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An epizootic in farmed, market-size rainbow trout in Spain caused by a strain of *Carnobacterium piscicola* of unusual virulence

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ABSTRACT: We report here the first description in Spain of a *Carnobacterium* strain causing important mortalities in market-size rainbow trout *Oncorhynchus mykiss*. Relevant clinical signs in affected fish were a pronounced bilateral exophthalmia with periocular hemorrhages, accumulation of ascitic fluid, and hemorrhages in the liver, swimbladder, muscle, and intestine. Taxonomic studies conducted in comparison with reference strains indicated that the present isolate (PT-31) was *Carnobacterium piscicola*. Agglutination assays demonstrated that this isolate was not serologically related to the reference strains. In addition, an analysis of the surface proteins revealed that different patterns occurred among the *C. piscicola* isolates. Although the immunoblotting assays supported the antigenic heterogeneity within this species, all strains did share 2 major antigenic proteins of 30 and 57 kDa. Interestingly, this immunoreactive 57 kDa protein is also produced by *Renibacterium salmoninarum* and *Corynebacterium aquaticum*. Regardless of the challenge method (injection or water-borne), isolate PT-31 proved to be highly pathogenic for rainbow trout ($LD_{50} < 5–6 \times 10^4$ cells), and the moribund fish displayed the external and internal signs observed in the natural disease. The inoculated strain could be recovered from various organs of both dead and surviving fish. The extracellular products (ECP) of strain PT-31 contained exotoxins lethal for rainbow trout ($LD_{50} = 4.5$ µg protein g^{-1} fish). However, the toxic ECP lacked enzymatic, cytotoxic, and hemolytic activities when tested *in vitro*. Histopathological examination of naturally and experimentally infected trout showed extensive and/or intensive lesions in most organs. The most marked tissue damage (acute hemorrhages and necrosis) was observed in eyes, kidney, liver, spleen, pancreas, and muscle. These findings should alert fish farmers to the existence of *C. piscicola* strains with a pathogenic capability higher than that previously recorded.

KEY WORDS: *Carnobacterium piscicola* · Rainbow trout · Serology · Taxonomy · Virulence

INTRODUCTION

Bacteria with characteristics resembling species of *Lactobacillus* or *Carnobacterium* have been isolated from seemingly healthy fish in routine microbiological surveys as well as from diseased fish (Table 1). In the majority of the disease cases, lactic acid bacilli have been recovered from chronically infected salmonids cultured in North America and mortality levels have been low. However, reports do indicate that the bacilli can also infect other commercially important species of fish such us striped bass, channel catfish, and carp (Michel et al. 1986, Baya et al. 1991).

The lactic acid bacteria form a biochemically and genetically heterogeneous group which have undergone substantial taxonomic revision. Although *Lactobacillus piscicola* (Hiu et al. 1984) was included in the genus *Carnobacterium* (Collins et al. 1987), some atypical lactobacilli isolated from salmonid fish were classified as new species: *Vagococcus salmoninarum* (Wallbanks et al. 1990) and *Lactococcus piscium* (Williams et al. 1990), on the basis of an analysis of their 16S rRNA sequences. However, the significance of these 2 last-named species for aquaculture remains to be determined.

With rare exceptions (Baya et al. 1991, Toranzo et al. 1993), attempts to experimentally reproduce disease or

Table 1. Reports of lactic acid bacilli isolated from apparently healthy and diseased fish

Fish group	Country of isolation	Bacteria described	Source
Healthy fish			
Salmonids	Canada	*Lactobacillus* sp.	Evelyn & McDermott (1961)
Salmonids	CIS	*Lactobacillus* sp.	Kvasnikov et al. (1977)
Salmonids	Denmark	*Lactobacillus* sp.	Knöchel (1981)
Salmonids	Australia	*Lactobacillus piscicola*	Humphrey et al. (1987)
Brown bullhead	USA	*Carnobacterium piscicola*	Baya et al. (1991)
Salmonids	USA	*Carnobacterium piscicola*	Starliper et al. (1992)
Salmonids	Iceland	*Carnobacterium piscicola* and *Lactobacillus* sp.	Stoffels et al. (1992)
Diseased fish			
Salmonids	USA	*Lactobacillus* sp.	Rucker et al. (1953)
Salmonids	USA	*Lactobacillus* sp.	Ross & Toth (1974)
Salmonids	Canada	*Lactobacillus* sp.	Cone (1982)
Salmonids	USA	*Lactobacillus piscicola*	Hiu et al. (1984)
Salmonids	USA	*Lactobacillus piscicola*	Herman et al. (1985)
Salmonids	Australia	*Lactobacillus piscicola*	Humphrey et al. (1987)
Salmonids and carp	France, Belgium	*Lactobacillus piscicola*	Michel et al. (1986)
Salmonids	USA	*Vagococcus salmoninarum*	Wallbanks et al. (1990)
Salmonids	USA	*Lactococcus piscium*	Williams et al. (1990)
Striped bass and channel catfish	USA	*Carnobacterium piscicola*	Baya et al. (1991)
Salmonids	USA	*Carnobacterium piscicola* and *Lactobacillus* sp.	Starliper et al. (1992)
Salmonids	Spain	*Carnobacterium piscicola*	Present study

mortality in fish with *Lactobacillus* or *Carnobacterium* strains have not been very successful (Ross & Toth 1974, Herman et al. 1985, Michel et al. 1986, Starliper et al. 1992). In addition, most of the isolations have been made from adult fish which had experienced stress, such as that associated with handling and spawning. These facts indicated that these microorganisms are generally opportunistic pathogens and that they possess low virulence.

