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Phenotypic and Genomic Studies of "*Cytophaga psychrophila*" Isolated from Diseased Rainbow Trout (*Oncorhynchus mykiss*) in France

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Five strains of gliding bacteria were isolated in France from farmed diseased rainbow trouts reared at low water temperature. The resemblance of these bacteria to the known fish pathogen "*Cytophaga psychrophila*" led to their comparative study with reference strain NCMB 1947 and with an American isolate. Morphological, physiological, and biochemical characteristics of the seven strains proved to be similar. Comparison of their DNA by the S1 nuclease DNA-DNA hybridization method showed that the seven strains formed a tight genomic species with DNA relatedness above 90%. This is the first identification of this fish pathogen in a European country. The main phenotypic characteristics differentiating this bacterium from other nonpathogenic gliding bacteria of fish origin include a poor gliding movement, yellow compact or weakly rhizoid colonies on solid media, and the presence of flexirubin-type pigments. The inability to metabolize any carbohydrates, the strong proteolytic activity, the absence of growth in more than 0.5% NaCl, and the tolerance to a maximum temperature of 25°C are also useful characteristics of this group of bacteria.

"*Cytophaga psychrophila*" was originally isolated in 1948 from diseased coho salmon (*Oncorhynchus kisutch*) by Borg (A. F. Borg, Ph.D. thesis, University of Washington, Seattle, 1948), who gave the first description of the bacterium and pointed out later that it was a new representative of the gliding bacteria family (4). Up to now, the species has been neither listed in the Approved Lists of Bacterial Names (19) and its supplement (12) nor validated since in the *International Journal of Systematic Bacteriology*; thus, its unrecognized status is indicated by enclosing the name in quotation marks.

"*C. psychrophila*" is the causative agent of a systemic infection affecting predominantly young salmonids in Northwest United States (22), Canada, and Alaska (R. A. Holt, Ph.D thesis, Oregon State University, Corvallis, 1987) at water temperatures ranging from 3 to 15°C (8) and classically designated by the names "cold-water disease," "low-temperature disease," and "peduncle disease." All species of salmonids are probably susceptible to the disease, particularly coho salmon; the condition has also been reported in chinook (*Oncorhynchus tshawytscha*) and sockeye (*O. nerka*) salmon and in rainbow (*Oncorhynchus mykiss* [formerly *Salmo gairdneri*]), brown (*S. trutta*), cutthroat (*S. clarkii*), and lake (*Salvelinus namaycush*) trout. Clinical signs of the disease are not pathognomonic and may involve spinal cord abnormalities, nervous forms, hepatic necrosis, splenic hypertrophy, exophthalmia, anemia, gill necrosis, melanose, and erosion of the peduncle area (16, 18, 22; Holt, Ph.D. thesis). Besides the infection occurring in young salmonids, some cases were occasionally observed in coho and chinook salmon spawners. At least two serotypes of "*C. psychrophila*" are known to exist (R. A. Holt, M. S. thesis, Oregon State University, Corvallis, 1972).

During the past 4 years, young rainbow trout mortalities occurring around 10°C and associated with a range of external signs were reported from several geographic areas in France (Aquitaine, Brittany, Touraine, Picardie), the troubles being constantly accompanied by the presence of numerous gram-negative bacteria (4 to 6 μm by 0.3 μm) in swollen spleens and also in kidneys. These bacteria failed to grow on Trypticase soy agar but grew on Anacker and Ordal medium at 18 to 20°C, suggesting that they were the causative agents of cold-water disease, "*C. psychrophila*," which had never been reported from Europe.

The aim of the investigations presented herein was to establish the indisputable identity of the French bacterial isolates by a comparative study with two strains of "*C. psychrophila*," one from the National Collection of Marine Bacteria (Aberdeen, Scotland) and another from the United States, on the bases of their morphological, physiological, and biochemical characteristics and of their DNA homology.

MATERIALS AND METHODS

Bacterial strains. Data on the bacterial strains are shown in Table 1.

