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# Investigation of several virulence properties among *Vibrio alginolyticus* strains isolated from diseased cultured fish in Tunisia

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ABSTRACT: We analysed 34 *Vibrio alginolyticus* strains isolated from gilthead sea bream *Sparus aurata* L. and sea bass *Dicentrarchus labrax* L. cultured in fish farms on the Tunisian Mediterranean coast for the presence of several virulence properties such as extracellular products (ECP) production, growth in iron-limiting conditions and survival in fish serum. The results obtained with different substrates showed that ECP of *V. alginolyticus* were hydrolytic. The virulence was correlated with the ability of strains to grow in the presence of non-immune fish serum or under conditions of iron limitation. We further examined the presence of virulence genes homologous to those in *V. cholerae* (*toxR*, *toxS*, *VPI* and *ace*); *toxR* was found in 16 *V. alginolyticus* strains and *toxS* in 17 strains out of 34 analysed. A positive amplification for the virulence pathogenicity island (VPI) was produced by 12 *V. alginolyticus* strains. Finally, the *ace* expected amplification fragment was found in 7 *V. alginolyticus* isolates. Thus, the pathogenicity of *V. alginolyticus* may be the result of a combination of all these factors.

KEY WORDS: *Vibrio alginolyticus* · Virulence genes · ECP production · Bacterial survival · Fish serum · *Dicentrarchus labrax* · *Sparus aurata*



## INTRODUCTION

Under intensive culture conditions, cultured fish are exposed to various stressors including bacterial pathogens. Among possible sources of bacterial infections, *Vibrio alginolyticus* is considered a marine fish and shellfish pathogen (Balebona et al. 1998a,b, Gómez-León et al. 2005). This species is often isolated as a causative agent of vibriosis in cultured gilthead sea bream *Sparus aurata* L. and sea bass *Dicentrarchus labrax* L. in Mediterranean coastal areas (Balebona et al. 1998b, Zorilla et al. 2003a,b, Ben Kahla-Nakbi et al. 2006).

A number of factors, such as iron availability in fish serum, which affects survival, have been implicated in contributing to disease caused by pathogenic *Vibrio alginolyticus*. Virulence also is related to the pathogen's ability to produce extracellular products (ECPs) (Lamas et al. 1994, Balebona et al. 1998b). According to Balebona et al. (1998a), these ECPs, which mostly consist of proteases, could facilitate the propagation of the bacteria by causing extensive host tissue damage, thereby degrading host proteins that provide readily available nutrients for bacterial growth.

*Vibrio cholerae* is known to be responsible for severe diarrhoeic disease that continues to be a global threat to human health. Different genes play a role in the expression of the potent cholera toxin (CT), which is encoded by the *ctxAB* genes on the filamentous phage CTXf along with genes encoding other virulence factors such as the Zonula occludens toxin (Zot), accessory cholera enterotoxin (Ace) and a core-encoded pilin (cep) (Colombo et al. 1994). The toxin coregulated pilus (TCP) also acts as a receptor for CTXf, which can infect nontoxigenic *V. cholerae*, leading to the emergence of new toxigenic strains (Waldor & Mekalanos

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1996). The *tcpA* gene is part of a pathogenicity island of about 39.5 kb known as the *V. cholerae* pathogenicity island (VPI) (Karaolis et al. 1998). The structural features of VPI are suggestive of a bacteriophage origin. This supports the concept that some pathogenic bacteria have evolved from non-pathogenic strains of the same species via horizontal transfer of virulence genes. The expression of TCP pathogenicity island and cholera toxin is mediated by the regulatory proteins ToxR and ToxS (Wong et al. 1998).

Pathogenic mechanisms are only clearly understood for *Vibrio cholerae* O1 and O139 and not for the majority of *Vibrio* species. Several studies showed a wide dissemination of *V. cholerae* and *V. parahaemolyticus* genes among environmental *V. alginolyticus* strains (Sechi et al. 2000, Xie et al. 2005, Masini et al. 2007) and *V. harveyi* strains (Bai et al. 2008).

Gilthead sea bream and sea bass are 2 of the main finfish species cultured with high economic value in the Mediterranean aquaculture industry and are the main aquacultural species in Tunisia. In many epizootic events *Vibrio alginolyticus* is often isolated from diseased fish cultured in Tunisia and is associated with mass mortality (Ben Kahla-Nakbi et al. 2006, 2007). Therefore, it is important to study the distribution of several virulence characteristics in *V. alginolyticus* strains isolated from diseased gilthead sea bream and sea bass cultured in Tunisia. These include ECP production, growth in iron-limiting conditions and survival in fish serum. We also investigated the presence of 4 *V. cholerae* virulence genes in the *V. alginolyticus* genome.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Thirty-four *Vibrio alginolyticus* strains were isolated from diseased gilthead sea bream and sea bass cultured (reared in tanks) on the Tunisian Mediterranean coast. The strains had previously been characterised and differentiated by molecular methods (Ben Kahla-Nakbi et al. 2006). Stock cultures were frozen at −80°C with 20% (vol/vol) glycerol, and the strains were routinely cultured on tryptic soy agar (TSA) or in tryptic soy broth (TSB) supplemented with 1% NaCl (TSA-1 and TSB1, respectively) (Difco) at 28°C.

