, and pH values was recorded following visual observation at 48 h.

RESULTS

Morphological studies

Wet mounts of infected tissue

Under the microscope, infected tissue was found to contain large numbers of long slender bacteria. The bacteria showed a slow gliding motility and sometimes gathered into 'columns'

Cultivation on AOA

The 9 strains produced yellow colonies of rather different sizes and shapes. All the strains except NCMB 1038 grew as rhizoid, flat colonies, adhering either strongly (H82/7, EK 28, NCMB 2248, 39/87, 44/87, P03/87) or weakly (DD3, IC8) to the agar. The colonies of NCMB 1038 were round, convex and non-adherent; their margins were sometimes feebly rhizoid. In some cases (H82/7, EK 28), different shapes were found on the same plate: some colonies were more rhizoid than others, and some were very small and umbonate. Under the stereomicroscope (× 20) with oblique transmitted illumination, colonies of the 9 strains appeared with small, yellow-blue, iridescent waves, sometimes with a fibrous or curly appearance. Under the microscope (× 100), the edges of the colonies appeared more or less rhizoid, with small groups of parallel bacteria moving to produce 'branches', arborizations, 'antlers', or curls (Fig. 1).

Cultivation in AOB

In shaken flasks, all strains except NCMB 1038 formed, in addition to the usual turbid culture, a thick ring of bacteria adhering to the glass at the edge of the liquid. Numerous filamentous tufts of bacteria also adhered to the base and sides of the flasks. Observation

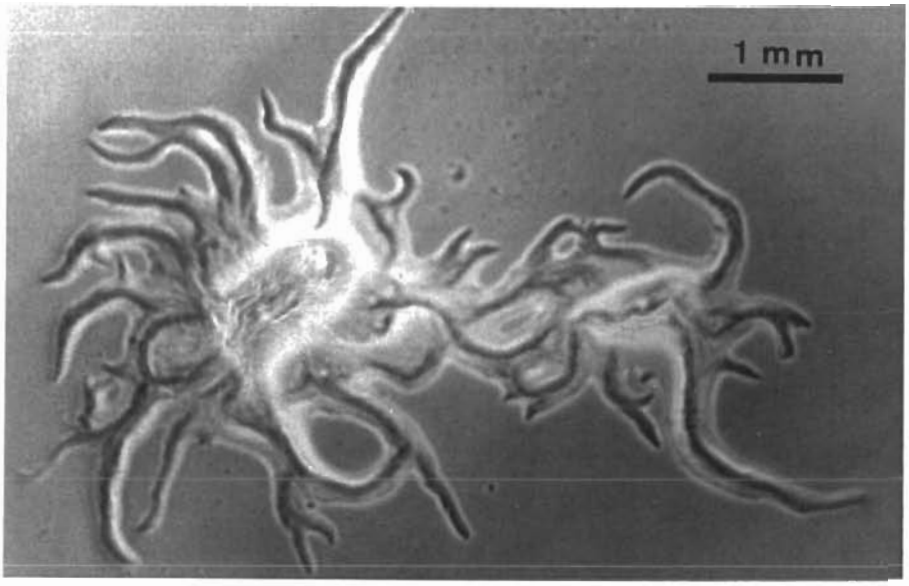

Fig. 1. *'Flexibacter columnaris'*. Typical rhizoid colony (strain NCMB 2248) on Anacker & Ordal Agar (48 h, 22 °C. Phase contrast microscopy)

