Vol. 8: 233–237, 1990 | DISEASES OF AQUATIC ORGANISMS Dis. aquat. Org. | Published July 12

NOTE

Flexibacter maritimus is the agent of 'black patch necrosis' in Dover sole in Scotland

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ABSTRACT: A *Flexibacter columnaris*-like bacterium (Strain NCMB 2158), isolated from Dover sole suffering from 'black patch necrosis' (BPN) in Scotland, was compared to *F. columnaris, F. psychrophilus, F. maritimus,* and to all other valid *Flexibacter* and *Cytophaga* species. Investigation of phenotypic characteristics showed the unidentified isolate to be more closely related to *F. maritimus* to than *F. columnaris*. DNA was subsequently extracted from each of the studied strains and compared using the DNA/DNA hybridization method. The very high DNA relatedness of Strain NCMB 2158 with 2 *F. maritimus* reference strains and its insignificant homology with all of the other strains confirmed that the Dover sole isolate belonged to the species *F. maritimus*. These results have resolved the bacterial aetiology of BPN and reveal for the first time the existence of *F. maritimus* elsewhere than in Japan.

During the 1970's, commercial production of Dover sole *Solea solea* juveniles at the Sea Fish Industry Authority, Marine Cultivation Unit, Hunterston, Ayrshire, Scotland, was adversely affected by a persistent disease known as 'black patch necrosis' (BPN). The skin lesions associated with the disease have been described by McVicar & White (1979): they begin as slight blistering of the skin surface or as dark areas between caudal and marginal fins. This darkening rapidly expands, and the loss of epithelial surface exposes haemorrhagic dermal tissues. The lesions develop into necrotic ulcers due to invasion by saprophytic organisms. In young fish, the condition can progress rapidly, causing mortalities up to 10% d^{-1}. The same authors noted a similarity to the lesions found in columnaris disease of freshwater fish and surmised an infectious aetiology. They failed to isolate any bacterial agent, but showed that no virus was involved and that the disease was strongly related to stress and particularly to skin surface conditions: the introduction of sand on tank floors resulted in a dramatic improvement, with the disease being virtually eliminated (McVicar & White 1982).

Subsequently, similar bacterial strains were repeatedly isolated from necrotic lesions of diseased Dover sole and one of them was compared with a reference *Flexibacter columnaris* strain (NCMB 1038): the isolate and the type culture were identical with respect to cell morphology, oxidase test, production of catalase and H_2S, gelatin liquefaction, degradation of tyrosine, and hydrolysis of casein, Tween 20, and starch. In addition, the isolates showed similar patterns of sensitivity to a range of antibiotic (Campbell & Buswell 1982). These results suggested that BPN was provoked by a *F. columnaris*-like bacterium (Campbell & Buswell 1981). However, the Dover sole isolate was shown to have an absolute requirement for seawater, whereas *F. columnaris* failed to grow on seawater-containing media. The role of the *F. columnaris*-like bacterium as the aetiological agent of BPN was demonstrated: the organism was repeatedly isolated from diseased tissue but not from healthy tissue; it was highly pathogenic to experimentally infected Dover sole, provoking 100% mortality within 96 h at 17.5°C; and it was reisolated from every infected fish (Campbell & Buswell 1982). A representative isolate was deposited in the National Collection of Industrial and Marine Bacteria (NCIMB, Aberdeen, Scotland) under the code NCMB 2158.

Despite the strong resemblance to the known fish pathogen *Flexibacter columnaris*, it is unlikely that the fish isolate belongs in this species: several strains of a given bacterial species may differ slightly in their NaCl tolerance, but there are few examples of bacteria able to grow as well in seawater as in freshwater. This was the opinion of Baxa et al. (1986) who stated that 'being

0177-5103/90/0008/0233/$ 03.00

of marine origin, the microorganism could not be considered as *F. columnaris*'. In fact, the bacterial isolate NCMB 2158 seemed closer phenotypically to *F. maritimus*, another fish-pathogenic species, than to *F. columnaris* (L. B. Perry, NCIMB, pers. comm.).

