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Pathogenicity of *Vibrio alginolyticus* for Cultured Gilt-Head Sea Bream (*Sparus aurata* L.)

M. CARMEN BALEBONA,[1] MANUEL J. ANDREU,[2] M. ANGELES BORDAS,[1] IRENE ZORRILLA,[1] MIGUEL A. MORIÑIGO,[1] AND JUAN J. BORREGO[1]*

Departments of Microbiology[1] and Cell Biology,[2] Faculty of Sciences, University of Malaga, Campus de Teatinos, 29071 Malaga, Spain

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The in vivo and in vitro pathogenic activities of whole cells and extracellular products of *Vibrio alginolyticus* for cultured gilt-head sea bream were evaluated. The 50% lethal doses ranged from 5.4×10^4 to 1.0×10^6 CFU/g of body weight. The strains examined had the ability to adhere to skin, gill, and intestinal mucus of sea bream and to cultured cells of a chinook salmon embryo cell line. In addition, the in vitro ability of *V. alginolyticus* to adhere to mucus and skin cells of sea bream was demonstrated by scanning electron microscopy. The biological activities of extracellular products of *V. alginolyticus* were hydrolytic activities; the products were able to degrade sea bream mucus. *V. alginolyticus* was cytotoxic for fish cell lines and lethal for sea bream. Moreover, the extracellular products could degrade sea bream tissues. However, experiments performed with the bath immersion inoculation technique demonstrated that *V. alginolyticus* should be considered a pathogen for sea bream only when the mucus layer is removed and the skin is damaged.

Gilt-head sea bream (*Sparus aurata* L.) is a marine fish with high economic value in the aquaculture industry of southern Europe. Several pathogenic microorganisms have been isolated from outbreaks affecting this fish species (5, 41, 52). One of these organisms, *Vibrio alginolyticus*, is frequently involved in epizootic outbreaks in cultured gilt-head sea bream in Mediterranean aquaculture, causing fish mortality and important economic losses (5, 15, 41). The natural disease caused by *V. alginolyticus* in sea bream includes the following symptoms: septicemia, hemorrhaging, dark skin, and ulcers on the skin surface in some cases. Internally, fish accumulate fluid in the peritoneal cavity and in some cases have hemorrhagic livers (5, 15).

V. alginolyticus, a ubiquitous organism in seawater, has been isolated from different marine organisms as part of the saprophytic microbiota (13). However, it has also been suggested that this species is a pathogen of several marine animals and humans (8, 29, 43). There is controversy about the precise role of *V. alginolyticus* as a fish pathogen (5). This species has been reported to be the causal agent of outbreaks of vibriosis in grouper (29) and sea bream (15) and also has been associated with other *Vibrio* species in high-mortality outbreaks related to abdominal swelling in larvae of several fish species (28, 37, 48). There is extensive knowledge concerning other fish-pathogenic *Vibrio* species, but the epidemiological, physiological, and virulence characteristics of *V. alginolyticus* have not been established yet. Knowledge of these characteristics should be very useful for designing adequate prophylactic and antimicrobial treatments.

The aim of this study was to characterize the virulence potential of *V. alginolyticus* strains responsible for outbreaks that lead to mortality in cultured gilt-head sea bream.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Eight *V. alginolyticus* strains were isolated from cultured gilt-head sea bream showing symptoms of disease, such as hemorrhagic fins and ulcers associated with high mortality. Two reference strains of *V. alginolyticus* (CECT 521 and ATCC 17749) were also included in the study.

Bacterial strains were cultured on tryptone soya agar and in tryptone soya broth (Oxoid Ltd., Basingstoke, Hampshire, England) supplemented with 1.5% NaCl (saline tryptone soya agar and saline tryptone soya broth, respectively) and were incubated at 22°C for 24 to 48 h.