Culture conditions. The liquid medium was Anacker and Ordal broth (AOB) (1), which contains 0.05% tryptone, 0.05% yeast extract, 0.02% sodium acetate, and 0.02% beef extract; the pH was adjusted to 7.2 to 7.4. The solid medium was Anacker and Ordal agar (AOA) enriched to 0.5% tryptone because the bacterial growth proved to be fastidious with 0.05% tryptone only. All of the bacterial cultures were incubated at 18 to 20°C.

Morphological studies. Colonies grown for 48 h on AOA were observed directly and by light microscopy (×20 and ×100). Cell mobility and gliding movement were assessed by phase-contrast microscope examination (×1,000) of 48-h liquid cultures in hanging drops. The cell morphology was observed after Gram staining.

Biochemical tests and physiological studies. The following characteristics were tested as described previously (3), with enriched AOA instead of AOA when necessary: presence of flexirubin-type pigments; absorption of Congo red; anaerobic growth; presence of cytochrome oxidase and catalase;

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TABLE 1. Origin of the seven strains of "*C. psychrophila*"

Strain	Affected host and organ sampled	Clinical signs	Geographic origin
NCMB 1947[a]	Young coho salmon (*O. kisutch*) kidney	Classical bacterial cold-water disease	Washington, United States
Holt SH3-81[b]	Young coho salmon kidney	Classical bacterial cold-water disease	Oregon, United States
TG 02/86[c]	Rainbow trout (*O. mykiss*) fry kidney	Deep ulcerated dorsal lesions	Picardie, France
TG 28/86	Adult rainbow trout skin lesion	Skin vesicles with ulceration of the underlying muscle	Touraine, France
LNPAA P01/88[d]	Rainbow trout fry spleen	Distended belly and melanose	Brittany, France
TG P02/88	Rainbow trout fry kidney	Melanose and spinning	Picardie, France
LNPAA P03/88	Rainbow trout fry spleen	Distended belly and melanose	Brittany, France

[a] NCMB, National Collection of Marine Bacteria, Aberdeen, Scotland. Strain NCMB 1947 was isolated by E. J. Ordal.
[b] Strain SH3-81 was kindly provided by R. A. Holt (Department of Microbiology, Oregon State University, Corvallis).
[c] TG, Strain isolated in Laboratoire d'Ichtyopathologie, Institut National de la Recherche Agronomique, 78850 Thiverval-Grignon, France.
[d] LNPAA, Strains kindly provided by F. Baudin-Laurencin, Laboratoire National de Pathologie des Animaux Aquatiques, IFREMER, Centre de Brest, 29263 Plouzané, France.

ability to reduce nitrates and to produce hydrogen sulfide; presence of a β-galactosidase; hydrolysis of cellulose, carboxymethylcellulose, chitin, starch, esculin, and agar; acid production from 49 carbohydrates studied in API 50 CH galleries (API System S.A., La Balme-les-Grottes, France); hydrolysis of gelatin, casein, and tyrosine; production of a brown color on tyrosine agar (probably due to an oxidized derivative of tyrosine); presence of arginine dihydrolase and of lysine and ornithine decarboxylases; hydrolysis of tributyrin, lecithin, and Tween 20 and 80; enzymatic activity tested in API ZYM galleries; sensitivity to 16 antibiotics and to the vibriostatic compound O/129; tolerance to temperature (3, 6, 10, 12, 15, 18, 20, 22, 25, 30, 33, 35, 37, and 40°C), NaCl concentration, and pH; and growth in Trypticase soy broth. In addition, DNase was tested by spot inoculation on a DNA-containing agar (Diagnostics Pasteur, Marnes-La-Coquette, France); DNA hydrolysis was visualized by flooding the plate with 1 N HCl.

DNA-DNA hybridization. Published methods were followed to extract, purify, and shear unlabeled DNAs of the seven strains (5). In vitro labeling of the strain NCMB 1947 DNA (nick translation) with tritium-labeled nucleotides (Amersham International, Amersham, England) and hybridization experiments between the labeled NCMB 1947 DNA and the seven unlabeled DNAs by the S1 nuclease-trichloroacetic acid method at 60°C were as described by Grimont et al. (7).