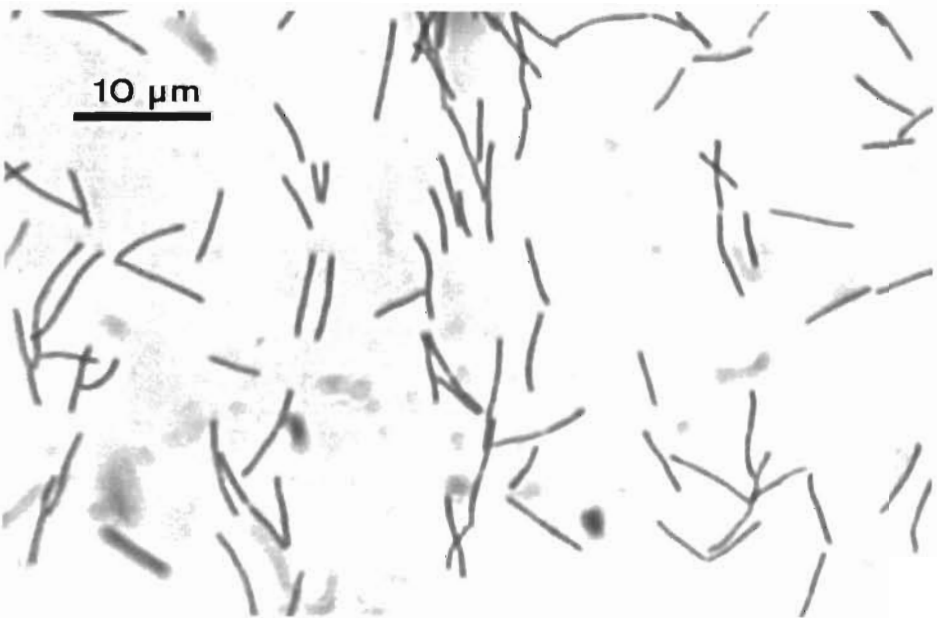

Fig. 2. *'Flexibacter columnaris'* Gram-stained 48 h old cells of strain 39/87 derived from Anacker & Ordal Broth

of a hanging drop of the broth under the microscope showed bacteria slowly gliding forward, then backward; they frequently bent and pivoted around one extremity fixed to the glass. On Gram staining, all 9 strains appeared as Gram-negative, slender, and rather long bacilli (Fig. 2); most of the cells were 3 to 8 µm long and 0.3 to 0.5 µm wide, but all strains also produced some longer cells (10 to 25 µm).

Biochemical tests

The main results are presented in Table 2.

There was no evidence of production of acid from any of the 49 carbohydrates tested in API 50 CH strips, even after 10 d.

The production of the 19 enzymes tested in API ZYM strips (quantified from 0 to 5) is presented in Table 3.

Physiological studies

As shown in Table 4, our 9 bacterial strains were able to grow with NaCl concentration below 1 %, at pH values above 6, and at 22 °C and 25 °C. The ability to grow at pH 6, and at 15 °C and 37 °C, depended on the strain.

No growth occurred in trypticase soy broth.

Table 2. *'Flexibacter columnaris'* Some characteristics of 6 reference strains and 3 test strains. –: negative; + positive; (+): weakly positive; 6/9 etc: number of positive strains/number tested

Flexirubin-type pigment	+	Tributyrin hydrolysis	+
Congo red test	+	Lecithin hydrolysis	+
Anaerobic growth	–	Tween 20 hydrolysis	+
Presence of cytochrome oxidase	+	Tween 80 hydrolysis	(+)
		Acid from fructose	4/9
Presence of catalase	+	galactose	1/9
Nitrate reduction	+	glucose	3/9
Production of H_2S	+	mannose	1/9
Presence of β-galactosidase (ONPG)	–	glycerol	–
		Sensitivity to	
Cellulose hydrolysis	–	ampicillin	+
Carboxymethylcellulose hydrolysis	–	cefalotin	+
		streptomycin	+
Chitin hydrolysis	–	gentamycin	–
Starch hydrolysis	–	neomycin	–
Aesculin hydrolysis	–	kanamycin	–
Agar hydrolysis	–	tetracycline	+
Gelatin hydrolysis	+	chloramphenicol	+
Casein hydrolysis	+	erythromycin	+
Tyrosine hydrolysis	1/9	polymyxin B	–
Brown pigment of tyrosine agar	6/9	novobiocin	+
		sulphonamides	3/9
Presence of arginine dihydrolase	–	trimethoprim	–
		nalidixic acid	+
Presence of lysine decarboxylase	–	furans	+
		actinomycin D	–
Presence of ornithine decarboxylase	–	Sensitivity to O/129	+

DISCUSSION

The 9 strains were similar to each other in size, shape, and gliding motility of bacteria. However, NCMB 1038 differed greatly from the other strains in that it produced round non-rhizoid colonies that failed to adhere to agar and to glass. A special leaflet edited by the National Collection of Industrial and Marine Bacteria (NCIMB) confirms that NCMB 1038 was initially a typical *'Flexibacter columnaris'* strain but that it became atypical (it lost its ability to produce rhizoid, adherent colonies) through subcultivation. The original (typical) form of NCMB 1038 was actually the source of strain ATCC 23463 (American Type Culture Collection) which, in turn, served as the source of strain NCMB 2248.