Bacterial strains isolated from diseased sea bream were identified biochemically by using the scheme described by Holt et al. (24). This scheme included 69 morphological, physiological, and biochemical tests (5), which were performed by the methods described by Smibert and Krieg (49). The *V. alginolyticus* strains tested exhibited high levels of homogeneity in their biochemical patterns; this was true for both strains isolated from fish and the *V. alginolyticus* culture collection strains used (ATCC 17749 and CECT 521). All of the strains were gram-negative motile rods that were oxidase positive, swarmed on saline tryptone soya agar plates, and were fermentative, arginine dihydrolase negative, lysine decarboxylase positive, and sensitive to vibriostatic agent O/129. Only slight differences were observed in the following tests: hydrolysis of esculin, utilization of D-sorbitol, growth in the presence of 8 and 10% sodium chloride, and sensitivity to erythromycin, kanamycin, amikacin, tobramycin, and gentamicin.

Extraction and characterization of extracellular products. Bacterial extracellular products were obtained by the technique described by Liu (32). Briefly, tubes containing 5 ml of saline tryptone soya broth were inoculated with one colony from a 24-h culture on saline tryptone soya agar and incubated at 22°C. A 0.2-ml portion of the culture was spread onto a sterile cellophane sheet overlaying a saline tryptone soya agar plate and incubated at 22°C for 48 h. Bacterial cells were harvested with a saline solution (pH 7), and the cell suspensions were centrifuged at $13,000 \times g$ and 4°C for 20 min. The supernatants were filtered through 0.45- and 0.2-μm-pore-size membrane filters and used as the crude extracellular product preparations. A number of enzymatic activities of the extracellular products were evaluated with the API ZYM system (BioMerieux, Madrid, Spain). Proteolytic, collagenolytic, lipolytic, phospholipolytic, amylolytic, and hemolytic activities were assayed on agarose plates (0.8% agarose in 0.1 M phosphate-buffered saline [PBS], pH 7) containing one of the following substrates: 1% (wt/vol) gelatin, 2% (wt/vol) skim milk, 1% (wt/vol) collagen, 1% (vol/vol) egg yolk, 5% blood, and 0.4% (wt/vol) starch. Elastolytic activity was tested by using the protocol described by Kothary and Kreger (26). Portions (20-μl) of extracellular product samples were inoculated into 2.5-mm-diameter wells in the plates. The total protein contents of the extracellular product samples were determined by the method described by Bradford (10) by using bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) as the standard.

Cytotoxicity tests. The toxicities of extracellular products were tested by using the following three fish cell lines: fathead minnow (FHM), chinook salmon embryo (CHSE-214), and *epithelioma papullosum cyprini* (EPC). The cells were grown as monolayers in 24-well culture plates (Nunc, A/S, Kamstrup, Roskilde, Denmark) at 18°C by using Eagle's minimum essential medium (Sigma) supplemented with 10% fetal calf serum (Sigma); the medium was inoculated with 0.1-ml serial dilutions of extracellular product samples. Cells inoculated with a saline solution were used as negative controls. Microtiter plates were incubated at 18°C, and the effects of extracellular products on cell monolayers were observed after 1, 6, and 24 h.

* Corresponding author. Mailing address: Department of Microbiology, Faculty of Sciences, University of Malaga, Campus de Teatinos, 29071 Malaga, Spain. Phone: 34-5-2131893. Fax: 34-5-2132000. E-mail: jjborrego@ccuma.uma.es.