G+C content of DNA. The moles percent G+C contents of the DNA of strains NCMB 1947, TG 02/86, and LNPAA P01/88 were determined from melting temperatures (T_m) by the equation of Owen et al. (14): G+C = (2.08 × T_m) − 106.4. The melting temperatures of 50-μg/ml DNA solutions in 0.1× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M trisodium citrate) were determined optically by thermal denaturation (10) in a Gilford spectrophotometer equipped with an automatic heating device that raised the temperature of the cell compartment from 60 to 90°C. The DNA of *Escherichia coli* K-12 was used as a standard (G+C content, 50.6 mol%) and was always dialyzed together with the tested DNA to avoid any difference in salt concentration.

RESULTS AND DISCUSSION

The only consistent studies formerly performed with "*C. psychrophila*" have been published by Borg (4; Borg, Ph.D. thesis), Pacha (15), Pacha and Porter (17), and more recently by Holt (R. A. Holt, M.S. and Ph.D. theses). Apart from some limited differences, the present study is in accordance with their results. Beside strain NCMB 1947, the only other "*C. psychrophila*" strain available from national collections is NCMB 1455. It was included in our study but proved to differ from the seven other strains by several morphological and biochemical characteristics; moreover, it had a very limited DNA relatedness with "*C. psychrophila*" NCMB 1947 (5% reassociation). Consequently, this strain, studied by Lewin and Lounsbery (9) as a representative "*C. psychrophila*," does not belong into this species. Holt (Ph.D. thesis) drew the same conclusion from biochemical and serological data.

In our study, the phenotypic and genomic characteristics of the seven strains proved very similar, with only some limited differences.

Morphological studies. (i) Culture on AOA. After 48 h at 18 to 20°C, the seven strains had similar circular, raised to convex, smooth and glossy colonies, 1 to 5 mm in diameter. All strains produced a bright yellow nondiffusible pigment. The colonies were usually compact with regular edges (Fig. 1), but most strains exhibited some more or less spreading colonies with uneven margins (Fig. 2); both types of colonies frequently coexisted on the same plate. The variable morphology of the colonies on agar had already been noticed by Pacha and by Holt. The colonies did not adhere to the agar. Under stereomicroscopic examination (×20) through oblique transmitted light, the colonies exhibited large yellow and blue iridescent waves. Under the microscope (×100),

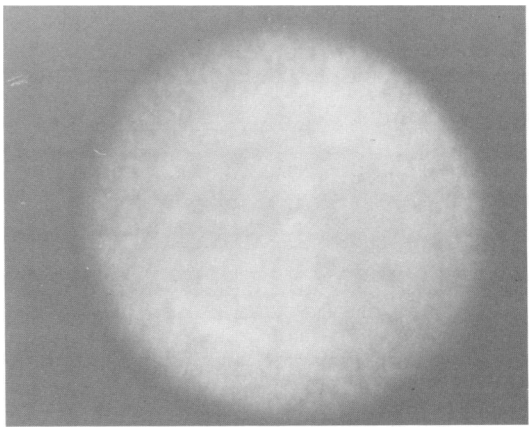

FIG. 1. "*C. psychrophila*" colony with regular edges; the diameter is 3 mm. Strain NCMB 1947 was grown on AOA for 7 days.

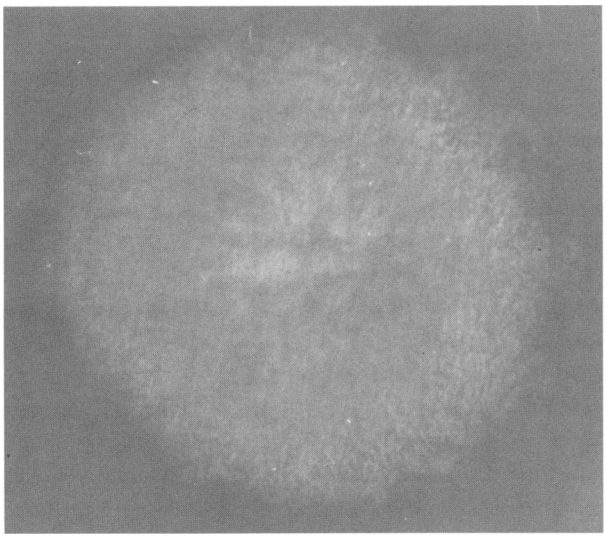

FIG. 2. "*C. psychrophila*" colony with spreading margins; the diameter is 3 mm. Strain Holt SH3-81 was grown on AOA for 7 days.

the margins appeared either entire or undulate depending on the morphology of the colony.

