

## Virulence factors of *Vibrio* P1, the causative agent of brown ring disease in the Manila clam, *Ruditapes philippinarum*

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### Abstract

Several virulence factors involved in the potential pathogenic capacity of *Vibrio* P1, the causative agent of brown ring disease (BRD) affecting cultured Manila clam adults (*Ruditapes philippinarum*), have been evaluated in comparison with other strains of several *Vibrio* species isolated from diseased clams. The importance of bacterial cell surface associated properties as virulence factors has been studied considering both non-specific and specific bacterial adhesion to clams. *Vibrio* P1 showed moderate hydrophobicity, but high affinity to bind to Congo Red dye and the presence of appendages, characterized as fimbriae or pili. All the strains of *Vibrio* P1 secreted haemolysis and cytotoxins, and were also strong exotoxin producers. The presence of a large 49.2-MDa plasmid in all the strains of *Vibrio* P1 may be used as an epidemiological marker, but its involvement in pathogenic mechanisms has not yet been established. Although in some *Vibrio* strains, iron-acquisition systems play an essential role in their pathogenicity, they do not seem to be an important factor in *Vibrio* P1, since this pathogen lacks siderophore-mediated iron transport mechanisms.

**Keywords:** *Vibrio*, virulence factors, Pathogenic bacteria, *Ruditapes philippinarum*, bacterial disease, marine molluscs, Spain.

*Factores de virulencia de Vibrio P1, el agente causal de la enfermedad del anillo marrón en almejas japonesas, Ruditapes philippinarum.*

### Resumen

Diferentes factores de virulencia implicados en la potencial capacidad patógena del *Vibrio* P1, el agente causal de la enfermedad del anillo marrón (BRD) que afecta a adultos de las almejas japonesas cultivadas (*Ruditapes philippinarum*), han sido evaluados en comparación con los presentados por otras cepas de especies de *Vibrio* aislados de las almejas enfermas. Se ha estudiado el papel de las propiedades de la superficie celular como factores de virulencia considerando tanto las adhesiones bacterianas específicas y no específicas a las almejas, *Vibrio* P1 mostraba moderada hidrofobicidad, pero poseía alta afinidad al colorante Rojo Congo y la presencia de apéndices celulares, caracterizados como fimbrias o pili. Todas las cepas de *Vibrio* P1 secretaban hemolisinas y citotoxinas, y eran fuerte productores de exotoxinas. La constante presencia de un gran plásmido de 49.2-MDa en todas las cepas de *Vibrio* P1 puede ser usada como un marcador epidemiológico, aunque su papel en los mecanismos patogénos todavía no se ha establecido. Aunque en algunas cepas de *Vibrio*, los sistemas de adquisición de hierro juegan un papel esencial en la patogenicidad, no parecen ser un factor importante para *Vibrio* P1, ya que este patógeno carece de sideróforos.

**Palabras-claves :** *Vibrio* P1, factores de virulencia, enfermedad del anillo marrón, *Ruditapes philippinarum*, enfermedad bacteriana, Moluscos marinos.

## INTRODUCTION

The acquisition of an infectious disease is basically the result of the imbalance between the microbial pathogenicity and the specific and non-specific defence mechanisms of the host. Therefore, microbial pathogenicity involves the presence of several mechanisms, named virulence factors, which may change the physiological conditions and even provoke a disease in the host (Isenberg, 1988).

Brown ring disease (BRD), first described in cultured *Ruditapes philippinarum* from Landela (Finistère, France) by Paillard *et al.* (1989), is a good experimental model to study host-pathogen interactions, since according to Paillard *et al.* (1994): (a) pathogen cultures and host breeding can be easily controlled; (b) experimental time to reproduce the symptoms throughout the test clams is relatively short (1 month); and (c) the response can be evaluated by stages and phases of the disease.

Clam defence reactions to microorganisms involve several internal and external systems, such as mollusc haemocytes (Fisher, 1986), circulating enzymes (Chu, 1988), shell nacrezation (Paillard and Maes, 1994), and embedding of the pathogen (Paillard *et al.*, 1994). In BRD, all the above mentioned defence mechanisms have been demonstrated. Even an activation of the melanization process by diseased clams has been suggested (Paillard *et al.*, 1994).

Paillard and Maes (1990) reported that the causative agent of BDR was a strain of *Vibrio* named P1. Although the mechanisms of response to *Vibrio* P1 by the affected clams are well documented, the virulence factors of this microbial pathogen are poorly understood. In the present work, we have studied several virulence factors, such as adhesion capabilities, toxin and exoenzymatic production and other virulence factors presented by *Vibrio* P1 and compared them with those of several *Vibrio* strains also isolated from diseased clams.

## MATERIALS AND METHODS

### Microorganisms

Twenty-two strains of *Vibrio* P1 and 32 strains isolated from affected clams belonging to *Vibrio harveyi* (9 strains), *V. pelagius* (11 strains) and *V. splendidus* (12 strains) were used to study comparatively their virulence factors. All the strains were cultured at 22°C for 24 h in tryptone soya broth (TSB) (Oxoid, Unipath Ltd., Basingstoke, Hampshire, England, UK) supplemented with 1.5% NaCl following the methodology described by Castro *et al.* (1992).

### Bacterial adhesion tests

To test the hydrophobic capabilities of the strains, the Salt Aggregation Test (SAT) was performed,

following basically the methodology specified by Lindahl *et al.* (1981) using ammonium sulfate solutions in 0.002 M phosphate buffered saline (PBS) (pH 6.8) as saline substratum. The hydrophobic criterion proposed by Santos *et al.* (1990) was used to classify the strains on the basis of their degree of hydrophobicity.

The protocol described by Kay *et al.* (1985) was applied to quantify bacterial binding to Congo Red. Briefly, several solutions of Congo Red dye (Panreac), at concentrations of 0, 5, 10, and 15 µg/ml, were added to the bacterial suspensions. After incubation at 22°C for 10 min, the mixtures were centrifuged (24 000 × g for 5 min) and the optical density (OD) (480 nm) was determined. The amount of dye linked to the bacteria is calculated from the difference of the total Congo Red remaining in the supernatant.

The method described by Ishiguro *et al.* (1985) was used to determine the uptake of Congo Red dye by the bacterial strains. Petri dishes with tryptone soya agar (TSA) (Oxoid) supplemented with 1.5% NaCl and 30 µg/ml of Congo Red were seeded with the bacterial strains. The presence of red colonies was indicative of a specific uptake of the dye.

The attachment of bacteria to erythrocytes (haemagglutination) has been proposed as an efficient "in vitro" system to demonstrate the bacterial adhesive activity (Jones and Isaacson, 1983; Santos *et al.*, 1990), and as a system to characterize the type of adhesions (Evans *et al.*, 1980). The haemagglutination test was carried out by mixing 20 µl of a bacterial culture (10<sup>9</sup> colony forming units/ml) and 20 µl of horse erythrocytes (BioMerieux Ibérica Madrid, Spain) on a slide, as described by Larsen and Mellergaard (1984). Negative controls consisted of erythrocytes and PBS, and bacterial suspensions and PBS. A positive haemagglutination result was considered to be the appearance of visible aggregates within 10 min.