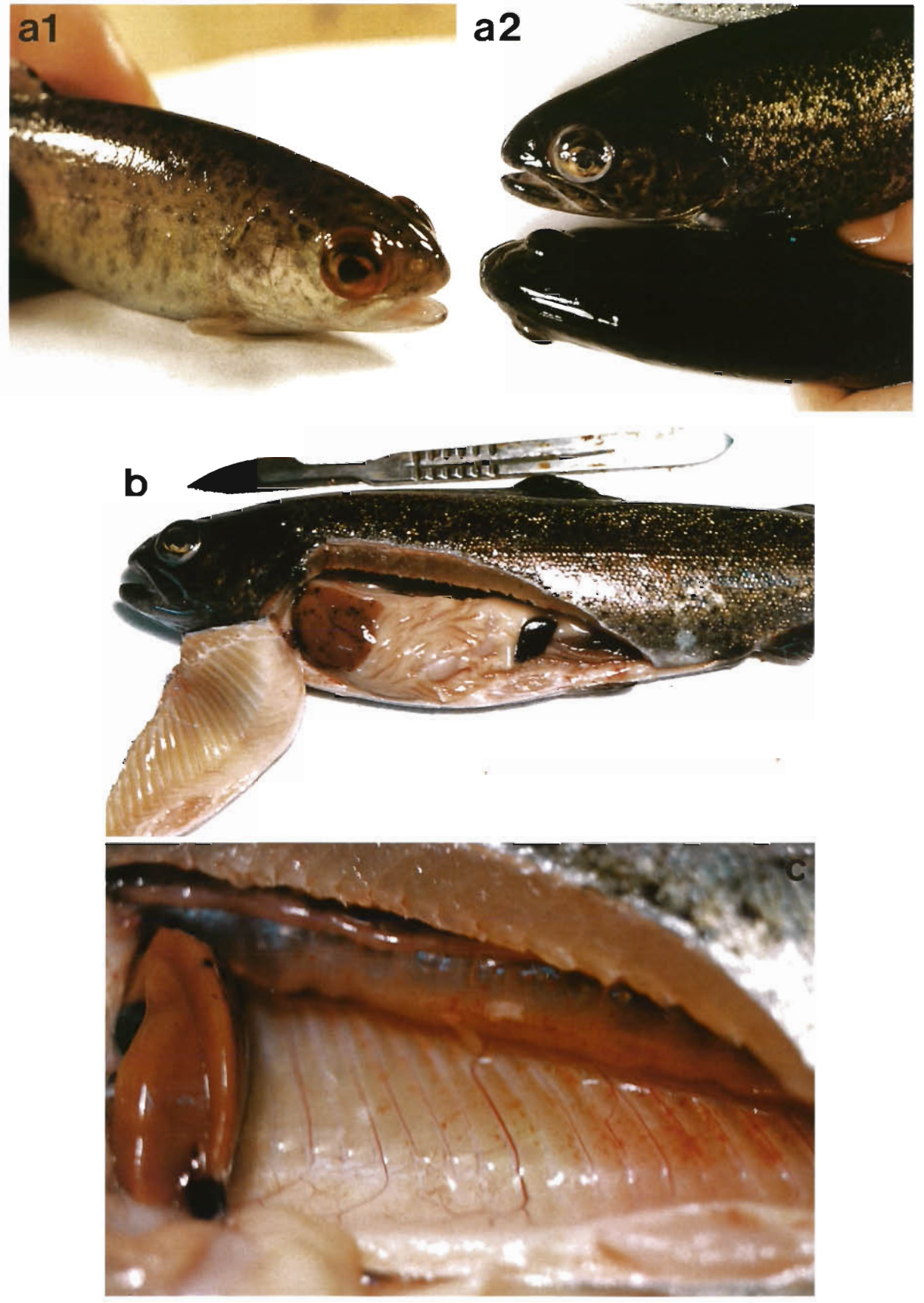
To date, there have only been a few reports of isolations of *Carnobacterium* from diseased fish in Europe (Michel et al. 1986) (Table 1). We therefore report on a recent case of *Carnobacterium piscicola* from Spain. The case was unusual because it represented an infection wth a highly virulent strain of *C. piscicola* and because it was associated with large and acute losses in market-size rainbow trout *Oncorhynchus mykiss*. Consequently, an extensive phenotypic and serological characterization of this microorganism was conducted. In addition, its pathobiological activities *in vitro* and *in vitro* were also evaluated.

MATERIALS AND METHODS

Description of the disease and microbiological examination. In July 1992, a disease outbreak causing high mortalities occurred in market-size (average weight 250 g) rainbow trout reared in a freshwater farm located in NW Spain. The fish were stocked in concrete tanks at a density of 20 kg m^{-2} and were being fed a commercial trout food. The epizootic was associated with a sudden rise in the water temperature (from 17 to 22°C). An initial treatment with oxolinic acid failed to control the disease. Therefore, therapy was changed to chloramphenicol which proved to be only moderately useful for controlling the epizootic. Fish losses over a 2 mo period amounted to about 25% of a total population of 600 000 rainbow trout.

Diseased fish exhibited a pronounced bilateral exophthalmia with periocular hemorrhages, and a general darkening of the body surface (Fig. $1a_1$, a_2). The most relevant internal clinical signs consisted of an accumulation of ascitic fluid in the peritoneal cavity and hemorrhages in the liver (showing strong

Fig. 1. External and internal clinical signs exhibited by rainbow trout *Oncorhynchus mykiss* suffering natural and experimental infections with *Carnobacterium piscicola* PT-31 strain: (a_1, a_2) pronounced bilateral exophthalmia with periocular hemorrhages, (b) general darkening of the body surface and marked petechiation in the liver, (c) hemorrhages in the musculature and swimbladder


a1
a2
b
c

pethechiae), swimbladder, musculature, and intestines (Fig 1b, c). In some moribund trout, the kidney, liver, and spleen were also swollen.

For bacterial isolation, samples of kidney, liver, spleen, and eye tissues were streaked onto tryptic soy agar (TSA, Difco Laboratories, Detroit, MI, USA), brain heart infusion agar (BHIA, Difco), and blood agar (BioMerieux, Madrid, Spain). Samples were also inoculated into tryptic soy broth (TSB, Difco). Plates and broth tubes were incubated at 25 °C for 48 to 72 h.

Tests for the possible presence of a viral agent were also performed following standard virological procedures (Amos 1985).

Taxonomic and serological characterization. Pure cultures of the isolated colonies were subjected to morphological, biochemical, and physiological tests (Hiu et al. 1984, Kandler & Weiss 1986, Collins et al. 1987). Acid production from carbohydrates was examined in phenol red broth base and purple broth base media, both supplemented with 0.5% yeast extract and the test sugar at a final concentration of 1%. Enzymatic (proteases, phospholipase, lipase, and amylase) and hemolytic activities were determined in plates as previously described (Baya et al. 1991). The final reading of the results was conducted after 7 d incubation at 25 °C.