**Extracellular products preparation.** Bacterial ECPs were produced by the cellophane overlay method as described by Liu (1957). Tubes containing 5 ml of TSB-1 were inoculated with one bacterial colony from a 24 h TSA-1 culture of each strain of *Vibrio alginolyticus* and incubated at 22°C for 18 h. A volume of 0.2 ml of this culture was transferred onto a sterile cellophane sheet placed on the surface of each TSA-1 plate. After incubation at 22°C for 48 h, cells were washed off the cellophane sheet using phosphate-buffered saline (PBS) (pH 7) and removed by centrifugation at 10 000 × *g* and 4°C for 30 min. The supernatant containing the ECPs was sterilized by filtration (0.22 µm) and stored at −80°C until use.

Enzymatic activities of the extracellular products were evaluated with the API Zym System (BioMérieux) composed of 19 enzymatic substrates. The activities of various other enzymes were determined following inoculation of 20 µl of ECPs onto agarose plates (0.8% agarose in 0.1 M PBS, pH 7) to which the following substrates had been added: 0.2% (wt/vol) starch for amylase, 1% (wt/vol) skim milk for caseinase, 1% (wt/vol) gelatin for gelatinase, 1% Tween 80 for lipase and 5% (vol/vol) egg yolk for phospholipase (lecithinase).

The expression of DNase and haemolysin activities was determined with whole viable cells. Bacteria grown overnight in TSA-1 at 22°C were spot-inoculated onto the plated assay media described below. DNase activity was studied using DNase test agar (DNase Agar, Scharlau Microbiogy). Production of haemolysin was detected on blood agar plates with 5% of gilthead sea bream and sea bass erythrocytes. Haemolysis was evident after incubation at 25°C for 24 h.

**Growth under iron-limiting conditions and siderophore production.** *Vibrio alginolyticus* strains were cultured in M9 minimal medium supplemented with 0.2% (wt/vol) Casamino Acids (CM9 medium) supplemented with 10 µM non-assimilable iron chelator 2,2-dipyrydil (Sigma), which makes iron unavailable to bacteria if they do not possess a high-affinity iron uptake system. The minimum inhibitory concentration (MIC) of 2,2-dipyrydil was determined in CM9 medium tubes containing increasing concentrations of the chelator and was defined as the lowest concentration at which no bacterial growth was observed.

**Serum sensitivity.** Gilthead sea bream and sea bass serum was collected by centrifugation of total blood at 3000 × *g* for 20 min at 4°C and stored at −20°C. Tests were conducted in 96-well microtitre dishes by mixing 50 µl of fresh serum or heat inactivated serum (44°C, 20 min) with 50 µl of bacteria suspension ($10^5$ CFU $ml^{-1}$) in PBS (pH 7). Assays were made by taking samples (10 µl) of the mixture (serum and bacteria) every 30 min for 3.5 h of incubation at room temperature (25°C). Viable counts were determined by drop plate on TSA-1. Survival tendency curves of the 34 strains of *Vibrio alginolyticus* were determined using an Excel tendency curve program.

**Detection of virulence genes.** The DNA extraction method was as follows: A 1.5 ml volume of overnight bacterial culture from each bacterial isolate was harvested by centrifugation at 8000 rpm for 5 min at

Table 1. PCR primers of virulence genes

| Target gene | PCR primer sequences (5′–3′) | Product size (bp) | Source |
|---|---|---|---|
| *tox*S | 5′-CCACTGGCGGACAAAATAACC-3′<br>5′-AACAGTACCGTAGAACCGTGA-3′ | 640 | GenBank L11929 |
| *tox*R | 5′-TTTGTTTGGCGTGAGCAAGGTTTT-3′<br>5′-GGTTATTTTGTCCGCCAGTGG-3′ | 595 | GenBank M21249 |
| *VPI* | 5′-GCAATTTAGGGGCGCGACGT -3′<br>5′-CCGCTCTTTCTTGATCTGGTAG-3′ | 680 | Sechi et al. (2000) |
| *ace* | 5′-GCTTATGATGGACACCCTTTA-3′<br>5′-TTTGCCCTGCGAGCGTTAAAC-3′ | 284 | Colombo et al. (1994) |

4°C, washed in saline solution 0.9% (wt/vol), re-centrifuged, and the cell pellet resuspended in 100 µl of distilled water. The mixture was boiled for 10 min, and centrifuged at 8000 rpm for 10 min to sediment the cell debris. Then, the DNA-containing supernatants were transferred to fresh microcentrifuge tubes.