### Scanning electron microscopy (SEM)

Manila clam specimens and bacterial cultures of *Vibrio* P1 were processed for visualization by scanning electron microscopy (SEM). The samples were fixed in 2.5% glutaraldehyde in cacodylate buffer (0.2 M, supplemented with 13.16 g NaCl, pH 7.3) for 4 h at 4°C. Then, the samples were washed in the same buffer, dehydrated in a graded alcohol series (25, 50, 70, 80, 96, 100°) and critical point processed. Prior to SEM visualization (SEM Jeol, Mod. JSM-840 at 15 kV), the samples were shadowed using vaporized gold.

### Haemolysin production

Haemolysin production was detected by using TSA supplemented with 1.5% NaCl to which 5% defibrinated horse blood (BioMerieux) was added, according to the methodology described by Mackman and Williams (1985).

### Lipopolysaccharide analysis

Lipopolysaccharide (LPS) extraction of the cell envelopes of the *Vibrio* strains tested was conducted following the method specified by Hitchcock and Brown (1983). Briefly, the bacteria were grown in saline TSA for 24 h at 22°C, then they were resuspended in 20 mM Tris-HCl buffer (pH 7.2), and were washed twice by centrifugation (at 24 000 × g for 5 min at 4°C). The cellular pellet was resuspended in the same buffer to an OD (650 nm) of 1.3. A volume of 1.5 ml of this suspension was centrifuged at 24 000 × g for 5 min, and the pellet was solubilized in 50 µl of sodium-dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer. Then, 5 µl of saturated bromophenol blue solution was added and the mix was boiled for 10 min. After colling in ice, 10 µl of a proteinase K (Sigma Chemical Co., St. Louis, MO, USA) solution in Tris-HCl (5 mg/ml) were added, and incubated at 65°C for 1 h. Then, the samples were cooled at room temperature, and electrophoresed in discontinuous polyacrylamide-SDS gels (4.5-12.5% and 4.5-15%), using the technique described by Laemmli (1970).

Electrophoresis was performed using a vertical Mini-Protean II apparatus (Bio-Rad), with 0.75-mm thick gels, at constant intensity of 10 mA in the stacking gel (4.5% acrylamide), and of 15 mA in the 12.5% acrylamide or 5-15% gradient acrylamide gels. In all the cases, 5 µl of sample were analysed per strain.

LPS was detected using a modification of the silver-staining procedure described by Tsai and Frasch (1982), which consists in the elimination of the 12 h fixation step, and an increase of the oxidation time (from 5 to 15 min), to eliminate the washing of LPS fractions with low numbers of fatty acids and to increase the method sensitivity, regardless of the acylation degree of LPS.

### Cytotoxic activities on fish cell lines

Cytotoxicity of *Vibrio* P1 extracellular products was tested using a fish cell line, developed in our Department, from fibroblasts of fin of gilt-head seabream (*Sparus aurata*). Cells were grown as monolayers in 24-well culture plates (Nunc) at 18°C and pH 7.4 using MEM medium (Gibco, Life Technologies Ltd., Paisley, UK) supplemented with Earle's salts (Gibco), 10% foetal calf serum (Sigma), 0.18% sodium bicarbonate, and an antibiotic solution (100 UI penicillin, 100 µg/ml streptomycin and 50 µg/ml gentamicin, all supplied by Sigma). For the toxicity tests, the cell line was inoculated with 0.1 ml of serial dilutions of extracellular products (ECP) sample. Wells inoculated with PBS were used as negative controls. Plates were incubated at 18°C and the effects of ECP on the monolayers were observed after 24 and 48 h, according to the method described by Toranzo *et al.* (1983b).

### Characterization of the extracellular products (ECP)

ECP were extracted by applying the cellophane technique described by Liu (1957). Briefly, tubes containing 5 ml of saline TSB were inoculated with one swab of a 24-h bacterial growth on saline TSA and incubated for 18 h at 22°C. A volume of 0.2 ml of the culture was spread on cellophane overlaid on a saline TSA plate and incubated at 22°C for 48 h. Bacterial cells were harvested in PBS (pH 7) and the cell suspensions centrifuged at 13,000 × g (20 min at 4°C). The supernatants were filtered through 0.45 µm pore size membrane filters and the filtrate was lyophilized and stored at 4°C. Total protein was measured in ECP samples following the method described by Bradford (1976) using bovine serum albumin as standard (Sigma).

Global enzymatic activities of ECP were evaluated by the use of the API ZYM system (BioMérieux). A 65-µl volume of each ECP sample was inoculated in each well and the gallery was incubated at 22°C for 18 h. The results were recorded following the manufacturer's instructions.

### Siderophore production and growth in iron-limiting conditions

The presence of siderophore in the bacterial supernatants was examined as described by Schwyn and Neilands (1987). Bacterial cultures grown in minimal M9 medium supplemented with 10 µg/ml of ethyldiamine-dihydroxyphenyl-acetic acid (EDDA) (Sigma) were centrifuged (8 000 × g for 20 min at 4°C), and 0.5 ml of the supernatant was mixed with 0.5 ml of a CAS solution. Then, the OD (630 nm) was determined using the same mixture with the medium without bacterial culture as control.

To detect the ability of the bacterial strains tested to grow in the presence of an iron-chelating agent, EDDA at several concentrations (between 0 and 50 µg/ml) was added to the minimal M9 medium modified by supplementing with 2% NaCl and 15 g/l glucose (Difco Laboratories, Detroit MI, USA). The growth of the strains in this medium was observed after incubation at 22°C for 5 days.

### Plasmid analysis of *Vibrio* P1 and restriction tests

The bacterial strains were cultured in saline TSA for 24 h at 22°C. Alkaline extraction method described by Birnboim and Doly (1979) as modified by Sambrook *et al.* (1989) was used for DNA plasmid isolation. RN-ase (Sigma type 1-A) digestion for 10 min at a concentration of 25 mg/ml was used to remove RNA from the lysates. Then, protein release from lysates was performed by addition of phenol-chloroform solution (1:1), agitation and centrifugation (12 000 × g for 5 min). Finally, DNA was concentrated in the phenolic phase, by addition of 2.5 volumes of

absolute ethanol and incubation at  $-30^{\circ}\text{C}$  for 10 min. After ethanol removal by centrifugation ( $12000 \times g$ ), the pellet was suspended in  $20 \mu\text{l}$  of Tris-acetate buffer, and supplemented with  $6 \mu\text{l}$  of running buffer (bromocresol purple, 0.125 g; glycerol, 25 g; Tris-HCl, 0.302 g; and distilled water, 50 ml; pH 7.9).

DNA suspensions were electrophoresed using 0.7% agarose gels in a horizontal apparatus (LKB 2013 Miniphor) and electrophoresis buffer (Tris-acetate, 4 mM, EDTA, 2 mM, pH 8). The gel was run at constant intensity of 45 mA for 3 h. The DNA bands were visualized by staining with ethidium bromide (0.5 mg/ml) for 30 min, destained in water, and photographed under 254 nm wave length UV-transilluminator (LKB 2011 Macrovue). *Escherichia coli* strains V517 and R40a were used as molecular mass markers in the same gel.

Purified plasmid bands of the *Vibrio* P1 strains were digested with restriction endonucleases *Eco*R1 and *Hind*III following the instructions of the manufacturer (Boehringer Mannheim). Lambda DNA digested with *Hind*III was used for molecular weight markers.

