

Toxic factors of *Vibrio* strains pathogenic to shrimp

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ABSTRACT: Vibriosis is a major disease problem in shrimp aquaculture. 'Syndrome 93' is a seasonal juvenile vibriosis caused by *Vibrio penaeicida* which affects *Litopenaeus stylirostris* in grow-out ponds in New Caledonia. This study assessed the toxic activities of extracellular products (ECPs) from *V. penaeicida*, *V. alginolyticus* and *V. nigripulchritudo* using *in vivo* injections in healthy juvenile *L. stylirostris* (= *Penaeus stylirostris*) and *in vitro* assays on shrimp primary cell cultures and the fish cell line epithelioma papulosum cyprini (EPC). Toxic effects of ECPs were demonstrated for all pathogenic *Vibrio* strains tested both *in vivo* and *in vitro*, but for shrimp only; no effect was observed on the fish cell line. ECP toxicity for New Caledonian *V. penaeicida* was found only after cultivation at low temperature (20°C) and not at higher temperature (30°C). This points to the fact that 'Syndrome 93' episodes are triggered by temperature drops. The assays used here demonstrate the usefulness of primary shrimp cell cultures to study virulence mechanisms of shrimp pathogenic bacteria.

KEY WORDS: *Vibrio* · *Vibrio penaeicida* · Shrimp · Extracellular products · Temperature

INTRODUCTION

Vibriosis is a major disease problem in shrimp aquaculture (Lightner 1988, Brock & LeaMaster 1992, Mohny et al. 1994). 'Syndrome 93' is a seasonal vibriosis caused by *Vibrio penaeicida* which affects juvenile *Litopenaeus stylirostris* (= *Penaeus stylirostris*) in grow-out ponds in New Caledonia. Little is known about virulence factors of *Vibrio* strains pathogenic to shrimp, despite extensive studies on other *Vibrio* species, including species pathogenic to humans and some species pathogenic to fish or shellfish (Brown & Roland 1984, Nottage & Birkbeck 1986, 1987, Fouz et al. 1994, Pedersen et al. 1997). As exotoxins often play a major role in the pathogenesis of bacterial diseases (Finlay & Falkow 1997), this study was aimed at assessing the toxic activities of extracellular products (ECPs) of *V. alginolyticus*, *V. penaeicida* and *V. nigripulchritudo* strains pathogenic to shrimp, using *in vivo* injections

in healthy juvenile *L. stylirostris*, and *in vitro* assays on shrimp primary cell cultures and the fish cell line epithelioma papulosum cyprini (EPC).

MATERIAL AND METHODS

Bacterial strains, culture conditions and preparation of toxic extracts. *Vibrio alginolyticus* strain SF 5, *V. penaeicida* strain AM 101 and *V. nigripulchritudo* strain AM 102 (Costa et al. 1998) were used in this study. Each strain was cultured in brain heart infusion (Bio Mérieux, France) because this rich medium has been shown to promote the expression of exotoxins in some *Vibrio* strains (Nishibuchi & Seidler 1983). Salinity of the culture medium was adjusted to 25 ppt by the addition of artificial seawater salt (Instant Ocean®). Cultures were carried out in 8 ml of medium placed in sloped 25 ml screw-capped culture tubes for 48 h at different temperatures (usually 25°C, see below) under continuous shaking (preliminary trials showed that 2 d old cultures had the most toxic supernatant fluids). The

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ECPs were prepared by filtration through 0.22 µm pore size filters (Sartorius) of culture supernatants after centrifugation at $5000 \times g$ for 10 min at 4°C, according to Newman & Feng (1982). Control preparations were prepared using the same protocol with sterile culture medium. Sterility of all preparations was checked by spreading 100 µl of each preparation onto marine agar (2216 E, Difco) petri dishes and incubating this for 48 h at $30 \pm 0.5^\circ\text{C}$.

Effect of temperature on *Vibrio penaeicida* ECPs. Sudden temperature drops are known to trigger Syndrome 93 episodes in New Caledonia. Therefore the toxicity of ECPs from New Caledonian *V. penaeicida* AM 101 was evaluated for cultures incubated at high ($30 \pm 0.5^\circ\text{C}$) and low ($20 \pm 1^\circ\text{C}$) temperatures. ECPs from *V. penaeicida* AM 101 cultured for 48 h at 20 and 30°C , and 22 h at 30°C were prepared as described above.

