Pathogenic activities of live cells and extracellular products of the fish pathogen *Pasteurella piscicida*

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The pathobiological activities *in vivo* and *in vitro* of live cells and extracellular products (ECP) of eleven *Pasteurella piscicida* strains of different origin were examined. Infectivity trials showed that *P. piscicida* did not possess strict host specificity since the majority of the isolates were virulent for gilthead seabream, rainbow trout and turbot, with LD₅₀ values ranging between 10³ and 10⁶ live cells. However, none of the strains tested were pathogenic for mice (LD₅₀ > 10⁸ cells). In addition, the ECP were strongly toxic for fish (LD₅₀ ranging from 1.0 to 4.6 µg protein per g fish), which clearly demonstrates their important role in the pathogenesis of pasteurellosis. All the ECP samples were cytotoxic for fish and homoiothermic cell lines, possessed notable phospholipase activity and displayed haemolytic activity for sheep, salmon and turbot erythrocytes (but not for trout erythrocytes). However, the production of proteolytic enzymes differed among the *P. piscicida* strains. Although no strain displayed elastase activity, five isolates (the Japanese and Italian strains) hydrolysed casein and gelatin. All these biological activities *in vivo* and *in vitro* were lost after heat treatment (100 °C for 10 min). The general enzymic patterns of both live cells and ECP evaluated by the API-ZYM system also revealed some variation among the *P. piscicida* isolates. Generally, whole cells showed a wider range of enzymic activities than ECP. The results presented here are important for the selection of strains in the development of effective polyvalent pasteurellosis vaccines containing both whole cells and ECP.

Introduction

Pasteurella piscicida has been, since 1963, one of the most important halophilic bacterial pathogens, causing significant losses in wild and farmed fish in Japan (Kubota *et al.*, 1970; Kimura & Kitao, 1971; Kusuda & Yamaoka, 1972; Ohnishi *et al.*, 1982; Egusa, 1983; Yasunaga *et al.*, 1984; Ueki *et al.*, 1990) and the USA (Sniezsko *et al.*, 1964; Paperna & Zwerner, 1976; Robohm, 1979; Hawke *et al.*, 1987). However, until 1990 there were no documented reports of fish pasteurellosis in Europe. Recently, we described the first pasteurellosis outbreak in juvenile gilthead seabream (*Sparus aurata*) cultured in the northwest of Spain (Toranzo *et al.*, 1991). Simultaneously, epizootic outbreaks occurred in France and Italy, affecting mainly sea bass (*Dicentrarchus labrax*) and gilthead (Baudin-Laurencin *et al.*, 1991; Ceschia *et al.*, 1991). In addition, we have demonstrated (Toranzo *et al.*, 1991; Magariños *et al.*, 1991, 1992) that, regardless of their origin and source of isolation, all the European, Japanese and American *P. piscicida* strains examined shared similar biochemical, serological and molecular characteristics. However, in contrast with other substantiated fish pathogens, the mechanism of virulence of *P. piscicida* and the nature of factors involved in the pathogenesis of pasteurellosis have not been studied. To our knowledge, the present work represents the first analysis of the possible role of extracellular products (ECP) in the development of this disease. The biological activities present in the ECP from *P. piscicida* isolates of different geographic origins were evaluated *in vivo* and *in vitro*. In addition, the virulence of *P. piscicida* for different host species was determined.

Methods

Bacterial strains. Eleven *P. piscicida* strains, isolated from different fish species and geographical locations, were used (Table 1). For comparison, two pasteurellas from mammalian sources (*P. multocida* CECT 902 and *P. haemolytica* CECT 924) were also included. The taxonomic position of all the isolates was confirmed following the

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Abbreviations: ECP, extracellular products; BHI, brain heart infusion; i.p., intraperitoneal(ly).

Table 1. Origin of *Pasteurella* isolates used.