## RESULTS

### Adhesion of *Vibrio* P1

All the strains of *Vibrio* P1 tested were moderately hydrophobic (table 1), although less than 25% of the strains of the other *Vibrio* species (*V. harveyi*, *V. pelagius* and *V. splendidus*) showed a high hydrophobic degree.

**Table 1.** – Hydrophobic degree of the strains of *Vibrio* P1, *V. harveyi*, *V. pelagius* and *V. splendidus* isolated from brown ring disease affected clams.

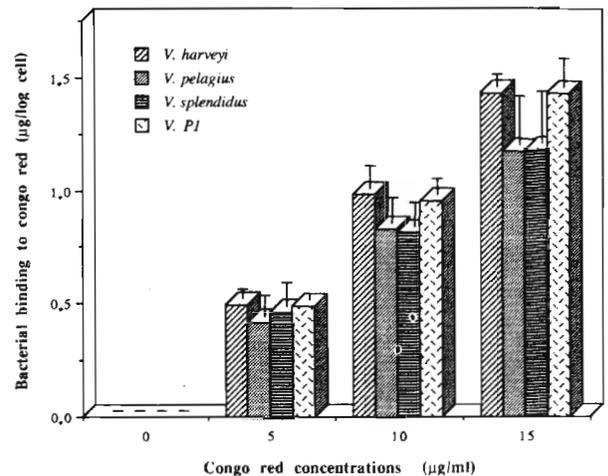
Hydrophobic degree <sup>a</sup>	SAT <sup>b</sup>	<i>V. harveyi</i>	<i>V. pelagius</i>	<i>V. splendidus</i>	<i>Vibrio</i> P1
Strong	0-1.0	22.2 <sup>c</sup>	18.2	25.0	0
Moderate	1.0-2.0	77.8	81.8	75.0	100
Low	2.0-4.0	0	0	0	0

<sup>a</sup> According to the Santos et al. (1990) criteria.

<sup>b</sup> Saline concentration intervals in the Salt Aggregation Test.

<sup>c</sup> Percentage of strains.

The main effect of *Vibrio* P1 on the affected clams is the presence of a conchiolin deposit in their shells. Therefore, the pathogen must adhere to the surface of the periostracum by means of specific structures (adhesins). In fact, the specific adhesive capability of *Vibrio* P1 has been demonstrated by means of the binding to Congo Red dye (structurally similar to uroporphyrin I), and direct electron microscopic visualization of pili.



**Figure 1.** – Congo red binding capabilities of several *Vibrio* strains isolated from diseased clams affected with brown ring disease.



**Figure 2.** – Scanning electron micrographs of *Vibrio* P1 filamentous appendages characterized as pilus-structures. Left:  $\times 7000$ ; Right:  $\times 20000$ .

High affinity capabilities of *Vibrio* P1 and *V. harveyi* strains to Congo Red are shown in figure 1. However, none of the strains tested showed uptake of Congo Red in the plate assay. On the other hand, the constant presence of filamentous appendages characterized as pili in *Vibrio* P1 is observed in scanning electron micrographs, both in the shell of contaminated clams with *Vibrio* P1 and in pure microbial cultures (fig. 2).

None of the *Vibrio* P1 strains tested showed haemagglutination of horse erythrocytes (table 2). Only a group of strains belonging to *V. splendidus* (16.6%) showed haemagglutination of horse erythrocytes.

### Bacterial toxins

One of the most important characteristics that defined a primary pathogen is its ability to synthesize toxins, which may be directly or indirectly responsible for damage to the host. We have detected two types of exotoxins in *Vibrio* P1 strains. First, a haemolysin which provokes lysis of horse erythrocytes. This haemolytic activity was also detected in different groups of species of *V. harveyi*, *V. pelagius* and *V. splendidus* (table 2).

**Table 2.** – Haemagglutination of horse erythrocytes, and haemolysin and siderophore production of *Vibrio* strains isolated from brown ring disease affected clams.

Bacteria	No. strains tested	Haemagglutination	Haemolysins	Siderophore
<i>V. harveyi</i>	4	– <sup>a</sup>	+ <sup>b</sup>	–
	2	–	+	+
	2	–	–	+
	1	–	–	–
<i>V. pelagius</i>	8	–	+	–
	3	–	–	–
<i>V. splendidus</i>	5	–	+	–
	3	–	+	+
	2	+	+	–
	2	–	–	–
<i>Vibrio</i> P1	22	–	+	–

<sup>a</sup> Negative test.

<sup>b</sup> Positive test.

The effects of the *Vibrio* P1 ECP included vacuolization of the cells at 18 h incubation, morphological alterations, such as rounding, shrinking, detaching and finally monolayer destruction (fig. 3).

The endotoxic fraction of the outer membrane layer of Gram-negative bacteria consists of the lipopolysaccharide fraction (LPS). In enteropathogenic bacteria, this fraction is an important virulence factor, which involves a specific immune response by the host.

The results obtained (fig. 4a) indicate that in the LPS electrophoretic profile of *Vibrio* P1 two regions may be distinguished. A region of high molecular mass (greater than 61 kDa) characterized by the presence of numerous bands is indicative of homogeneous polysaccharide chains in length. The other region possessed intermediate molecular mass (between 57 and 25 kDa), in which at least 8 bands are detected. This bacterial LPS preparation showed the typical ladder-like pattern, indicating O-specific side chains with variable length or sugar chains with incomplets o-acetyl substitution, considered as smooth (S) LPS. Only the LPS electrophoretic profile of *V. splendidus* showed characteristics similar to those of *Vibrio* P1 (fig. 4b).

### Bacterial exoenzymes

We have studied the exoenzymatic content of the extracellular products (ECP) of *Vibrio* P1, as well as their role in the pathogenicity of this microorganism. Extracellular enzymatic composition of *Vibrio* P1 varied depending on the strains studied. All the strains tested ( $n=22$ ) were negative for trypsin,  $\alpha$ - and  $\beta$ -galactosidase, and  $\beta$ -glucuronidase activities (table 3). The exoenzymatic activities of the ECP of other species studied are given in table 4.

More homogeneous enzymatic activity patterns were detected in the strains of *V. harveyi*, *V. pelagius* and *V. splendidus*. In all the strains negative activities were recorded for lipase,  $\alpha$ - and  $\beta$ -galactosidase,  $\alpha$ - and  $\beta$ -glucosidase,  $\alpha$ -mannosidase, and  $\alpha$ -fucosidase.

### Other virulence factors

None of the strains of *Vibrio* P1 demonstrated an iron-uptake system (table 2). However, this ability was frequently detected in several strains of *V. harveyi* (44.4%) and *V. splendidus* (25%). On the other hand, almost all the strains could grow in presence of 10  $\mu\text{g/ml}$  of EDDA (fig. 5) but only *V. harveyi* showed high growth capacity at high EDDA concentrations ( $>30 \mu\text{g/ml}$ ). *Vibrio* P1 strains had the lowest ability to compete with EDDA for iron-sequestering.

