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Characterization of *Vibrio alginolyticus* Isolates from Diseased Cultured Gilthead Sea Bream, *Sparus aurata*

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Abstract

Biochemical, pathogenical, antigenical, and molecular characteristics of *Vibrio alginolyticus* isolates from diseased cultured gilthead sea bream juveniles (4 and 20 g) were determined by laboratory challenge experiments. Intraperitonal challenge resulted in development of the disease but immersion did not. Electrophoresis of outer membrane protein (OMP) and lipopolysaccharides (LPS) was performed using sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE). Molecular typing of *Vibrio* isolates was conducted using random amplification of polymorphic DNA (RAPD). According to SDS-PAGE of surface antigens of the isolates, profiles of both OMP and LPS were heterogeneous. According to RAPD, isolated bacteria varied genetically. The findings reveal differences among *V. alginolyticus* isolates from diseased cultured gilthead sea bream in Turkey.

Introduction

Vibriosis is the most significant disease of cultured sea bream in the Mediterranean (Colorni et al., 1981; Paperna, 1984; Balebona et al., 1998a) and Aegean (Akayli and Timur, 2002, 2004) areas. The widely distributed *Vibrio alginolyticus* is an important pathogen that causes severe vibriosis in cultured gilthead sea bream, *Sparus aurata* L. (Colorni et al., 1981; Balebona et al., 1998a; Zorrilla et al., 2003). However, many strains of *V. alginolyticus* are avirulent and could be used as probiotic strains (Austin et al., 1995). As wide variation exists among the strains, differentiating between harmful and beneficial strains would help to find ways to control infection.

Biochemical characteristics and outer membrane protein (OMP) profiling are valuable methods for typing and differentiating

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between bacterial fish pathogens. Lipopolysaccharides (LPS) and OMP are thought to relate to antigenic determinants in gram-negative bacteria (Zorrilla et al., 2003).

Specific, sensitive, and rapid methods for detecting and identifying pathogenic microorganisms are needed to control bacterial infections in intensive fish culture. Random amplification of polymorphic DNA (RAPD) can be used to compare microorganisms at the interspecies and intraspecies levels with high discrimination (Welsh and McClelland, 1990). RAPD has been used for intraspecies characterization of *Vibrio harveyi* (Pujalte et al., 2003), *V. parahaemolyticus* (Najiah et al., 2003), and *V. vulnificus* (Gutacker et al., 2003) isolated from diseased fish.

The few reports on *V. alginolyticus* infection in cultured gilthead sea bream in Turkey focus on the isolation and identification of the etiological agent (Akayli and Timur, 2002, 2004). Some studies concentrate on the intraspecific characterization of *V. alginolyticus* strains isolated from cultured sea bream (Balebona et al., 1998b; Zorrilla et al., 2003). However, no attention has been given to the molecular characterization of the bacteria. In the present work, we applied several typing methods to twelve strains of *V. alginolyticus* isolated from different epizootic outbreaks affecting cultured gilthead sea bream in Turkey and compared those strains with five reference strains.

Materials and Methods

Bacteria. Bacteria were isolated from gilthead sea bream showing clinical signs of vibriosis. The fish were collected from different farms along the southwestern coast of Turkey. The biochemical, pathogenical, antigenical, and molecular properties of twelve bacteria isolates were examined and compared to five representative reference strains (Table 1).

Biochemical analyses. The isolates were recovered on TSA (T-TSA) and TSB (T-TSB) supplemented with 1% NaCI for 2 days at 22°C. The physiological and biochemical characterization of the isolates and the reference strains was carried out using conventional bacteriological methods (Alsina and Blanch, 1994; Holt et al., 1994) and API 20E and API 50CH test kits (MacDonell et al., 1982). Phenotypes of the isolates were compared using approximately 40 morphological, phenotypical, and biochemical tests described by Alsina and Blanch (1994), Holt et al. (1994), and Austin and Austin (1999).

Table1. Sources of reference strains used in this study.

Bacteria	*Source*
V. alginolyticus	CECT 521[a]
V. damsela (=*Photobacterium damselae)*	CECT 626[a]
V. ordalii	CECT 582[a]
V. anguillarum (=*Listonella anguillarum)* serotype 01	IMR[b]
V. anguillarum serotype 02	IMR[b]

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Pathogenicity assay. Four isolates were tested for pathogenicity in sea bream juveniles. For each isolate, two sizes of 30 fish each were challenged. The first size (avg 4 g) was challenged by bacterial suspension in a bath with approximately 10^7 cells/ml for 60 min. The second (avg 20 g) was injected intraperitonally with the same bacterial dose. There were two replicates of each challenge, a total of 480 fish.

Electrophoresis. Outer membrane protein (OMP) and lipopolysaccharides (LPS) profiles of four of the twelve Turkish isolates were analyzed by electrophoresis using sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE) in a Mini-Protein II unit (BioRad). OMP and LPS from bacteria cultures of the isolates were obtained in tryptic soy broth (TSB) supplemented with 1.5% NaCI (TSAS) after 24 h incubation at 22°C.