Enzymatic activities associated with ECPs. Enzymatic activities of ECPs were evaluated using API-zym test kits (Bio Mérieux, France). Each strip was filled with 65 µl of the ECP to be tested. The strip was then incubated for 4.5 h at 30°C . Readings were performed according to the manufacturer's instructions.

Cell lines. The fish cell line EPC, originating from carp epidermal herpes virus-induced hyperplastic lesions, was provided by CNEVA (Laboratoire de Pathologie des Animaux aquatiques, Brest, France). Shrimp cells were prepared from 15 juvenile (10 to 15 g) female penaeid shrimp *Litopenaeus stylirostris* supplied by IFREMER-COP (Tahiti, French Polynesia).

The culture media consisted of (1) fish cells: Eagle's minimal essential medium (MEM; Sigma) buffered by 0.16 M Tris-HCl, pH 7.4 and supplemented with 10% foetal bovine serum (FBS) plus 200 i.u. penicillin and 200 µg streptomycin ml^{-1} ; (2) shrimp cells: M 199 Medium (Sigma). All media (1×) in powder were reconstituted with 0.1 M NaCl and 0.01 M Tris buffer and pH was adjusted to 8.0 with HCl. The routine holding media (HM) consisted of 1 × M 199 medium with 1% of antibiotic-antimycotic solution (Sigma) containing 10^4 units ml^{-1} penicillin, 10 mg ml^{-1} streptomycin, 25 µg ml^{-1} amphotericin and supplemented with 10% FBS (Sigma). All heat-stable salt solutions were sterilized by autoclaving for 30 min at 121°C . Heat-labile media were filter sterilized using sterile 0.22 µm pore size filters (OSI, France).

Cell isolation and primary cultures. Primary cell cultures of ovary cells and haemocytes from *Litopenaeus stylirostris* were obtained as previously described (Le Groumellec et al. 1995). Briefly, shrimp were anesthetized by being dipped in cold 5% hypochloride solution for 5 min. The haemolymph (100 to 500 µl) was withdrawn from the ventral sinus using a 1 ml syringe, 26 gauge needle, containing an equal volume of anti-

coagulant solution (30 mM sodium citrate, 100 mM glucose, 350 mM NaCl, and 10 mM EDTA; pH 7). Ovaries were dissected out, washed twice in sterile medium (1×) with antibiotics (4 or 10×) during 1 h and then minced into pieces less than 1 mm in diameter with Pascheff scissors. Ovary fragments were directly transferred to 35 × 10 mm culture dishes (Nunclon Delta) containing 1.5 ml HM. Cultures were incubated at $26 \pm 1^\circ\text{C}$ and observed with a Leitz DMIL inverted microscope. Cell viability was determined using the trypan blue (1%) dye exclusion test.

In vitro assays for detection of toxic activities. The effect of the ECPs of the different *Vibrio* strains was assayed *in vitro* on shrimp ovarian and haemocyte primary cell cultures and EPC cell line cultures. Before determining the cytopathic effects of ECPs to primary cell cultures, the culture medium was replaced with fresh HM supplemented with antibiotics and FBS. Either an ECP preparation or control medium (5 µl) was then added to the cell culture dishes (1.5 ml). After 20 h of incubation for shrimp cells and 48 h for EPC cells, the cultures were fixed and stained using Giemsa's stain (30 s). The preparations were then observed for cytopathic effects using normal light microscopy. Each test was performed with 3 replicates.

In vivo assays for detection of toxic activities. Healthy juvenile *Litopenaeus stylirostris* (10 to 20 g mean weight) originating from the experimental Station d'Aquaculture de Saint Vincent (IFREMER, New Caledonia) were injected intramuscularly with 50 µl of either an ECP preparation or control medium. Between 5 and 7 injected shrimp were placed in 30 l aquaria filled with filtered seawater ($25 \pm 2^\circ\text{C}$; 35 ± 2 ppt salinity). Each experiment was conducted in at least 3 aquaria (i.e. 15 to 21 shrimp). Mortality was recorded over a 3 d period. Total haemocyte counts (THC) were performed as described by Le Moullac et al. (1997) on surviving C-stage animals at the end of this 3 d period. When mortality was excessive, a similar assessment of THC was conducted after a shorter period post injection.

Results of both mortality and THC measurements were analysed using StatView® software.