Strain	Origin	Donor*
<i>P. piscicida</i>		
DI 21	<i>Sparus aurata</i> (gilthead seabream), Spain	A. E Toranzo & B. Magariños
DI 71	<i>Sparus aurata</i> (gilthead seabream), Spain	A. E Toranzo & B. Magariños
DI 91	<i>Sparus aurata</i> (gilthead seabream), Spain	A. E Toranzo & B. Magariños
10831	<i>Dicentrarchus labrax</i> (sea bass), France	F. Baudin-Laurencin
IT-1	<i>Sparus aurata</i> (gilthead seabream), Italy	G. Giorgetti
IT-2	<i>Sparus aurata</i> (gilthead seabream), Italy	G. Giorgetti
MP-7801	<i>Seriola quinqueradiata</i> (yellowtail), Japan	T. Kitao
MZS-8001	<i>Seriola quinqueradiata</i> (yellowtail), Japan	T. Kitao
EPOY-8803-II	<i>Epinephelus akaara</i> (red grouper), Japan	K. Muroga
ATCC 17911	<i>Roccus americanus</i> (white perch), USA	ATCC
ATCC 29690	<i>Seriola quinqueradiata</i> (yellowtail), Japan	ATCC
<i>P. haemolytica</i>		
CECT 924	Ovine	CECT
<i>P. multocida</i>		
CECT 902	Bovine	CECT

* Donor addresses: F. Baudin-Laurencin, Laboratoire National de Pathologie des Animaux Aquatiques, Brest, Plouzané, France; G. Giorgetti, Laboratorio di Ittiopatologia, Istituto Zooprofilattico Sperimentale delle Venezie, Italy; T. Kitao, Department of Fisheries, Faculty of Agriculture, Miyazaki University, Japan; K. Muroga, Faculty of Applied Biological Science, Hiroshima University, Japan; ATCC, American Type Culture Collection, Rockville, Maryland (USA); CECT, Colección Española de Cultivos Cultivos Tipo, Valencia, Spain.

procedures of West & Colwell (1984), Fouz *et al.* (1990) and Toranzo *et al.* (1991). Serological characterization was performed by slide agglutination test as previously described (Toranzo *et al.*, 1987a). Experimental cultures were prepared on Brain Heart Infusion Agar (BHIA, Difco) with 2% (w/v) NaCl added (BHIA-2) from cultures stored frozen at -70°C in Tryptic Soy Broth (TSB, Difco) with 15% (v/v) glycerol. In the case of clinical pasteurellas the same culture medium without salt was employed.

In all the assays, strains *Vibrio anguillarum* R-82 and *Aeromonas hydrophila* B-32, which possess pathogenic activities for fish and mammals (Toranzo *et al.*, 1987b; Santos *et al.*, 1991a) were used as controls.

Pathogenicity assays. In order to determine the host susceptibility range of *P. piscicida*, infectivity trials were conducted using different fish species and mice.

(i) **Virulence for fish.** The 50% lethal dose (LD_{50}) assays were performed by intraperitoneal (i.p.) injection as previously described (Toranzo *et al.*, 1983a), using fingerling gilthead seabream (8 g), rainbow trout (*Oncorhynchus mykiss*) (10 g) and turbot (*Scophthalmus maximus*) (5 g). Groups of six fishes were inoculated i.p. with bacterial doses ranging from 10^2 to 10^8 c.f.u. Control fish received 0.1 ml phosphate-buffered saline (PBS, pH 7.4). Fish were maintained at 22°C with aeration during the course of the experiment (two weeks). The LD_{50} was calculated by the method of Reed & Muench (1938). Fish surviving the challenge were killed and reisolation of the inoculated strain was attempted to test for the possible carrier state.

(ii) **Mouse pathogenicity.** To determine the degree of virulence of *P. piscicida* for homoiothermic animals, a mouse virulence assay was performed. Briefly, between 5 and 10 BALB/C mice (10–12 weeks old, 21–25 g) were inoculated i.p. with doses ranging from 10^4 to 10^8 c.f.u. of each *P. piscicida* strain. Mortalities were recorded daily and the LD_{50} calculated 7 d after inoculation according to Reed & Muench (1938). Strains displaying an $\text{LD}_{50} < 10^7$ c.f.u. were considered as virulent (Daily *et al.*, 1981).

Preparation of the extracellular products (ECP) from the *Pasteurella* strains. The cellophane plate technique of Liu (1957) was used. Briefly,

sterilized cellophane sheets were placed on the surface of BHIA plates and inoculated by spreading 0.5 ml of a 24 h old broth culture of each strain with a sterile swab. The temperature of incubation was 22°C (*P. piscicida*) or 37°C (clinical pasteurellas). After incubation for 48 h, cells were washed off the cellophane with a minimum volume of PBS. All the cell suspensions were centrifuged at $10000g$ for 30 min at 4°C .