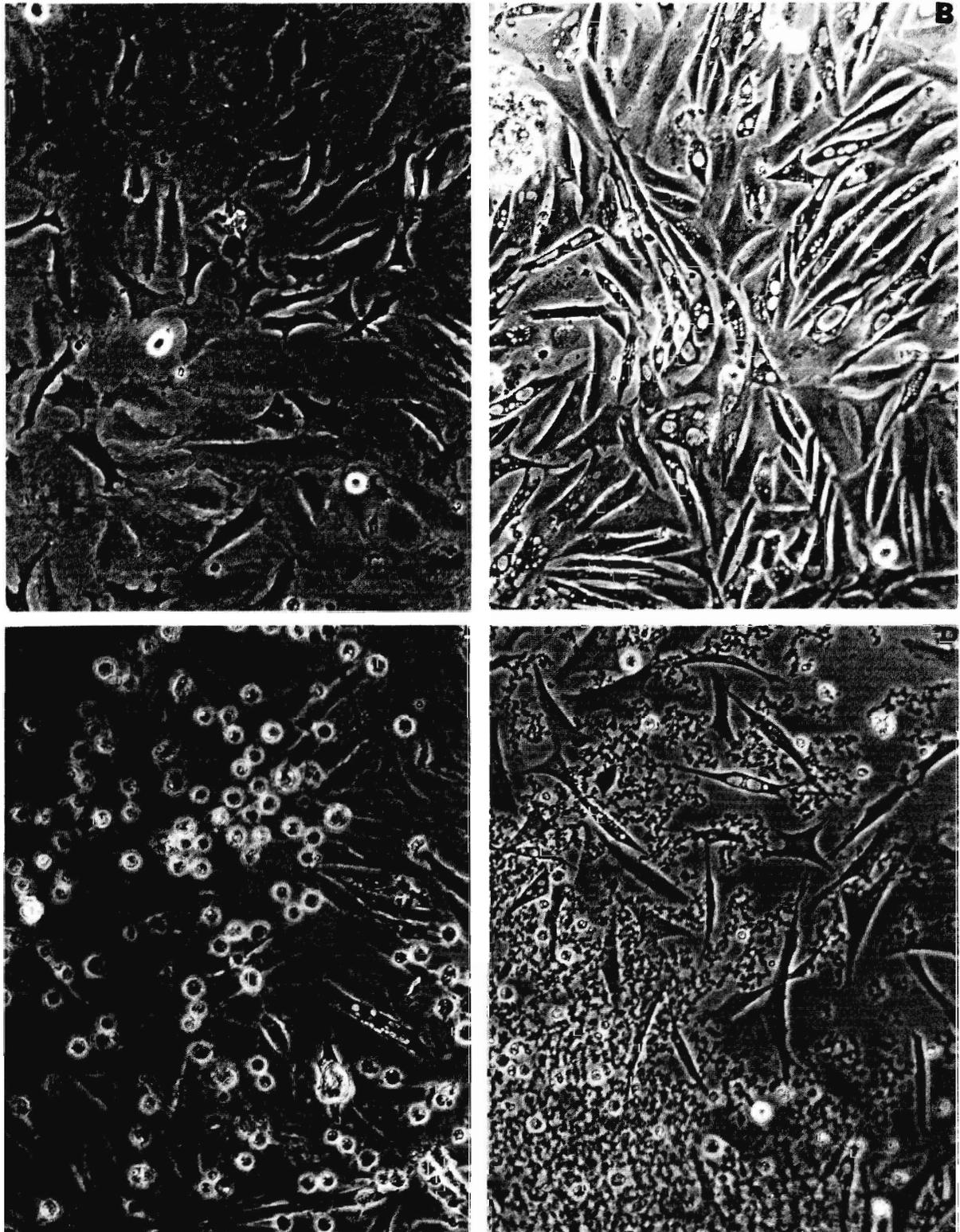
Large plasmids may contain genes which code for several virulence factors (such as pili, toxin synthesis or siderophore). The results obtained from the plasmid analysis of the *Vibrio* P1 strains showed that all the strains harboured the same plasmid profile, consisting of the presence of two bands of 52.8 and 40.2 MDa in the electrophoretic gels. However, the presence of two bands may be due to the plasmid methodology used, since one plasmid can suffer conformational changes in the supercoiled DNA, and the bands of approximately 52.8 and 40.2 MDa correspond to open-circular (oc) and close-circular (cc) forms. The endonuclease restriction analysis of the plasmid content of the strains, using *EcoRI* and *HindIII*, clearly show that all the strains of *Vibrio* P1 possessed the same cleavage patterns, made up of 9 fragments with *EcoRI* digestion and 10 fragments with *HindIII* digestion, and confirms the existence of only one plasmid of estimated molecular mass of 49.2 MDa.

To establish the relationship between the plasmid presence and the virulence of the strain, several curing experiments using acridine orange and ethidium bromide were performed. Unfortunately, the plasmidless-cured strains obtained lost their growth capacity both in broth and agar media. Therefore, to date we have not been able to test the virulence of the plasmidless *Vibrio* P1.

## DISCUSSION

### Adhesion

The bacterial adhesion or attachment to cells and tissues of the host in an important virulence factor for many pathogenic bacteria (Gibbons, 1977), and is known to be the initial step of many infections. This process requires the interaction of specialized complementary molecules in a ligand-receptor fashion between surfaces of bacteria and substratum. This interaction typically



**Figure 3.** – Cytotoxic effects of extracellular products of *Vibrio P1* on a gilt-head sea bream cell line culture. (A) Negative control inoculated with phosphate buffered saline (PBS). (B, C and D) Progressive steps of cytopathic effects provoked by *Vibrio P1* extracellular products (ECPs),  $\times 300$ .

**Table 3.** – Exoenzymatic activities of the extracellular products of *Vibrio* P1 strains.

Enzymes	<i>Vibrio</i> P1 groups <sup>a</sup>						Global (n = 22)
	1	2	3	4	5	6	
Alkaline phosphatase	100 <sup>b</sup>	100	100	100	80.0	33.3	86.4
Esterase	100	85.7	0	75.0	60.0	100	72.7
Esterase-lipase	100	100	100	100	80.0	100	95.5
Lipase	0	0	0	0	0	66.6	9.1
Leucine-arylamidase	100	100	100	100	0	66.6	72.7
Valyl-arylamidase	100	0	0	25.0	0	66.6	18.2
Cystine-arylamidase	0	28.6	0	0	0	0	9.1
Trypsin	0	0	0	0	0	0	0
$\alpha$ -chymotrypsin	100	85.7	50.0	75.0	0	100	63.6
Acid phosphatase	100	85.7	100	50.0	100	33.3	77.3
Phosphohydrolase	0	100	100	100	60.0	100	86.4
$\alpha$ -galactosidase	0	0	0	0	0	0	0
$\beta$ -galactosidase	0	0	0	0	0	0	0
$\beta$ -glucuronidase	0	0	0	0	0	0	0
$\alpha$ -glucosidase	100	0	0	25.0	0	0	9.1
$\beta$ -glucosidase	0	0	0	0	0	33.3	4.5
N-acetyl- $\beta$ -glucosaminidase	0	100	100	50.0	0	100	63.6
$\alpha$ -mannosidase	0	0	0	50.0	0	0	9.1
$\alpha$ -fucosidase	0	0	0	50.0	0	0	9.1

<sup>a</sup> Group 1 (n=1): *Vibrio* P1 B1090 (type strain); Group 2 (n=7): *Vibrio* P1 strains 8.3, 8.4, 8.5, 8.6, 8.7, 8.17, 8.19; Group 3 (n=2): *Vibrio* P1 strains 2.1, 2.3; Group 4 (n=4): *Vibrio* P1 strains 9.3, 9.4, 9.5, 9.7; Group 5 (n=5): *Vibrio* P1 strains IS1, IS5, IS7, IS8, IS9; Group 6 (n=3): *Vibrio* P1 strains 11.1, 11.2, 11.4.