RESULTS

In vivo assays

With *Vibrio* strains cultured at 25°C

Survivals of ECP-injected shrimp are presented in Fig. 1. The ECPs from *Vibrio nigripulchritudo* AM 102 was lethal to *Litopenaeus stylirostris*: all injected shrimp died within a 24 h period following injection ($\chi^2 = 56.46$; $p < 0.001$). Lethal toxicity was not demonstrated *in vivo*

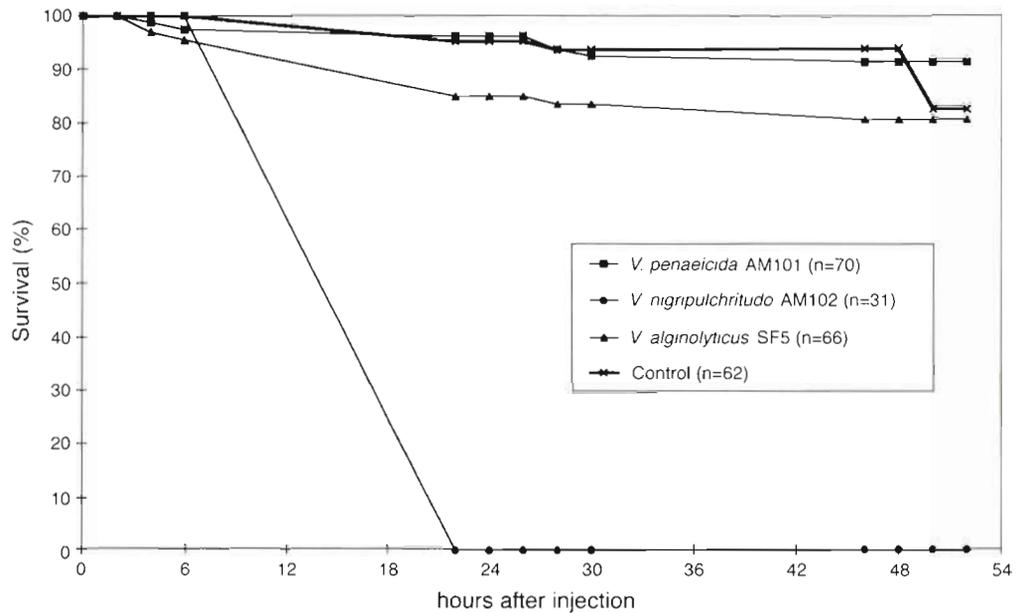


Fig. 1. Survival of juvenile *Litopenaeus stylirostris* injected with extracellular products (ECPs) from *Vibrio* strains cultured at 25°C. Values in the parentheses indicate the number of shrimp for each preparation

for *V. penaeicida* AM 101 ($\chi^2 = 3.37$; $p = 0.12$) and *V. alginolyticus* SF 5 ($\chi^2 = 0.08$; $p = 0.78$) cultured at 25°C. Total haemocyte counts did not demonstrate any toxic activity for *V. penaeicida* AM 101 and *V. alginolyticus* SF 5 supernatants ($F = 0.10$; $p = 0.91$). The THC from control and *V. nigripulchritudo* ECP-injected animals are presented in Fig. 2. A significant decrease in THC was observed in *V. nigripulchritudo* ECP-injected shrimp 3 h after injection ($t = 5.14$; $p < 0.01$). At the end of the experiment, the surviving shrimp showed a

3-fold decrease in THC compared to control shrimp ($t = 3.52$; $p < 0.01$). This result suggested the possible presence of haemocytolytic activity in the ECPs from *V. nigripulchritudo* AM 102.

With *Vibrio penaeicida* cultured at 20 or 30°C

The effect of culture temperature on *Vibrio penaeicida* AM 101 ECP toxicity is shown in Table 1. Cultures