LPS samples were prepared by Proteinase K digestion of whole-cell lysates as described by Hitchcock and Brown (1983). They were fractionated by 18% SDS-PAGE (Laemmli, 1970) and visualized with Silver Stain Plus (Merck-159437) following the manufacturer's instructions. OMP was determined using the method described by Arda and Ertan (2004). The samples were separated by 12.5% SDS-PAGE (Laemmli, 1970). The gels were fixed and stained with 0.1% Coomassie brilliant blue (Merck-112553).

Random amplification of polymorphic DNA. RAPD was used to characterize the bacteria to the genotype level. Genomic DNA was extracted using the High Pure PCR Template Preparation Kit (Roche Cat. No.1796828). The reaction procedure was modified from Sudheesh et al. (2002), Valle et al. (2002), and Najiah et al. (2003). Two randomly designed 10-mer oligonucleotide primers (Operon Technologies CA/USA), OPC-05 (5' GATGACCGCC 3') and OPC-19 (5' GTTGCCAGCC 3'), were used for amplification. These primers were preliminarily tested in PCR cycles at different temperatures. PCR reactions were run in a PHC-3 thermocycler (Techno, Princeton, N.J.), using the following program: denaturation step at 94ºC for 5 min, 45 cycles consisting of 94ºC for 1 min, 34ºC for 1 min, and 72ºC for 2 min, and a final cycle consisting of 72ºC for 15 min. The RAPD products were electrophoresized using a Fisher Biotech Small Horizontal Gel System.

Results

Biochemical analyses. All the isolates shared the same general characteristics and were classified as typical *V. alginolyticus* (Table 2). All had gram-negative rods and were motile, sensitive to vibriostat (O/129), cytochrome oxidase positive, and fermentative by O/F glucose test. They all produced acid from sucrose, grew at 37°C, and swarming growth developed on the surface of the solid medium (TSA). Some isolates responded negatively to ornithine decarboxylase, indol production, and citrate reaction.

Pathogenicity assay. The bath challenge did not lead to development of the disease while intraperitonal injection did cause vibriosis. Infected fish were moribund seven days after injection. Their skin was dark with superficial lesions and their abdomens were swollen with ascetic fluid.

Electrophoresis. SDS-PAGE analysis of the four isolates showed that the structures of LPS and OMP were heterogeneous (Fig. 1). The major OMP had molecular masses ranging 33-37 kDa while LPS bands ranged 20-34 kDa.

Random amplification of polymorphic DNA. Using two primers, the PCR amplification technique successfully differentiated between the twelve *Vibrio* strains, as validated by RAPD. The RAPD-PCR profiles of the four strains showed genetic variation (Fig. 2).

Discussion

The phenotypic identification of genera and species of *Vibrionaceae* is problematic, mainly because of great variability of diagnostic phenotypic features such as arginine dihydrolase, indole production, and carbon utilization (Austin and Lee, 1992; Austin et al., 1997). Our isolates had similar biochemical profiles and were all typed as *V. alginolyticus*. There was a close relationship between our *V. alginolyticus* isolates and those described by Alsina and Blanch (1994), Holt et al. (1994), and Balebona et al. (1998a). Some of our strains responded negatively to ornithine decarboxylase, indol production, and citrate reaction. In general, however, our results showed that the biochemical profile allows a low degree of discrimination among isolates.

Our pathogenicity results were similar to those of Balebona et al. (1998ab) and Zorrila et al. (2003), i.e., *V. alginolyticus* is an important pathogen for gilthead sea bream. While Paperna (1984) isolated strains of *V. alginolyticus* from this fish, he did not perform pathogenicity studies.

There were several differences in OMP profiles, as reported by Zorrilla et al. (2003). Likewise, the LPS profiles were heterogeneous. Although we have no other reports on LPS profiles with which to compare, we conclude that SDS-PAGE and silver staining of purified LPS is a useful tool for determining antigenical properties of isolates.

Table 2. Selected biochemical characteristics of twelve *Vibrio alginolyticus* isolates and one reference strain.

Characteristic	*Isolate no.*												*Reference*
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*CECT 521*
Swarming colonies	+	+	+	+	+	+	+	+	+	+	+	+	+
H_2S	+	+	+	v	+	+	+	+	+	+	+	+	+
Arginine dihydrolase	-	-	-	-	-	-	-	-	-	-	-	-	-
Lysine decarboxylase	+	+	+	+	+	v	+	+	+	+	+	+	+
Ornithine decarboxylase	+	-	+	-	+	-	-	+	+	-	+	+	+
Indole	+	-	+	+	-	-	+	+	+	-	+	+	+
Hydrolysis of aesculine	-	-	-	-	+	-	-	-	-	-	-	-	-
Methyl red test	+	+	+	+	+	+	+	+	+	+	+	+	+
Voges-Proskuer	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate	+	+	+	-	+	+	-	+	+	+	-	+	+
Urea	+	+	+	+	+	+	v	+	+	+	+	+	+
Acid from salicin	+	v	+	+	+	+	+	+	+	+	+	+	-
Growth on 0% (w/v) sodium chloride	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth on 7% (w/v) sodium chloride	+	+	+	+	+	+	+	+	+	+	+	+	+

