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Pathogenicity of atypical *Aeromonas salmonicida* in Atlantic salmon compared with protease production

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B. GUNNLAUGSDÓTTIR AND B.K. GUDMUNDSDÓTTIR. 1997. The pathogenicity of extracellular products (ECPs) from 24 atypical *Aeromonas salmonicida* strains was studied with respect to: lethality in Atlantic salmon, pathogenic effect in muscle, haemolytic activity, cytotoxicity in two fish cell lines and proteolytic activities. Furthermore, the relationship between lethality of ECPs and mortality caused by bacterial challenge was examined. Correlation was demonstrated between the pathogenic properties and proteolytic activities of the ECPs. Cytolytic (GCAT) activity comparable with that of the typical reference strain used (NCMB 1102) was not detected in ECPs of any of the atypical strain tested. An extracellular metallo-caseinase, AsaP1, was linked with lethal toxicity and a strong pathogenic effect. Furuncular-like lesions were produced by ECPs containing AsaP1 activity. One strain produced a lethal toxin which was neither caseinolytic nor with GCAT comparable activity. The examined atypical strains form at least three distinct groups based on different virulence mechanisms and extracellular proteases.

INTRODUCTION

According to Bergey's Manual of Determinative Bacteriology (1994), the Gram-negative bacterium *Aeromonas salmonicida* is classified into four subspecies: subsp. *salmonicida*, subsp. *achromogenes*, subsp. *masoucida* and subsp. *smithia*. *Aeromonas salmonicida* subsp. *salmonicida* is the causal agent of furunculosis in salmonids and is referred to as the typical strain. Other strains are referred to as atypical strains. These are known to cause ulcerative or generalized diseases in a wide variety of both freshwater and marine fish (Austin and Austin 1993). The classification of these bacteria is a controversial issue, partly because of the growing number of strains which cannot be classified under any of the existing subspecies. Tentatively, these are generally termed atypical strains (Wiklund *et al*. 1994).

Atypical and typical isolates of *Aer. salmonicida* are reported to share cell-associated virulence factors such as the A-layer protein, outer membrane proteins and the LPS component (Chart *et al*. 1984; Evenberg *et al*. 1985; Austin and Austin 1993; Toranzo and Barja 1993; Hirst and Ellis 1994). On the other hand, exotoxins produced by typical and atypical strains are found to differ in nature. Glycerophospholipid : cholestrol acyltransferase (GCAT) has been described as the major lethal exotoxin and cytolysin of typical *Aer. salmonicida*, but a 20 kDa metallo-caseinase (AsaP1) with no cytolytic activity as the major lethal toxin of some atypical strains (Lee and Ellis 1990, 1991; Gudmundsdóttir *et al.* 1990).

It has been demonstrated by several workers that extracellular products (ECPs) of *Aer. salmonicida* can produce symptoms associated with pathogenicity when injected into fish (Munro *et al*. 1980; Ellis *et al*. 1981). Proteases, haemolysins and other cytotoxins are considered to play a major role in the virulence of the bacterium (reviewed by Ellis 1991).

The proteolytic properties of ECPs from a collection of both typical and atypical strains have been studied by Gudmundsdóttir (1996). Significant differences were detected in the extracellular proteases produced by typical and atypical *Aer. salmonicida* strains and within atypical strains. The results indicate that the 32 strains examined formed six protease groups with all the typical strains belonging to one group. The aim of this study was to investigate, in Atlantic salmon, the pathogenicity of the atypical strains examined by Gudmundsdóttir (1996) and compare their proteolytic activity. The following pathogenic properties of ECPs were examined: pathogenic effect in muscle, lethality in Atlantic

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salmon, haemolytic activity to horse and salmon erythrocytes and cytotoxic activity in two fish cell lines. Furthermore, the efficacy of bacterial challenge using strains representative of each protease group was studied.

MATERIALS AND METHODS

Experimental fish

Atlantic salmon (*Salmo salar* L.) fingerlings with an average weight of 40 g were used to estimate pathogenic effect and lethal activity. Prior to injection, the fish was anaesthetized by immersion in benzocaine at a concentration of 40 mg l^{-1} and marked with alcian blue dot spots. The fish were kept in basins with a continuous flow of 15°C fresh water during the experiment. Atlantic salmon (*Salmo salar* L.) weighing approximately 200 g were used as a source of erythrocytes.

Bacterial strains

The strains used in the study are listed in Table 1. All 24 atypical *Aer. salmonicida* strains, together with the reference strains of *Aer. salmonicida* subsp. *achromogenes* (NCMB 1110) and *Aer. salmonicida* subsp. *salmonicida* (NCMB 1102), were used in a previous study by Gudmundsdóttir (1996). The strains were routinely grown on blood agar at 22°C. Stock cultures were stored in brain heart infusion broth (BHI, Oxoid, Basingstoke, UK) containing 15% glycerol at −80°C.