Drug sensitivity of the isolates was determined by the disc diffusion method on Mueller-Hinton agar (Oxoid, Ltd, Basingstoke, Hamsphire, UK) using the following chemotherapeutic agents (μg $disc^{-1}$): penicillin G (10), ampicillin (10), tetracycline (30), oxytetracycline (30), chloramphenicol (30), erythromycin (15), streptomycin (10), oxolinic acid (2), flumequine (3), trimethoprim-sulfamethoxazole (23.75-1.25), nitrofurantoin (300), and furazolidone (100).

Because preliminary tests indicated that the present isolates (representative strain PT-31) were Gram-positive organisms belonging to the genus *Carnobacterium*, the following reference strains of *C. piscicola* were included in the taxonomic and serological studies: type strain ATCC 35586 (B-270^T) isolated from diseased trout *Salmo clarki* in Oregon, USA (Hiu et al. 1984); strains HB 426 and HB 569L isolated in Maryland from epizootics in cultured striped bass *Morone saxatilis* and channel catfish *Ictalurus punctatus*, respectively; and strain RB 498 recovered from asymptomatic wild brown bullhead *I. nebulosus* in Chesapeake Bay (Baya et al. 1991).

Working cultures of all the strains were maintained in tubes of soft agar (casitone, 0.1%; yeast extract, 0.3%; NaCl, 1%; agar, 0.3%, pH 7.2) under mineral oil. For long-term preservation, cultures were frozen at −70 °C in TSB with 15% (v/v) glycerol.

The serological relationships among the *Carnobacterium piscicola* isolates were examined by slide agglutination tests as we previously described (Toranzo et al. 1987) using rabbit antisera raised against *C. piscicola* strains ATCC 35586 and HB 426. In addition, cross-quantitative agglutination tests were performed in microtiter plates using serial 2-fold dilutions of 25 μl aliquots of the antisera. The agglutination titer was recorded as the reciprocal of the highest dilution of the antiserum which gave a positive reaction after overnight incubation with the antigen at 30 °C.

Because, using the western blot technique, we have recently detected the presence of a common immunoreactive protein of about 57 kDa among strains of *Renibacterium salmoninarum*, *Corynebacterium aquaticum*, and *Carnobacterium piscicola* (Bandín et al. 1993), agglutination assays were also conducted with antisera raised against the reference strain of *R. salmoninarum* ATCC 33209 (Lea 1-74^T) and *C. aquaticum* RB 968 BA (Baya et al. 1992).

Virulence for fish. A representative strain from the present outbreak, *Carnobacterium piscicola* PT-31, and the reference strain of *C. piscicola* (ATCC 35586) were tested for pathogenicity in fingerling rainbow trout (10 g) maintained at 20 ± 2 °C in freshwater aquaria with aeration. Two challenge methods were employed: intraperitoneal (i.p.) injection as previously described (Toranzo et al. 1983b), and water-borne exposure. After fish stocks were determined by culture to be free of *C. piscicola* or other bacterial species, infectivity trials were conducted using a range of bacterial doses (6 fish being used per dose). Mortalities were recorded daily over a 3 wk period, and the degree of virulence (50% lethal dose) was calculated by the Reed & Müench method (1938). Control fish were exposed to 0.85% saline using the same challenge procedures.

From each moribund or dead fish, samples of kidney, liver, spleen, and brain were streaked directly onto BHIA plates in order to reisolate the challenge strain. In addition, kidney samples were taken from some of the dead fish as well as from the survivors. These kidney samples were homogenized, diluted in 0.85% saline, and plated on BHIA in order to determine the number of viable *Carnobacterium piscicola* cells present [colony-forming units (CFU) g^{-1} of tissue].

Histopathological studies. Samples of kidney, liver, spleen, pancreas, intestine, brain, and eye taken from moribund trout in both the natural and the experimental infections were fixed in 4% buffered formalin for 24 h. The fixed samples were then embedded in paraffin and 5 μm sections were prepared and stained with hematoxylin and eosin, and by the Gram and Giemsa methods.

Analysis of surface proteins and western blotting. Membrane proteins from all the *Carnobacterium piscicola* strains were prepared basically as described

(Toranzo et al. 1983b). Briefly, bacterial cultures grown on TSB were centrifuged at 7000 × *g* for 10 min at 4°C. The resulting cell pellets were resuspended in 3 ml of 10 mM Tris-HCl buffer (pH 8.0) with 0.3% added NaCl, and the cells were disrupted by sonic treatment (Branson sonifier 250). After centrifugation at 10 000 × *g* for 1 min, the supernatant fluids were transferred to other tubes and centrifuged again for 60 min at 20 000 × *g* at 4°C. The precipitates were suspended in distilled water, and these suspensions were frozen at −30°C until used. Samples were electrophoresed in sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) at constant current (20 mA) (Laemmli 1970). The proteins were stained with Coomassie brilliant blue R (Sigma Chemical Co., St. Louis, MO), and the molecular weights (MW) were determined by comparison with a mixture of MW markers (Bio-Rad). In the SDS-PAGE, membrane proteins of the Gram-positive fish pathogens *Renibacterium salmoninarum* ATCC 33209 and *Corynebacterium aquaticum* RB 968 BA were also included for comparative purposes.

After electrophoresis, proteins were electroblotted from the gel onto nitrocellulose (NC) membranes (0.45 µm, Bio-Rad) using a modification of the method of Towbin et al. (1979). The membranes were then separately reacted with the antisera raised against *Carnobacterium piscicola* ATCC 35586, *C. piscicola* HB 426, and *Renibacterium salmoninarum* ATCC 33209. NC membranes were incubated for 1 h with control or immune rabbit serum diluted 1:1000. After washing, membranes were incubated for 1 h with goat anti-rabbit IgG-alkaline phosphotase conjugate (Bio-Rad) diluted 1:3000. Bands were visualized by incubating NC membranes in 0.1 M carbonate buffer (pH 9.8) containing tetrazolium blue (0.3 mg ml^{-1}) and 5- bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (0.15 mg ml^{-1}).

***In vivo* and *in vitro* biological activities of extracellular products.** The extracellular products (ECP) of the Spanish isolate *C. piscicola* PT-31 and the reference strain ATCC 35586 were obtained using the cellophane plate technique (Liu 1957) with slight modifications (Santos et al. 1992). The protein concentrations of the ECP samples were determined by the method of Bradford (1976) using bovine serum albumin as standard.