The supernatants were filtered through a $0.45\text{ }\mu\text{m}$ pore-size membrane (Millipore), freeze-dried and reconstituted in PBS to a final volume of 10 ml. All ECP samples were stored at -30°C until use. The protein concentration of each ECP sample was determined by the method of Bradford (1976), using bovine serum albumin as standard. The lipopolysaccharide (LPS) content of ECP was evaluated by the method of Keler & Novotny (1986), using LPS from *Escherichia coli* serotype O128:B12 (Sigma) as standard.

Proteolytic, phospholipase and haemolytic activities. For the detection of the total proteolytic activity present in the ECP samples a general proteolytic substrate (Azocoll, Sigma) was employed. The assay was performed following the manufacturer's instructions (Sigma). One unit of protease activity produced an A_{520} reading of 1.0 after 30 min incubation at 37°C .

Caseinase and gelatinase activities were determined 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% (w/v) sodium caseinate (Difco) or 1.5% (w/v) gelatin (Oxoid), respectively. Drops ($10\text{ }\mu\text{l}$) of each ECP were placed on the plates and incubated at 22°C for 24–48 h. To enhance cleared zones, plates of gelatin were flooded with 15% (w/v) mercuric chloride in 20% (v/v) HCl. One unit of caseinase or gelatinase activity was defined as that amount which produced a zone of clearing equal in area to that produced by $1\text{ }\mu\text{g}$ trypsin (Sigma) (Bandín *et al.*, 1991). Elastase activity was assayed on elastin medium (elastin, 1 g l^{-1} ; yeast extract, 2 g l^{-1} ; tryptone, 2 g l^{-1} ; cysteine hydrochloride, 0.3 g l^{-1} ; agar 15 g l^{-1} ; 0.02 M -Tris/HCl; pH 8.0). Elastase activity was calculated as the reciprocal of the highest dilution of ECP producing elastin degradation after 72 h incubation. The production of phospholipase was quantified on plates of BNA containing 1% (v/v) egg yolk emulsion (Oxoid). Plates were incubated for 48 h, and the titre was defined as the reciprocal of the highest

dilution of crude ECP producing opaque zones around the inoculum (10 µl).

Haemolytic activity was evaluated by the standard radial diffusion method using BHIA medium with 5% (v/v) sheep, trout or turbot erythrocytes added. One unit of haemolytic activity was defined as that which produced a zone of clearing equal in area to that produced by 0.1% sodium dodecyl sulphate (SDS) (Bandín *et al.*, 1991).

The general enzymic activities of the extracellular products and live cells of *Pasteurella* spp. were also evaluated by using the API-ZYM system (bioMérieux) at 22 °C and following the manufacturer's instructions. The strips were incubated at 22 °C (*P. piscicida*) or 37 °C (clinical pasteurellas).

The stability of all these activities was assessed by assay after heating the ECP samples at 100 °C for 10 min.

Cytotoxicity assays. The ECP preparations were assayed for cytotoxicity as previously described (Toranzo *et al.*, 1983b) in the following fish and homiothermic cell lines: CHSE-214 (Chinook salmon embryo), RTG-2 (rainbow trout gonad), EPC (epithelioma papulosum cyprine), FHM (fathead minnow peduncle), HeLa (human cervix epithelioid) and Vero (African green monkey kidney). Monolayers grown in 24-well plates (Costar) were inoculated with 100 µl of serial dilutions of each sample and incubated at 18 °C (fish cell lines) or 37 °C (homiothermic cell lines). Total or partial destruction of monolayers within a 2 d period was considered as a positive cytotoxic effect. Results were expressed as the minimal amount of protein necessary to produce a cytotoxic response. Samples heated at 100 °C for 10 min were also tested.