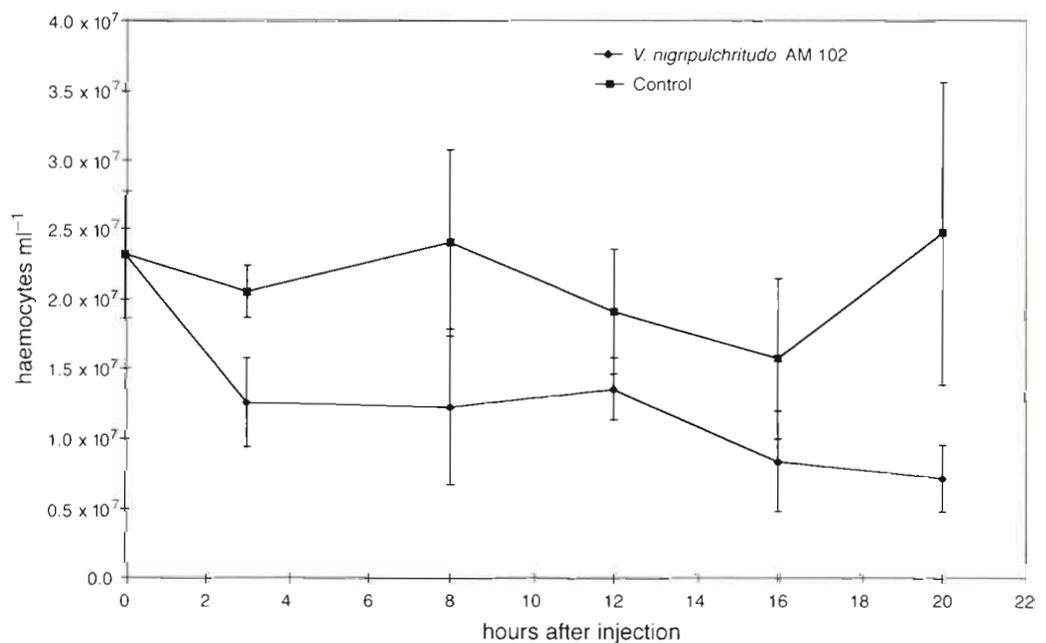


Fig. 2. Total haemocyte counts of different juvenile *Litopenaeus stylirostris* following injection with either ECPs from cultured *Vibrio nigripulchritudo* AM 102 or control medium. Each value corresponds to the average THC from 5 to 10 C-stage shrimp. Bars: standard deviation

Table 1. Survival of *Litopenaeus stylirostris* injected with ECPs from *Vibrio penaeicida* AM 101 cultured at different temperatures

Culture temperature (°C)	Initial cell concentration (CFU ml ⁻¹) (culture duration [h])	No. of injected shrimp (no. of aquaria)	Survival of ECP-injected shrimp (%)
30	10 ^{8.2} (48 h)	42 (6)	95
30	10 ^{7.9} (22 h)	42 (6)	100
20	10 ^{7.9} (48 h)	42 (6)	0 ^a
Control	0	21 (6)	95

^aMedian time to death: 12.5 h

Table 2. Cytopathological effects on cell cultures incubated with ECPs of shrimp-pathogenic *Vibrio* strains that were cultured at 25°C (Giemsa's stain). EPC: epithelioma papulosum cyprini

ECP origin	Fish cell line EPC	<i>Litopenaeus stylirostris</i> ovary cells	<i>Litopenaeus stylirostris</i> haemocytes
<i>V. penaeicida</i> AM 101	Normal	Altered cell structure, naked nuclei, karyorrhexis	Obliteration of cellular boundaries, karyorrhexis
<i>V. nigripulchritudo</i> AM 102	Normal	Fragmented cytoplasm, karyorrhexis and marginated chromatin	Naked nuclei and fragmented cytoplasm, obliteration of cellular boundaries, flocculated cytoplasm
<i>V. alginolyticus</i> SF 5	Normal	Cells surrounded by lipid drops	Fragmented cytoplasm and marginated chromatin
Control (no ECPs)	Normal	Normal	Normal

incubated at 30°C grew faster, but their ECPs (either same culture duration or same initial cell density) showed no lethal activity when injected into healthy juvenile shrimp, whereas 100% mortality was induced by injecting shrimp with ECPs from cultures of *V. penaeicida* AM 101 incubated at 20°C. The toxic factor was demonstrated to be heat-labile because heating the ECPs (80°C; 10 min) considerably reduced its lethality. However, when another clone of this same strain, *V. penaeicida* AM 101, was cultured at 20 or 30°C, low mortalities were obtained (Ph. Haffner pers. comm.).

Enzymatic activities associated with ECPs

All tested ECPs showed strong to moderate alkaline phosphatase, esterase, esterase lipase, phosphohydro-lase, N-acetyl-β glucosaminidase and acid phosphatase activities. *Vibrio penaeicida* ECPs showed light trypsin activity. No enzymatic pattern of ECPs could be linked to the toxic effects. Furthermore, these patterns were unaffected by culture temperatures.