The primer pairs used for PCR assays (Table 1) were performed as described in previous studies (Colombo et al. 1994, Sechi et al. 2000). Amplification reactions were performed in a 25 µl final volume with 5 µl green Go *Taq* buffer (5×), 200 µM of deoxynucleoside triphosphate (dNTP), 25 pM of each primer (Table 1) and 1 U of Go *Taq* DNA polymerase (Promega). The mixtures were incubated for 5 min at 94°C, followed by 35 cycles of amplification. Except for the primer annealing temperature, each cycle consisted of denaturation at 94°C for 40 s, annealing for 40 s and primer extension at 72°C for 1 min, and the mixtures were kept at 72°C for 10 min. The annealing temperature was 58°C for *toxR* and *toxS* and 62°C for *VPI* and *ace*. The amplification products were visualized after electrophoresis at 90 V on 1% agarose gel stained with ethidium bromide (0.5 µg $ml^{-1}$), which was visualized under UV transillumination and photographed using Gel Doc XR apparatus (Bio-Rad). All PCR positive strains had the presence of the virulence genes confirmed by repeating the PCR 3 times independently.

Table 2. *Vibrio alginolyticus*. Positive strains for various enzyme expression in extracellular products

| Enzyme | No. (%) of positive strains |
|---|---|
| Gelatinase | 34 (100) |
| Amylase | 21 (61.76) |
| Lecithinase | 32 (94.11) |
| Lipase | 4 (11.76) |
| Caseinase | 16 (73.52) |
| Alkaline phosphatase | 30 (88.23) |
| Esterase(C4) | 17 (50) |
| Esterase lipase C8 | 22 (64.70) |
| Lipase (C14) | 11 (32.35) |
| Leucine arylamidase | 28 (82.35) |
| Valine arylamidase | 13 (38.23) |
| Cystine arylamidase | 9 (26.47) |
| Trypsine | 17 (50) |
| α-chymotrypsine | 11 (32.35) |
| Acide phosphatase | 26 (76.47) |
| Naphtol-AS-BI-phosphohydrolase | 11 (32.35) |
| α-galactosidase | 0 |
| β-galatosidase | 2 (5.58) |
| β-glucorinidase | 0 |
| α-glucosidase | 0 |
| β-glucosidase | 2 (5.58) |
| N-acetyl-β-glucosaminidase | 21 (61.76) |
| α-mannosidase | 0 |
| α-fucosidase | 0 |

**Statistical analysis.** The cross table test (SPSS v. 14.0, Statistical Package for the Social Sciences) was used to evaluate the presence of correlation between every studied virulence factor. p-values $<0.05$ were considered significant. Counts of culturable cells from the experiments of serum survival were processed for variance analysis using the procedure of the general linear model with repeated measures available in the SPSS package.

## RESULTS

### Enzymatic activity

The results obtained from the different tested substrates showed that the ECP of the isolated *Vibrio* were hydrolytic. Indeed the enzymatic activities characterized by Api ZYM showed that all the strains produced a phosphatase alkaline, but they were negative for α-galactosidase, β-glucoronisidase, α-glucosidase, α-mannosidase and α-fucosidase. However, we noted a difference of production between the strains for esterase(C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase and naphtol-AS-BI-phosphohydrolase (Table 2).

When other substrates were added to prepared mediums, the majority of the strains presented a positive gelatinase and lecithinase; however, 21 strains

presented an amylase activity and 16 strains showed a caseinase activity, whereas, only 4 strains, which had been isolated from gilthead sea bream, had a positive lipase activity (Table 3). An interrelationship exists ($p = 0.015$) between the origin of the strains and the expression of this enzyme.

For DNase production, we noted that 47.1% (16 strains) were positive, and for haemolysin expression, 64.7% of the strains of *Vibrio alginolyticus* were haemolytic for sea bass blood erythrocytes whereas 61.8% were haemolytic for sea bream blood erythrocytes. We also noted that no interrelationship existed between the origin of the strains and the haemolysis of the type of erythrocytes.

