Vol. 21: 103–108, 1995 | DISEASES OF AQUATIC ORGANISMS Dis. aquat. Org. | Published March 9

Response of *Pasteurella piscicida* and *Flexibacter maritimus* to skin mucus of marine fish

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ABSTRACT: The antibacterial activity present in the skin mucus of turbot *Scophthalmus maximus*, seabream *Sparus aurata* and seabass *Dicentrarchus labrax* against *Pasteurella piscicida* and *Flexibacter maritimus* was evaluated. Using assays on agar plates, none of the mucus samples from the above fish showed any antibacterial activity against *F. maritimus* isolates. Turbot mucus inhibited the growth of the *P. piscicida* but mucus from seabream and seabass did not. Assays in liquid systems to determine the survival of the above pathogens in the presence of skin mucus corroborated the results obtained by the agar plate method. The bactericidal properties of the mucus were lost after heat treatment at pH 3.5 and all skin mucus samples displayed activity against *Staphylococcus aureus* ATCC 25923, a strain resistant to lysozyme. These findings indicated that thermolabile substances other than lysozyme were responsible for the antibacterial activity in mucus of marine fish. Enzymatic and heat treatments of the mucus also showed that factors other than complement were involved and that the active component(s) was likely a glycoprotein. Regardless of the source of isolation and degree of virulence, all *P. piscicida* and *F. maritimus* strains adhered strongly to the skin mucus of the 3 fish species tested. Taking all of the foregoing results into consideration, it appears that whereas a possible portal of entry for *F. maritimus* into the fish body is the skin, in *P. piscicida* another pathway must be involved.

KEY WORDS: *Pasteurella piscicida* · *Flexibacter maritimus* · Marine fish · Skin mucus Antibacterial activity · Adhesiveness

INTRODUCTION

Bacterial adhesion to tissue surfaces of fish is important during the initial stages of infection (Thune et al. 1993, Toranzo & Barja 1993). However, fish body surfaces are covered with a layer of mucus with antibacterial properties that protects against colonization by potential pathogens (Harrel et al. 1976, Austin & McIntosh 1988). These antimicrobial effects of body mucus are therefore valuable to fish as a first line of defense against infection.

Pasteurellosis and flexibacteriosis, caused respectively by the halophilic bacteria *Pasteurella piscicida* and *Flexibacter maritimus*, are fish diseases that cause problems in marine aquaculture. *P. piscicida* is one of the main agents responsible for economic losses in cultured yellowtail *Seriola quinqueradiata* in Japan. Indeed, since 1990 this bacterium has also caused serious disease outbreaks in the culture of seabream *Sparus aurata* and seabass *Dicentrarchus labrax* in European countries (reviews by Toranzo et al. 1991, Kitao 1993, Kusuda & Salati 1993). *F. maritimus* is widely distributed among marine fish cultured mainly in Japan and Europe (Campbell & Buswell 1982, Wakabayashi et al. 1986, Bernardet et al. 1990). In Spain, pathological problems attributable to this microorganism have increased considerably during the last few years and it poses a threat to the culture of turbot (*Scophthalmus maximus*) and salmon (*Salmo salar* and *Oncorhynchus kisutch*) (Devesa et al. 1989, Pazos et al. 1993, Toranzo et al. 1993).

The mode of transmission and route of infection involved in these diseases are still uncertain. The importance of skin as a portal of entry for *Pasteurella piscicida* and *Flexibacter maritimus* has not been explored. Consequently, the role of the external mucus

layer as a barrier to infection is not known. In the present study, the antibacterial activity of skin mucus from different marine fish species against several *P. piscicida* and *F. maritimus* isolates was studied and the nature of the biologically active substance(s) in the mucus was investigated. In addition, the ability of both pathogens to adhere to glass surfaces coated with mucus from the fish was also determined.