<sup>b</sup> Percentage of positive strains.

**Table 4.** – Exoenzymatic activities of the extracellular products of several strains of *V. harveyi*, *V. pelagius* and *V. splendidus*.

Enzymes	<i>V. harveyi</i>	<i>V. pelagius</i>	<i>V. splendidus</i>
Alkaline phosphatase	100 <sup>a</sup>	100	100
Esterase	100	100	100
Esterase-lipase	100	100	100
Lipase	0	0	0
Leucine-arylamidase	100	100	100
Valyl-arylamidase	100	100	20.0
Cystine-arylamidase	100	50.0	0
Trypsin	100	0	20.0
$\alpha$ -chymotrypsin	100	0	0
Acid phosphatase	100	100	100
Phosphohydrolase	0	100	20.0
$\alpha$ -galactosidase	0	0	0
$\beta$ -galactosidase	0	0	0
$\beta$ -glucuronidase	0	0	20.0
$\alpha$ -glucosidase	0	0	0
$\beta$ -glucosidase	0	0	0
N-acetyl- $\beta$ -glucosaminidase	100	100	100
$\alpha$ -mannosidase	0	0	0
$\alpha$ -fucosidase	0	0	0

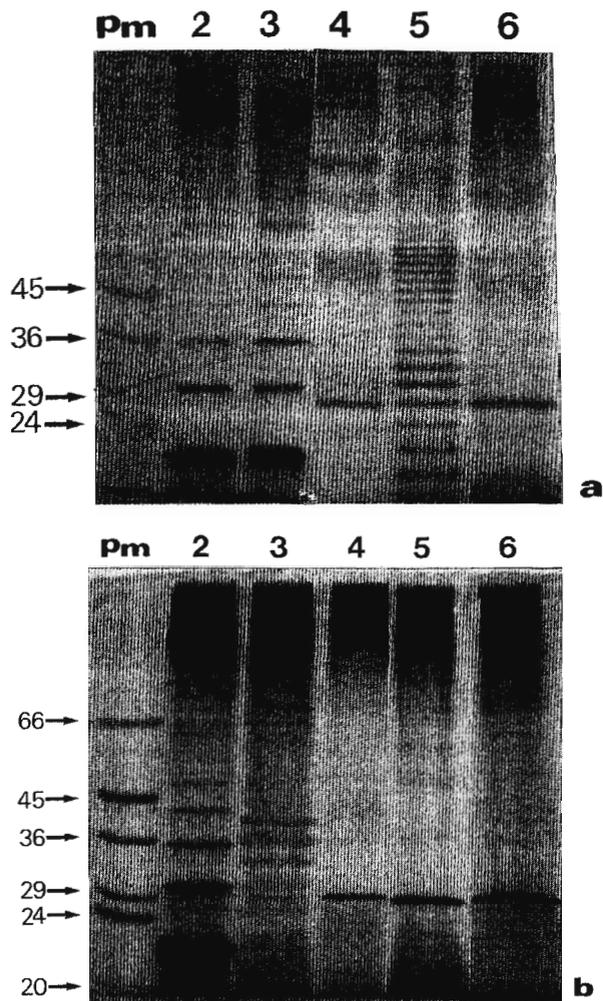
<sup>a</sup> Percentage of positive strains.

occurs between surface proteins and carbohydrate-containing molecules of the eucaryotic cell membrane or glycocalyx (Beachy, 1981).

Several authors have pointed out that prior to the bacterial adhesion it is necessary that bacteria may maintain their position along a mucosal surface by establishing small numbers of non-

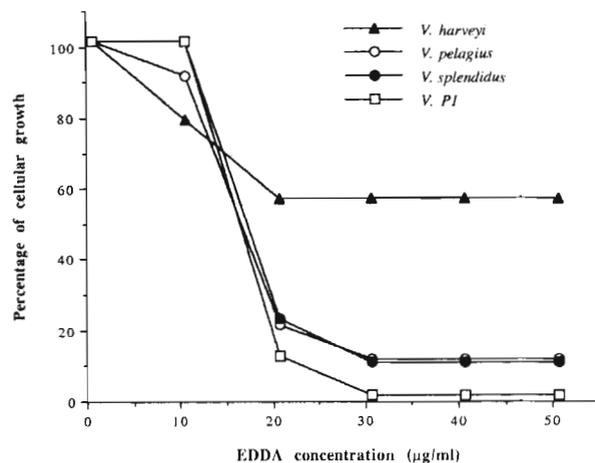
covalent bonds between the bacterial and mucosal surfaces (Arp, 1988), depending on several physical mechanisms, the hydrophobic interactions being the most significant (Rosenberg and Kjelleberg, 1989). Thus, the contribution of these interactions to the virulence of fish pathogens has been recorded in *Renibacterium salmoninarum* (Daly and Stevenson, 1987). However, the moderate hydrophobicity detected in the strains of *Vibrio* P1 does not seem to indicate that this type of interactions would be essential in the bacterial adhesion and colonization of the clam tissues. Similar results have been reported by Santos *et al.* (1990), who found contradictory results studying the relationship between the hydrophobicity and the virulence of several fish bacterial pathogens.

The bacterial binding to Congo red aromatic dye has been used to discriminate virulent and non-virulent strains of Gram-negative bacteria (Payne and Finkelstein, 1977; Ishiguro *et al.*, 1985). This discrimination is based on the fact that Congo red is structurally and conformationally similar to protoporphyrin IX and hemin (Janda *et al.*, 1991), proteins involved in the iron transport. None of the *Vibrio* strains tested produced red colonies on the Congo red plates, but a high ability to bind the dye was obtained in the spectrophotometric assays by the strains of *Vibrio* P1 and *V. harveyi*. The bacterial binding to Congo red apparently proceeds by at least two mechanisms; one non-specific, by means of hydrophobic interactions; and another specific, by means of stacked aggregates (Kay *et al.*, 1985). On the basis of the moderate hydrophobic capabilities of



**Figure 4.** – Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of lipopolysaccharide fraction (LPS) of several *Vibrio* lysates digested by proteinase K and stained with ammonical silver. (A): Lanes 2 to 6: *Vibrio* P1 strain B1090, *Vibrio* P1 strain Is7, *V. anguillarum* R-82, *V. anguillarum* ATCC 19264 and *V. anguillarum* 6062A, respectively. (B): Lanes 2 to 6: *Vibrio* P1 strain B1090, *V. splendidus* CECT 528, *V. splendidus* ATCC 25914, *V. tubiashii* CECT 631 and *V. pelagius* ATCC 25916, respectively. Molecular weight markers (Pm) in kDa are given in lane 1.

the *Vibrio* P1 strains, we think that the binding to Congo red proceeds by a salt-independent mechanism of surprisingly high affinity. It is possible that Congo red bound to the high-affinity site acts as a nucleation site for further binding at higher dye concentrations. However, what is the physiological significance of the specific interaction to Congo red by *Vibrio* P1 strains? In our opinion, their implication with the iron-transport is a remote possibility, but protoporphyrin IX is a precursor of uroporphyrin I, the prominent porphyrin pigment of the inner clam shells, and some bivalves conjugate uroporphyrin I with the calcareous material of their shells (Fox, 1974); thus, this would explain the higher affinity by the clam shells of *Vibrio* P1 compared to other marine *Vibrio* strains.