+ = possesses characteristic, - = lacks characteristic, v = variable results

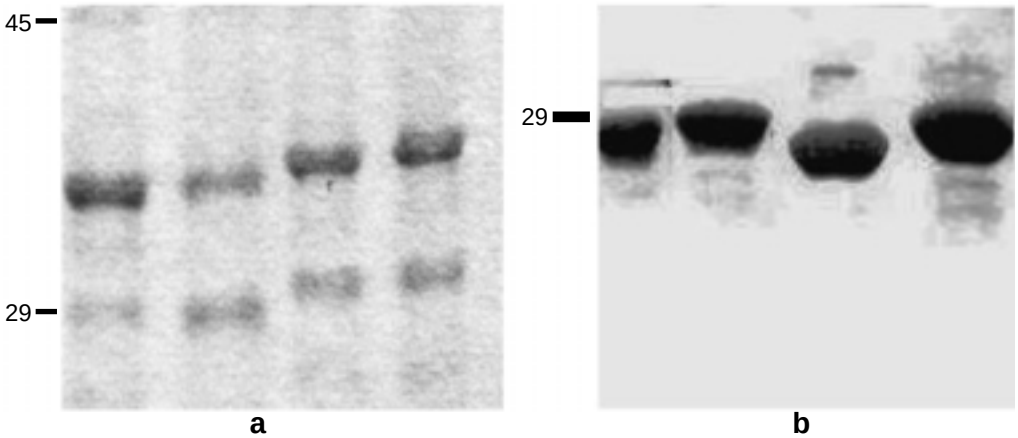

Fig. 1. SDS-PAGE of (a) outer membrane protein (OMP) and (b) lipopolysaccharides (LPS) from four *Vibrio alginolyticus* strains isolated from diseased gilthead sea bream cultured in Turkey.

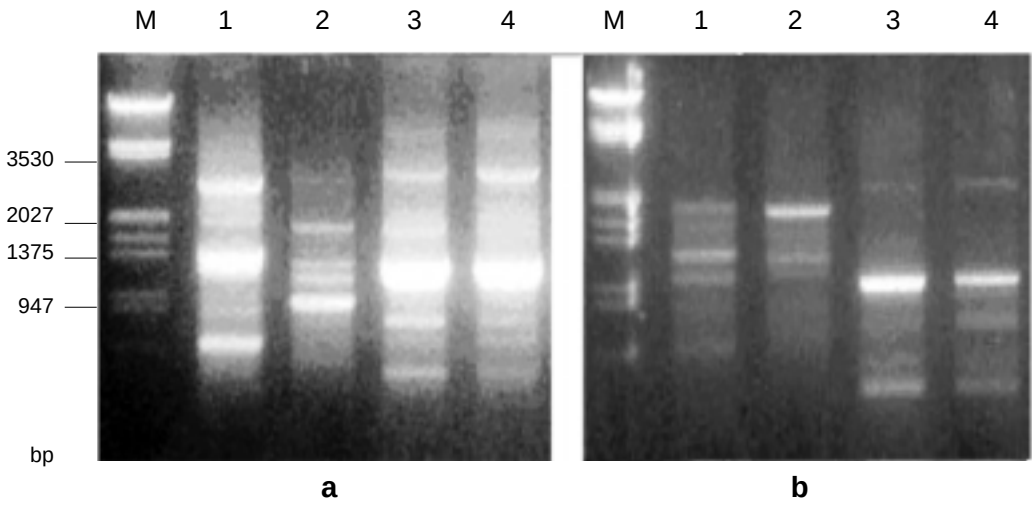

Fig. 2. RAPD-PCR profiles of four strains of *Vibrio alginolyticus* (lanes 1-4) generated after amplification with primers (a) OPC-05 and (b) OPC-19, compared to a DNA molecular mass marker, λDNA/EcoRI+HindIII (lane M).

Varying genetic profiles were obtained from RAPD analysis in our study, similar to the findings of Sudheesh et al. (2002) for *V. alginolyticus* isolates from cultured shrimp. Similarly, there were genetic variations in *V. harveyi* (Pujalte et al., 2003), *V. parahaemolyticus* (Najiah et al., 2003), and *V. vulnificus* (Gutacker et al., 2003) isolated from diseased fish.

In conclusion, the isolates of *V. alginolyticus* were phenotypically and antigenically similar, but molecularly heterogeneous. RAPD was the most discriminating typing method, although biochemical and antigenical profiles were complementary for intraspecific characterization of *V. alginolyticus* isolates in gilthead sea bream.

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