MATERIALS AND METHODS

Strains. Strains of *Pasteurella piscicida* and *Flexibacter maritimus* from different sources were used in this work (see Table 1). Pathogenic isolates of *Vibrio anguillarum* (R-82 and RG-111) and *V. damsela* (ATCC 33539) (Toranzo & Barja 1993) were included in the study for comparative purposes. *Staphylococcus aureus* ATCC 25923, a strain resistant to lysozyme, was used as a control. The *P. piscicida* strains were cultured on Brain Heart Infusion Agar (BHIA) (Oxoid, Ltd, Basingstoke, Hampshire, UK) supplemented with NaCl to a final concentration of 2% (BHIA-2) and the *F. maritimus* isolates were cultured on a *F. maritimus* medium (FMM) (peptone, 0.5%; sodium acetate, 0.001%; yeast extract, 0.5%; and agar, 1.5%; prepared in seawater) as described by Pazos et al. (1993).

Infectivity trials. After fish stocks were determined to be free of bacterial pathogens by microbiological analysis of the internal organs, infectivity trials were performed as previously described (Magariños et al. 1992b) in fingerling turbot, seabream, and seabass (average weights 8 to 10 g). The challenge was administered as an intraperitoneal injection of 0.1 ml of bacterial suspension containing from 10^3 to 10^8 cells (10 fish per dose). Control fish were similarly injected with saline solution. Mortalities were attributed to the inoculated bacterium if the injected organism was recovered in pure culture from the internal organs. The degree of virulence (LD_{50}, lethal dose 50%) was determined by the Reed & Müench (1938) method. In the case of *Flexibacter maritimus*, this assay was only performed in turbot using a single dose (between 10^6 and 10^7 cells per fish).

Bactericidal assay of the surface mucus. The mucus was scraped from the skin of 8 healthy turbot, 8 seabream, and 8 seabass with a glass slide which was passed along the animals from the caudal peduncle to the operculum. The mucous material was dissolved in sterile seawater or saline solution (SS: 0.85% NaCl) (0.1 ml mucus per 2 ml seawater or SS), mixed thoroughly, filter sterilized, and stored at −30°C until used.

The antibacterial activity of the different skin mucus preparations was tested by 2 different procedures. The assays were conducted in triplicate and the means and their associated standard deviation (SD) values were calculated.

(1) Assay on solid media: The classical disc diffusion method on agar plates was employed (Fouz et al. 1990). Bacteria were resuspended in SS or seawater to a concentration of 10^5 to 10^6 cells ml^{-1} and then seeded on plates of BHIA-2 or FMM, depending on the strain tested. After an absorption period of 15 min, sterile 6 mm diameter discs impregnated with 20 µl of the mucus preparations were applied to the agar plates. Incubation was at 25°C for 24 h for *Pasteurella piscicida, Vibrio anguillarum,* and *V. damsela* or 72 h for *Flexibacter maritimus,* and at 37°C for 24 h for *Staphylococcus aureus.* The antibacterial activity was evident as a zone without bacterial growth around the disc.

The presence of lysozyme activity in the skin mucus was evaluated microbiologically on mucus preparations that had been heated to 100°C for 10 min following adjustment to various pH values (3 to 9) (Takahashi et al. 1986).

(2) Survival and growth ability of the strains in skin mucus: This assay was conducted only with turbot mucus because in the previous plate assay this was the only mucus to show an antimicrobial effect. The isolates were inoculated at a concentration of approximately 10^5 cells ml^{-1} (*Pasteurella piscicida, Vibrio damsela* and *V. anguillarum*) or 10^6 cells ml^{-1} (*Flexibacter maritimus*) in sterile seawater supplemented with skin mucus (100 µl ml^{-1}). At different times, samples (0.1 ml) were taken and spread onto plates of BHIA-2 or FMM media. The number of colonies was counted after incubation at 25°C for 24 or 72 h (depending on the strain tested). For reference purposes, viable counts were also made on bacteria suspended in seawater alone.