Grouping of the strains into six different protease groups

The 26 strains used in the study were assigned to six different protease groups according to substrate specificity, sensitivity to phenyl methyl sulphonyl fluoride (PMSF) and 1, 10-phenanthroline (OPA) inhibition, and their substrate SDS-PAGE profile detected in their ECPs in a previous study (Gudmundsdóttir 1996). The grouping is described in Table 1. The only difference between protease groups 2 and 3, as well as 5 and 6, was PMSF-sensitive caseinase activity. PMSF-sensitive caseinase activity was always relatively low and therefore, the assignment of strains to protease group 2 or 3 and 5 or 6, respectively, may be a reflection of the sensitivity of the assay method.

Extracellular products (ECPs)

ECPs were produced by the cellophane overlay method described by Gudmundsdóttir (1996) using BHI agar supplemented with 5% new-born calf serum at 22°C. The bacteria and ECPs were washed off the cellophane overlay with minimal amounts of phosphate buffered saline (PBS, pH 7·2) when cells were adequately grown (72–144 h). The protein concentration was measured with the Bio-Rad (Bio-Rad Laboratories Ltd, Bradford, UK) protein assay using bovine serum albumin as a standard. The preparations were aliquotted and stored at −80°C until used.

Analysis of ECPs activities

Caseinase activity. Caseinase activity of ECPs was determined as described by Gudmundsdóttir (1996) using azocasein as a substrate; 50 μl of enzyme sample were incubated with 450 μl of 1% (w/v) solution of azocasein in 0·06 mol l^{-1} phosphate buffer (pH 7·2) at 22°C for 60 min. In reagent blanks, PBS was used instead of enzyme sample. The reaction was stopped by adding 0·5 ml of 10% (w/v) trichloroacetic acid (TCA). After 30 min, the precipitate was pelleted by centrifugation and a 0·5 ml aliquot of the supernatant fluid was added to 0·5 ml of 1 mol l^{-1} NaOH. Released azodye was measured spectrophotometrically at 450 nm (A_{450}) against a reagent blank. The assay was performed in triplicate. One caseinase unit was defined as an increase of 0·001 in A_{450} under the assay conditions.

Gelatinase assay. Gelatinase detection was performed by a radial diffusion method described by Gudmundsdóttir (1996) using 3% gelatin (Difco, Detroit, USA) in 1% agarose gels. Proteolytic activity was determined from a standard curve using trypsin (bovine pancreas type III, Sigma, St Louis, USA). One unit of gelatinase activity was defined as equivalent to that of 1 μg trypsin.

Haemolytic titration. Haemolytic activity of ECPs against horse and salmon red blood cells (RBC) was estimated as previously described (Gudmundsdóttir *et al.* 1990); 100 μl two-fold serial dilutions of ECPs in PBS were added to 100 μl of 1% (v/v) RBC suspension in PBS and incubated for 20 h at 22°C. PBS was used instead of the enzyme solution as a negative control. ECP of the reference strain of *Aer. salmonicida* subsp. *salmonicida* (NCMB 1102), prepared as previously described, was used as a positive control. One unit haemolytic activity (HU) was defined as the dilution causing 50% haemolysis.

Cytotoxic effect. Two cultured cell lines were used to evaluate the cytotoxic effect: Bluegill fry (BF-2) and *Epithelioma papillosum cyprini* (EPC). Confluent cell monolayers were prepared in 96-well, flat-bottomed microtitre plates containing 200 μl growth medium supplemented with 10% foetal bovine serum (300 ml H_2O, 50 ml Tris buffer (pH 7·7), 50 ml Tryptose Phosphate Broth (ICN Biomedicals Inc., Costa Mesa, USA), 40 ml 10 × Glasgow modification of Eagle's medium (ICN Biomedicals Inc.), 10 ml 5% L-glutamine solution, 1 ml of

Table 1 Grouping of *Aeromonas salmonicida* strains after proteolytic activities detected in their extracellular products (ECPs)*