Toxicity of ECP in vivo. The lethal effects of exotoxins produced by the *P. piscicida* strains for fish and mammals were evaluated in rainbow trout, turbot and mice by i.p. inoculation with 0.1 ml of each preparation of ECP. Groups of six fish, maintained under the conditions described above for the virulence assays of live cells, were used per dose (serial twofold dilutions of the ECP). Mortalities were monitored over 7 d and the LD₅₀ (expressed as µg ECP protein per g body weight of fish) was calculated by the method of Reed & Muench (1938). In the case of mice, groups of between 5 and 10 animals were inoculated only with the undiluted ECP samples and the lethal effects expressed as number of dead mice/total number of inoculated animals. Control fish and mice were injected with 0.1 ml PBS. The samples exhibiting lethal effects for fish or mice were also assayed after heating (100 °C for 10 min).

Detection of vascular permeability factor. The presence in the ECP of dermonecrotic activity was determined as described by Olivier *et al.* (1981). Briefly, New Zealand White rabbits (1 kg body weight) were inoculated intradermally with 0.1 ml of each ECP sample. Twenty hours after inoculation, the rabbits were killed and skinned. The presence of haemorrhagic and/or oedematous zones with a diameter greater than 0.8 cm was considered as a positive result. All the ECP were inoculated in triplicate and the standard deviation (SD) of the results was calculated. Positive samples were also inoculated after heating at 100 °C for 10 min.

Results

Pathogenicity for fish and mammals

To determine whether *P. piscicida* possesses host specificity, virulence assays were performed in different fish species and mice. With the exception of two strains (EPOY-8803-II and ATCC 29690), the *P. piscicida* isolates were virulent for gilthead seabream, rainbow

trout and turbot with, LD₅₀ values ranging from 10³ to 10⁶ cells (Table 2). Interestingly, trout and turbot proved to be the most susceptible species to pasteurellosis under laboratory conditions. Although, in general, no clinical signs were apparent in the moribund fish, the inoculated strains were always reisolated in pure culture from the internal organs of all dead fish. However, attempts to recover *P. piscicida* from surviving fish were unsuccessful.

The pathogenicity assays in mice demonstrated that none of the *P. piscicida* strains could be considered as virulent since they displayed LD₅₀ values higher than 4 × 10⁷ live cells (Table 2). Only the *P. multocida* isolate was pathogenic for mice, with an LD₅₀ < 2.0 × 10³ cells. Therefore no relationship could be established between pathogenicity for fish and for mice in the *Pasteurella* species.

Biological activities of the ECP in vitro

The protein and LPS content of ECP from *P. piscicida* isolates was different depending on the extraction procedure. In general, all ECP obtained using the cellophane plate method showed notable levels of protein (0.3–3.4 mg ml⁻¹) and LPS (0.9–2.3 mg ml⁻¹) (Table 3). The production of proteolytic enzymes differed among the strains since only the Japanese and Italian isolates were able to hydrolyse casein and gelatin. No strain exhibited elastase activity. However, all the ECP samples were cytotoxic for fish and homiothermic cell lines, producing, in general, degenerative changes manifested by pyknotic nuclei, clusters of round cells, shrinking, dendritic elongations and finally cell detachment. The minimal amount of protein necessary to produce a cytotoxic response varied among the isolates and ranged from 0.09 µg ECP protein (Spanish, French and American strains) to 17 µg (Japanese and Italian isolates). These results indicated no correlation between the proteolytic activities tested and cytotoxicity.

All the ECP from *P. piscicida* possessed notable phospholipase activity (300–400 U ml⁻¹), which was not present in the clinical pasteurellas. However, the haemolytic activity was variable according to the erythrocytes employed. Whereas the majority of strains produced haemolysins against sheep and turbot erythrocytes, haemolytic activity for trout erythrocytes was not detected (Table 3).

The enzymic activities of the ECP of *Pasteurella* strains were also tested by using the API-ZYM system and the results were compared with the activities exhibited by the live cells (Table 4). In general, live cells of the different *P. piscicida* strains displayed a very similar pattern except for α-glucosidase and N-acetyl-β-glucosaminidase activities. The *P. piscicida* live cells

Table 2. Susceptibility of gilthead seabream, turbot and rainbow trout to live cells of *P. piscicida*.