The total proteolytic activity present in the ECP was determined using a non-specific protease substrate (Azocoll, Sigma) following the manufacturer's instructions. One unit of protease activity produced an absorbance reading of 1.0 at 520 nm in 30 min at 37°C. The production of caseinase, gelatinase, phospholipase, and lipase activities was quantified in plates by a radial diffusion method using a basal nutrient agar (BNA) (peptone, 4 g l^{-1}; yeast extract, 1 g l^{-1}; agar, 15 g l^{-1}) containing 1% of the appropriate substrate: gelatin (Oxoid), sodium caseinate (Difco), egg yolk emulsion (Oxoid), or Tween 80 (Sigma) (Baya et al. 1992). Elastase activity was assayed on elastin medium (Santos et al. 1992). Ten µl of each ECP was placed on the plates and incubated at 25°C for 24 to 72 h until final reading of the results (Baya et al. 1992). Hemolytic activity was measured in microtiter plates using a 2% suspension of sheep erythrocytes.

The ECP preparations were assayed for cytotoxicity, as previously described by Toranzo et al. (1983a), in the following fish cell lines: CHSE-214 (chinook salmon embryo), EPC (epithelioma papulosum cyprini), and RTM (rainbow trout muscle). Results were expressed as the minimal amount of ECP protein needed to produce detectable monolayer damage within a 2 d period.

The lethal effects of the *Carnobacterium piscicola* exotoxins were evaluated by i.p. and intramuscular (i.m.) inoculation of rainbow trout (10 g). Serial 2-fold dilutions of ECP samples were tested (0.1 ml of each dilution of each ECP preparation per fish). Groups of 6 fish, maintained under the conditions described above for the virulence assays of live cells, were used per dose. The lethal dose 50% (LD_{50}) was expressed as µg ECP protein g^{-1} body wt of fish.

RESULTS AND DISCUSSION

Phenotypic characterization of the causative organism

Microbiological examination of diseased trout revealed the presence of a single colony type from all of the tissues sampled. Colonies on TSA plates were white, round, convex, and less than 2 mm in diameter after 48 to 72 h incubation at 25°C. The features of the isolates obtained from the sampled fish were consistent with those of the reference strains of *Carnobacterium piscicola* tested in parallel (Table 2). All were Gram-positive facultatively anaerobic, nonmotile, non-spore-forming straight rods that occurred singly or in short chains. They did not produce cytochrome oxidase, catalase, or indole, and did not reduce nitrate. All isolates were positive for the methyl red and Voges-Proskauer tests but negative for the citrate reaction; they failed to produce H_2S on triple sugar iron agar or gas from glucose or gluconate. In addition, all strains failed to grow on acetate agar and possessed the enzyme arginine dihydrolase, 2 important tests for differentiating *Carnobacterium* species from *Lactobacillus*, *Vagococcus*, and *Lactococcus* species (Collins et al. 1987, Wallbanks et al. 1990, Williams et al. 1990, Baya et al. 1991, Montel et al. 1991).

Table 2. Biochemical and physiological characteristics of the *Carnobacterium piscicola* isolated in this study in comparison with those exhibited by other reference strains. +: positive; (+): weak positive results; –: negative; α: α-hemolytic activity; R: resistant; S: sensitive; I: intermediate

Test	*Carnobacterium piscicola* isolates				
	Present study	Reference strains			
	PT-31	ATCC 35586	HB-426	HB-569L	RB-498
Oxidase	–	–	–	–	–
Catalase	–	–	–	–	–
Methyl red	+	+	+	+	+
Voges-Proskauer	+	+	+	+	+
Indole production	–	–	–	–	–
Nitrate reduction	–	–	–	–	–
H_2S on TSI	–	–	–	–	–
Citrate utilization	–	–	–	–	–
Gas from glucose	–	–	–	–	–
Gas from gluconate	–	–	–	–	–
ONPG (β-galactosidase)	+	+	+	+	+
Arginine dihydrolase	+	+	+	+	+
Lysine decarboxylase	–	–	–	–	–
Ornithine decarboxylase	–	–	–	–	–
Enzyme production:					
Gelatinase	–	–	–	–	–
Caseinase	–	(+)	–	+	+
Elastase	–	–	–	–	–
Phospholipase	–	–	–	–	–
Lipase	–	–	–	–	–
Amylase	–	–	–	–	–
Urease	–	–	–	–	–
Hemolysis:					
Sheep erythrocytes	+ α	(+) α	(+) α	(+) α	(+) α
Trout erythrocytes	+ α	–	–	–	–
Acid from:					
D-mannitol	+	+	+	+	+
Inulin	+	+	+	+	+
D-sorbitol[a]	(+)	(+)	(+)	(+)	(+)
Inositol	–	–	–	–	–
Glycerol	–	(+)	(+)	–	–
L-arabinose	–	–	+	–	+
Sucrose	+	+	+	+	+
Lactose[a]	–	(+)	–	(+)	+
D-ribose	+	+	+	+	+
D-fructose	–	+	+	+	+
Amygdalin	+	+	+	+	+
Esculin	+	+	+	+	+
Growth in:					
0% NaCl	+	+	+	+	+
3% NaCl	+	+	+	+	+
5% NaCl	(+)	+	+	+	+
8% NaCl	–	–	–	–	–
Growth at:					
4 °C	–	+	+	+	+
15 °C	+	+	+	+	+
37 °C	+	+	+	+	+
42 °C	–	–	–	–	–
Growth on inhibitory media:					
Acetate agar	–	–	–	–	–
KF Streptococcus agar	+	+	+	+	+
McConkey agar	–	–	–	–	–
Resistance/sensitivity to (μg disc^{-1}):					
Penicillin G (10)	S	R	S	S	S
Ampicillin (10)	S	R	S	S	S
Tetracycline (30)	R	S	S	R	R
Oxytetracycline (30)	R	S	S	R	R
Chloramphenicol (30)	I	I	I	I	I
Erythromycin (15)	S	S	S	I	S
Streptomycin (10)	R	R	R	R	R
Oxolinic acid (2)	R	R	R	R	R
Flumequine (3)	R	R	R	R	R
Trimethoprim (23.75)-sulfamethoxazole (1.25)	R	R	S	R	R
Nitrofurantoin (300)	R	R	R	R	R
Furazolidone (100)	R	R	R	R	R

[a] Weak reactions for acid production from carbohydrates in phenol red broth base medium were clearly negative using the purple broth basal medium