### Growth under iron-limiting conditions

To evaluate the ability of *Vibrio alginolyticus* strains to grow in low-iron environments, the MICs of 2,2-dipyrydil were determined. The strains were able to grow in CM9 minimal medium supplemented with 10 µM 2,2-dipyrydil. The MIC of this chelator ranged from 100 to 3000 µM (Table 3).

### Survival in fish serum

The strains of *Vibrio alginolyticus* were tested for their ability to resist the bactericidal activity of gilthead

Table 3. Results of tests for different virulence genes and virulence factors. MIC: minimum inhibitory concentration of 2,2-dipyrydil (µM); SEL: sea bass serum; SED: gilthead sea bream serum; +: positive; –: negative; A: strains that resisted the bactericidal action of the serum and whose numbers remained constant during 4 h test period; B: strains that showed a resistance and were capable of proliferating; C: other strains that showed sensitivity to the serum and decreased percentage survival (see Fig. 1 for trend curve profiles A, B and C, and text for further explanation)

| Strain | Virulence genes | | | | MIC | Survival in fish serum | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *tox*R | *tox*s | *VPI* | *ace* | (µM) | SEL | Profile | SED | Profile |
| *Dicentrarchus labrax* | | | | | | | | | |
| S1 | – | – | – | – | 3000 | + | B | + | B |
| S2 | – | + | – | – | 3000 | + | B | + | B |
| S3 | + | – | – | – | 1300 | + | B | + | B |
| S4 | + | + | + | + | 150 | + | A | + | C |
| S5 | + | – | + | – | 2000 | + | A | + | A |
| S6 | – | – | + | – | 1300 | + | A | + | A |
| S7 | + | + | + | + | 1300 | + | A | + | A |
| S17 | – | + | – | – | 300 | + | B | + | B |
| S18 | – | – | – | – | 1400 | + | A | + | A |
| S19 | + | + | – | – | 1300 | + | A | + | B |
| S23 | – | + | + | + | 1300 | + | A | + | A |
| S24 | + | + | – | – | 2000 | + | A | + | A |
| S25 | + | + | + | – | 150 | + | C | – | – |
| S26 | + | + | + | – | 1300 | + | A | + | A |
| S27 | – | – | – | – | 1300 | + | A | + | A |
| S28 | + | + | – | + | 3000 | + | A | + | A |
| S30 | – | + | – | – | 1300 | + | A | + | A |
| S31 | – | – | – | + | 1600 | + | A | + | A |
| S32 | – | – | – | – | 1300 | + | A | + | A |
| S36 | – | – | – | – | 1300 | + | A | + | A |
| *Sparus aurata* | | | | | | | | | |
| S38 | + | – | – | + | 2000 | + | A | + | A |
| S8 | + | + | – | – | 1400 | + | A | + | A |
| S9 | – | – | – | – | 800 | + | A | + | A |
| S10 | + | + | + | – | 100 | + | A | + | A |
| S11 | + | + | + | – | 100 | + | A | + | A |
| S12 | – | – | – | – | 100 | + | A | + | A |
| S13 | + | + | + | – | 800 | + | A | + | A |
| S14 | + | + | – | – | 3000 | + | A | + | A |
| S15 | – | – | – | – | 800 | + | A | + | A |
| S16 | + | + | + | – | 800 | + | A | + | A |
| S20 | – | – | – | – | 100 | + | A | + | A |
| S21 | – | – | – | + | 100 | + | A | + | A |
| S22 | – | – | – | – | 100 | + | A | + | A |
| S34 | – | – | + | – | 1600 | + | A | + | A |

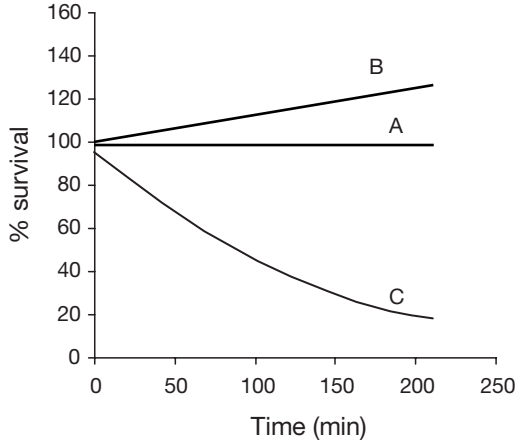


Fig. 1. *Vibrio alginolyticus*. Tendency curves of survival of 34 strains in the serum of sea bass and gilthead sea bream. Trend curve Profiles A, B and C are described in Table 3

sea bream and sea bass natural serum in a plate plaque assay. Only 2 strains isolated from sea bass (S25 and S4) were found to be sensitive. The survival tendency curves of the strains of *V. alginolyticus* in the serum of the 2 fishes are shown in Fig. 1. The equations of the curves were determined from the percentage survival of the 34 strains *V. alginolyticus* during 4 h incubation in sera. The strains showed 3 different response profiles: (A) strains that resisted the bactericidal action of the serum and their number remained constant during the 4 h period; (B) strains that showed a resistance and were capable of proliferating; and (C) other strains that showed sensitivity to the serum and whose percentage survival decreased (Fig. 1, Table 3).