Effect of enzymatic and heat treatments on the antimicrobial activity of mucus. The effect of various enzymes on the antibacterial activity of the skin mucus of turbot (against *Pasteurella piscicida* and *Staphylococcus aureus*) and of seabass and seabream (against *S. aureus*) was evaluated using solutions (1 mg ml^{-1}) in SS of the following enzymes (Sigma): proteinase K, trypsin, β-galactosidase, achromopeptidase, lysozyme, β-amylase, and lipase. Mucus preparations were mixed with the enzyme solutions (ratio 1:1) and incubated for 1 h at 37°C. The residual activities of the mucus preparations were assayed by the paper disk diffusion method on BHIA-2 plates previously seeded with the *P. piscicida* or with *S. aureus.* Disks impregnated separately with untreated mucus and enzyme solutions were included as controls.

To determine the possible involvement of complement in the antibacterial activity, the mucus preparations were heated as follows: 47°C for 30 min, 56°C for

20 min and 80°C for 10 min. Following this, the mucus preparations were assayed for antibacterial activity by the disc diffusion/agar plate method.

Adherence to skin mucus. The assay was performed by the method of Krovaceck et al. (1987). Mucus preparation (50 µl) was placed on glass slides and left overnight to air-dry following fixation for 20 min with absolute methyl alcohol. Ten ml of bacterial suspensions (2×10^5 cells ml^{-1}) in SS or seawater was added to mucus-coated slides in petri plates. The plates were incubated at room temperature for 1 h with continuous gentle shaking and washed several times with sterile seawater or SS. The slides were then air-dried at room temperature for 12 to 14 h, fixed with absolute methyl alcohol (20 min at room temperature), and stained with crystal violet for 2 min. Following this, the slides were washed and air-dried. Finally, the slides were observed under the light microscope and the number of bacteria attached to 1 mm^2 of the mucus-coated glass slides was determined. The assays were performed in duplicate.

To rule out possible influence of the alcohol fixation treatment on adherence and, hence, to simulate more natural conditions, we also repeated the adherence experiments using only air-dried mucus. Control tests using uncoated glass slides were performed. In addition, strains of *Vibrio harveyi* and *V. fischeri* (kindly supplied by J. J. Borrego, Departamento de Microbiología, Facultad de Ciencias, Universidad de Málaga, Spain), lacking the ability to adhere to fish mucus, were used as negative controls.

RESULTS

Bactericidal assay

Table 1 summarizes the findings with the various mucus samples and bacteria tested. All *Flexibacter maritimus* strains tested resisted the antimicrobial activity of skin mucus from the different fish species evaluated. All isolates of *Pasteurella piscicida* were sensitive to the antibacterial action of turbot mucus but were resistant to the action of seabream and seabass skin mucus. The strains of *Vibrio anguillarum* tested showed similar results to *P. piscicida*; *V. damsela* was resistant to all mucus samples; and the *Staphylococcus aureus* strain was susceptible to all of the mucus samples.

The *Staphylococcus aureus* ATCC 25923, employed as an indicator for the presence of lysozyme, was very sensitive to the action of all of the mucus samples assayed. However, the sensitivity of this bacterium to the different skin mucus samples disappeared when the samples were heated at 100°C in the acidic buffer (pH 3.5). These results were obtained whether or not the mucus was dissolved in seawater or SS.