In their carbohydrate fermentation patterns, the isolates from the present epizootic (representative strain PT-31) differed from the reference strains mainly in their inability to ferment fructose (a substrate attacked by all of the reference strains) (Table 2). However, after a prolonged incubation period (more than 7 d) this reaction became weakly positive. In all strains, variations were found in the fermentation of sorbitol and lactose depending on the basal medium utilized (phenol red broth or purple broth). This is important because the typical *Carnobacterium piscicola* strains are considered to be sorbitol-negative using the purple broth base medium (Hiu et al. 1984, Collins et al. 1987). In summary, the discrepancies among the results obtained by different authors in the sugar reactions (Cone 1982, Hiu et al. 1984, Herman et al. 1985, Michel et al. 1986, Baya et al. 1991, Starliper et al. 1992, Stoffels et al. 1992) can be attributed, as we previously reported (Baya et al. 1991), to variations in the basal medium used, time of reading, and methodology (conventional plate and tube tests versus commercial API-50 CHL system). However, in the present study, regardless of the basal medium utilized, all strains fermented mannitol and inulin, 2 characteristics useful for differentiating *Carnobacterium* from *Vagococcus* and *Lactococcus* and the only reliable criteria for separating the related *Carnobacterium* species, *C. picicola* and *C. divergens* (Collins et al. 1987, Wallbanks et al. 1990, Williams et al. 1990, Montel et al. 1991).

The physiological and enzymatic characteristics of our isolate PT-31 were similar to those exhibited by the reference strains, thus also supporting its assignation to the species *Carnobacterium piscicola*. None of the strains grew at 42 °C, and the salinity range for growth was from 0 to 5% NaCl. The strains failed to produce the enzymes gelatinase, elastase, phospholipase, lipase, amylase, and urease but exhibited an α-hemolytic activity with sheep erythrocytes. Only our strain PT-31 displayed hemolytic activity against fish erythrocytes (Table 2).

Table 3. Characteristics useful in differentiating *Lactobacillus*, *Carnobacterium*, *Vagococcus*, and *Lactococcus* species. Data compiled from information obtained in the present study and that reported by other authors (Collins et al. 1987, Wallbanks et al. 1990, Williams et al. 1990, Baya et al. 1991, Montel et al. 1991). V: variable reaction among the isolates

Test	*Lactobacillus* spp.	*Carnobacterium piscicola*	*Carnobacterium divergens*	*Vagococcus salmoninarum*	*Lactococcus piscium*
Growth in acetate agar	+	–	–	–	–
Arginine dihydrolase	V	+	+	–	–
Acid production from:					
Mannitol	V	+	–	–	+
Inulin	V	+	–	–	–
Production of H_2S	–	–	–	+	–
Hydrolysis of starch	V	–	–	+	+
Major cellular fatty acids	cis-vaccenic	oleic	oleic	oleic	cis-vaccenic

Because lactic acid bacteria form a heterogeneous biochemical group of some importance to aquaculture, we have listed several tests in Table 3 that should be useful for the rapid differentiation of species that make up this biochemical group.

The bacterial isolate responsible for the present epizootic proved to be resistant to most of the chemotherapeutic agents commonly used in aquaculture such as oxytetracycline, the quinolones, the nitrofurans, and the potentiated sulfonamides. Chloramphenicol exhibited only an intermediate activity (Table 2). The drug-resistant pattern of our isolate was consistent with the results obtained on the affected farm where a quinolone (oxolinic acid) proved ineffective and chloramphenicol proved moderately effective. In general, regardless of the source of isolation, all of the *Lactobacillus-Carnobacterium* strains seemed to be sensitive to erythromycin (Michel et al. 1986, Baya et al. 1991). Unfortunately, to date, this antibiotic has only been used for controlling bacterial kidney disease caused by *Renibacterium salmoninarum* (Evelyn et al. 1986, Elliott et al. 1989, Brown et al. 1990).

The negative results obtained in the virological analysis using different fish cell lines suggested that a concomitant viral infection was not involved in the epizootic.

Serological and antigenic characteristics

The results of agglutination assays indicated that the *Carnobacterium piscicola* PT-31 isolate was not serologically related to the reference strains ATCC 35586 and HB-426 (titers < 10). In addition, no cross-reactions were detected with the antisera raised against the *Renibacterium salmoninarum* or *Corynebacterium aquaticum* strains (Table 4).

Table 4. Slide agglutination and cross-agglutination titers of the *Carnobacterium piscicola* isolates

Antigen	Source of rabbit antiserum:			
	Carnobacterium piscicola		*Renibacterium salmoninarum*	*Corynebacterium aquaticum*
	ATCC 35586	HB-426	ATCC 33209	RB 968BA
Carnobacterium piscicola				
PT-31	– (< 10)	– (< 10)	– (< 10)	– (< 10)
ATCC 35586	+ (1280) [a]	– (< 10)	– (< 10)	– (< 10)
HB-426	(+) (80)	+ (1280)	– (< 10)	– (< 10)
HB-569 L	– (< 10)	– (< 10)	– (< 10)	– (< 10)
RB-498	– (< 10)	– (< 10)	– (< 10)	– (< 10)
Renibacterium salmoninarum				
ATCC 33209	– (< 10)	– (< 10)	+ (5120)	– (< 10)
Corynebacterium aquaticum				
RB 968BA	– (< 10)	– (< 10)	– (< 10)	+ (2048)

[a] Agglutination titer expressed as the reciprocal of the highest dilution of antiserum that gave a positive reaction after an overnight incubation with the antigen at 30 °C