Strain	Virulence for fish (LD ₅₀)*			Pathogenicity for mice (LD ₅₀)*
	Seabream	Turbot	Trout	
<i>P. piscicida</i>				
DI 21	+ (1.6 × 10 ⁵)	+ (<1.6 × 10 ⁴)	+ (<6.3 × 10 ³)	– (>2.0 × 10 ⁸)
DI 71	+ (2.1 × 10 ⁵)	+ (5.0 × 10 ³)	+ (<8.3 × 10 ³)	– (>1.0 × 10 ⁸)
DI 91	+ (1.4 × 10 ⁵)	+ (<7.6 × 10 ³)	+ (<6.0 × 10 ³)	– (>4.0 × 10 ⁷)
10831	+ (8.2 × 10 ⁴)	+ (<4.7 × 10 ³)	+ (1.7 × 10 ⁴)	– (>2.0 × 10 ⁸)
IT-1	+ (3.7 × 10 ⁵)	+ (<2.0 × 10 ⁴)	+ (2.3 × 10 ⁴)	– (>2.0 × 10 ⁸)
IT-2	+ (3.0 × 10 ⁵)	+ (<3.0 × 10 ⁴)	+ (5.2 × 10 ⁴)	– (>4.0 × 10 ⁸)
MP-7801	+ (1.0 × 10 ⁵)	+ (<5.0 × 10 ³)	+ (1.6 × 10 ⁵)	– (>2.0 × 10 ⁸)
MZS-8001	+ (2.0 × 10 ⁵)	+ (<6.0 × 10 ³)	+ (1.2 × 10 ⁵)	– (>3.0 × 10 ⁸)
EPOY-8803-II	– (>2.0 × 10 ⁷)	– (>2.0 × 10 ⁷)	– (>2.0 × 10 ⁷)	– (>8.0 × 10 ⁷)
ATCC 17911	(+) (4.7 × 10 ⁶)	+ (3.3 × 10 ⁵)	(+) (3.1 × 10 ⁶)	– (>2.0 × 10 ⁸)
ATCC 29690	– (2.3 × 10 ⁷)	– (>2.0 × 10 ⁷)	– (>6.3 × 10 ⁷)	– (>6.0 × 10 ⁷)
<i>P. haemolytica</i>				
CECT 924	ND	ND	– (>3.0 × 10 ⁸)	– (>4.0 × 10 ⁷)
<i>P. multocida</i>				
CECT 902	ND	ND	– (>1.0 × 10 ⁸)	+ (<2.0 × 10 ³)
Controls				
<i>A. hydrophila</i>				
B-32	ND	ND	+ (3.2 × 10 ⁴)	+ (1.0 × 10 ⁶)
<i>V. anguillarum</i>				
R-82	+ (7.3 × 10 ⁵)	+ (4.0 × 10 ⁴)	+ (8.0 × 10 ²)	– (5.0 × 10 ⁷)

* Median lethal dose 50% (LD₅₀) is expressed as the number of bacteria needed to kill 50% of inoculated animals. Symbols: +, virulent strain; –, avirulent strain; (+) weakly virulent strain. ND, Not determined.

Table 3. Biological activities in vitro of the ECP from the *Pasteurella* strains obtained by the cellophane plate technique

Strain	Total protein (mg ml ⁻¹)	LPS content (mg ml ⁻¹)	Proteolytic activity (U ml ⁻¹)*	Enzyme activities (U ml ⁻¹)†				Haemolytic activity (U ml ⁻¹)‡			Cytotoxic activity for cells	
				Gelatinase	Caseinase	Elastase	Phospholipase	Sheep	Trout	Turbot	Fish	Mammal
<i>P. piscicida</i>												
DI 21	2.8	1.1	0.5	–	–	–	400	50	–	140	+	+
DI 71	3.4	1.2	1.3	–	–	–	300	50	–	160	+	+
DI 91	2.7	1.0	1.2	–	–	–	400	50	–	170	+	+
10831	3.1	0.9	0.8	–	–	–	300	66	–	150	+	+
IT-1	0.4	1.0	3.1	134	94	–	400	50	–	140	+	+
IT-2	0.3	ND	3.0	138	87	–	400	50	–	140	+	+
MP-7801	0.5	1.0	7.8	138	87	–	300	111	–	180	+	+
MZS-8001	0.4	2.3	4.1	134	94	–	400	50	–	170	+	+
EPOY-8803-II	2.0	0.7	6.9	138	62	–	400	50	–	–	+	+
ATCC 17911	0.3	1.0	0.2	–	–	–	400	50	–	120	+	+
ATCC 29690	1.5	ND	0.3	–	–	–	400	122	–	–	+	+
<i>P. haemolytica</i> CECT 924	0.3	1.3	0.3	–	–	–	–	111	ND	–	–	+
<i>P. multocida</i> CECT 902	0.4	1.7	0.7	123	70	–	–	–	ND	ND	+	+
Controls												
<i>A. hydrophila</i> B-32	0.5	0.5	13.7	192	112	800	200	100	125	70	+	+
<i>V. anguillarum</i> R-82	0.8	3.1	13.6	156	94	1600	–	–	125	100	+	+