Flexibacter maritimus was isolated in Japan by Hikida et al. (1979) from species of marine fish suffering from a disease first described by Masumura & Wakabayashi (1977). A detailed study of 15 strains was published by Wakabayashi et al. (1984) who showed that the bacterium had an absolute requirement for seawater. These authors demonstrated the pathogenicity of the bacterium and they proposed a new species, *'F. marinus'*. However, it was the opinion of Austin & Allen-Austin (1985) that 'a detailed comparison to existing species remains to be done'. Wakabayashi et al. (1986) validated the new species under the name *F. maritimus* and proposed a type strain (NCMB 2154^T) but these authors did not investigate the molecular biology of their strains. Baxa et al. (1987) confirmed the

Table 1. *Cytophaga* and *Flexibacter* strains included in this study. Abbreviations are: ATCC: American Type Culture Collection, Rockville, USA; NCIB, NCMB: National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland; DSM: Deutsche Sammlung von Mikroorganismen, Göttingen, FRG; JCM: Japanese Collection of Microorganisms, Tokyo, Japan; Holt: R. A. Holt, Department of Microbiology, Oregon State University, USA; Wakabayashi: H. Wakabayashi, Department of Fisheries, Faculty of Agriculture, University of Tokyo, Tokyo, Japan; Farkas: J. Farkas, Fisheries Research Institute, Szarvas, Hungary; TG: Laboratoire d'Ichtyopathologie, Insitut National de la Recherche Agronomique, Thiverval-Grignon, France; LNPAA: Laboratoire National de Pathologie des Animaux Aquatiques, IFREMER, Centre de Brest, Plouzané, France

Name as received	Strain	Source
C. aquatilis	DSM 2063^T	Gills of diseased salmon, Michigan
C. arvensicola	JCM 2836^T	Soil, Osaka, Japan
C. fermentans[a]	NCMB 2218^T	Marine mud, California
C. flevensis	DSM 1076^T	Lake Ijsselmeer, The Netherlands
C. heparina	NCIB 9290^T	Soil
C. hutchinsonii	NCIB 9469^T	Soil
C. johnsonae	DSM 2064^T	Soil or mud, Rothamsted or Cambridge, England
C. latercula[a]	NCMB 1399^T	Outflow of marine aquarium, California
C. lytica[a]	NCMB 1423^T	Beach mud, Limon, Costa Rica
C. salmonicolor[a]	NCMB 2216^T	Marine mud, California
'C. allerginae'	ATCC 35408	Water in industrial air-cooling unit, United States
F. aggregans[a]	NCMB 1443^T	Beach sand, Tema, Ghana
F. aurantiacus	NCMB 1382^T	Garden soil, Minnesota
F. canadensis	ATCC 29591^T	Soil, Canada
F. flexilis	NCMB 1377^T	Lily pond, San Jose, Costa Rica
F. litoralis[a]	NCMB 1366^T	Outflow of marine aquarium, California
F. polymorphus[a]	ATCC 27820^T	Decaying ascidian, La Paz, Mexico
F. roseolus	NCMB 1433^T	Hot spring, Agua Caliente, Costa Rica
F. ruber	NCMB 1436^T	Hot spring, Geysir, Iceland
F. sancti	NCMB 1379^T	Buenos Aires, Argentina
F. tractuosus[a]	NCMB 1408^T	Sand, Nhatrang, Vietnam
F. maritimus[a]	NCMB 2154^T	Diseased red sea bream (*Pagrus major*) kidney, Hiroshima, Japan
	NCMB 2153	Diseased black sea bream (*Acanthopagrus schlegeli*) kidney, Hiroshima, Japan
	NCMB 2158	Dover sole (*Solea solea*) skin lesions, Scotland
F. columnaris	NCMB 1038	Salmonid, United States
	NCMB 2248^T	Morphological variant of strain NCMB 1038
	Holt DD3-69	Adult chinook salmon (*Oncorhynchus tshawytscha*) gill lesions, Oregon
	Holt IC8-69	Young catfish (*Ictalurus* sp.) kidney, Idaho
	Wakabayashi EK28	Japanese eel (*Anguilla japonica*) gill lesions, Japan
	Farkas H82/7	Carp (*Cyprinus carpio*) skin ulcer, Hungary
	TG 39/87	Adult black bullhead (*Ictalurus melas*) skin ulcer, France
	TG 44/87	Brown trout fry (*Salmo trutta*) skin lesions, France
F. psychrophilus	NCMB 1947^T	Coho salmon (*Oncorhynchus kisutch*) kidney, Washington
	Holt SH3-81	Coho salmon kidney, Oregon
	TG 02/86	Rainbow trout fry (*Oncorhynchus mykiss*) kidney, France
	TG 28/86	Adult rainbow trout skin lesions, France
	LNPAA P01/88	Rainbow trout fry spleen, France
	TG P02/88	Rainbow trout fry spleen, France
	LNPAA P03/88	Rainbow trout fry spleen, France