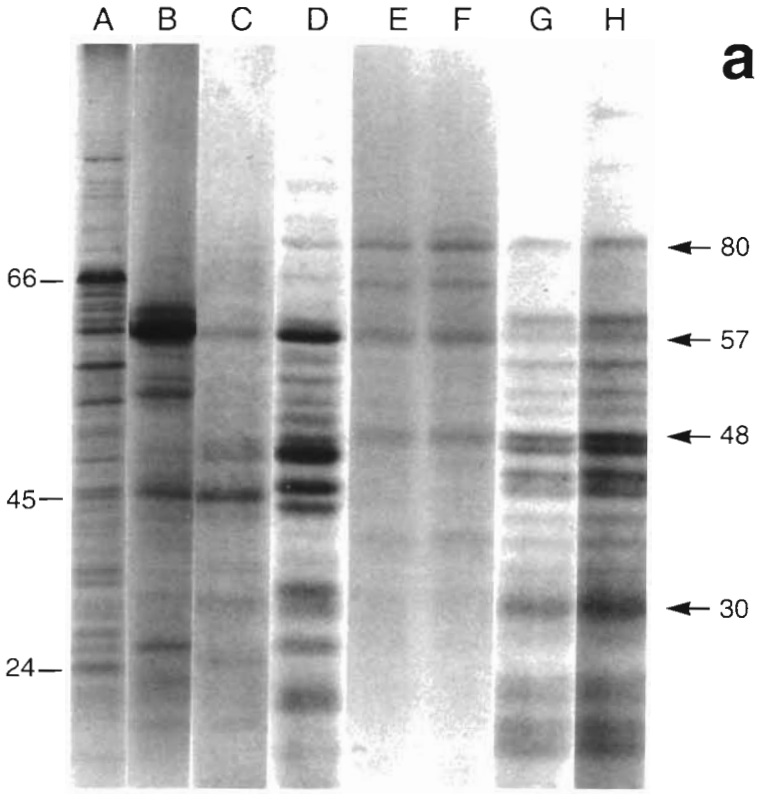
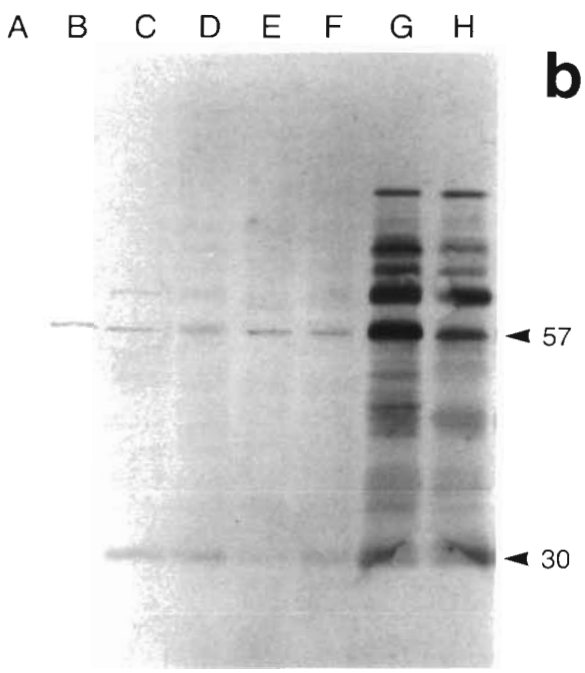
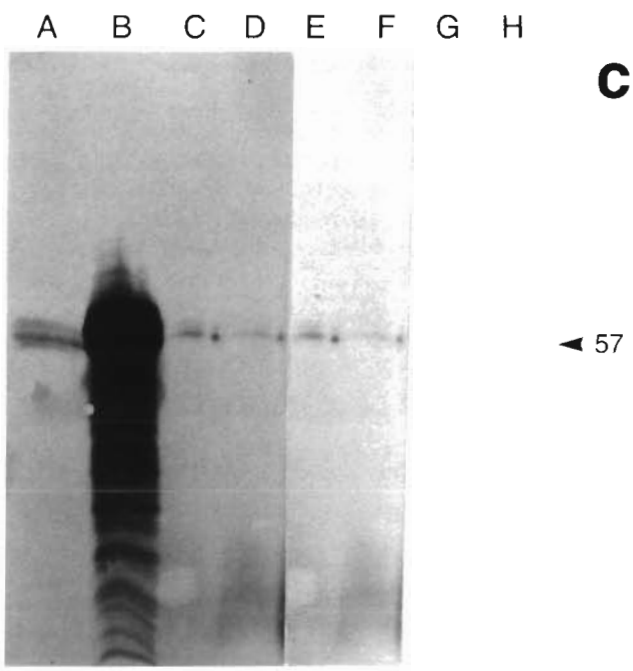


Fig. 2. SDS-PAGE of cell-surface proteins of *Carnobacterium piscicola*, *Renibacterium salmoninarum*, and *Corynebacterium aquaticum* strains (a), and the corresponding western blots using rabbit antisera raised against *C. piscicola* ATCC 33568 (b), and *R. salmoninarum* ATCC 33209 (c). Lanes: (A) *C. aquaticum* RB 968 BA; (B) *R. salmoninarum* ATCC 33209; (C) *C. piscicola* RB-498; (D) *C. piscicola* HB-569L; (E & F) *C. piscicola* PT-31 and PT-32 (isolates in the present outbreak); (G) *C. piscicola* HB-426; (H) *C. piscicola* ATCC 35586. Numbers on the left indicate the molecular weight of protein standards in kDa. Arrows show cell surface proteins shared by most of the *C. piscicola* strains. Arrowheads indicate the major antigenic protein bands common to the isolates





The analysis of surface proteins revealed that our isolate possessed a protein pattern different from those exhibited by the *Carnobacterium piscicola* reference strains, although some bands were shared in common by most straints (e.g. 80, 57, 48, 30 kDa) (Fig 2a). Only the ATCC 35586 and HB-426 strains showed an essentially identical profile which was supported by immunoblotting using the respective homologous antisera. In addition, these western blot assays indicated that all the *C. piscicola* isolates did share at least

Table 5. Susceptibility of rainbow trout *Oncorhynchus mykiss* to live cells and extracellular products (ECP) of *Carnobacterium piscicola*. Qualitative appearance of reisolated bacteria by streaking organs directly onto BHIA plates: ++, exuberant bacterial growth; +, moderate growth; –, no growth. LD_{50}: number of viable cells or µg ECP protein g^{-1} fish needed to kill 50% of the inoculated fish. Values in parenthesis show the mean CFU g^{-1} kidney tissue. NA: not applicable

Strain	Live cells						ECP	
	LD_{50}	Reisolation from dead fish				Recovery from kidney of survivors	Lethal effects	LD_{50}
		Kidney	Liver	Spleen	Brain			
PT-31	$< 5.0 \times 10^4$ (i.p. injection)	++ (2.3×10^8)	++	++	+	+ (5.0×10^3)	+	4.5
	$< 6.0 \times 10^4$ (water-borne)	++	++	++	+	+		
ATCC 35586	2.6×10^6 (i.p. injection)	++ (8.0×10^7)	+	+	–	+ (7.5×10^2)	+	10
	$> 5.0 \times 10^7$ (water-borne)	NA	NA	NA	NA	–		

2 major antigenic bands of about 30 and 57 kDa (Fig. 2b). Interestingly, this immunoreactive 57 kDa protein was also produced by the *Renibacterium salmoninarum* and *Corynebacterium aquaticum* reference strains. Similarly, using antiserum from *R. salmoninarum* ATCC 33209, all the *C. piscicola* strains exhibited the antigenic 57 kDa band (Fig. 2c). These results support our preliminary findings (Bandín et al. 1993) and, therefore, allow us to reaffirm that any immunodiagnostic procedure relying on the specific detection of *R. salmoninarum* 57 kDa antigen can suffer from false positive reactions with other Gram-positive bacteria.