(ii) Culture in AOB. In hanging drops, the gliding movement was usually very slow and difficult to see. In most cases, it could only be determined by observing the position of the bacteria in the same field of the microscope at intervals of several minutes. The strains producing more or less spreading colonies on agar (i.e., strain Holt SH3-81) exhibited a more rapid gliding movement, although slower than most other gliding bacteria. This poor gliding ability is a striking characteristic when "*C. psychrophila*" is compared with the other fish-pathogenic members of the order Cytophagales, "*Flexibacter columnaris*" (3) and *Flexibacter maritimus* (21). However, no special mention about the gliding movement was made my other authors; this tends to prove that they did not consider it to be particularly difficult to observe.

After Gram staining, all strains appeared as gram-negative, slender and rather short rods; most of them were 1 to 5 μm long and 0.3 to 0.5 μm wide, with a limited number of longer cells (8 to 12 μm). In older cultures, some cells had thickened ends, as already noticed by Holt (Ph.D. thesis).

Biochemical tests. The results of the biochemical tests are presented in Table 2, and the production of the 19 enzymes tested in API ZYM galleries is presented in Table 3. Even after 10 days, there was no evidence of acid production from any of the 49 carbohydrates tested in API 50 CH galleries.

Holt considered that his strains did not contain flexirubin pigments. However, in the present study, the seven strains exhibited a clear change of color from bright yellow to orange when two loopfuls of bacteria collected on agar were deposited side by side on a glass slide with a white background and one of them was flooded with 20% KOH.

Pacha, Pacha and Porter, and Holt considered "*C. psychrophila*" as devoid of cytochrome oxidase, as determined by the method of Gaby and Hadley (6). Spreading fresh cultures (2 to 3 days) onto oxidase test disks or flooding the disks into 1-ml heavy bacterial suspensions in distilled water, we could obtain positive results with all tested strains. Thus, the cytochrome oxidase of "*C. psychrophila*" seems rather difficult to detect and needs a highly sensitive method.

The reaction of our strains with hydrogen peroxide was constantly weak and slow, thus confirming Holt's observations.

Concerning the use of proteins and carbohydrates, our results were consistent with those of Borg, Pacha, Pacha and Porter, and Holt: "*C. psychrophila*" was highly proteolytic but showed no ability to degrade either simple or complex carbohydrates. The enzyme production pattern as tested in API ZYM galleries appeared very similar for the seven strains, with only minor quantitative variations. In spite of

TABLE 2. Characteristics of two "*C. psychrophila*" reference strains and five test strains[a]

Property	Reaction[a]	Property	Reaction
Flexirubin-type pigment	+	Lecithin hydrolysis	+
Congo red test	−	Tween 20 hydrolysis	+
Anaerobic growth	−	Tween 80 hydrolysis	+
Presence of cytochrome oxidase	(+)	DNA hydrolysis	+
Presence of catalase	(+)	Susceptibility to:	
Nitrate reduction	−	Ampicillin	+
Production of H_2S	−	Cephalothin	+
Presence of β-galactosidase[b]	−	Streptomycin	+
Cellulose hydrolysis	−	Gentamicin	−
Carboxymethylcellulose hydrolysis	−	Neomycin	−
Chitin hydrolysis	−	Kanamycin	6/7
Starch hydrolysis	−	Tetracycline	+
Esculin hydrolysis	−	Chloramphenicol	+
Agar hydrolysis	−	Erythromycin	+
Gelatin hydrolysis	+	Polymyxin B	−
Casein hydrolysis	+	Novobiocin	+
Tyrosine hydrolysis	+	Sulfonamides	2/7
Brown color on tyrosine agar	−	Trimethoprim	−
Presence of arginine dihydrolase	−	Nalidixic acid	4/7
Presence of lysine decarboxylase	−	Furans	+
Presence of ornithine decarboxylase	−	Actinomycin D	5/7
Tributyrin hydrolysis	+	O/129	+

[a] −, All strains negative; +, all strains positive; (+) weakly positive; 6/7, six positive strains out of seven strains tested.
[b] *o*-Nitrophenyl-β-D-galactopyranoside test.