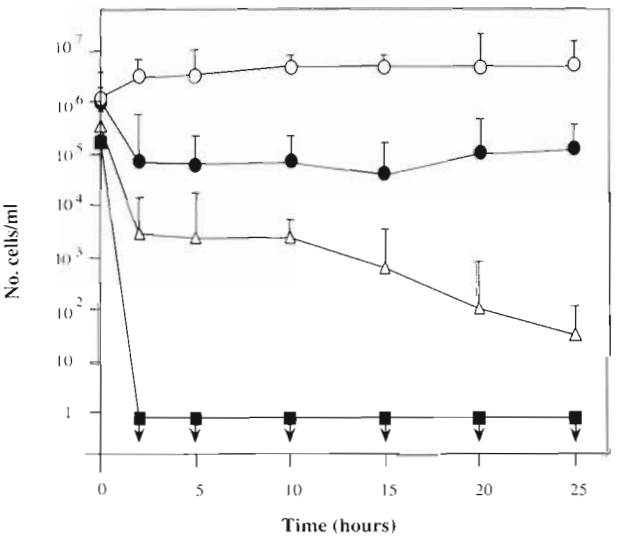

Fig. 1. Survival of representative strains of *Pasteurella piscicida* and *Flexibacter maritimus* in preparations of skin mucus of turbot *Scophthalmus maximus*. (■) *P. piscicida* DI 21; (●) *F. maritimus* NCIMB 2154. *Vibrio anguillarum* R-82 (△) and *V. damsela* ATCC 33539 (○) isolates were included for comparison. Arrowheads indicate values below the detection limit. Vertical bars represent standard deviations

Fig 1 is representative of the survival observed when the various *Flexibacter maritimus* and *Pasteurella piscicida* isolates were suspended in seawater containing turbot mucus. The *F. maritimus* isolates all survived the exposure, although initially they experienced a slight drop in the population of viable cells. In contrast, turbot mucus exerted a strong and rapid antimicrobial effect on the *P. piscicida* isolates, no viable *P. piscicida* cells being detectable within 2 h. Indeed, by 30 min, no viable *P. piscicida* cells were detectable (data not shown). The effect of turbot mucus on the *Vibrio anguillarum* strains was less marked than on *P. piscicida* and was negative on the *V. damsela* isolate. All isolates survived well in the controls (seawater) maintaining the same or higher viable cell populations than present at time zero (data not shown).

Properties of the antibacterial molecule(s)

The effect of heat and enzymatic treatments on the antibacterial substance(s) in the mucus is shown in Table 2. The antibacterial activity was completely lost on treatments at 80°C for 10 min. Moreover, the mucus dissolved in SS (free of Ca^{2+} and Mg^{2+}) showed the same antibacterial activity as that of mucus dissolved in seawater. These data suggest that the antibacterial

Table 1. Effect of the skin mucus from different marine fish against *Pasteurella piscicida* and *Flexibacter maritimus* strains