[a] Halophile or marine species requiring media supplemented with NaCl or seawater for growth

Table 2. Phenotypic characteristics differentiating *F. maritimus* from *F. columnaris*, *F. psychrophilus*, and from all other valid *Flexibacter* and *Cytophaga* species. Symbols are +: all strains give a positive reaction; (+): all strains give a weak positive reaction; –: all strains give a negative reaction; d: different reactions; x/21: no. strains giving a positive reaction per no. strains tested

Tests	*F. maritimus* (3 strains)[a]	*F. columnaris* (8 strains)[a]	*F. psychrophilus* (7 strains)[a]	*Flexibacter* spp. and *Cytophaga* spp.[b]
Gliding motility in AOB[c]	+	+	(+)	18/21
Morphology of colonies on AOA[c]	Pale yellow, flat, irregular	Greenish yellow, flat, rhizoid	Bright yellow, convex, circular[d]	[d]
Adherence to the agar	(+)	+	–	–
Flexirubin-type pigments[e]	–	+	+	7/21
Conro red absorption[f]	+	+	–	1/21
Oxidase reaction[g]	+	+	(+)	+
Catalase production	+	+	(+)	13/21
NO_3 reduction[h]	+	+	–	6/21
H_2S production[i]	–	+	–	6/21
ONPG test[j]	–	–	–	14/21
Hydrolysis of				
CMC[k]	–	–	–	12/21
Chitin[l]	–	–	–	5/21
Starch[m]	–	–	–	17/21
Gelatin[n]	+	+	+	16/21
Lecithin[o]	+	+	+	7/21
Tyrosine[p]	+	–	+	16/21
Brown pigment on tyrosine agar	+	(+)	–	8/21
Growth in trypticase-soy broth	–	–	–	11/21
Tolerance to[q]				
Temperature	15 to 34° C	10 to 37° C[r]	6 to 22° C	[d]
NaCl conc.	No growth with NaCl only	0 to 0.5 %	0 to 0.5 %	[d]
Carbohydrate metabolism				
API ZYM[s]	0	0	0	0 to 7[t]
API 50CH[u]	0	0	0	0 to 34[v]