TABLE 1. LD_{50}s of *V. alginolyticus* strains, abilities to grow under iron-limiting conditions, and hydrolytic and cytotoxic activities of the extracellular products

Strain	LD_{50} (CFU/g of fish)	CAS agar ratio[a]	MIC of EDDHA (μM)	Hydrolytic activities						Cytopathic effects on:		
				Caseinase	Gelatinase	Elastase	Collagenase	Phospholipase	Amylolytic	CHSE-214 cells	EPC cells	FHM cells
CAN	5.4×10^4	1.1	1,000	+	+	−	+	+	+	+	+	+
AO35	6.2×10^4	1.3	1,000	+	+	−	+	+	+	+	+	+
DP1-HE-4	2.0×10^5	1.1	1,000	+	+	−	+	+	+	+	+	+
16	1.0×10^6	1.1	800	+	+	−	+	+	+	+	+	+
110	1.0×10^5	1.1	1,000	+	+	−	+	+	+	+	+	+
121	NT[b]	1.1	1,000	+	+	−	+	+	+	+	+	+
139	5.2×10^4	1.3	1,000	+	+	−	+	+	+	+	+	+
21	NT	1.1	800	+	+	−	+	+	+	+	+	+
ATCC 17749	NT	NT	NT	+	+	−	+	+	+	+	+	+
CECT 521	NT	NT	NT	+	+	−	+	+	+	+	+	+

[a] Ratio of the orange halo diameter to the colony diameter on CAS agar.
[b] NT, not tested.

Virulence for fish. We determined 50% lethal doses (LD_{50}s) for the strains included in this study with gilt-head sea bream weighing between 5 and 10 g. Whole bacterial cells and extracellular products obtained as described above were assayed separately.

LD_{50}s of bacterial cells were determined after intraperitoneal inoculation of groups of five fish with bacterial doses ranging from 10^2 to 10^8 CFU. In all cases, groups of control fish were inoculated with 0.1 ml of sterile PBS (pH 7.4). The fish were kept at 22°C for 2 weeks and observed for pathological signs. Bacteriological analyses of dead fish were carried out in all the cases; death was considered caused by inoculated bacteria only if the strain used for inoculation was isolated in pure culture. Surviving fish were killed and cultured to determine whether they were possible carriers. In both cases, samples were taken from the liver, the spleen, and the kidneys and cultured in saline tryptone soya broth and on saline tryptone soya agar. After incubation at 22°C for 5 days, the colonies that grew were confirmed to be pure cultures and were identified by using conventional techniques (24).

The virulence of the extracellular products was determined by intraperitoneal and intramuscular inoculation of groups of five fish with 0.1-ml serial dilutions of the extracellular products prepared as described above.

The samples used to study the histopathological effects of extracellular products were taken from the inoculation zones of gilt-head sea bream specimens where symptoms appeared. The samples were fixed in Bouin's solution for 18 to 20 h and subsequently were dehydrated with a graded alcohol-xylene series. Dehydrated samples were embedded in toluene-paraffin and stained with hematoxylin-eosin (50).

Surface hydrophobicity and hemagglutinating activity. The cell surface hydrophobicities of *V. alginolyticus* strains were evaluated by the salt aggregation test (31). Portions of bacterial suspensions in 0.002 M PBS were mixed on glass slides with equal volumes of ammonium sulfate solutions (pH 6.8) whose concentrations ranged from 4 to 0.01 M. The mixtures were gently rocked for 2 min at 20°C, and a reaction that caused aggregation was considered a positive reaction.

The ability to agglutinate gilt-head sea bream and horse erythrocytes was determined by the methods of Toranzo et al. (51). Hemagglutination was considered negative if visible agglutination was not observed within 10 min. The same technique was used to test the ability of the strains to agglutinate yeast cells.

Adhesion. The ability to adhere to mucus of gilt-head sea bream was also tested. Fish were anesthetized with MS-222 (Sigma) and sacrificed by anesthetic overdose, and blood was removed by caudal puncture. Skin, gill, and intestinal mucus suspensions were obtained as follows: (i) skin mucus was collected from the body surfaces of gilt-head sea bream specimens by scraping the skin with a spatula and then diluted in sterile artificial seawater (3); (ii) gill mucus was obtained by excising the gill arches and soaking them in sterile artificial seawater for 2 h at 4°C with occasional shaking (33), and the resulting suspension was diluted in sterile artificial seawater; and (iii) intestinal mucus was prepared from large and small intestines of sea bream that had been deprived of food for 48 h by using a modification of the protocol described by Olsson et al. (39). The intestines were removed and placed in sterile petri dishes containing 0.01 M PBS (pH 7.2), and then they were opened with a scalpel. The inner intestinal surfaces were gently scraped with a rubber spatula, and the mucus obtained from the intestinal lumen was diluted in an equal volume of PBS.