Strains	Origin	Donor[a]	Pathogenicity[b]	Source of mucus		
				Turbot	Seabream	Seabass
P. piscicida						
DI 21	Seabream, Spain	A. E. Toranzo	10^3–10^5	S (11 ± 0.5)[c]	R	R
DI 71	Seabream, Spain	A. E. Toranzo	10^3–10^5	S (13 ± 1)	R	R
B 32	Seabream, Spain	J. Borrego	10^3–10^4	S (12 ± 1)	R	R
B 51	Seabass, Spain	J. Borrego	10^3–10^5	S (11 ± 0.2)	R	R
10831	Seabass, France	F. Baudin-Laurencin	10^3–10^4	S (11 ± 0.5)	R	R
IT-1	Seabream, Italy	G. Giorgetti	10^3–10^5	S (12 ± 0.5)	R	R
MP-7801	Yellowtail, Japan	T Kitao	10^3–10^5	S (12 ± 1)	R	R
EPOY-8803-II	Red grouper, Japan	K. Muroga	$>10^8$	S (12 ± 2)	R	R
ATCC 29690	Yellowtail, Japan	ATCC	$>10^8$	S (13 ± 0.5)	R	R
ATCC 17911	White perch, USA	ATCC	10^3–10^6	S (12 ± 0.2)	R	R
P-3333	Amberjack, Japan	R. Kusuda	10^4–10^5	S (14 ± 1)	R	R
ATLIT 2	Striped bass, Italy	A. Colorni	10^4–10^5	S (17 ± 0.4)	R	R
F. maritimus						
NCIMB 2153	Black seabream, Japan	NCIMB	NT	R	R	R
NCIMB 2154	Red seabream, Japan	NCIMB	$>3 \times 10^6$	R	R	R
NCIMB 2158	Dover sole, UK	NCIMB	$<1 \times 10^7$	R	R	R
386	Red seabream, Japan	H. Wakabayashi	NT	R	R	R
394	Red seabream, Japan	H. Wakabayashi	NT	R	R	R
SP 9.1	Atlantic salmon, Spain	A. E. Toranzo	$<1 \times 10^7$	R	R	R
SE 30.1	Pacific salmon, Spain	A. E. Toranzo	NT	R	R	R
RA 79.1	Turbot, Spain	A. E. Toranzo	NT	R	R	R
RP 67.1	Turbot, Spain	A. E. Toranzo	$<8 \times 10^6$	R	R	R
RP 70.1	Turbot, Spain	A. E. Toranzo	NT	R	R	R
RPM 539.1	Turbot, Spain	A. E. Toranzo	$>1 \times 10^7$	R	R	R
RPM 562.1	Turbot, Spain	A. E. Toranzo	$<1 \times 10^6$	R	R	R
Controls:						
Vibrio anguillarum						
R-82 (O1)[d]	Turbot, Spain	A. E. Toranzo	10^2–10^4	S (10 ± 0.5)	R	R
RG-111 (O2)	Turbot, Spain	A. E. Toranzo	10^3–10^4	S (11 ± 0.2)	R	R
V. damsela						
ATCC 33539	Damselfish, USA	ATCC	10^3–10^4	R	R	R
Staphylococcus aureus						
ATCC 25923	Clinical isolate, USA	ATCC	NT	S (18 ± 0.5)	S (18 ± 0)	S (17 + 0)

[a] ATCC: American Type Culture Collection, Rockville, MD, USA; NCIMB: National Collections of Industrial and Marine Bacteria, Aberdeen, Scotland, UK

[b] The pathogenicity of *P. piscicida* and *Vibrio* controls is expressed as the range of LD_{50} values on different fish species (seabream, turbot and trout) (Magariños et al. 1992b, Toranzo & Barja 1993). NT: not tested

[c] R: resistant; S: sensitive. Values in parentheses indicate the diameter (mean ± SD) of inhibition zones in mm

[d] Serotype of the *V. anguillarum* strains used

activity in mucus was not due to complement but rather to other thermolabile substance(s).

The antibacterial activity exhibited by the various preparations against *Pasteurella piscicida*, *Vibrio anguillarum* and *Staphylococcus aureus* was totally eliminated by treatment with proteinase K and β-galactosidase and partially eliminated by trypsin and achromopeptidase. None of the other nonproteolytic enzymes had any effect. Table 2 illustrates the results for turbot mucus but the same findings held true for seabass and seabream mucus. These results suggest that the antibacterial substance is a glycoprotein.

Adherence assays

All *Pasteurella piscicida* and *Flexibacter maritimus* isolates adhered strongly to the turbot, seabream, and seabass skin mucus, values for attached cells ranging from 10^2 to 10^3 cells mm^{-2} depending on the strain. Differences between replicates were always lower than 0.5%. *F. maritimus* strains also showed some (slight) adherence to uncoated slides, but *P. piscicida* strains did not bind at all to uncoated glass surfaces. As expected, the *Vibrio* strains used as negative controls showed no adherence to coated or uncoated slides.