**Figure 5.** – Growth of the *Vibrio* strains tested in presence of several concentrations of ethyldiamine-dihydroxyphenyl-acetic acid (EDDA).

For many pathogenic bacterial strains, mucosal attachment is mediated by specific bacterial structures called fimbria or pili. Pili are considered as relevant adhesins or colonization factors in several enteropathogens (Jann *et al.*, 1981; Isaacson, 1988). In addition, Chart (1983) demonstrated a correlation between virulence for fish and the existence of *V. anguillarum* multiflagellate forms, suggesting that additional appendages may be involved in chemotaxis and/or penetration of mucosal surfaces.

Scanning electron microscopic studies revealed the constant presence of pilus-structures on *Vibrio* P1 cell surface. These structures are involved in the specific adhesion to the clam surfaces (*fig. 2*), but may also mediate agglutinating properties, such as haemagglutination of erythrocytes. Surprisingly, none of the *Vibrio* P1 strains showed haemagglutination of horse erythrocytes, and only 16.6% of the strains of *V. pelagius* were haemagglutinating. This apparent contradiction may be explained by the fact that: (i) both pathogenic and environmental strains of *Vibrio* possessed agglutinating properties (Larsen and Mellergaard, 1984); and (ii) the haemagglutination depends on the presence of specific receptors on the erythrocyte surface, and such receptors contain oligosaccharides which varied in function of the animal species (Jones and Freter, 1976). The inability of *Vibrio* P1 to agglutinate horse erythrocyte does not imply the lack of this property for other animal species erythrocytes; in addition, it must be considered that not all the adhesins produced haemagglutination, and not all the haemagglutinins played an important role in the pathogenicity (Santos *et al.*, 1990).

### Bacterial toxins

More than 50% of the *Vibrio* strains tested isolated from affected clams synthesize haemolysins, which suggests that this capability is widely distributed among the environmental *Vibrio* strains (Kreger and

Lockwood, 1981). These toxins play a significant, though not yet fully defined, role in the pathogenesis of isolates from human and fish infections (Welch *et al.*, 1981; Inamura *et al.*, 1984); but its significance in invertebrate pathogenesis is not well known yet, although Nottage and Birkbeck (1987) described haemolysins synthesized by *Vibrio* strains involved in the proteolysis of the gill tissue of mussels.

Endotoxins are heat-stable toxic components that elicit a wide range of pathophysiological and pharmacological effects on susceptible hosts. It becomes clear that virtually all species of Gram-negative bacteria produced endotoxins as part of their cell walls, characterized as lipopolysaccharides (LPS). The polysaccharide component of LPS plays an important role in virulence *in vivo*, because smooth isolates with the repeated polysaccharide side chains are more virulent than rough or semirough variants that lack of O antigen. This may be because surface components assist bacterial penetration of the host tissue or help the bacteria to resist immune defence mechanisms, such as phagocytosis. All the strains tested, in the present study, showed a smooth LPS (S-LPS), which potentially confers on them several virulence properties (Bradley, 1979). In the case of BRD, the LPS fraction seems not to be related to the induction and development of the clam disease.

### Bacterial exoenzymes

The extracellular products (ECP) produced by bacterial pathogens may contain different biological activities, such as proteases, cytolysins, haemolysins, siderophores, esterases and phospholipases (Campbell *et al.*, 1990; Ellis, 1991). All of them are considered as putative virulence factors because they caused the toxic effects involved with the infections provoked by the producing-bacteria (Price *et al.*, 1989). In addition, these virulence factors allow the bacteria to survive, to proliferate and to invade the host tissues (Toranzo *et al.*, 1983b; Ellis, 1991); by means of the production of two main effects, toxicity and tissue hydrolysis (Campbell *et al.*, 1990).

The exoenzymatic content of ECP from the strains studied (grouped according to their geographical site of isolation) varied inter- and intraspecifically (table 3). This high intraspecific heterogeneity of these biological activities has been reported previously in *Vibrio* strains (Balebona, 1994) and other fish pathogens (Nieto and Ellis, 1991).

The ability to degrade protein substrates represents an important advantage for the bacteria to obtain suitable nutrients from the microenvironment to multiply in the host tissue. This capability is associated with the bacterial exoenzyme production. In the case of BRD, Paillard *et al.* (1994) suggested that *Vibrio* P1 can use N-acetylglucosamine which is a precursor of chitin in the shell matrix. This fact has led these authors to propose a hypothesis on how the conchiolin deposit is formed in affected clams. However, the

N-acetylglucosaminase activity was observed in only 63.6% of the *Vibrio* P1 strains tested, and must be considered that other exoenzymes may play a more significant role in the host tissue hydrolysis, such as esterase-lipases, phosphatases and phosphohydrolases.

In addition, Elston *et al.* (1982), describing a conchiolin infection by strains of *Vibrio* sp. affecting oysters and clams, suggested that the close association of bacteria producing a variety of acidic metabolites would be inimical to normal deposition of calcium carbonate in newly elaborated shell.

On the other hand, we have demonstrated that ECP of *Vibrio* P1 is also involved in certain cytotoxic activities, demonstrated by the fact that these ECP produced cellular alterations and cytopathic effects on fish cell-line monolayers. These results must be understood in the sense that a high cytotoxic activity of ECP does not imply a high virulence of the bacterial strain, since Santos *et al.* (1987) observed that several ECP of *Aeromonas* strains provoked cytotoxic effects on fish cell lines independently of their virulence capability.

### Siderophore production and plasmid content

The pathogenic species of *Vibrio* possess specific mechanisms which allow them to take in extracellular iron and gives them a higher survival capability both in host tissues and in aquatic environments (Crosa, 1979; Otto *et al.*, 1992). One of these mechanisms is mediated by the production of low-molecular mass compounds (400-1000 Da), which exhibit a high affinity with ferric ion (Griffiths, 1987), named siderophores. According to their structure, the siderophores are classified into two groups: hydroxamates and phenolate-catecholate, the latter of which has only been detected in bacteria (Neilands, 1981). However, the contribution of the siderophore-dependent iron-uptake systems to the virulence of some pathogenic bacteria is still unclear. Thus, for several pathogens, including *V. anguillarum* and *V. vulnificus*, the role of siderophores in their pathogenicity is very significant, but by other *Vibrio* species this direct relationship has not been clearly established (Wright *et al.*, 1981; Walter *et al.*, 1983). Several studies showed that concentrations of 10  $\mu$ M of EDDA avoid the growth of bacteria lacking an iron-sequestering system (Mazoy and Lemos, 1991). All the strains tested, in the present study, except several strains of *V. harveyi*, could grow in limited-iron conditions, which indicates the potential uptake of iron ions for the tested microorganisms. However, this ability has been quantitatively different in the strains studied, ranging between 10 and 90% for *Vibrio* P1 and *V. pelagius* strains, respectively, at 20  $\mu$ M EDDA, and 60% of the strains of *V. harveyi* at increasing EDDA concentrations (30, 40, and 50  $\mu$ M) (fig. 5). For this reason, the siderophore production does not seem to be a principal mechanism in the bacterial pathogenicity of bivalves.