Skin mucus, intestinal mucus, and gill mucus were purified by two centrifugations at 20,000 × *g* for 30 min at 4°C, followed by filtration of the final supernatant through 0.45- and 0.2-μm-pore-size filters. The final mucus suspensions contained between 800 and 1,000 μg of protein per ml, as estimated by the method of Bradford (10).

The abilities of the strains to adhere to skin mucus, intestinal mucus, and gill mucus were evaluated by the methods described by Krovacek et al. (27). Bacterial suspensions in saline solutions (ca. 10^8 CFU/ml) were placed in petri dishes containing mucus-coated slides, incubated at 20°C for 1 h with continuous gentle shaking, and washed thoroughly in saline solutions. The mucus-coated glass slides were glass slides on which 50 μl of a mucus suspension had been spread in the center. These slides were air dried overnight, fixed with methanol, and stained with crystal violet before they were observed by light microscopy. Plain glass slides without mucus were used as controls.

Samples of skin tissue from the dorsal region of gilt-head sea bream were processed for scanning electron microscopy. After bacterial adhesion assays were performed as described above, samples were fixed with 2.5% glutaraldehyde in 0.05 M cacodylate buffer (Sigma) (pH 7.2) for 12 to 24 h at 4°C, dehydrated with a graded ethanol series (50, 70, 80, and 96% ethanol), critical point dried, coated with gold, and observed with a model JSM-840 scanning electron microscope (JEOL, Tokyo, Japan).

The abilities of *V. alginolyticus* strains to adhere to two fish cell lines, CHSE-214 and EPC, were determined by the method of Krovacek et al. (27). Briefly, fish cells were grown on polystyrene slides (Thermanox Lux^R; Miles Scientific, Division of Miles Laboratories, Inc., Naperville, Ill.) and fixed with methanol. Fixed cells were incubated with bacterial suspensions for 1 h at 20°C with gentle shaking. After the slides were washed to remove nonadherent cells, they were fixed with methanol and stained with crystal violet.

Bacterial growth under iron-limiting conditions. The abilities of *V. alginolyticus* strains to grow under iron-limiting conditions were evaluated by using minimal M9 agar supplemented with different concentrations of ethylenediamine-di-(*o*-hydroxyphenylacetic acid) (EDDHA) as an iron chelant. The bacteria were grown on M9 agar plates supplemented with 10 μM EDDHA to decrease the iron contents of the cells. Strains grown under these conditions were streaked onto M9 agar supplemented with different concentrations of EDDHA to determine the MIC of the iron chelant. The MIC was the lowest concentration of EDDHA that inhibited bacterial growth.

Production of siderophores was investigated by performing the universal assay (47) with chrome azurol S (CAS) agar. Bacterial cultures previously grown on M9 agar were inoculated onto CAS agar plates and incubated at 20°C for 3 days. Siderophore production was considered positive if the ratio of the orange halo diameter to the colony diameter was greater than 1.

Ability to grow by using mucus as a sole nutrient source. The abilities of bacterial strains to use skin mucus, intestinal mucus, and gill mucus as sole nutrient sources were determined by the methods described by Bordas et al. (9). Briefly, 0.2 M PBS (pH 7.2) was mixed with 1% agarose and supplemented with 2 ml of a mucus suspension containing 1 mg of protein per ml, and this preparation was poured into petri dishes. Bacterial strains were streaked onto this medium and incubated at 22°C for 20 h before the plates were observed for bacterial growth. Plates containing 0.2 M PBS (pH 7.2) supplemented with 1% agarose were used as controls.