Protease group	Proteolytic activity†	Strain	Host	Country of isolation
1	PMSF-sensitive caseinase (P1) PMSF-sensitive gelatinase (P1) OPA-sensitive gelatinase	NCMB 1102		Type strain
2		NCMB 1110		Type strain
		1777/92	*Anarhichas lupus*	Norway
		2656/92	*Hippoglossus hippoglossus*	Norway
	OPA-sensitive caseinase (AsaP1)	M45/89	*Salvelinus alpinus*	Iceland
	PMSF-sensitive caseinase	S226/90	*Salmo trutta*	Iceland
	OPA-sensitive gelatinase	M283/89	*Salmo salar*	Iceland
		T233/91	*Gadus morhua*	Iceland
		T3-A1	*Melanogrammus aeglefinus*	Iceland
		265-87	*Salmo salar*	Iceland
3	OPA-sensitive caseinase	908/81	*Salmo salar*	Norway
	OPA-sensitive gelatinase	2013/81	*Salmo salar*	Norway
4	PMSF-sensitive caseinase	26F1-24	*Platichthys flesus*	Finland
	PMSF-sensitive gelatinase	921203-2/3	*Platichthys flesus*	Denmark
		920720-2/5	*Scopthalmus maximus*	Denmark
5	PMSF-sensitive caseinase	298/89	*Salvelinus alpinus*	Sweden
	OPA-sensitive gelatinase	420/88	*Salmo trutta*	Sweden
		261/89	*Salmo trutta*	Sweden
6		No. 1	*Salmo trutta*	Finland
		No. 2	*Salmo trutta*	Finland
		No. 3	*Thymallus thymallus*	Finland
	OPA-sensitive gelatinase	1977/88	*Salmo salar*	Norway
		329/89	*Salmo salar*	Sweden
		860613-1/1	*Salmo salar*	Faroe Islands
		850319-1/4	*Ammodytes lancea*	Denmark
		920225-1/2	*Anguilla anguilla*	Denmark

* Gudmundsdóttir 1996; † Sensitive, >10% of the activity resisted treatment; PMSF, Phenyl methyl sulphonyl fluoride; OPA, 1,10-phenanthroline; NCMB, National Collection of Marine Bacteria; NCMB 1102, type strain for *Aer. salmonicida* subsp. *salmonicida*; NCMB 1110, type strain for *Aer. salmonicida* subsp. *achromogenes*.

penicillin (3%)/streptomycin (2·5%) solution and 2 ml of 7% $NaHCO_3$ solution). The reaction system was prepared by removing 100 μl from each well and adding 100 μl enzyme solution containing 35 μg of ECP-protein diluted in PBS, or PBS as a control. The cells were incubated at 15°C and examined microscopically for cytotoxic effects after 2, 25 and 45 h. The cytotoxic effect was graded 0, +, ++ or +++ (Fig. 1)

Pathogenicity and toxicity assays

Pathogenic effect of ECPs. Two fish were injected intramuscularly (i.m.) with 0·2 ml of ECPs, each containing 100 μg protein, or PBS as a control. They were injected in the dorsal musculature, lateral to the dorsal fin. Dead fish were collected at 1, 24, 48 and 72 h post-injection. Survivors were killed 72 h post-injection. Gross pathology was observed around the site of injection and graded 0, +, ++ or +++ according to the severity of the changes (Fig. 2).

50% lethal dose (LD_{50}) of ECPs. Lethal activity of ECPs was determined by intraperitoneal (i.p.) injection with 0.1 ml of ECPs diluted in PBS. Two-fold dilutions were used and six fish were injected with each dilution, or PBS as a control.

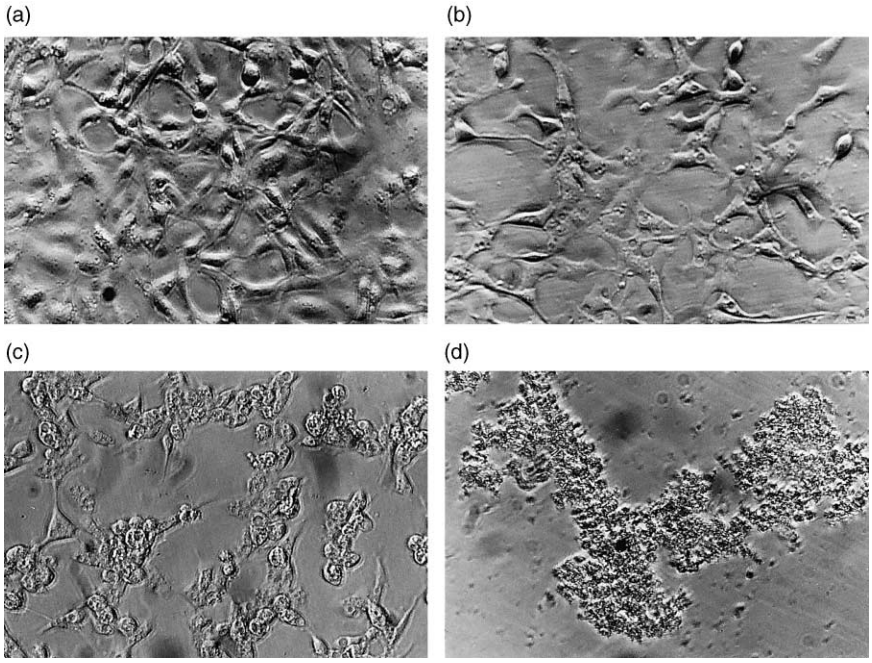

Fig. 1 Grading of cytotoxic effect on Bluegill fry cells (BF-2) incubated with atypical *Aeromonas salmonicida* extracellular products (ECPs) 0, no effect (a); +, notable degeneration and detachment of cells (b); ++, pronounced degeneration and detachment of cells (c); +++, over 25% cell lysis (d)