The majority of strains, regardless of origin, can survive in the sera of the sea bass and gilthead sea bream and adopt Profile A or B (Fig. 1). Only 60% of cells from strain S25 were resistant to the serum of the sea bass and none the cells survived in the serum of sea bream, with the cell number falling towards 0 in the first 30 min of contact with the serum. The strain S4 were resistant to a level of 53% of original cell number in the serum of gilthead sea bream adopting the Profile C and was unaffected by sea bass serum. To see whether the bactericidal effect of the serum of sea bream was due to the effect of the complement, we deactivated this serum by heat and incubated the bacteria (S25) again in this serum; we noted that these bacteria had 100% survival.

### Distribution of virulence genes

The PCRs of 4 virulence genes (*toxS*, *toxR*, *VPI* and *ace*) were made for the 34 *Vibrio alginolyticus* strains. The expected PCR product for *toxS* was amplified from the chromosomes of 17 of the 34 *V. alginolyticus* strains analysed (Fig. 2, Table 3). Sixteen strains were positive for amplification using the *toxR* primers (Fig. 3, Table 3). Presence of the VPI was also tested and 12 *V. alginolyticus* strains generated the amplification product of the size expected, as shown in (Fig. 2, Table 3). The virulence gene *ace* was amplified by PCR using specific primers previously reported (Colombo et al. 1994). Only 7 *V. alginolyticus* produced the amplification product of the expected size for the *ace* gene. Only strains S4 and S7 gave amplification products for the 4 virulence genes.

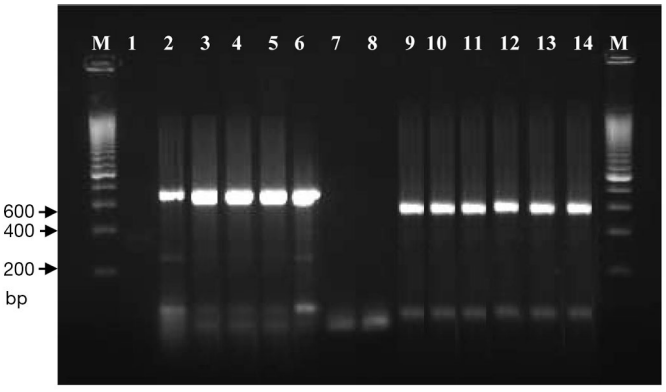


Fig. 2. *Vibrio alginolyticus*. Agarose gel electrophoresis of 1% agarose of the amplification products of isolates obtained with PCR for the *VPI* (Lanes 2–8) and PCR for the *toxS* (Lanes 9–14). Lane 1: negative control; Lane 2: S4; Lane 3: S5; Lane 4: S6; Lane 5: S7; Lane 6: S10; Lane 7: S27; Lane 8: S28; Lane 9: S19; Lane 10: S23; Lane 11: S24; Lane 12: S25; Lane 13: S26; Lane 14: S8; M: molecular weight marker 200 bp (Promega). Table 3 shows virulence test result strains listed above

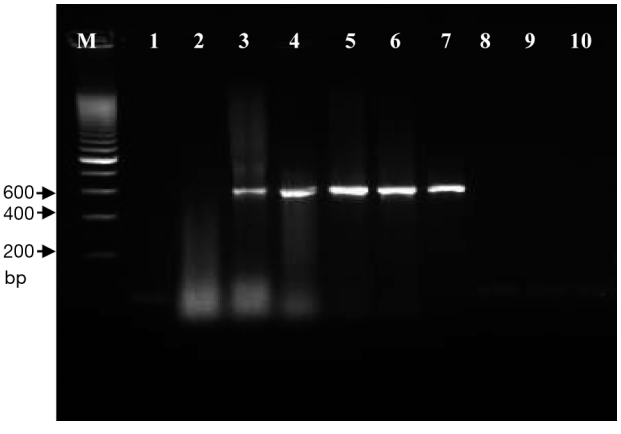


Fig. 3. *Vibrio alginolyticus*. Agarose gel electrophoresis of 1% agarose of the amplification products of *toxR* gene. Lane 1: negative control; Lane 2: S20; Lane 3: S3; Lane 4: S4; Lane 5: S5; Lane 6: S24; Lane 7: S25; Lane 8: S21; Lane 9: S22; Lane 10: S34; M: molecular weight marker 200 bp (Promega). Table 3 shows virulence test result strains listed above