ND, Not determined.

* One unit of protease activity produced an *A*₅₂₀ reading of 1.0 after 30 min incubation at 37 °C.

† One unit of caseinase or gelatinase activity was defined as that which gave a zone of clearing equal in area to that produced by 1 µg trypsin; one unit of phospholipase activity was expressed as the reciprocal of the highest dilution of crude ECP producing opaque zones; one unit of elastase was defined as the reciprocal of highest dilution producing elastin degradation.

‡ One unit of haemolytic activity was expressed as that which gave a zone of clearing equal in area to that produced by 0.1% SDS.

Table 4. Enzyme activities displayed by live cells and ECP samples of *Pasteurella* strains

Strain		Enzymic activities*									
		2	3	4	6	10	11	12	13	16	18
<i>P. piscicida</i>											
DI 21	Live cells	+	+	+	+	—	+	+	—	—	+
	ECP	+	—	—	—	—	+	—	—	—	—
DI 71	Live cells	+	+	+	+	—	+	+	—	—	+
	ECP	+	—	—	—	—	+	—	—	—	—
DI 91	Live cells	+	+	+	+	—	+	+	—	—	+
	ECP	+	—	—	—	—	+	—	—	—	—
10831	Live cells	+	+	+	+	—	+	+	—	—	+
	ECP	+	—	—	—	—	+	—	—	—	—
IT-1	Live cells	+	+	+	+	—	+	+	—	+	—
	ECP	+	—	—	+	—	+	—	—	+	—
IT-2	Live cells	+	+	+	+	—	+	+	—	+	—
	ECP	+	—	—	+	—	+	—	—	+	—
MP 7801	Live cells	+	+	+	+	—	+	+	—	—	+
	ECP	+	—	—	—	—	+	—	—	—	—
MZS 8001	Live cells	+	+	+	+	—	+	+	—	—	+
	ECP	+	—	—	—	—	+	—	—	—	—
EPOY 8803-II	Live cells	+	+	+	+	—	+	+	—	+	—
	ECP	+	—	—	—	—	+	—	—	+	—
ATCC 17911	Live cells	+	+	+	+	—	+	+	—	—	—
	ECP	+	—	—	—	—	+	—	—	—	—
ATCC 29690	Live cells	+	+	+	+	—	+	+	—	—	—
	ECP	+	—	—	—	—	+	—	—	—	—
<i>P. haemolytica</i>	Live cells	+	+	+	+	+	+	+	+	—	—
CECT 924	ECP	+	+	+	+	+	+	+	—	—	—
<i>P. multocida</i>	Live cells	+	+	+	+	+	+	+	+	—	—
CECT 902	ECP	+	+	+	+	+	+	+	—	—	—

* Numbers indicate API code: 2, alkaline phosphatase; 3, esterase; 4, esterase-lipase; 6, leucine arylamidase; 10, α -chymotrypsin; 11, acid phosphatase; 12, phosphoamidase; 13, α -galactosidase; 16, α -glucosidase; 18, *N*-acetyl- β -glucosaminidase. +, Positive response; —, negative response. All the strains failed to produce lipase, valine arylamidase, cystine arylamidase, trypsin, β -galactosidase, β -glucuronidase, β -glucosidase, α -mannosidase and α -fucosidase.