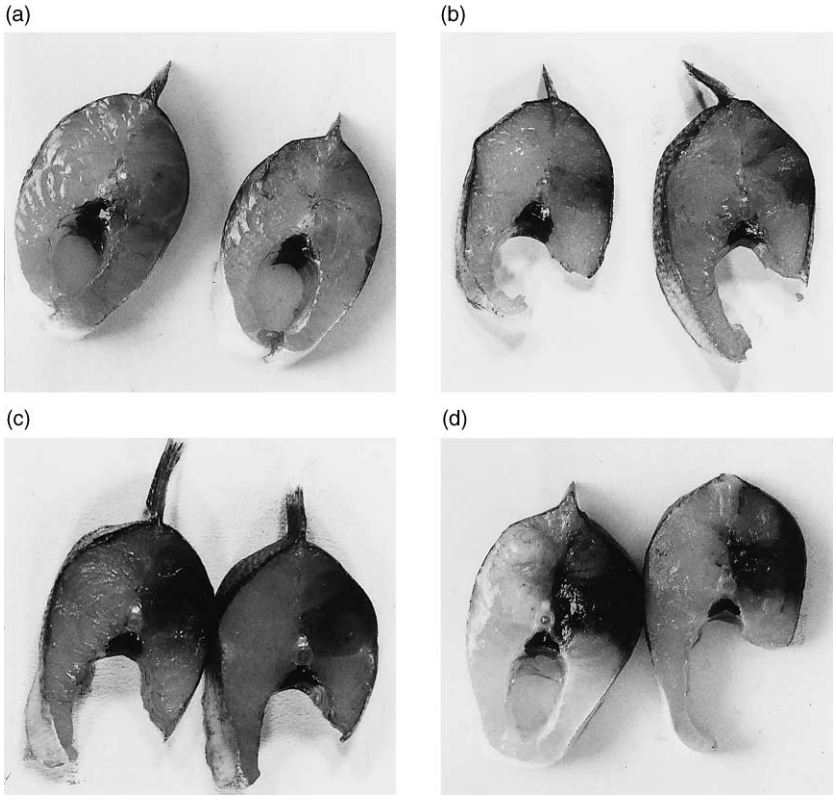

Fig. 2 Grading of pathogenic effect detected in Atlantic salmon (*Salmo salar* L.) after intramuscular injection of atypical *Aeromonas salmonicida* extracellular products (ECPs). 0, a slight bruise caused by the injected volume (a); +, notable bleeding (b); ++, pronounced bleeding (c); +++, extensive bleeding and liquefied necrosis (d)

The survivors were sacrificed 30 d post-injection. The LD_{50} was calculated according to Reed and Muench (1938).

Estimation of LD_{50} for the bacterium in Atlantic salmon. Eight strains were chosen as representatives of the five protease groups containing atypical isolates (Table 1). Before estimating the lethal dose, the strains were passaged twice in fish and reisolated from kidneys on blood agar. Passaged bacteria were grown in 50 ml of BHI supplemented with 5% new-born calf serum at 22°C. After incubation for 16 h, the cells were washed once and diluted with sterile PBS. For challenge, ten-fold dilutions of the bacteria ranging from 10 to 10^9 cfu per fish were used and six fish injected with each dilution. Colony forming units (cfu) were confirmed using the drop plate technique of Miles and Misra (1938). The LD_{50} was calculated according to the method of Reed and Muench (1938). Deaths were recorded daily for 30 d and the cause of death confirmed by reisolating the bacteria from kidney samples on blood agar. Kidney smears from survivors were also sampled and inoculated onto blood agar plates.

RESULTS

Haemolytic activity and cytotoxic effect of ECPs

The results are shown in Table 2. Haemolytic activity was detected neither against horse nor salmon red blood cells (RBC) in any of the ECPs belonging to protease groups two and three. On the other hand, weak haemolytic activity against both horse and salmon RBC was detected in all ECPs belonging to protease group four. The ECPs in protease groups five and six exhibited more diverse properties. When haemolytic activity was detected in protease groups five and six, it was very weak, only 2–14 HU mg^{-1} ECP protein. The highest haemolytic titre against horse erythrocytes (91 HU mg^{-1} ECP protein) was detected in ECP of strain 921203-2/3 in protease group four. None of the atypical strains tested produced haemolytic activity against salmon erythrocytes comparable to that of the positive control strain (2381 HU mg^{-1} ECP protein).