## DISCUSSION

Vibriosis has been a particularly devastating disease in the marine environment, affecting many species of fish. Although most studies have focused on *Vibrio anguillarum* as an etiological agent, other members of the genus *Vibrio*, such as *V. alginolyticus*, have also been implicated in epizootics of cultured and wild marine fishes throughout the world as well as with disease in larval and juvenile bivalve molluscs and crustaceans (Zorilla et al. 2003a, Xie et al. 2005, Cai et al. 2006, Jayaprakash et al. 2006). The knowledge of virulence properties of this emergent species could be useful for designing adequate prophylactic and antimicrobial treatments.

Adhesion and hydrolytic activities are essential factors for the infection and disease symptoms of *Vibrio alginolyticus* (Balebona et al. 1998a). Hydrolytic activities have been considered virulence factors because they allow bacteria to survive, proliferate and invade host tissues. Proteolytic activities of extracellular products have been correlated with the pathogenicity of many other *Vibrio* and *Aeromonas* species (Esteve et al. 1993, Aguirre-Guzmán et al. 2004). Our results indicated that most of the isolates of *V. alginolyticus* in the present study produced gelatinase, lipase, DNase and caseinase, all probable virulence factors that might help the organisms to invade and proliferate. Recently, Wang et al. (2007) demonstrated that secretion of extracellular protease and haemolytic activity material as well as siderophore are under the regulation of LuxO and σ54, and the potential quorum sensing systems in *V. alginolyticus.*

A comprehensive search for virulence factors among vibrios by many workers has unequivocally revealed the role of plasmids coding for iron chelators, apart from haemolysins, in initiating infectious mortality (Toranzo et al. 1983, Zorilla et al. 2003b). Survival and proliferation of the pathogen in the host should be enhanced by the ability of the microrganism to obtain iron from chelated sources. Our results showed that all isolated strains of *Vibrio alginolyticus* grew under iron-limiting conditions, and the MICs of 2,2-dipyrydil were higher for many strains (21 strains > 1000 µM) (Table 3), which indicated that there were greater levels of siderophore activity or higher iron affinities in these strains.

Resistance to the bactericidal mechanisms of normal serum appears to be an important contributor to the virulence of fish-pathogenic vibrios (Amaro et al. 1997). Complement activity in fish serum is known to play an important role in the defence against bacterial pathogens (Ellis 1989). Isolated *Vibrio alginolyticus* showed a bactericidal resistance to the sea bass and gilthead sea bream sera. Bacterial resistance to complement-mediated killing by either of the 2 pathways may occur because the bacterium avoids initiating complement activation or because activated complement fails to damage the bacterium (Muschel & Larsen 1970). Smooth strains of gram-negative bacteria carry long polysaccharide side chains (the O antigen) in their lipopolysaccharide (LPS) structures. They are more resistant to serum complement-mediated killing than rough strains, which lack the O-antigen side chains (Muschel & Larsen 1970). The LPS structure of gram-negative bacteria, which functions as a molecular and physical barrier for the cell, may thus influence the bactericidal effect of the complement system and cause resistance to serum killing (Tomás et al. 1986, Boesen et al. 1999).

Recent studies reported the wide dissemination of different virulence genes such as the genes homologous to those of *Vibrio parahaemolyticus* and *V. cholerae* among environmental *Vibrio* species (*V. parahaemolyticus*, *V. cholerae* non-O1, *V. mimicus*, *V. hollisae*, *V. fluvialis* and *V. alginolyticus*) (Nishibuchi et al. 1996, Sechi et al. 2000, Xie et al. 2005). The mobility of virulence genes leads to new pathogenic strains by successful transfer (Hentschel et al. 2000, Faruque & Nair 2002).

Our results indicate that most of the *Vibrio alginolyticus* strains isolated from diseased gilthead sea bream and sea bass possess homologues to some *V. cholerae* virulence genes such as *toxR*, *toxS*, *VPI* or *ace*. These data support those of Xie et al. (2005) and their suggestion that *V. alginolyticus* may be an important reservoir of many known virulence genes of other *Vibrio* species in the aquatic environment and may explain the emergence of this species as a new pathogenic strain. The genes *toxR* and *toxS* not only regulate expression of the virulence factor genes (*ctx* and *tcp* in *V. cholerae*), but also regulate 'housekeeping' genes; this partly explains the large presence of these genes in the different *Vibrio* strains (Sechi et al. 2000).