Table 2. Activity of the turbot *Scophthalmus maximus* mucus against *Pasteurella piscicida*, *Vibrio anguillarum*, and *Staphylococcus aureus* after the enzymatic and heat treatment. +: positive; (+): moderate; –: negative

Inhibition treatment	*P. piscicida* (8 strains)	*V. anguillarum* R-82	*S. aureus* ATCC 25923
Enzymes			
Proteinase K	–	–	–
Trypsin	(+) (8)[a]	(+) (7)	(+) (10)
β-Galactosidase	–	–	–
Achromopeptidase	(+) (9)	(+) (7)	(+) (9)
β-Amylase	+ (14)	+ (10)	+ (16)
Lipase	+ (14)	+ (11)	+ (17)
Lysozyme	+ (14)	+ (11)	+ (17)
Temperature			
47°C, 30 min	+ (14)	+ (10)	+ (17)
56°C, 20 min	(+) (7)	(+) (6)	(+) (9)
80°C, 10 min	–	–	–
Mucus without treatment	+ (14)	+ (10)	+ (17)

[a]Values in parentheses indicate the diameter of inhibition zones in mm

Adherence of the strains was not affected by the salt solution in which the mucus was dissolved (SS or seawater). In addition, the mucus fixation method did not influence the ability of the strains to bind to coated slides.

DISCUSSION

Pasteurella piscicida and *Flexibacter maritimus* constitute 2 phenotypically, serologically and molecularly homogeneous taxa (Wakabayashi et al. 1986, Bernardet et al. 1990, Toranzo et al. 1991, Magariños et al. 1992a, b, Pazos et al. 1993) and the results of this study confirm that homogeneity. Constituent strains of each taxon were uniform in their responses to the antibacterial effects of mucus and in their ability to attach to mucus.

The bactericidal assays demonstrated that skin mucus of turbot, seabream, and seabass did not contain compounds that inhibited the growth of *Flexibacter maritimus* isolates, a result similar to that reported by Al-Harbi & Austin (1992) for a fish-pathogenic *Cytophaga*-like bacterium. This resistance to the antibacterial effects of mucus could help to explain the fact that the primary site of infection by *F. maritimus* is the body surface, where ulcerative skin lesions are the lesions typically produced (Campbell & Buswell 1982, Devesa et al. 1989, Toranzo et al. 1993). With *Pasteurella piscicida*, all strains were susceptible to turbot mucus but not to seabream and seabass mucus. These observations may well explain why all disease outbreaks in Europe due to *P. piscicida* have to date occurred in seabream and seabass (Ceschia et al. 1990, Baudin-Laurencin et al. 1991, Toranzo et al. 1991) but never in turbot (Toranzo et al. 1993).

Specific and nonspecific antimicrobial factors including complement, lysozyme, C-reactive protein, immunoglobulins, proteases, lectin-like molecules, and glycoproteins have been reported to occur in mucus (Alexander & Ingram 1992). These substances may function as natural defence factors by preventing the colonization of fish surfaces by microorganisms and the penetration of bacteria into the body.

It is well recognized that the temperatures for inactivation of fish complement range from 42 to 50°C (depending on the fish species) and that the activation of the complement system is dependent on the presence of Ca^{2+} and Mg^{2+} (Sakai 1992). In our study, the activities of the different mucus preparations were inhibited at temperatures higher than 56°C. Further, mucus dissolved in saline solution (free of divalent cations) showed the same antibacterial properties as mucus dissolved in seawater. These data indicate that the antibacterial activities of the skin mucus from the fish species tested were not due to complement.

In the present work, the loss of mucus activity after heat treatment at pH 3.5 and the sensitivity displayed by *Staphylococcus aureus* (ATCC 25923) demonstrated that lysozyme was not the main factor responsible for the activity of mucus. Overall, the data indicated that other thermolabile antimicrobial substances exist in the skin mucus of marine fishes. These data are in accordance with those described by Takahashi et al. (1987), Austin & McIntosh (1988) and Fouz et al. (1990). In fact, the results of the enzymatic treatments revealed that the antimicrobial molecules were totally inhibited by proteinase K and by β-galactosidase, suggesting that they are glycoproteins.