TABLE 3. Production of 19 enzymes by two "*C. psychrophila*" reference strains and five test strains[a]

Enzymes	Production of enzyme by strain:						
	NCMB 1947	Holt SH3-81	TG 02/86	TG 28/86	LNPAA P01/88	TG P02/88	LNPAA P03/88
Control	0	0	0	0	0	0	0
Alcaline phosphatase	5	5	5	5	5	5	5
Esterase (C_4)	2	2	1	1	2	2	2
Esterase lipase (C_8)	4	3	3	4	3	3	3
Lipase (C_{14})	1	1	1	1	1	1	1
Leucine arylamidase	5	5	5	5	5	5	5
Valine arylamidase	2	2	1	2	1	1	1
Cystine arylamidase	1	1	1	1	0	0	0
Trypsin	0	0	0	0	1	1	1
α-Chymotrypsin	0	0	0	0	0	0	0
Acid phosphatase	3	4	2	5	3	3	3
Naphthol-AS-BI-phosphohydrolase	2	2	2	5	3	3	3
α-Galactosidase	0	0	0	0	0	0	0
β-Galactosidase	0	0	0	0	0	0	0
β-Glucuronidase	0	0	0	0	0	0	0
α-Glucosidase	0	0	0	0	0	0	0
β-Glucosidase	0	0	0	0	0	0	0
N-Acetyl-β-glucosaminidase	0	0	0	0	0	0	0
α-Mannosidase	0	0	0	0	0	0	0
α-Fucosidase	0	0	0	0	0	0	0

[a] The API ZYM micromethod was used. Numbers indicate relative amounts of enzyme produced, estimated by intensity of the color.

the strong proteolytic activity of our strains for gelatin, casein, and tyrosine, there was no evidence of trypsin and α-chymotrypsin production. The negative results for all of the enzymes involved in carbohydrate metabolism appearing in API ZYM galleries (α- and β-galactosidases, β-glucuronidase, α- and β-glucosidases, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase) were in accordance with the inability to produce acid from any carbohydrates in API 50 CH galleries and to hydrolyze any polysaccharides tested in agar (cellulose, carboxymethylcellulose, chitin, starch, esculin, and agar itself).

Our seven strains proved able to hydrolyze tyrosine, but Pacha and Otis (E. J. Otis, M.S. thesis, University of Rhode Island, Kingston, 1984) observed variable reactions for tyrosine among the "*C. psychrophila*" strains they studied. Our strains, as well as Pacha's, were able to hydrolyze tributyrin, whereas all the strains studied by Holt and by Otis were not. This difference was probably not related with the method, because Pacha, Holt, and Otis used the same medium and we used a different one (13).

The only differences among our strains concerned the susceptibility to kanamycin, nalidixic acid, actinomycin D, and sulfonamides. The susceptibility of the present strains to chloramphenicol and erythromycin confirmed previous observations by other authors, but there were discrepancies in the cases of neomycin, polymyxin B, kanamycin, streptomycin, and sulfonamides. The use of very different concentrations could account for the difference noticed in the case of sulfonamides but not for the other drugs, because identical concentrations were used by all authors. However, the difficulty in assessing any significance to these results must be stressed: the diffusion method used to test the susceptibility to antibiotics was performed in conditions (18 to 20°C, 48 to 72 h) quite different from those recommended by the World Health Organization, and the results could thus be appreciated only in a qualitative way (11).