Results in Tables 2, 3, and 4 show that our isolates 39/87, 44/87, and P03/87 were biochemically and physiologically similar to the 6 tested reference strains. Nevertheless, some differences occurred among the 9 strains. For example, P03/87 was the only strain degrading tyrosine and only 6 strains produced a distinct brown diffusible pigment on tyrosine agar.

Differences in acid production from carbohydrates on AOA slants were more difficult to interpret because, in some cases, an acidification occurred in inoculated control tubes lacking the added carbohydrate. This suggests that the medium contained other degradable components and indicates that AOB is not a particu-

Table 3. Production of 19 enzymes by 6 *'Flexibacter columnaris'* reference strains and 3 test strains, as demonstrated by API ZYM micromethod. Amounts of enzymes produced, noted from 0 to 5, are deduced from the intensities of the colours

Enzymes	Strain								
	NCMB 1038	NCMB 2248	H82/7	EK28	DD3	IC8	39/87	44/87	P03/87
Control	0	0	0	0	0	0	0	0	0
Alkaline phosphatase	5	5	5	5	5	5	4	4	5
Esterase (C4)	2	2	2	2	3	3	2	1	2
Esterase lipase (C8)	3	3	4	3	4	4	3	3	3
Lipase (C14)	1	1	0	1	0	0	0	0	1
Leucine arylamidase	5	4	5	4	5	4	4	4	5
Valine arylamidase	4	4	2	4	4	3	4	4	5
Cystine arylamidase	1	1	1	2	0	0	1	1	1
Trypsin	3	3	0	3	0	0	3	3	2
α-Chymotrypsin	2	1	0	2	0	0	1	2	2
Acid phosphatase	4	4	3	3	3	2	3	4	4
Naphtol-AS-BI-phosphohydrolase	5	4	3	2	2	2	2	3	3
α-Galactosidase	0	0	0	0	0	0	0	0	0
β-Galactosidase	0	0	0	0	0	0	0	0	0
β-Glucuronidase	0	0	0	0	0	0	0	0	0
α-Glucosidase	0	0	0	0	0	0	0	0	0
β-Glucosidase	0	0	0	0	0	0	0	0	0
N-acetyl-β-glucosaminidase	0	0	0	0	0	0	0	0	0
α-Mannosidase	0	0	0	0	0	0	0	0	0
α-Fucosidase	0	0	0	0	0	0	0	0	0

Table 4. *'Flexibacter columnaris'* Growth of 6 reference strains and 3 test strains under various environmental conditions. −: negative; +: positive; (+): weakly positive

	Strain								
	NCMB 1038	NCMB 2248	H82/7	EK28	DD3	IC8	39/87	44/87	P03/87
Growth at:									
15 °C	(+)	(+)	(+)	−	(+)	+	(+)	+	−
22 °C	+	+	+	+	+	+	+	+	+
25 °C	+	+	+	+	+	+	+	+	+
37 °C	−	−	+	+	+	+	+	+	−
Growth with:									
0 % NaCl	+	+	+	+	+	+	+	+	+
0.5 % NaCl	+	+	+	+	+	+	+	+	+
1.0 % NaCl	−	−	−	−	−	−	−	−	−
2 % NaCl	−	−	−	−	−	−	−	−	−
3 % NaCl	−	−	−	−	−	−	−	−	−
Growth at:									
pH 4	−	−	−	−	−	−	−	−	−
5	−	−	−	−	−	−	−	−	−
6	−	−	−	−	+	(+)	(+)	+	+
7	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+	+	+
11	+	+	+	+	+	+	+	+	+

larly suitable medium for carrying out tests on carbohydrate metabolism.

Concerning sensitivity to antibiotics, the only difference noted among the strains was with the sulphonamides: 3 strains were sensitive (NCMB 1038, DD3, EK 28) and 6 were resistant. With the sulphonamides, the diameter of the inhibition zone was difficult to measure accurately, because the transition from growth to no growth was very gradual. Moreover, the significance of the results is difficult to assess because the experimental conditions required by many freshwater bacteria (22 °C, 48 h) are quite different from those specified in the World Health Organization instructions (Michel & Bassalert 1982). The results suggest that some strains may have come in contact with sulphonamides in the past.