Table 5. Toxic activities for fish and mammals of ECP from *Pasteurella* strains

Strain	Toxicity for fish (LD ₅₀)*		Time to death of fish (h)	Toxicity for mice†	Time to death of mice (h)	Dermonecrotic factor‡
	Trout	Turbot				
<i>P. piscicida</i>						
DI 21	+	+	6–48	+	24	+
DI 71	+	+	24–72	ND	ND	+
DI 91	+	+	24–72	+	6–24	+
10831	+	+	48	+	24	+
IT-1	+	+	24–72	+	24–48	+
IT-2	ND	+	24–48	+	24–48	ND
MP-7801	+	+	6–48	+	6–24	+
MZS-8001	+	+	24–48	+	6–24	—
EPOY 8803-II	+	+	6–24	+	6–24	+
ATCC 17911	+	+	72–96	+	24–48	—
ATCC 29690	+	+	6–24	+	24–48	—
<i>P. haemolytica</i> CECT 924	ND	ND	ND	—	NA	+
<i>P. multocida</i> CECT 902	—	—	NA	—	NA	—
Controls						
<i>A. hydrophila</i> B-32	+	ND	6–8	+	6–48	+
<i>V. anguillarum</i> R-82	+	ND	6–8	+	6–48	+

ND, Not determined; NA, not applicable.

* Median lethal dose (LD₅₀) is expressed as μ g protein (g fish)⁻¹ needed to kill 50% of the inoculated fish.

† Number of mice dead/number of inoculated animals.

‡ Oedematous and/or haemorrhagic areas greater than 0.8 cm in diameter were considered as a positive test result. Each sample was tested three times.

always exhibited more activities than their respective ECP. In the majority of ECP samples, esterase, lipase, leucine arylamidase, phosphoamidase and *N*-acetyl- β -glucosaminidase activities were not detected. The live cells and ECP of *P. multocida* and *P. haemolytica* had an identical pattern but different from those exhibited by *P. piscicida* strains. In the ECP of clinical pasteurellas only the α -galactosidase activity was absent (Table 4).

The proteolytic, cytotoxic, haemolytic and phospholipase activities of the ECP samples were all lost after heating at 100 °C for 10 min.

Toxicity of the ECP for fish and mice

The ECP from *P. piscicida* strains, extracted by the cellophane method, were strongly toxic for rainbow trout, causing mortality between 6 and 72 h after i.p. inoculation. In general, the LD₅₀ ranged from 1.7 to 4.6 (for trout) and from 1.0 to 3.0 (for turbot) μ g protein (g fish)⁻¹ (Table 5). Similarly, all the ECP were lethal for mice and the mortalities occurred before 48 h. In contrast, the ECP from clinical pasteurellas were not toxic for fish and mice. The lethal effects were lost after heating the ECP samples at 100 °C for 10 min.

Presence of dermonecrotic factor

In this assay, seven of the ten *P. piscicida* strains tested produced haemorrhagic and/or oedematous zones at the inoculation site. Of the clinical pasteurellas, only the ECP from *P. haemolytica* isolate possessed dermonecrotic activity (Table 5). As with the other toxic and enzymic activities, dermatotoxicity was lost after heating.

Discussion

Hitherto, there have been no reports implicating the ECP of *P. piscicida* in the pathogenesis of the disease caused by this micro-organism. In this study, we examined the biological activities *in vivo* and *in vitro* of live cells and ECP of *P. piscicida* strains with different geographical origins and sources of isolation.

The virulence assays demonstrated that the majority of *P. piscicida* strains were pathogenic for all fish species challenged, with LD₅₀ values ranging between 10³ and 10⁶ cells. It is important to note that, under laboratory conditions, the virulent strains were more pathogenic for turbot and rainbow trout than for seabream. Therefore, although *P. piscicida* did not seem to exhibit host specificity, we cannot rule out the possibility that the two non-virulent strains for the fish species tested (EPOY-8803-II and ATCC 29690) can maintain their pathogenic

capability for their original hosts (see Table 1). These results are in accordance with those obtained by Toranzo *et al.* (1987b) and Santos *et al.* (1991a) for *Vibrio anguillarum* and indicate that pasteurellosis may be a risk for marine fish species in which this disease has not been described. On the other hand, none of the strains can be considered as virulent for mice since they all displayed LD₅₀ values greater than 10⁸ c.f.u.