[a] See Table 1 for details
[b] Type strains of 10 valid *Flexibacter* species and of 11 *Cytophaga* species (see Table 1)
[c] AOB and AOA: Anacker & Ordal Broth and Agar (Anacker & Ordal 1955). Marine strains were cultivated in AOB and AOA prepared with artificial seawater (Reichenbach & Dworkin 1981) instead of distilled water
[d] Most *F. psychrophilus* strains may produce both compact colonies with regular edges and more or less spreading colonies with uneven margins
[e] Detected by the KOH test (Reichenbach et al. 1974)
[f] Detection of an extracellular galactosamine glycan (McCurdy 1969)
[g] Commercially prepared test paper (Diagnostics Pasteur, Marnes-la-Coquette, France)
[h] 0.1 % potassium nitrate AOB tubes (Bullock 1972)
[i] Lead acetate paper (Pacha 1968)
[j] *o*-nitrophenyl-β-D-galactopyranoside commercially prepared test paper (Diagnostics Pasteur)
[k] 3 % carboxymethylcellulose AOB (modified from Lewin & Lounsbery 1969)
[l] 20 % chitin AOA (Reichenbach & Dworkin 1981)
[m] 0.2 % soluble starch AOA (Bullock 1972)
[n] Film method (Le Minor & Piechaud 1963)
[o] Precipitate on 5 % sterile egg yolk AOA (modified from Cowan & Steel 1974)
[p] 0.5 % L-tyrosine AOA (Bullock 1972)
[q] Limit values for growth in AOB
[r] Strains NCMB 1038 and NCMB 2248 do not grow above 35° C
[s] No. positive reactions (hydrolysis of the substrates) for the 8 carbohydrate substrates in API ZYM galleries (API System, La Balme-les-Grottes, France)
[t] Type strains of 6 species (*F. roseolus, F. ruber, F. litoralis, F. polymorphus, C. latercula, C. hutchinsonii*) behave like the 3 fish-pathogenic species (no positive reaction). The type strains of the 15 other species give 1 to 7 positive reactions
[u] No. positive reactions (production of acid from 49 carbohydrates) in API 50CH galleries (API System) with Ammonium Salt Sugar broth (modified from Cowan & Steel 1974)
[v] Type strains of 4 species (*F. roseolus, F. ruber, F. litoralis, F. polymorphus*) behave like the 3 fish-pathogenic species (no positive reaction). Type strains of the 17 other species give 4 to 34 positive reactions

homogeneity of a group of *F. maritimus* strains using numerical and molecular taxonomy methods, but did not compare *F. maritimus* to other *Flexibacter* and *Cytophaga* species. Indeed, until very recently, *F. maritimus* had been isolated exclusively in Japan, from several marine fish species.

In the course of an extensive study of gliding bacteria isolated from diseased fish in France, a comparison of their morphological, physiological, and biochemical characteristics with those of the type strains of all valid *Flexibacter* and *Cytophaga* species led to the first identification of *F. columnaris* (Bernardet 1989) and of *F. psychrophilus* (*'C. psychrophila'*) (Bernardet & Kerouault 1989) in France. Molecular studies using the DNA/DNA hybridization method proved both species to be distinct from all other valid species. The 2 species were described and validated, and type strains designated (Bernardet & Grimont 1989).

Previously described methods (see Table 2 footnotes) were used to compare Strain NCMB 2158 isolated from Dover sole with the 2 *Flexibacter maritimus* reference strains deposited in the NCIMB (NCMB 2153 and NCMB 2154^T) and with all other species and strains included in the study (Table 1). Results of the phenotypic investigations (Table 2) showed Strain NCMB 2158 to be identical to *F. maritimus*, while several differences were noted in comparisons with *F. columnaris*. This similarity was unequivocally confirmed by the very high DNA relatedness among the 3 strains: *F. maritimus* NCMB 2154^T and the unidentified isolate NCMB 2158 were more than 73% related to *F. maritimus* NCMB 2153 and only 0 to 8% related to all of the other organisms studied (Bernardet & Grimont 1989). These 3 strains should thus be considered as belonging to the same bacterial species, according to the definition of this taxon provided by Wayne et al. (1987).

The following conclusions have been drawn from these results: (1) Comparison of the DNA from *Flexibacter maritimus* with all of the other *Flexibacter* and *Cytophaga* species confirms that *F. maritimus* is a genomic species, thus justifying the proposal of a new species by Wakabayashi et al. (1986). (2) The aetiology of 'black patch necrosis' of Dover sole in Scotland is resolved: the bacterial agent is *F. maritimus*. (3) This is the first identification of *F. maritimus* elsewhere than in Japan. It is more than likely that this bacterium exists in other locations in Europe, because diseases closely resembling 'black patch necrosis' frequently occur in farmed marine fish in several European countries.

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Responsible Subject Editor: Dr T. Evelyn, Nanaimo, B.C., Canada

Manuscript first received: September 29, 1989
Revised version accepted: April 10, 1990