All strains were highly sensitive to O/129, inhibition zones being about 30 mm wide. It is worthy of note that they were all resistant to trimethoprim. Thus, unlike the findings with *Vibrio* spp. reported by Richard (1987), there was no correlation between sensitivity to O/129 and trimethoprim among isolates of *'Flexibacter columnaris'*. Richard found that certain species of *Vibrio* that were resistant to trimethoprim were also resistant to O/129, due, perhaps, to structural similarities between the 2 drugs.

Results of the API enzyme assays, summarized in Table 3, show the only differences among the 9 strains occur with trypsin and α-chymotrypsin: 3 strains (H82/7, DD3 and IC8) failed to produce these enzymes. It is worth noting that there was no correlation between presence or absence of these enzymes and hydrolysis of gelatin and casein: these proteins were degraded by all strains. The inability to degrade carbohydrates in API 50 CH strips and in agar (fructose, galactose, glucose, mannose, glycerol, cellulose, carboxymethylcellulose, chitin, starch, aesculin, and agar itself) was confirmed by the absence of the enzymes involved in hydrolysis of certain carbohydrates using API ZYM strips (α and β-galactosidases, β-glucuronidase, α and β-glucosidases, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase).

Our results with respect to *'Flexibacter columnaris'* agree quite well with those of other authors for this organism (Pacha & Porter 1968, Mitchell et al. 1969, Wakabayashi et al. 1970, Bootsma & Clerx 1976, Christensen 1977, Farkas & Olàh 1980, Pyle & Shotts 1980, Campbell & Buswell 1982, Farkas 1984). However, there are slight differences. For example, nitrate reduction is considered by most authors to be negative, but Wakabayashi et al. (1970) found it to be positive, as we did.

Although some atypical reactions could occur, the different methods used to demonstrate acid production from carbohydrates gave negative results. Likewise, most authors have considered that *'Flexibacter columnaris'* is unable to carry out this degradation.

Presence of a lipase was revealed by tributyrin hydrolysis. Pacha & Porter (1968) and Farkas & Olàh (1980), using 0.1 % tributyrin AOA, failed to find

hydrolysis by *'Flexibacter columnaris'*. On the other hand, our 9 strains gave positive results on the medium of Mourey & Kilbertus (1976), with clearing of the medium under and around the colonies, probably thanks to the emulsification of tributyrin by polyvinylic alcohol which results in tributyrin microdrops that are more readily degraded by bacteria. Concerning Tween 80, Campbell & Buswell (1982) found NCMB 1038 to be unable to hydrolyze it; our strains hydrolyzed it, but always more weakly and slowly than Tween 20.

Morphological, physiological, and biochemical characteristics clearly identify our isolates as *'Flexibacter columnaris'*. Some characteristics of *'F. columnaris'* are quite different from those of other species of gliding bacteria commonly isolated from diseased fish and thus could prove important in distinguishing it from opportunistic species not known to be pathogenic. The most significant characteristics of this organism are as follows: it produces strongly adhering and rhizoid flat yellow colonies on solid media; it reacts with the Congo red dye; it produces flexirubin-type pigments and H_2S; and it reduces nitrates. Other important features are: its lack of β-galactosidase (ONPG test) and of other enzymes involved in carbohydrate metabolism (API ZYM strips); its inability to attack carbohydrates on API 50 CH strips; its failure to degrade starch and aesculin; its rapid and intense hydrolysis of lecithin; and its inability to grow in trypticase soy broth or in AOB containing more than 0.5 % NaCl.

The finding of *'Flexibacter columnaris'* in France is significant in that this is the first report of this serious fish pathogen in this country. Unfortunately, it is presently impossible to state how the pathogen made its way into the country. Additional studies are in progress to enhance our abilities to recognize *'F. columnaris'*. The DNA from each strain has been extracted and purified in order to perform DNA/DNA hybridization tests. Moreover, the production of antisera against our strains is planned. The antisera should greatly facilitate the identification of the pathogen and permit serological comparisons among the isolates.

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