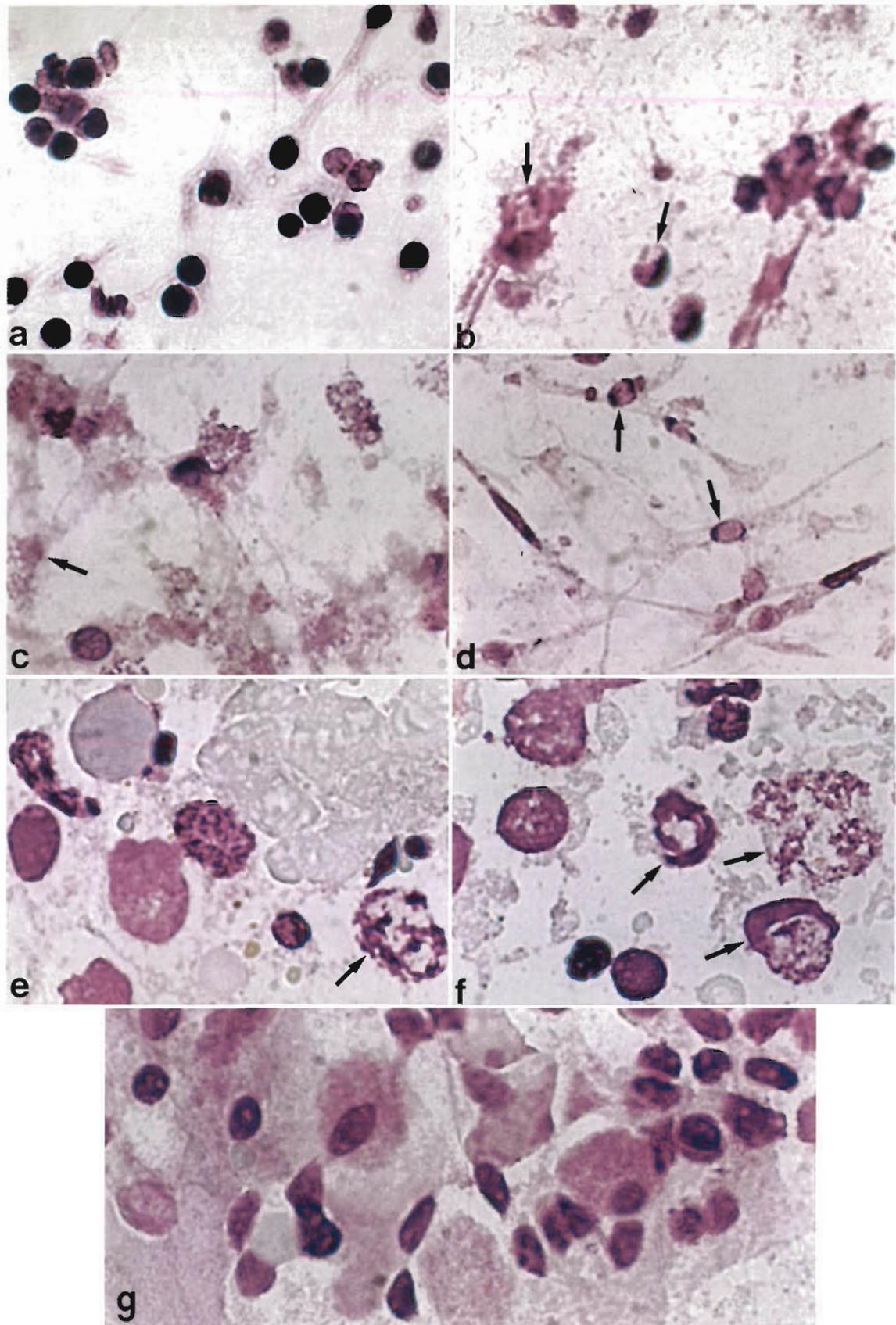
In vitro assays

None of the ECPs of *Vibrio* strains pathogenic to shrimp were cytotoxic for the fish cell line EPC. By contrast, cytopathic effects caused by ECPs from *Vibrio* strains pathogenic to shrimp were observed in cell monolayers derived from haemocytes and ovarian tissue (Fig. 3). All ECPs induced cytopathic effects in haemocyte primary cell cultures. Obliteration of cellular boundaries, karyorrhectic nuclei and marginated chromatin were noticed after Giemsa staining (Fig. 3, Table 2). In primary cultures of ovarian cells, the most significant cytopathic effects were induced by ECPs from *V. penaeicida* AM 101 and *V. nigripulchritudo* AM 102; ECPs from *V. alginolyticus* SF 5 induced much less of a cytopathic effect in these cultures (Fig. 3, Table 2).