All the ECPs tested possessed some cytotoxic activity. The ECPs of strains in protease group two and three showed the lowest cytotoxic effect and those of protease group four, the highest for the cell lines tested. The only ECP that caused acute lysis was the positive control (*Aer. salmonicida* subsp. *salmonicida* strain NCMB 1102) which lysed the EPC cells completely in only 2 h. In general, the BF-2 cell line was less susceptible to the cytotoxic effect of ECPs than the EPC cell line.

Pathogenic and toxic effects of the ECPs in Atlantic salmon

The results are shown in Table 3. All protease groups contained ECPs, producing a notable pathogenic effect in Atlantic salmon. The pathogenic changes were restricted to the site of injection with the exception of three strains. The fish injected with ECPs of strains no. 2, no. 3 and 329/89 had blood-stained ascites in addition to the changes noted at the site of injection. When the survival time was not the same, stronger pathogenic effects were detected in the fish that survived longer. The most severe pathogenic changes (+++) were detected when ECPs containing AsaP1 activity from strains belonging to protease groups two and three were injected.

All except one of the ECPs containing AsaP1 activity (protease groups two and three) were lethal to the fish, with LD_{50} doses between 7 and 58 μg ECP protein per fish. The amount of caseinase activity in the LD_{50} dose ranged from 1·3 to 4·6 caseinase units per fish. The two ECPs (M45/89 and 265-87) that caused acute death (1 h) after i.m. injection also contained comparatively high amounts of caseinase activity. The non-lethal ECP of strain 2013/81 had a very low caseinase activity compared to the other ECPs in protease groups two and three.

None of the ECPs in protease groups four and five were lethal for the salmon, but pathological changes around the site of injection were always detected in the fish. The ECPs belonging to protease group six caused weak (+) or no pathogenic effect (0). Strain 329/89 was the only strain belonging to protease group six that secreted a lethal ECP. The lethal dose was within the same range of ECP protein concentration as the lethal ECPs of protease groups two and three, but the survival time was longer.

Virulence of bacteria for Atlantic salmon

The results are shown in Table 3. The two strains tested from protease group two (M45/89 and T233/91) were virulent for the salmon with LD_{50} of $7{\cdot}0 \times 10^2$ and $1{\cdot}2 \times 10^4$ cfu per fish, respectively. The representative strain for protease group three, 2013/81, was only lethal when a very high concentration of the bacterium was injected and the LD_{50} dose was $1{\cdot}8 \times 10^8$ cfu per fish. Strain 26F1-24 from protease group four could not be fish-passaged as it was not reisolated from salmon after repeated i.p. injections with $> 10^8$ cfu. Strain 298/89, representing protease group five, was virulent ($LD_{50} = 1{\cdot}1 \times 10^3$ cfu per fish) although it did not secrete lethal toxic products *in vitro*. LD_{50} was calculated for three strains from protease group six. The results show that the only strain of the group producing lethal toxic ECP (329/89) was the one with the highest LD_{50} dose. The other two strains tested (no. 1 and 860613-1/1) were virulent for Atlantic salmon ($LD_{50} = 1{\cdot}4 \times 10^4$ and $5{\cdot}2 \times 10^4$ cfu per fish).

Aer. salmonicida was reisolated from all fish killed in the LD_{50} tests. Furuncular-like lesions or ulcers could not be detected after challenge by i.p. injection. On the other hand, general signs of disease were observed such as petechial haem-

Table 2 Haemolytic and cytotoxic properties of *Aeromonas salmonicida* extracellular products (ECPs)

		Haemolytic activity HU/mg protein		Cytotoxic effect on					
				EPC cells			BF-2 cells		
*Protease group	ECP of strain	H-RBC	S-RBC	2 h	25 h	45 h	2 h	25 h	45 h
1	NCMB 1102	149	2381	+++	+++	+++	++	+++	+++
2	NCMB 1110	0	0	+	++	+++	+	++	+++
	1777/92	0	0	0	++	++	0	+	+
	2656/92	0	0	+	+	+	0	0	0
	M45/89	0	0	0	+	+	0	+	+
	S226/90	0	0	0	+	+	0	0	+
	M283/89	0	0	0	+	+	0	0	+
	T233/91	0	0	+	++	+++	+	++	++
	T3-A1	0	0	0	+	+	0	0	+
	265-87	0	0	0	+	+	0	+	++
3	909/81	0	0	0	+	+	0	0	0
	2013/81	0	0	0	+	+	0	0	0
4	26F1-24	8	8	++	+++	+++	+	+	+
	921203-2/3	91	11	++	+++	+++	++	+++	+++
	920720-2/5	28	14	++	+++	+++	+	++	+++
5	298/89	8	4	+	+++	+++	0	+	+++
	420/88	0	2	0	+++	+++	0	+	++
	261/89	0	0	0	+++	+++	0	+	++
6	No. 1	0	3	0	+++	+++	0	+	+++
	No. 2	0	0	+	++	++	0	+	++
	No. 3	0	0	+	++	++	+	++	+++
	1977/88	0	14	0	++	+++	0	+	+
	329/89	0	14	+	+++	+++	0	+	+
	860613-1/1	0	0	0	++	++	0	++	++
	850319-1/4	0	0	0	0	+	0	+	++
	920225-1/2	11	0	++	+++	+++	+	+++	+++