Regarding ECP, we found that all the *P. piscicida* strains produced notable levels of extracellular proteins and LPS. In addition, all these ECP were lethal for turbot and trout, with LD₅₀ values ranging from 1.0 to 4.6 μ g (g fish)⁻¹. These LD₅₀ values are comparable to those reported for other fish pathogens such as *Aeromonas* (Thune *et al.*, 1982; Ellis & Stapleton, 1988; Santos, 1991; Santos *et al.*, 1988) and *Vibrio* (Kodama *et al.*, 1984; Inamura *et al.*, 1985; Santos, 1991) or *Serratia* species (Baya *et al.*, 1992).

A striking finding was that, although the *P. piscicida* strains were unable to proliferate within the homoiothermic host, all the ECP contained toxins with lethal effects for mice and, therefore, with a strong activity at 37 °C. However, not all the ECP tested possessed dermatotoxic activity when injected into rabbit skin. Therefore, in contrast to other fish pathogens such as *Edwardsiella tarda* (Ullah & Arai, 1983) and *Yersinia ruckeri* (Romalde *et al.*, 1992), dermatotoxins are not the most important virulence factors in *P. piscicida*. Interestingly, although the *P. multocida* strain was highly virulent for mammals (LD₅₀ < 10³ live cells), its ECP had neither lethal effects for mice nor dermatotoxic activity in rabbits. In fact, it was recently reported that toxigenic strains of *P. multocida* produce a toxin (PMT) located in the cytoplasm which is not secreted by living intact cells (Idali *et al.*, 1991).

The proteolytic activity of the ECP from *P. piscicida* was variable among the isolates, with no correlation with the total protein content. Moreover, only the Italian and most of the Japanese strains displayed gelatinase and caseinase activities. The use of the API ZYM system also revealed slight variations among the isolates. In addition, in this system the ECP displayed fewer enzymic activities than the live cells, which suggests that many of these activities were associated with the cell envelope or that some substrates were internalized.

Regardless of the origin of the cell lines (fish or mammals), all the ECP samples from *P. piscicida* showed cytotoxic effects. Therefore, no relationship exists between cytotoxins and the other enzymic activities tested. Similar results have been found in *Vibrio anguillarum* (Toranzo *et al.*, 1983b), *Yersinia ruckeri* (Romalde *et al.*, 1990) and *Renibacterium salmoninarum* (Bandín *et al.*, 1991).

A common characteristic of all the ECP from *P.*

piscicida (but not the other *Pasteurella* strains) was the presence of a thermolabile phospholipase activity. The role of phospholipases as the main extracellular toxin responsible for virulence has been clearly demonstrated in fish pathogens such as *Aeromonas salmonicida* (Lee & Ellis, 1990), which produces a glycerophospholipid cholesterol acyltransferase (GCAT), and in the halophilic bacterium *Vibrio damsela*, which secretes a potent thermolabile cytotoxin (damselysin) with phospholipase D activity (Kreger, 1984; Kreger *et al.*, 1987).

All the biological activities of *P. piscicida* *in vivo* and *in vitro* were lost after heat treatment, which suggests that toxicity for fish and mammals is not solely associated with the LPS content of the ECP.

We have clearly demonstrated that the ECP from *P. piscicida* are involved in the pathogenesis of pasteurellosis when the bacteria have the capacity to successfully invade and proliferate in host tissues. In fact, it was reported in other fish pathogens (Allan & Stevenson, 1981; Kodama *et al.*, 1984; Chabot & Thune, 1991; Ellis, 1991; Santos, 1991) that the biological activities present in the ECP can contribute in the development of the disease in terms of bacterial nutrition or as aggressins enabling the bacteria to counteract the host's defence systems.

In addition, although we have reported that *P. piscicida* constitutes a homogeneous phenotypic, serological and genetical taxon (Toranzo *et al.*, 1991; Magariños *et al.*, 1991, 1992), the present results indicated that the ECP composition differs among strains, which could be important for planning effective vaccination programmes. The results obtained in this work, and the fact that the use of whole cell bacterins is not very effective in the control of pasteurellosis outbreaks (Wakabayashi *et al.*, 1977; Fukuda & Kusuda, 1985; Hamaguchi & Kusuda, 1989), suggest that the formulation of a polyvalent vaccine must include a mixture of whole cells and ECP. We have already demonstrated (Santos *et al.*, 1991b) the efficacy of this type of vaccine in the control of vibriosis.

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