The negative results in the cross-agglutination assays among the isolates may be due to the possibility that the majority of common membrane proteins are not exposed to the antibodies or that they occur in such low amounts as to be unmeasurable in agglutination reactions, or to a combination of both effects.

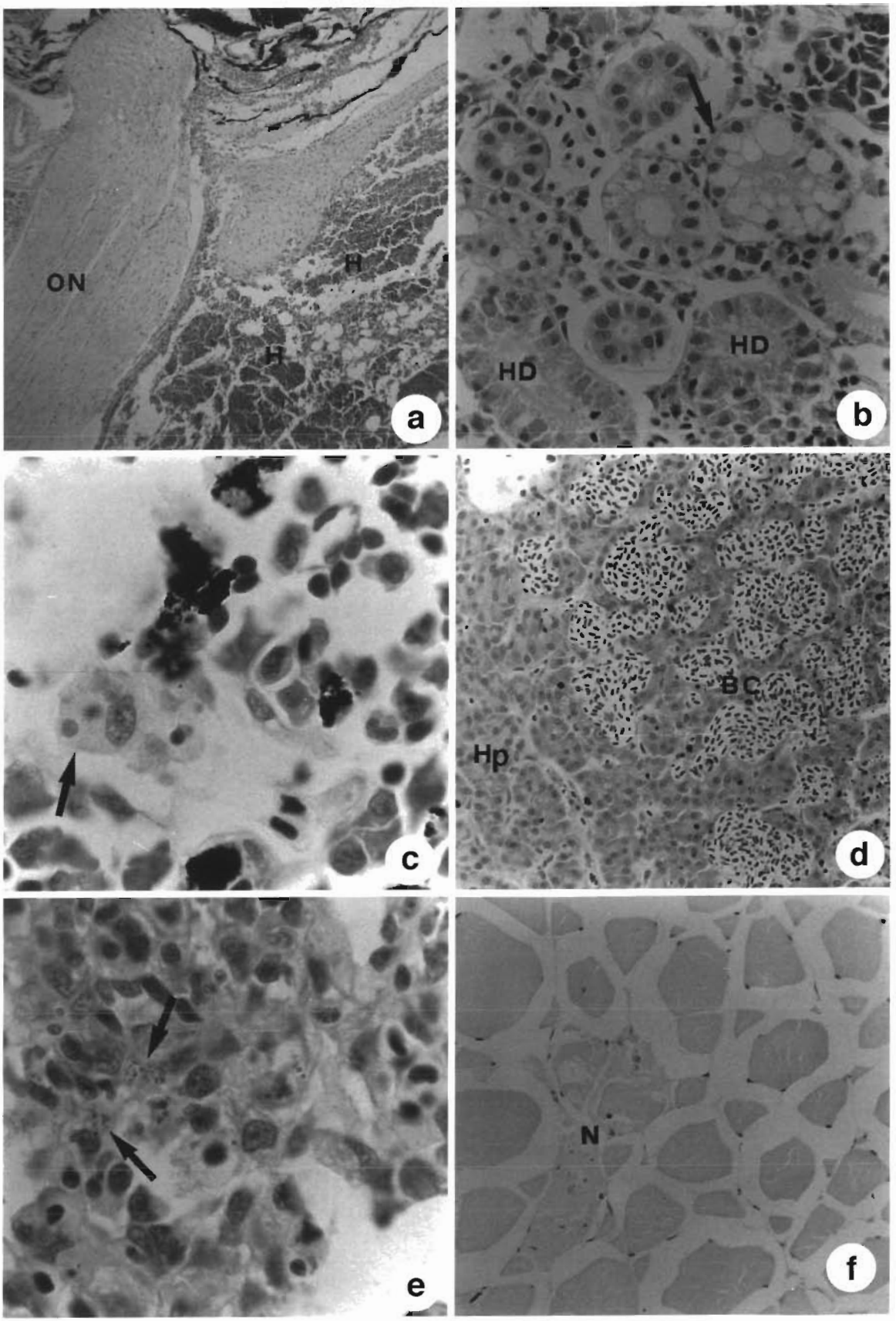
All of these findings indicate that *Carnobacterium piscicola* is an antigenically heterogeneous species. This makes serological diagnosis of the disease difficult but this variability could be used in epidemiological studies to trace sources of infection.

Pathobiological activities of live cells and extracellular products

Virulence tests conducted by i.p. inoculation and water challenge indicated that our isolate PT-31 was highly pathogenic for rainbow trout, with a mean LD_{50} value of $< 5 \times 10^4$ cells by injection and $< 6 \times 10^4$ by water challenge. Mortalities occurred within 3 to 7 d after challenge (Table 5). It is noteworthy that all of the moribund fish exhibited most of the external and internal clinical signs of the natural disease (i.e. pronounced exophthalmia, abdominal swelling with accumulation of ascitic fluid, and hemorrhages in the liver, intestine and musculature). In contrast, the reference strain *Carnobacterium piscicola* ATCC 35586 exhibited a low degree of virulence, being only pathogenic by i.p. injection ($LD_{50} = 2.6 \times 10^6$ cells). Mortalities occurred more slowly (between Day 4 and Day 12) and the dead fish did not exhibit any apparent clinical signs. This finding is in agreement with our previous reports in which this ATCC strain was also tested for comparative purposes (Baya et al. 1991, Toranzo et al. 1993).