RESULTS AND DISCUSSION

Virulence of *V. alginolyticus* strains for fish. Epizootic outbreaks caused by *V. alginolyticus* have been reported in gilt-head sea bream (41) and in juvenile turbot (*Scophthalmus maximus* L.) (2). In this study, intraperitoneal inoculation of gilt-head sea bream with different doses of *V. alginolyticus* strains yielded LD_{50}s ranging from 5.4×10^4 to 1.0×10^6 CFU/g of body weight (Table 1). Thus, the strains used can be considered highly virulent for gilt-head sea bream on the basis of the criteria established by Mittal et al. (36) and Santos et al. (45).

V. alginolyticus was reisolated in pure culture from all mor-

ibund and dead fish after the challenge. The organisms were reisolated from spleens, kidneys, and livers. The time to death ranged from 1 to 5 days after the challenge. The fish apparently were not carriers because it was not possible to isolate *V. alginolyticus* from live fish 2 weeks after the challenge.

Studies carried out with several strains of *Photobacterium damselae* (formerly *Vibrio damsela*) revealed LD_{50}s ranging from 3×10^4 to more than 10^8 CFU/g of fish when intraperitoneal inoculation was used (20). On the other hand, the LD_{50}s of *Vibrio vulnificus* biotype 2 for eels ranged from 2.6×10^1 to 1.4×10^4 CFU per fish when intraperitoneal inoculation was used (1). In the case of *Vibrio anguillarum* O1, the LD_{50}s for *Salmo salar* ranged from 5.1×10^3 to more than 10^8 CFU/g of fish (42).

In a previous study, we determined the pathogenicity of *V. alginolyticus* CAN and two strains of *V. anguillarum* (DC12R9 and DC7R2) for sea bream by the bath immersion inoculation technique. The results obtained showed that the LD_{50} of *V. alginolyticus* CAN varied depending on fish skin integrity; the LD_{50}s were more than 2×10^7 CFU/ml for fish with intact surface layers and less than 2×10^3 CFU/g for fish with the mucus layer removed or the skin damaged.

Ability to grow under iron-limiting conditions. Iron is an essential element for the growth of microorganisms, but very low concentrations are present in the host tissues and the iron is frequently chelated with proteins. Microorganisms have developed diverse mechanisms for acquiring iron in vivo, including the production of siderophores that can remove iron from chelating host proteins, such as transferrin and lactoferrin (11, 40). The presence of siderophores has been reported by different authors to be a very important virulence factor for fish pathogens, including *Photobacterium damselae* subsp. *piscicida* (34), *V. anguillarum* (22, 30), *V. vulnificus* (7), and *Aeromonas* spp. (18). In this work, all of the strains of *V. alginolyticus* tested were positive for the presence of siderophores on CAS agar. In addition, the strains assayed were also able to grow under iron-limiting conditions, and the MIC of EDDHA ranged from 800 to 1,000 μM (Table 1). These results indicate that *V. alginolyticus* strains can obtain iron from high-affinity compounds and suggest that they could also obtain iron from iron-chelating proteins of the host. The lack of a relationship between the ratio of the orange halo diameter to the colony diameter detected on CAS agar and the ability to grow on M9 agar supplemented with different concentrations of EDDHA, as observed in this study, was also reported by Esteve and Amaro (18) for *Aeromonas* species and by Magariños et al. (34) for *P. damselae* subsp. *piscicida*. This lack of a relationship could be due to the different sensitivities of the methods or to the presence of iron-chelating mechanisms other than siderophores that cannot be detected in CAS agar.