The strains of *Pasteurella piscicida* and *Flexibacter maritimus* (regardless of their origin and degree of virulence) adhered strongly to glass slides coated with mucus from all of the fish species tested. However, because *P. piscicida* was highly sensitive to the antimicrobial action of turbot skin mucus, the bacterium would likely be rapidly killed after attachment to the surface of turbot, thus negating turbot skin as a portal of entry. These findings indicate that a strong bacterial adhesion to an organ or tissue does not necessarily result in the occurrence of disease. Obviously, for prognostic purposes, it is worth knowing whether the mucus associated with a given organ or tissue is bactericidal.

The homogeneity in the adherence patterns exhibited by the strains of *Flexibacter maritimus* and *Pas-*

teurella piscicida found in this study was similar to that found for strains of *Vibrio damsela* (Fouz 1993) and *V. vulnificus* biotype 2 (Amaro et al. 1993). In contrast, strains of *Aeromonas hydrophila* and *V. anguillarum* described by Krovaceck et al. (1987) were heterogeneous in this property.

The results reported here suggest collectively that a possible portal of entry for *Flexibacter maritimus* into the fish body can be the skin. However, with *Pasteurella piscicida* the pathways of entry may vary depending on the host. With some fish, infection may follow ingestion of the pathogen. Regardless of the route of entry (invasion) employed by a pathogen once it has succeeded in invading the fish, its success in establishing an infection or causing a disease will depend on other factors, including its ability to neutralize or evade the immune system of the fish and its ability to scavenge the nutrients required for its growth.

Acknowledgements. This investigation was supported by Grants MAR 91-1133-C02-01 and AGF-94-136-C03-01 from the Comisión Interministerial de Ciencia y Tecnología (CICYT), and XUGA 20004A94 from Xunta de Galicia, Spain, and by EUREKA project No. EU-374 between Spain and Norway. B.M. and F.P. thank the Xunta de Galicia for Research Fellowships. The kind donation of the bacterial strains used in this study is gratefully acknowledged.

LITERATURE CITED

Alexander JB, Ingram GA (1992) Noncellular nonspecific defence mechanism of fish. In: Faisal M, Hetrick FM (eds) Annual review of fish diseases. Pergamon Press, New York, p 249–279

Al-Harbi AH, Austin B (1992) Influence of skin and gut mucus from turbot (*Scophthalmus maximus* L.) on the survival of a fish-pathogenic *Cytophaga*-like bacterium (CLB). Bull Eur Ass Fish Pathol 12:80–82

Amaro C, Biosca EG, Fouz B, Esteve C, Garay E (1993) Experimental induction of vibriosis produced by *V. vulnificus* biotype 2 in eels by immersion. Abstracts, 6th Int Conf Eur Ass Fish Pathol, Brest, France. European Association of Fish Pathologists, Brest, p 18

Austin B, McIntosh D (1988) Natural antibacterial compounds on the surface of rainbow trout, *Salmo gairdneri*. J Fish Dis 11:275–277

Baudin-Laurencin F, Pepin JF, Raymond JC (1991) First observation of an epizootic of pasteurellosis in farmed and wild fish of the French mediterranean coasts. Abstracts, 5th Int Conf Eur Ass Fish Pathol, Budapest, Hungary. European Association of Fish Pathologists, Budapest, p 1

Bernardet JF, Campbell AC, Buswell JA (1990) *Flexibacter maritimus* is the agent of 'black patch necrosis' in Dover sole in Scotland. Dis aquat Org 8:233–237

Campbell AC, Buswell JA (1982) An investigation into the bacterial aetiology of 'black patch necrosis' in Dover sole, *Solea solea* L. J Fish Dis 5:495–508

Ceschia G, Giorgetti G, Bovo G (1990) Grave epizoozia da *Pasteurella piscicida* in specie eurihaline del Nord Adriatico. Boll Soc Ital Patol Ittica 4:11–17

Devesa S, Barja JL, Toranzo AE (1989) Ulcerative skin and fin lesions in reared turbot, *Scophthalmus maximus* (L.). J Fish Dis 12:323–333