Although both of the challenge strains were recovered in pure culture from kidney, liver, and spleen of moribund trout, only in the case of our isolate PT-31 could the challenge organisms be reisolated from the brain. In addition, we recovered the inoculated bacterial strains from the kidney of survivor fish sacrificed 30 d after challenge, indicating that *Carnobacterium piscicola* can be harboured by the fish in a carrier state. Although in most of the challenge experiments conducted with *Lactobacillus* or *Carnobacterium* species, the microorganisms showed low virulence or failed to produce mortalities (Ross & Toth 1974, Herman et al. 1985, Michel et al. 1986, Baya et al. 1991, Starliper et al. 1992), the carrier state could usually also be established in the fish populations using these microorganisms.

To evaluate the possible role of exotoxins in the virulence mechanisms of *Carnobacterium piscicola* PT-31 for rainbow trout, we determined the biological activities of its ECP in comparison with those shown by the reference ATCC strain. The total proteolytic activity of the ECP of each of the strains was very low (0.1 and 0.3 U ml^{-1}, respectively) and caseinase, gelatinase, lipase, phospholipase and amylase activities were not detected. Moreover, cytotoxins or hemolysins were not detected in the ECP samples. This absence of enzymatic activities is similar to that reported for the ECP from *Renibacterium salmoninarum* (Bandín et al. 1989, Toranzo & Barja 1993). However, unlike *R. salmoninarum*, *C. piscicola* produced exotoxins with lethal effects for fish; mortalities in treated fish occurred within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24 h after i.p. or i.m. inoculation of the ECP. In contrast with the virulence assays with the live cells, the dead ECP-treated fish did not exhibit any significant clinical signs; only fish inoculated i.m. showed any adverse effects (an internal hemorrhagic zone around the injection site). The LD_{50} values of the ECP from PT-31 and ATCC strains were 4.5 and 10 µg protein g^{-1} fish, respectively (Table 5), which were comparable to those reported for other fish pathogens (Toranzo & Barja 1993).

Our *in vitro* tests did not help to account for the strong pathogenicity of our *Carnobacterium piscicola* isolate. However, we cannot rule out the possibility that there was *in vivo* production of different enzymes and/or toxins needed by the invading bacterium to proliferate in the specific tissues of fish. In addition, the rapidity with which the mortalities occurred following ECP injection could indicate the involvement of a neurotoxin.

Histopathological examination

Tissues of moribund rainbow trout taken from fish suffering the natural or experimental infection with *Carnobacterium piscicola* PT-31 were histologically examined to evaluate the changes produced in the different organs. This bacterial strain caused extensive and intensive pathological changes in the eyes, kidney, liver, spleen, pancreas, and muscle (Fig. 3). The eyes showed an intensive periocular hemorrhaging, and all of the adjacent orbital adipose tissue was markedly hemorrhagic and infiltrated with leucocytes (Fig. 3a). However, no obvious lesions were observed in the brain. In the kidney, a great number of renal tubules exhibited hyaline droplet degeneration, and the epithelium of some of these tubules was necrotic and vacuolated (Fig. 3b). In addition, many macrophages containing phagocytized erythrocytes were detected in the kidney (Fig. 3c). Mild localized hemorrhages were present in this organ. The liver showed extensive congestion in sinusoids, many blood lacunae being present (Fig. 3d). Hepatic cords were disorganized and in certain areas the hepatocytes were undergoing necrosis. The pulp of the spleen was edematous, hemorrhagic, and infiltrated with leucocytes (Fig. 3e). The intestine showed necrosis of the mucous epithelium, and the acinar cells of the pancreas were also necrotic. However, no lesions were evident in the stomach and pyloric caeca. Red and white musculature underwent necrosis (Fig. 3f)

Although Gram-positive bacilli were observed in all of the organs examined, the highest bacterial numbers were found within the splenocytes and macrophages of the spleen (Fig. 3e) and in the necrotic pancreatic tissues.

These findings differ from those decribed in our recent paper dealing with the histophatological lesions produced by other *Carnobacterium piscicola* strains in rainbow trout and striped bass (Toranzo et al. 1993). These strains of *C. piscicola* caused only mild lesions in spleen and kidney. In addition, no damage to the eyes and musculature was observed. The present *C. piscicola* isolate PT-31 is thus clearly much more pathogenic and virulent than the other strains reported to date. It is noteworthy that the hemorrhages in the eyes displayed by our isolate were as severe as those seen in streptococcosis (Boomker et al. 1979, Miyazaki 1982, Baya et al. 1990) or in the recently described fish infection by *Corynebacterium aquaticum* (Baya et al. 1992)

CONCLUDING REMARKS

This report deals with the first case of a *Carnobacterium piscicola* infection in Spain. Heavy losses in cultured rainbow trout were experienced during the infection. At present, the source of the infection on the affected farm is unknown. The mortalities were most likely triggered by a sudden increase of water temperature because the fish had not experienced any other environmental or management stress. In contrast with previous reports, the present *C. piscicola* isolate exhibited a high degree of virulence in challenge experiments, a virulence that was supported by the strong histopathological changes that it caused in most of the fish organs examined. All of these findings warrant a reevaluation of the general assumption that the Gram-positive bacilli belonging to *Lactobacillus-Carnobacterium* species are opportunistic bacteria of low pathogenicity, and we hope that the information presented here serves to alert fish farmers to the exis-

Fig. 3. *Oncorhynchus mykiss* infected with *Carnobacterium piscicola* PT-31 strain. Histopathological lesions observed in the different organs of rainbow trout suffering the natural disease and the experimental i. p. challenge with live cells. (a) Intensive hemorrhage (H) in the adipose tissue adjacent to the eye. ON: optical nerve. H&E, 50×. (b) Hyaline droplet degeneration (HD) in the renal tubules of the kidney. Necrosis and vacuolation of the epithelium of some tubules (arrow). H&E, 320×. (c) Red blood cells engulfed by macrophages in the kidney (arrow). H&E, 1000×. (d) Extensive blood congestion (BC) in the sinusoids of the liver with blood lacunae being present. Normal aspect of the liver hepatocytes (Hp). H&E, 300×. (e) Edematous pulp of the spleen showing bacteria within the splenocytes (arrows). H&E, 1000×. (f) Necrosis (N) in the muscle. H&E, 300×

tence of strains possessing a range of virulence wider than that previously considered within these bacterial groups.

Because *Carnobacterium piscicola* strains are highly resistant to the majority of the drugs commonly employed in aquaculture, we urge that consideration be given to using erythromycin for controlling *C. piscicola* infections.

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