Several virulence factors of bacteria are coded in plasmid genes, such as adhesive properties, toxin production and iron-sequestering systems (Toranzo *et al.*, 1983a; Crosa, 1980; Tomalsky *et al.*, 1985). Interestingly, all the strains of *Vibrio* P1 harboured a very conservative plasmid of 49.2 MDa, which strongly suggests that this plasmid may be related to very important physiological and/or pathological functions of these strains (Stanisich, 1990). Certain strains of *Vibrio* P1 which were kept at  $-20^{\circ}\text{C}$  were tested repeatedly for plasmid composition in a 3-year period. No changes could be observed in relation to its plasmid profile and biochemical characteristics. Compared to the plasmid profiles of other *Vibrio* strains tested, the plasmid profile of *Vibrio* P1 seems to be specific for this species. Crosa *et al.* (1980) demonstrated the implication of a plasmid of similar

molecular mass, designed pJMI (47 MDa), in the coding of a very efficient iron-sequestering system that enables the invasion of *V. anguillarum* to host tissues. The genetic loci of pilus adhesins in *E. coli* are located in plasmids ranging 50- and 60-MDa (Isaacson, 1988). In addition, heat-labile and heat-stable enterotoxins and cytotoxins are coded in large plasmids (Ellwood and Shirpley, 1980). However, to demonstrate the implication of the 49.2-MDa plasmid harboured by *Vibrio* P1 in its virulence, plasmid transfer between positive and negative strains, together with curing experiments, must be carried out. As the results obtained by Wards *et al.* (1991), all our attempts of curing and transfer of the plasmid have been unsuccessful. Therefore, at present we cannot confirm definitively the relationship between the plasmid and the virulence of *Vibrio* P1.

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#### REFERENCES

- Arp L. H. 1988. Bacterial infections of mucosal surfaces: An overview of cellular and molecular mechanisms In: Virulence mechanisms of bacterial pathogens. J. A. Roth ed. American Society for Microbiology, Washington, D.C., 3-27.
- Balebona M. C. 1994. Caracterización y mecanismos de virulencia de cepas del género *Vibrio* aisladas de patologías de doradas (*Sparus aurata* L.) cultivadas. Ph. D. Thesis, Univ. Malaga, Spain.
- Beachy E. H. 1981. Bacterial adherence: adhesin-receptor interactions mediating the attachment of bacteria to mucosal surfaces. *J. Infect. Dis.* **143**, 325-345.
- Birnboim H. C., J. Doly 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7**, 1513-1523.
- Bradley S. G. 1979. Cellular and molecular mechanisms of action of bacterial endotoxins. *Ann. Rev. Microbiol.* **33**, 67-94.
- Bradford M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.
- Campbell C. M., D. Duncan, N. C. Price, L. Stevens 1990. The secretion of amylase, phospholipase and protease from *Aeromonas salmonicida*, and the correlation with membrane-associated ribosomes. *J. Fish Dis.* **13**, 463-474.
- Castro D., E. Martínez-Manzanares, A. Luque, B. Fouz, M. A. Moriño, J. J. Borrego, A. E. Toranzo 1992. Characterization of strains related to the brown ring disease outbreaks in Southwestern Spain. *Dis. Aquat. Org.* **14**, 229-236.
- Chart H. 1983. Multiflagellate variants of *Vibrio anguillarum*. *J. Gen. Microbiol.* **129**, 2193-2197.
- Chu F. E. 1988. Humoral defense factors in marine bivalves. *Am. Fish. Soc., Spec. Publ.* **18**, 178-188.
- Crosa J. H. 1979. *Vibrio anguillarum*: an iron uptake mechanism as a factor of virulence. *Fish. Health. News* **8**, 7-9.
- Crosa J. H. 1980. A plasmid associated with virulence in the marine fish pathogen *Vibrio anguillarum* specifies an iron-sequestering system. *Nature* **284**, 566-568.
- Crosa J. H., L. L. Hodges, M. H. Schiewe 1980. Curing of a plasmid is correlated with an attenuation of virulence in the marine fish pathogen *Vibrio anguillarum*. *Infect. Immunol.* **27**, 897-902.
- Daly J. G., W. M. R. Stevenson 1987. Hydrophobic and haemagglutinating properties of *Renibacterium salmoninarum*. *J. Gen. Microbiol.* **133**, 3575-3580.
- Ellis A. E. 1991. An appraisal of the extracellular toxins of *Aeromonas salmonicida* ssp. *salmonicida*. *J. Fish Dis.* **14**, 265-277.
- Ellwood D. C., P. L. Shipley 1980. Plasmid-mediated factors associated with virulence of bacteria to animals. *Ann. Rev. Microbiol.* **34**, 465-496.
- Elston R., E. Elliott, R. R. Colwell 1982. Conchiolin infection and surface coating *Vibrio*: shell fragility, growth depression and mortalities in cultured oysters and