Fouz B (1993) Caracterización de *Vibrio damsela*: nuevo patógeno en acuicultura marina. Doctoral dissertation, Universidad de Santiago de Compostela

Fouz B, Devesa S, Gravninger K, Barja JL, Toranzo AE (1990) Antibacterial action of the mucus of turbot. Bull Eur Ass Fish Pathol 10:56–59

Harrell LW, Etlinger HM, Hodgins HO (1976) Humoral factors important in resistance of salmonid fish to bacterial disease. II. Anti-*Vibrio anguillarum* activity in mucus and observations on complement. Aquaculture 7:363–370

Kitao T (1993) Pasteurellosis. In: Inglis V, Roberts RJ, Bromage NR (eds) Bacterial diseases of fish. Blackwell, Oxford, p 159–165

Krovaceck K, Faris A, Ahne W, Hånsson J (1987) Adhesion of *Aeromonas hydrophila* and *Vibrio anguillarum* to fish cells and to mucus-coated glass slides. FEMS microbiol Lett 42:85–89

Kusuda R, Salati F (1993) Major bacterial diseases affecting mariculture in Japan. In: Faisal M, Hetrick FM (eds) Annual review of fish diseases. Pergamon Press, New York, p 69–85

Magariños B, Romalde JL, Bandín I, Fouz B, Toranzo AE (1992a) Phenotypic, antigenic and molecular characterization of *P. piscicida* strains isolated from fish. Appl environ Microbiol 58:3316–3322

Magariños B, Santos Y, Romalde JL, Rivas C, Barja JL, Toranzo AE (1992b) Pathogenic activities of the live cells and extracellular products of the fish pathogen *Pasteurella piscicida*. J gen Microbiol 138:2491–2498

Pazos F, Santos Y, Núñez S, Toranzo AE (1993) Characterization of *Flexibacter maritimus* isolated in northwest of Spain. Abstracts, 6th Int Conf Eur Ass Fish Pathol, Brest, France. European Association of Fish Pathologists, Brest, p 8

Reed LJ, Müench M (1938) A simple method of estimating fifty percent end points. Am J Hyg 27:493–497

Sakai DK (1992) Repertoire of complement in immunological defense mechanism of fish. In: Faisal M, Hetrick FM (eds) Annual review of fish diseases. Pergamon Press, New York, p 223–247

Takahashi Y, Itami T, Konegawa K (1986) Enzymatic properties of partially purified lysozyme from the skin mucus of carp. Bull Jap Soc scient Fish 52:1209–1214

Takahashi Y, Kajiwaki T, Itami T, Okamoto T (1987) Enzymatic properties of the bacteriolytic substances in the skin mucus of yellowtail. Nippon Suisan Gakk 53:425–431

Thune RL, Stanley LA, Cooper RK (1993) Pathogenesis of Gram-negative bacterial infections in warmwater fish. In: Faisal M, Hetrick FM (eds) Annual review of fish diseases. Pergamon Press, New York, p 37–68

Toranzo AE, Barja JL (1993) Virulence factors of bacteria pathogenic for cold water fish. In: Faisal M, Hetrick FM (eds) Annual review of fish diseases. Pergamon Press, New York, p 5–36

Toranzo AE, Barreiro S, Casal JF, Figueras A, Magariños B, Barja JL (1991) Pasteurellosis in cultured gilthead seabream (*Sparus aurata*): first report in Spain. Aquaculture 99:1–15

Toranzo AE, Novoa B, Romalde JL, Nuñez S, Devesa S, Mariño E, Silva R, Martínez E, Figueras A, Barja JL (1993) Microflora associated with healthy and diseased turbot (*Scophthalmus maximus*) from three farms in Northwest Spain. Aquaculture 114:189–202

Wakabayashi H, Hikida M, Masumura K (1986) *Flexibacter maritimus* sp. nov., a pathogen of marine fishes. Int J syst Bacteriol 36:396–398

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

Manuscript first received: May 2, 1994
Revised version accepted: November 10, 1994