TABLE 4. DNA relatedness among "*C. psychrophila*" strains as determined by the S1 nuclease method at 60°C

Source of unlabeled DNA	% Reassociation with labeled DNA from strain NCMB 1947
NCMB 1947	100
Holt SH3-81	92
TG 02/86	103
TG 28/86	105
LNPAA P01/88	105
TG P02/88	107
LNPAA P03/88	90

Physiological studies. No growth occurred in Trypticase soy broth. In AOB, the seven strains grew well from 10 to 20°C, but the growth was scant and slow at 6, 22, and 25°C. There was no growth at 3°C and above 25°C. Borg as well as Pacha and Porter considered "*C. psychrophila*" unable to grow at 25°C, but, at the same temperature, Holt observed that 18 strains among the 28 he studied were able to grow.

Considering the tolerance to NaCl concentration, our strains developed without difficulty in liquid medium without NaCl or with 0.5% NaCl but did not grow at higher concentrations. Pacha found that some strains were able to grow with 1% NaCl, but the upper limit of tolerance to NaCl was 0.8% for most strains; all 28 strains studied by Holt grew with 1% NaCl but in a liquid medium different from AOB.

In AOB buffered at different pHs (4 to 11), the seven strains grew well at pH 7 and slightly at pH 5, 6, and 8; there was no growth at pH 4 and above pH 8.

DNA-DNA hybridization. The DNA relatedness results obtained with labeled reference DNA from strain NCMB 1947 are shown in Table 4. The seven "*C. psychrophila*" strains form a tight genomic species more than 90% related to strain NCMB 1947. Hybridization rates slightly above 100% are not uncommon in the case of very similar strains.

G+C content of DNA. The DNA base compositions of the three tested strains were as follows: 32.5 mol% for NCMB 1947 and 33.8 mol% for TG 02/86 and LNPAA P01/88. These results (mean, 33.4 mol%) are quite similar to those of Holt, averaging 34.3 mol%.

With regard to morphological, physiological, and biochemical characteristics, our five isolates therefore appeared fairly similar to American strains NCMB 1947 and Holt SH3-81 and to the strains used by other authors in previous studies. This similarity was definitely confirmed by the very high DNA relatedness among the seven strains.

This work constitutes the first study of DNA homology among this fish-pathogenic gliding bacterium. Moreover, this is the first isolation and identification of "*C. psychrophila*" in France and in Europe; until now, this dangerous fish pathogen had only been isolated in Oregon, Washington, Idaho, New Hampshire, Michigan, Alaska, and Canada (British Columbia). It is presently impossible to have a definite opinion on how it was introduced in France; however, several authors have shown *Cytophaga* spp. to adhere at the surface of salmonid eggs (2, 20, 23), and Borg (4) reported a probable example of the transportation of "*C. psychrophila*" on eggs in Washington State. The intense

trade of eggs taking place between the United States and Europe could probably help the spreading of the disease in spite of recommendation by Holt of egg disinfection procedures with iodine compounds; it is also possible that the bacterium existed in Europe long ago and remained unnoticed because of its poor growth on classical media and of its low thermal optimum.

As genotypic studies are not easy to perform, it is worthwhile to list the main phenotypic characteristics allowing clear differentiation between "*C. psychrophila*" and other members of the order *Cytophagales* commonly associated with the saprophytic microflora of the fish. The most significant characteristics of this species include a poor gliding movement in liquid culture and a weak and inconstant spreading on agar (resulting in bright yellow compact colonies without or with limited rhizoid aspect), the presence of flexirubin-type pigments, and weak positive cytochrome oxidase and catalase activities. Other important features are lack of β-galactosidase (*o*-nitrophenyl-β-D-galactopyranoside test) and of all enzymes involved in carbohydrates metabolism (API ZYM galleries), no action on carbohydrates in API 50 CH galleries, no degradation of polysaccharides (cellulose, carboxymethylcellulose, starch, esculin, chitin), strong proteolytic activity (gelatin, casein, tyrosine), no growth in Trypticase soy broth or in AOB containing more than 0.5% NaCl, good growth from 10 to 20°C and slow and weak growth at 6 and 25°C, and susceptibility to ampicillin, cephalothin, and streptomycin.

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