Hydrolytic activities of *V. alginolyticus* extracellular products in vitro. The results obtained with different substrates showed that extracellular products of *V. alginolyticus* are very hydrolytic. Samples of extracellular products contained from 366 to 722 μg of protein per ml. The enzymatic activities of the extracellular products of *V. alginolyticus* strains characterized with the API ZYM system produced the same pattern for several activities. Thus, all of the strains were positive for alkaline phosphatase and acid phosphatase but negative for lipase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, α-mannosidase, and α-fucosidase. However, differences among the strains were detected in the esterase, esterase-lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chemotrypsin, naphthol-AS-BI-phosphohydrolase, β-glucosidase, and *N*-acetyl-β-glucosaminidase activities, some of which were not being detected in all of the strains. The API ZYM system has been used in several studies for discrimination of related microorganisms which exhibited high levels of homogeneity in their conventional biochemical patterns (7, 23). A high degree of phenotypic homology was observed among the *V. alginolyticus* strains isolated from cultured sea bream (4). No relationship between the origins of the strains and the enzymatic activities detected with the API ZYM system was observed.

On the contrary, all of the *V. alginolyticus* strains had the same enzymatic activities when additional substrates were assayed. Thus, caseinase, gelatinase, amylolytic, phospholipolytic, and collagenase activities were detected in all of the strains. However, elastase activity was not detected in the extracellular products (Table 1).

Hydrolytic activities have been considered virulence factors because they allow bacteria to survive, proliferate, and invade host tissues (12, 17). Proteolytic activities of extracellular products have been correlated with the pathogenicity of *V. anguillarum* (25, 35) and *Aeromonas salmonicida* (17).

Cytotoxic activities of extracellular products. All extracellular product samples were cytotoxic for CHSE-214, EPC, and FHM cells (Table 1). Cell monolayers were destroyed by 10-fold-diluted samples within 1 h. The morphological changes consisted mainly of cell elongation, rounding, shrinking, detachment, and finally monolayer destruction. High numbers of vesicles were observed in affected FHM cells. Dendritic formations were also observed in EPC and CHSE-214 cells.

No differences in the origins of the strains or the mortality rates caused by the strains were related to the cytotoxic effects of the extracellular products. In all cases, important degenerative changes were observed when extracellular products were inoculated. The effects detected in fish cell lines probably corresponded to the presence of hydrolytic activities in the extracellular products.

However, no clear relationship between the cytotoxicity of extracellular products of different *Vibrio* and *Aeromonas* species and the virulence of the strains, as expressed by the LD_{50} for fish, has been reported (3, 20, 44, 53). The presence of cytotoxic effects in cell lines can be related to the virulence of *V. alginolyticus* for fish.

Virulence of extracellular products for fish. Three strains that were responsible for the most important epizootic outbreaks detected in Spain (strains AO35, CAN, and DP1-HE-4) were used to study the ability of *V. alginolyticus* strains to adhere to host surfaces and the ability of their extracellular products to damage host tissues.

Extracellular products of these strains were inoculated into gilt-head sea bream. Intramuscular inoculation resulted in the appearance of hemorrhagic areas that evolved into ulcers on the external body surfaces similar to those observed in the gilt-head sea bream specimens from which the strains were isolated. The symptoms observed in intraperitoneally inoculated fish consisted of inactivity, anorexia, erratic swimming, and liquefaction of internal organs, with important degradation of the intestines, liver, and spleen. Thus, extracellular products of *V. alginolyticus* play an important role in the pathology of this bacterial species in sea bream. Lee (29) obtained similar results for extracellular products of one *V. alginolyticus* strain isolated from grouper that synthesized a protease responsible for some of the symptoms displayed by diseased fish.