- clams (*Crassostrea gigas*, *Ostrea edulis* and *Mercenaria mercenaria*). *J. Fish Dis.* **5**, 265-284.
- Evans D. J., Jr., D. G. Evans, L. S. Young, J. Pitt 1980. Hemagglutination typing of *Escherichia coli*: definition of seven hemagglutination types. *J. Clin. Microbiol.* **12**, 225-242.
- Fisher W. S. 1986. Structure and function of oyster hemocytes In: Immunity in invertebrates. M. Brehelin ed. Springer-Verlag Berlin, 25-35.
- Fox D. L. 1974. Biochromes. Occurrence, distribution and comparative biochemistry of prominent natural pigments in the marine world. In: Biochemical and biophysical perspectives in marine biology, Vol. 1. D.C. Malins, J.R. Sargent eds. Academic Press, London, 170-210.
- Gibbons R. J. 1977. Adherence of bacteria to host tissue In: Microbiology. D. Schlessinger ed. American Society for Microbiology, Washington, D.C., 395-406.
- Griffiths E. 1987. The iron-uptake systems of pathogenic bacteria. In: Iron and infection: Molecular, physiological and clinical aspects. J. J. Bullen, E. Griffiths eds. John Wiley & Sons, Chichester, 69-137.
- Hitchcock P. J., T. M. Brown. 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. *J. Bacteriol.* **154**, 269-277.
- Inamura H., K. Muroga, T. Nakai 1984. Toxicity of extracellular products of *Vibrio anguillarum*. *Fish Pathol.* **19**, 89-96.
- Isaacson R. E. 1988. Molecular and genetic basis of adherence for enteric *Escherichia coli* in animals. In: Virulence mechanisms of bacterial pathogens. J. A. Roth ed. American Society for Microbiology, Washington, D.C., 28-44.
- Isenberg H. D. 1988. Pathogenicity and virulence: another view. *Clin. Microbiol. Rev.* **1**, 40-53.
- Ishiguro E. E., T. Ainsworth, T. J. Trust, W. W. Kay 1985. Congo red agar, a differential medium for *Aeromonas salmonicida*, detects the presence of the cell surface protein array involved in virulence. *J. Bacteriol.* **164**, 1233-1237.
- Janda J. M., S. L. Abbott, S. Kroske-Bystrom, W. K. W. Cheung, C. Powers, R. P. Kokka, K. Tamura 1991. Pathogenic properties of *Edwardsiella* species. *J. Clin. Microbiol.* **29**, 1997-2001.
- Jones D., R. E. Isaacson 1983. Proteinaceous bacterial adhesins and their receptors. *Crit. Rev. Microbiol.* **10**, 229-260.
- Jones G. W., R. Freter 1976. Adhesive properties of *Vibrio cholerae*. Nature of the interaction with isolated rabbit brush-border membranes and human erythrocytes. *Infect. Immunol.* **14**, 240-245.
- Kay W. W., B. M. Philipps, E. E. Ishiguro, T. J. Trust 1985. Porphyrin binding by the surface array virulence protein of *Aeromonas salmonicida*. *J. Bacteriol.* **164**, 1332-1336.
- Kreger A., D. Lockwood 1981. Detection of extracellular toxin(s) produced by *Vibrio vulnificus*. *Infect. Immunol.* **33**, 583-590.
- Laemmli U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.
- Larsen J. L., S. Møllergaard 1984. Agglutination typing of *Vibrio anguillarum* isolates from diseased fish and from the environment. *Appl. Environ. Microbiol.* **47**, 1261-1265.
- Lindahl M., A. Faris, T. Wadstrom, S. Hjerten 1981. A new test based on salting out to measure relative surface hydrophobicity of bacterial cells. *Biochim. Biophys. Acta* **677**, 471-476.
- Liu P. V. 1957. Survey of hemolysin production among species of *Pseudomonas*. *J. Bacteriol.* **74**, 718-727.
- Mackman N., P. H. Williams 1985. Detection of  $\alpha$ -haemolysin by clinical isolates of *Escherichia coli* In: The virulence of *Escherichia coli*. M. Sussman ed. The Society for General Microbiology, Academic Press, London, 425-427.
- Mazoy R., M. L. Lemos 1991. Iron-binding proteins and heme compounds as iron sources for *Vibrio anguillarum*. *Curr. Microbiol.* **23**, 221-226.
- Neilands J. B. 1981. Microbial iron compounds. *Ann. Rev. Biochem.* **50**, 715-731.
- Nieto T. P., A. E. Ellis 1991. Heterogeneity of extracellular proteases produced by different isolates of *Aeromonas hydrophila* and *A. sobria* pathogenic for fish. *J. Fish. Dis.* **14**, 229-235.
- Nottage A. S., T. H. Birkbeck 1987. The role of toxins in *Vibrio* infections of bivalve molluscs. *Aquaculture* **67**, 244-246.
- Otto B. R., A. M. J.J. Verweij-van Vught, D. H. MacLaren 1992. Transferrins and heme-compounds as iron sources for pathogenic bacteria. *Crit. Rev. Microbiol.* **18**, 217-233.
- Paillard C., P. Maes 1990. Etiologie de la maladie de l'anneau brun chez *Tapes philippinarum*: pathogénicité d'un *Vibrio* sp. *C. R. Acad. Sci. Paris* **310**, 15-20.
- Paillard C., P. Maes 1994. Brown ring disease in the manila clam *Ruditapes philippinarum*: establishment of a classification system. *Dis. Aquat. Org.* **19**, 137-146.
- Paillard C., L. Percelay, L. Le Pennec, D. Le Picard 1989. Origine pathogène de « l'anneau brun » chez *Tapes philippinarum* (Mollusque, Bivalve). *C. R. Acad. Sci. Paris* **309**, 235-241.
- Paillard C., P. Maes, R. Oubella 1994. Brown ring disease in clams. *Ann. Rev. Fish Dis.* **4**, 219-240.
- Price N. C., L. Stevens, D. Duncan, M. Snodgrass 1989. Proteases secreted by strains of *Aeromonas salmonicida*. *J. Fish Dis.* **12**, 223-232.
- Rosenberg M., V. Kjelleberg 1989. Hydrophobicity interactions: role in bacterial adhesion. *Adv. Microbiol. Ecol.* **1**, 353-359.
- Sambrook J., E. F. Fritsch, T. Maniatis 1989. Molecular Cloning: A Laboratory Manual, 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
- Santos Y., A. E. Toranzo, C. P. Dopazo, T. P. Nieto, J. L. Barja 1987. Relationship among virulence for fish, enterotoxigenicity, and phenotypic characteristics of motile *Aeromonas*. *Aquaculture* **67**, 29-39.
- Santos Y., I. Bandin, T. P. Nieto, D. W. Bruno, A. E. Ellis, A. E. Toranzo 1990. Comparison of the cell surface hydrophobicity of bacterial fish pathogens by different procedures. In: Pathology in Marine Science. F.O. Perkins, T.C. Chen ed. Academic Press, New York, 101-115.
- Schwyn B., J. B. Neilands 1987. Universal chemical assay for the detection and determination of siderophores. *Anal. Biochem.* **160**, 47-56.
- Stanisich V. A. 1990. Identification and analysis of plasmids at genetic level. In: Plasmid Technology. J. Grinstead, P.M. Bennet ed. Academic Press, London, 11-47.

- Tolmasky M. E., L. A. Actis, A. E. Toranzo, J. L. Barja, J. H. Crosa 1985. Plasmids mediating iron uptake in *Vibrio anguillarum* strains isolated from turbot in Spain. *J. Gen. Microbiol.* **131**, 1989-1997.
- Toranzo A. E., J. L. Barja, R. R. Colwell, F. M. Hetrick 1983a. Characterization of plasmids in bacterial fish pathogens. *Infect. Immunol.* **39**, 184-192.
- Toranzo A. E., J. L. Barja, S. A. Potter, R. R. Colwell, F. M. Hetrick, J. H. Crosa 1983b. Molecular factors associated with virulence of marine vibrios isolated from striped bass in Chesapeake Bay. *Infect. Immunol.* **39**, 1220-1227.
- Tsai C. M., C. E. Frasch 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal. Biochem.* **119**, 115-119.
- Walter M. A., S. A. Potter, J. H. Crosa 1983. Iron uptake system mediated by *Vibrio anguillarum* plasmid pJM1. *J. Bacteriol.* **155**, 880-887.
- Wards B. J., H. H. Patel, C. D. Anderson, C. W. de Lisle 1991. Characterization by restriction endonuclease analysis and plasmid profiling of *Vibrio ordalii* strains from salmon (*Oncorhynchus tshawytscha* and *Oncorhynchus nerka*) with vibriosis in New Zealand. *New Zealand J. Mar. Freshw. Res.* **25**, 345-350.
- Welch R. A., E. P. Dellinger, B. Minshew, S. Falkow 1981. Haemolysin contributes to virulence of extra-intestinal *E. coli* infections. *Nature* **294**, 665-667.
- Wright A. C., L. M. Simpson, J. D. Oliver 1981. Role of iron in the pathogenesis of *Vibrio vulnificus* infections. *Infect. Immunol.* **34**, 503-508.