* Protease group numbers are as presented by Gudmundsdóttir (1996); HU, haemolytic units; H-RBC, horse red blood cells; S-RBC, salmon red blood cells; EPC, *Epithelioma papillosum cyprini*; BF-2, bluegill fry.

orrhages and haemorrhages in the eyes, at the base of the fins and at the vent. In the peritoneal cavity, punctuate or larger haemorrhages were observed and accumulation of ascites was observed in three strains (2013/81, 329/89 and 860613-1/1). *Aer. salmonicida* was not isolated from fish surviving the challenge.

DISCUSSION

In this study, a correlation was demonstrated between the pathogenic properties of ECPs isolated from 24 atypical *Aer. salmonicida* strains and their proteolytic activities. Cytolytic (GCAT) activity against salmon erythrocytes comparable with that of the typical reference strain used (NCMB 1102) was not detected in ECPs of any of the atypical strains tested. An extracellular metallo-caseinase, AsaP1, was linked with lethal toxicity and a strong pathogenic affect. One strain, 329/89, isolated from Atlantic salmon in Sweden, produced a lethal toxin which was neither caseinolytic nor strongly haemolytic; this is a new finding regarding *Aer. salmonicida* exotoxins.

The proteolytic properties of all the strains used have been described by Gudmundsdóttir (1996) in a study where *Aer. salmonicida* strains were divided into six groups (protease groups 1–6) according to proteolytic properties detected in their ECPs. In the same study, all typical strains examined

Table 3 Pathogenic effect and LD_{50} in Atlantic salmon (*Salmo salar* L.) injected with atypical *Aeromonas salmonicida* extracellular products (ECPs) or infected with the bacterium

Protease* group no.	ECP of strain	Proteolytic units injected		Pathogenic effect ($n = 2$)	Survival time (h)	LD_{50}, ECP μg protein	LD_{50}, ECP caseinase units	LD_{50}, bacterium cfu
		Caseinase	Gelatinase					
2	NCMB 1110	18	15	+++/+++	24/24	17	3·0	ND
	1777/92	5	23	+++/+++	48/NL	58	2·7	ND
	2656/92	19	23	+++/+++	24/24	7	1·3	ND
	M45/89	35	38	+/++	1/24	12	4·1	$0{\cdot}7 \times 10^2$
	S226/90	20	18	+++/+++	24/24	14	2·8	ND
	M283/89	22	8	+++/+++	24/24	15	3·3	ND
	T233/91	21	22	+++/+++	24/24	22	4·6	$1{\cdot}2 \times 10^4$
	T3-A1	16	18	++/++	24/24	14	2·2	ND
	265-87	33	23	+/++	1/24	7	2·3	ND
3	909/81	10	12	+/+++	24/NL	17	1·7	ND
	2013/81	2	21	++/++	NL	NL	-	$1{\cdot}8 \times 10^8$
4	26F1-24	27	13	+/+	NL	NL	-	NL
	921203-2/3	30	27	++/++	NL	NL	-	ND
	920720-2/5	2	16	+/+	NL	NL	-	ND
5	298/89	3	8	++/++	NL	NL	-	$1{\cdot}1 \times 10^3$
	420/88	2	6	++/++	NL	NL	-	ND
	261/89	8	7	++/+	NL	NL	-	ND
6	No. 1	0	12	+/+	NL	NL	-	$1{\cdot}4 \times 10^4$
	No. 2	0	16	+/+	NL	NL	-	ND
	No. 3	0	14	+/+	NL	NL	-	ND
	1977/88	0	33	0/0	NL	NL	-	ND
	329/89	0	6	+/+	72/72	12	0	$2{\cdot}1 \times 10^7$
	860613-1/1	0	5	0/0	NL	NL	-	$5{\cdot}2 \times 10^4$
	850319-1/4	0	4	0/0	NL	NL	-	ND
	920225-1/2	0	4	+/+	NL	NL	-	ND

* Protease group numbers are as presented by Gudmundsdóttir (1996); cfu, colony forming units; LD_{50}, 50% lethal dose; NL, not lethal; ND, not determined.

belonged to a separate group (protease group 1) according to these criteria. In the present study, pathogenic properties of 24 atypical *Aer. salmonicida* strains, and the type strain of *Aer. salmonicida* subsp. *achromogenes* (NCMB 1110), were examined by measuring haemolytic activity against horse and salmon erythrocytes, cytotoxicity in two fish cell lines, pathogenic effects in muscle tissue and lethality in Atlantic salmon. Furthermore, mortality caused by bacterial challenge was examined for strains representative of each of the five protease groups.