DISCUSSION

Toxicity of ECPs was demonstrated for shrimp-pathogenic *Vibrio* strains by *in vitro* assays. The inevitable

Fig. 3. *In vitro* assays for detection of toxic activities with ECPs of shrimp-pathogenic *Vibrio* strains cultured at 25°C; cell sheets derived from haemocytes and ovarian tissue of *Litopenaeus stylirostris* (×800; Giemsa staining). Arrows show nuclei in the process of karyolysis. (a) Control haemocytes ('exotoxin control'); (b–d) haemocytes with ECP of (b) *V. penaeicida* (AM 101), (c) haemocytes with ECP of *V. nigripulchritudo* (AM 102), and (d) *V. alginolyticus* (SF 5); (e) ovarian cells with ECP of *V. penaeicida* (AM 101) and (f) *V. nigripulchritudo* (AM 102); (g) control ovarian cells ('exotoxin control')



contamination of the ECPs by some lipopolysaccharide (LPS) might be responsible for some of the alterations observed in haemocyte primary cell cultures, possibly via activation of the prophenoloxidase system (Söderhäll & Häll 1984). On the other hand, the cytopathic effects observed in ovarian cells were most probably due to the toxic effect of other components of the ECPs, even though some haemocytes were inevitably present in these cultures. Using *in vivo* assays, ECP toxicity was demonstrated only for the very pathogenic *V. nigripulchritudo* AM 102 and for *V. penaeicida* AM 101 cultured at cooler temperatures (20°C). Their ECPs were lethal for shrimp. The toxic factor present in *V. penaeicida* AM 101 was demonstrated to be heat-labile and might correspond to an exotoxin since LPSs are relatively heat-stable. Haemolysins have been demonstrated in various *Vibrio* strains (Rodrigues et al. 1993), including strains pathogenic to fish (Toranzo et al. 1983) and shellfish (Nottage & Birkbeck 1987), and have frequently been correlated with virulence (Kreger 1984, Fouz et al. 1993, Chakraborty et al. 1997, Fiore et al. 1997). Proteases have also been implicated as virulence factors in various *Vibrio* strains pathogenic to fish (Norqvist et al. 1990) or shrimp (Lee et al. 1997, Liu & Lee 1999). The toxic activities we describe here seem to be specific, as no cytopathic effect of *Vibrio* strains pathogenic to shrimp was noted with the fish cell line tested. These *in vitro* assays demonstrate the usefulness of primary cell cultures from penaeid shrimp in the study of virulence mechanisms and especially the cytotoxic factors of shrimp bacterial pathogens. Thus, *in vitro* assays appear more sensitive than *in vivo* assays. A wide range of enzymatic activities were demonstrated in the ECPs but it was not possible to link one specific enzymatic pattern with the toxic activity. Though most of these exoenzymes are considered as putative virulence factors by some authors (Borrego et al. 1996), their presence in culture supernatants could not be related to virulence in the present study.

The excretion of exotoxins has been demonstrated to be phase-dependent in various bacterial species or isolates, including *Pseudomonas aeruginosa* (Liu 1957) and *Vibrio vulnificus* (Kreger & Lockwood 1981). We also found that ECP toxicity for some *V. penaeicida* clones was phase- or temperature-dependent. Temperature is often associated with regulation of virulence factors in bacterial pathogens (Finlay & Falkow 1997). However, virulence often increases when the temperature increases. In this study, ECP virulence was observed when the temperature decreased. The physiological significance of this phenomenon remains to be studied, but could correspond to an adaptive response to cold temperatures, as demonstrated in *V. vulnificus* by Bryan et al. (1999). It was not possible here to determine whether *V. penaeicida* AM 101 ECP

toxic factors were not produced at higher temperatures or whether they were simply degraded in the culture broth by an exoenzyme excreted (or active) at higher temperatures. However the occurrence of ECP toxicity in cultures incubated at low temperatures seems to correlate with the occurrence of Syndrome 93 episodes after temperature drops.

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