The results of lethal concentration ($LD_{50}$) tests obtained previously (Ben Kahla-Nakbi et al. 2006) showed that the $LD_{50}$ values ranged from $5.01 \times 10^4$ to $6.20 \times 10^7$ CFU fish$^{-1}$. According to these results a correlation could be seen between virulence genes and pathogenicity for strains S7, S8, S10 and S19. However, strains S36 and S22 were pathogenic to fish, but they did not possess any detectable virulence gene. Thus, the pathogenicity seems to be a strain-specific expression in *Vibrio alginolyticus* strains. Baffone et al. (2005) reported that the high diversity of strains belonging to the same species may explain the strain-specific expression of virulence factors and, consequently, the different data obtained from various geographic areas.

However, we did not find any correlation between the expression of certain virulence factors and the pos-

session of the studied virulence genes. Xie et al. (2005) also reported that no correlation between the presence and absence of the virulence genes used to investigate *Vibrio alginolyticus* and its virulent strains. This suggests that the pathogenicity mechanism of *V. alginolyticus* might be different from other pathogenic *Vibrio* species or the virulence may be caused by multivirulent factors.

In conclusion, *Vibrio alginolyticus* may posses a specific pathogenic mechanism for sea bass and gilthead sea bream that requires the presence of other, as yet unexamined, virulence factors. Although adhesion and hydrolytic activities are essential factors for the infection and disease symptoms, virulent strains possess at least 2 others important properties that presumably enable them to proliferate in a non-immune host. They are able to resist the bactericidal activity of normal serum and have a high-affinity iron-sequestering system that allows growth under conditions of iron limitation. Though the possession of virulence genes does not directly reflect the pathogenicity of the strains in the present study, the expression of these genes must be investigated under natural conditions.

## LITERATURE CITED


Aguirre-Guzmán G, Mejia Ru›z H, Ascencio F (2004) A review of extracellular virulence product of *Vibrio* species important in diseases of cultivated shrimp. Aquac Res 35: 1395–1404

Amaro C, Fouz B, Biosca EG, Macro-Noales E, Collado R (1997) The lipopolysaccharide O side chain of *Vibrio vulnificus* serogroup E is a virulence determinant for eels. Infect Immun 65:2475–2479

Baffone W, Vittoria E, Campana R, Citterio B, Casaroli A, Perfelici L (2005) Occurence and expression of virulence related properties by environmental *Vibrio* spp. in *in vitro* and *in vivo* systems. Food Control 16:451–457

Bai F, Pang L, Qi Z, Chen J, Austin B, Zhang XH (2008) Distribution of five *Vibrio* virulence-related genes among *Vibrio harveyi* isolates. J Gen Appl Microbiol 54:71–78

Balebona MC, Andreu MJ, Bordas MA, Zorrilla I, Moriñigo MA, Borrego JJ (1998a) Pathogenicity of *Vibrio alginolyticus* from cultured gilt-head sea bream (*Sparus aurata* L.). Appl Environ Microbiol 64:4269–4275

Balebona MC, Zorrilla I, Moriñigo MA, Borrego JJ (1998b) Survey of bacterial pathologies affecting farmed gilt-head sea bream (*Sparus aurata* L.) in southwestern Spain from 1990 to 1996. Aquaculture 166:19–35

Ben Kahla-Nakbi A, Chaieb K, Besbes A, Zmantar T, Bakhrouf A (2006) Virulence and enterobacterial repetitive intergenic consensus PCR of *Vibrio alginolyticus* strains isolated from Tunisian cultured gilthead sea bream and sea bass outbreaks. Vet Microbiol 117:321–327

Ben Kahla-Nakbi A, Besbes A, Bakhrouf A, Alcaide E (2007) Characterization and virulence properties of *Vibrio* strains isolated from diseased gilthead sea bream (*Sparus aurata*) cultured in Tunisia. Bull Eur Assoc Fish Pathol 27:90–99

Boesen HT, Pedersen K, Larsen JL, Koch C, Ellis AE (1999) *Vibrio anguillarum* resistance to rainbow trout (*Oncorhynchus mykiss*) serum: role of O-antigen structure of lipopolysaccharide. Infect Immun 67:294–301

Cai J, Han H, Song Z, Li C, Zhou J (2006) Isolation and characterization of pathogenic *Vibrio alginolyticus* from diseased postlarval abalone, *Haliotis diversicolor supertexta* (Lischke). Aquac Res 37:1222–1226

Colombo MM, Mastrandrea S, Santona A, de Andrade AP, Uzzau S, Rubino S, Cappuccinelli P (1994) Distribution of the *ace, zot* and *ctxA* Foxin genes in clinical and environmental *Vibrio cholerae.* J Infect Dis 170:750–751