Most of the caseinase activity in ECPs of strains in protease groups 2 and 3 is caused by the lethal toxic metallo-protease, AsaP1. Strains in other protease groups were not found to produce this enzyme (Gudmundsdóttir 1996). In this study, the presence of AsaP1 in ECPs correlated with the lack of haemolysin, low cytotoxic effect, severe pathogenic effect and lethality (Tables 2 and 3). The quantity of caseinase activity injected was associated with the survival time of the fish (Table 3). The only non-lethal ECP with AsaP1 activity (2013/81) possessed very low caseinase activity, and ECPs that caused the most acute death (M45/89, 265-87) also contained the highest caseinase activity injected (Table 3). Although the LD_{50} dose of ECP proteins injected differs substantially, the caseinase activity in the LD_{50} dose differs far less, indicating that the main lethal factor is the caseinase. This confirms earlier data describing AsaP1 as a major lethal toxin of an *Aer. salmonicida* subsp. *achromogenes* strain (Gudmundsdóttir *et al.* 1990), but the exact mode of the lethal action still remains to be elucidated. The relationship between the presence of AsaP1 and the lethal and pathogenic effect is

clear and consistent, although the presence of undetected potential pathogenic factors in the ECPs cannot be excluded. Good correlation was also found between lethal toxicity of ECPs of strains from protease groups 2 and 3 produced *in vitro* and mortality caused by bacterial challenge.

The furuncular-like lesions caused by typical *Aer. salmonicida* strains can be produced by i.m. injection of ECPs (Ellis *et al.* 1981). It has been demonstrated that the P1 protease (70 kDa serine protease) alone can produce muscle liquefaction and haemorrhage when injected i.m., and furthermore, that the lesions are more extensive when a haemolytic factor (lysing fish erythrocytes) is included (Fyfe *et al.* 1989, 1996; Lee and Ellis 1989, 1991). The haemolytic factor (GCAT) appears to be the major lethal toxin of *Aer. salmonicida* subsp. *salmonicida* (Lee and Ellis 1990, 1991; Huntly *et al.* 1992; Eggset *et al.* 1994). In the present study, the muscle liquefaction and massive bleeding caused by i.m. injection of ECPs with AsaP1 activity, resemble the furuncular-like lesions that are linked with the disease caused by typical *Aer. salmonicida* strains. However, as neither P1 protease nor GCAT activity is present in those ECPs, the lesions are produced in a different way.

In the ECPs of protease group 4, the presence of a PMSF-sensitive protease, comparable to P1 of typical strains, is connected with the presence of haemolysin, a strong cytotoxic effect, a notable to pronounced pathogenic effect and lack of lethality (Tables 2 and 3). Still, the haemolytic activity detected is very low compared to the positive control (the type strain of *Aer. salmonicida* subsp. *salmonicida*, NCMB 1102), especially against fish erythrocytes (below 15 and 2381 HU/mg protein, respectively, Table 2). Although these ECPs contain P1 comparable proteolytic activity, tissue liquefaction could not be detected in fish muscle (Table 3). This contradicts the perviously mentioned results of Fyfe *et al.* (1988) and Lee and Ellis (1991), indicating that there is either qualitatively or quantitatively a difference between the enzymes produced by the strains in protease group 4 and typical *Aer. salmonicida* strains. The strains in protease group 4 were the only cytochrome oxidase-negative isolates used in this study and they all originated from wild flatfish (Table 1). In spite of a relatively high content of protease activity, their ECPs were not lethal for the fish (Table 3). The representing strain from protease group 4 (26F1-24) was also avirulent in Atlantic salmon. That these strains can be distinguished from the other strains both according to protease profiles (Gudmundsdóttir 1996) and pathological properties observed in this study, supports results presented by Wiklund *et al.* (1994) and Wiklund (1995), suggesting that cytochrome oxidase-negative variants isolated from flounder are not virulent in salmonids and may form a new subspecies of *Aer. salmonicida*.

The presence of an OPA-sensitive gelatinase activity in ECPs of protease group 5 and 6 cannot be connected with any of the examined pathogenic traits (Tables 2 and 3). The ECP with the highest gelatinase activity (1977/88) did not produce any detectable pathogenic effect in the fish, while the one (920225-1/2) with the lowest, did. Furthermore, neither haemolytic nor cytotoxic activity showed a correlation with gelatinase activity. The strains chosen as representative of protease groups 5 and 6 for bacterial challenge caused mortality of the salmon but unexpectedly, strain 329/89 producing a lethal toxic ECP was the least virulent of the strains tested. This cannot be explained by different host specificity as the strain was originally isolated from Atlantic salmon. An alternative explanation could be that the doses of ECPs injected were artificially high compared with those released by the bacterium growing in the host.