Ellis AE (1989) The immunology of teleosts. In: Roberts RJ (ed) Fish pathology. Bailliere-Tindall, London, p 135–152

Esteve C, Biosca EG, Amaro C (1993) Virulence of *Aeromonas hydrophila* and some other bacteria isolated from European eels *Anguilla anguilla* reared in fresh water. Dis Aquat Org 16:15–20

Faruque SM, Nair GB (2002) Molecular ecology of toxigenic *Vibrio cholerae.* Microbiol Immunol 46:59–66

Gómez-León J, Villamil L, Lemos ML, Novoa B, Figueras A (2005) Isolation of *Vibrio alginolyticus* and *Vibrio splendidus* from aquacultured carpet shell clam (*Ruditapes decussatus*) larvae associated with mass mortalities. Appl Environ Microbiol 71:98–104

Hentschel U, Stelnert M, Hacker J (2000) Common molecular mechanisms of symbiosis and pathogenesis. Trends Microbiol 8:226–231

Jayaprakash NS, Pai SS, Philip R, Singh SB (2006) Isolation of a pathogenic strain of *Vibrio alginolyticus* from necrotic larvae of *Macrobrachium rosenbergii* (de Man). J Fish Dis 29:187–191

Karaolis DK, Johnson JA, Bailey CC, Boedeker EC, Kaper JB, Reeves PR (1998) A *Vibrio cholerae* pathogenicity island associated with epidemic and pandemic strains. Proc Natl Acad Sci USA 95(6):3134–3139

Lamas J, Santos I, Bruno D, Toranzo AE, Anderson R (1994) A comparison of pathological changes caused by *Vibrio anguillarum* and its extracellular products in rainbow trout (*Oncorynchus mykiss*). Fish Pathol 29:79–89

Liu PV (1957) Survey of hemolysin production among species of *Pseudomonas.* J Bacteriol 74:718–727

Masini L, De Grandis G, Principi F, Mengarelli C, Ottaviani D (2007) Research and characterization of pathogenic vibrios from bathing water along the Conero Riviera (Central Italy). Water Res 41:4031–4040

Muschel LH, Larsen LJ (1970) The sensitivity of smooth and rough Gram-negative bacteria to the immune bactericidal reaction. Proc Soc Exp Biol Med 133:345–348

Nishibuchi M, Janda JM, Ezaki T (1996) The thermostable direct hemolysin gene (tdh) of *Vibrio hollisae* is dissimilar in prevalence to and phylogenetically distant from the tdh genes of other vibrios: implications in the horizontal transfer of the tdh gene. Microbiol Immunol 40:59–65

Sechi LA, Dupre I, Deriu A, Fadda G, Zanetti S (2000) Distribution of *Vibrio cholerae* virulence genes among different *Vibrio* species isolated in Sardinia, Italy. J Appl Microbiol 88:475–481

Tomás JM, Benedí VJ, Ciurana B, Jofre J (1986) Role of capsule and O antigen in resistance of *Klebsiella pneumoniae* to serum bactericidal activity. Infect Immun 54:85?89

Toranzo AE, Barja JL, Colwell RR, Hetrick FM (1983) Characterization of plasmids in bacterial fish pathogens. Infect Immun 39:184–192

Wang Q, Liu Q, Ma Y, Rui H, Zhang Y (2007) LuxO controls extracellular protease, haemolytic activities and siderophore production in fish pathogen *Vibrio alginolyticus.* J Appl Microbiol 103:1525–1534

Waldor MK, Mekalanos JJ (1996) Lysogenic conversion by

a filamentous phage encoding cholera toxin. Science 272:1910–1914

Wong SM, Carroll PA, Rahme LG, Ausubel FM, Calderwood SB (1998) Modulation of expression of the ToxR regulon in *Vibrio cholerae* by a member of the two-component family of response regulators. Infect Immunol 66:5854–5861

Xie ZY, Hu CQ, Chen C, Zhang LP, Ren CH (2005) Investigation of seven *Vibrio* virulence genes among *Vibrio alginolyticus* and *Vibrio parahaemolyticus* strains from the coastal mariculture systems in Guangdong, China. Lett Appl Microbiol 41:202–207

Zorrilla I, Chabrillón M, Arijo S, Díaz-Rosales P, Martínez-Manzanares E, Balebona MC, Moriñigo MA (2003a) Bacteria recovered from diseased cultured gilthead sea bream (*Sparus aurata* L.) in southwestern Spain. Aquaculture 218:11–20

Zorrilla I, Moriñigo MA, Castro D, Balebona MC, Borrego JJ (2003b) Intraspecific characterization of *Vibrio alginolyticus* isolates recovered from cultured fish in Spain. J Appl Microbiol 95:1106–1116

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