The lethal ECP produced by strain 329/89 did not contain caseinase activity and the gelatinolytic activity detected was very low. Furthermore, only a low haemolytic titre for salmon red blood cells (S-RBC) was detected and the cytotoxic effect was no stronger than in other ECPs of protease groups 5 and 6. This indicates that strain 329/89 secretes a lethal toxic factor that has neither a detectable caseinolytic, nor strong haemolytic activity; this is a new finding regarding extracellular lethal toxins of *Aer. salmonicida*. In order to identify the nature of this new toxin, further studies are needed. Exotoxins that lack haemolytic or proteolytic activities have been reported to be virulence factors of other related Gram-negative fish pathogenic bacteria. An extracellular acetylcholinesterase has been identified as an exotoxin of some fish pathogenic *Aer. hydrophila* strains (Nieto *et al.* 1991). The toxic acetylcholinesterase was not proteolytic and it was not cytotoxic against a salmon embryo cell line. When the toxin was injected into trout, no gross pathology was produced, but the behaviour of the fish suggested that it might be acting on the central nervous system. Furthermore, undetermined extracellular toxic substances with neither haemolytic nor proteolytic activities have been reported to be virulence factors of *Vibrio anguillarum* (Toranzo and Barja 1993).

As GCAT is known to be extremely haemolytic for salmonid erythrocytes (Lee and Ellis 1990; Huntly *et al.* 1992; Eggset *et al.* 1994), the results presented indicate that it may be absent from the ECPs of the type strain for *Aer. salmonicida* subsp. *achromogenes*, NCMB 1110, and all the 24 atypical strains tested in this study. The low haemolytic activity recorded could be the result of membrane-damaging factors other than specific haemolysins.

The observed difference in cytotoxic effect of the ECPs is not very distinct but there appears to be a relationship between detectable haemolytic titres and high cytotoxicity, as in protease group four. Lowest cytotoxic effect was observed in protease groups two and three where haemolytic activity was not detected. Acute lysis was only caused by ECP of the positive control strain (NCMB 1102) which also contained high haemolytic activity. Cell lysis was, however, observed in the absence of haemolytic activity. This dem-

onstrates that at least two different factors are responsible for the membrane-damaging activities detected in the ECPs (Table 2). Many phospholipases show membrane-damaging activities. A phospholipase C activity, which differs from the phospholipase A activity associated with the GCAT enzyme, has been detected in *Aer. salmonicida* ECP, which was not found to correspond with haemolytic activity (Campbell *et al.* 1990).

The poor correlation between lethality of ECPs and virulence of live bacteria in protease groups five and six (Table 3) reveals the complex nature of the virulence mechanisms of *Aer. salmonicida*. It is possible that cell-associated, rather than extracellular factors are of major importance for the invasiveness of these strains. Moreover, virulence factors which are not secreted *in vitro* could be produced *in vivo* or vice versa, as bacterial virulence factors can be expressed as a sequential response to the environment (Di Rita and Mekalanos 1989).

Two strains that were primarily isolated from non-salmonid fish were used in the LD_{50} study. The one belonging to protease group two, which was isolated from cod, was virulent for salmon whereas the one belonging to group four was not. This indicates that at least the strains in protease group two are not species specific, although those of group four probably are. However, the bacteria are known to produce different diseases in different hosts, as has been demonstrated in cod (*Gadus morhua* L.) and Atlantic salmon by Morrison *et al.* (1984).

In the present study, all strains were grown to stationary phase before the ECPs were harvested. This was done to minimize growth phase affects, as the growth rate of the isolates was quite different. Measures were taken to avoid the effect of storage. Thus, all procedures were performed using ECPs produced and stored under identical conditions.

In order to retrieve virulence possibly lost during repeated sub-cultivation on agar slants, the bacterial strains chosen for the challenge experiments were passaged twice prior to the estimation of LD_{50}.

According to the results of this study, the atypical strains examined form at least three distinct groups based on different virulence mechanisms. Pathogenic properties of strains in protease groups two and three were comparable. The strains in protease group four, which were distinct from the others by membrane-damaging effects and virulence for Atlantic salmon, form a distinct group. The strains belonging to protease groups five and six form the third group ; the only difference observed between pathogenic properties of these strains was that the ECPs of group five strains produced pronounced pathogenic effect (++) in muscle tissue, whereas those of group six were notable (+) or zero (0). This is in good accordance with the previous subdivision of these strains into five protease groups, where the division between protease groups two and three and five and six, respectively, was based only on the detection of a PMSF-sensitive caseinase in amounts close to the assay limits (Gudmundsdóttir 1996).

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