Fish mortality was observed 72 h after inoculation of extracellular products. The lytic effects were detected soon after intramuscular inoculation, and a necrotic zone appeared in the area surrounding the inoculation site after 6 h. Histological studies showed that the effects of extracellular products on host tissues consisted of gradual degradation of the muscle tissue in the inoculation zone (Fig. 1A), as well as necrosis,

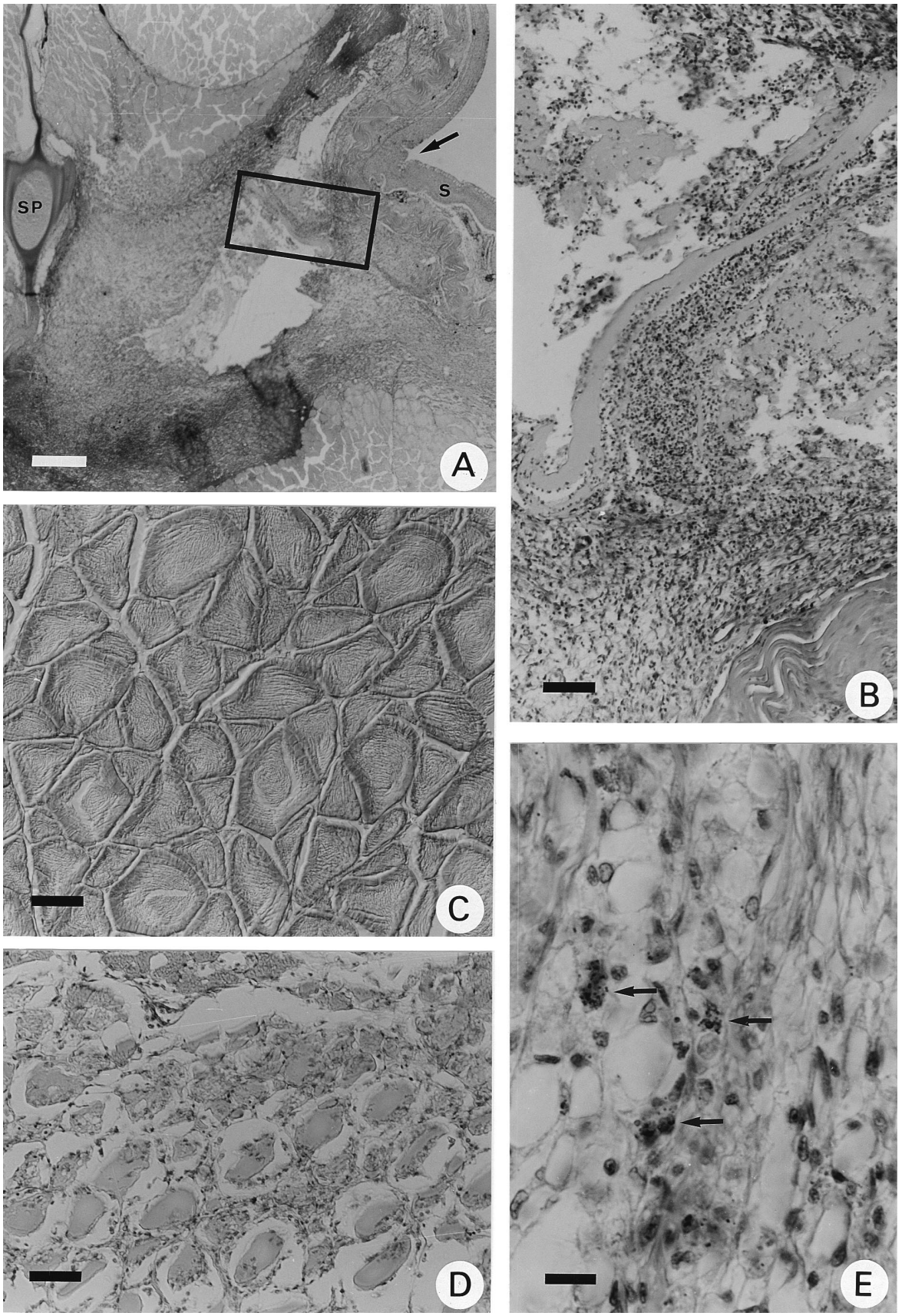

FIG. 1. Photomicrographs of hematoxylin-eosin-stained sections from sea bream, showing the histopathological effects of injection of *V. alginolyticus* extracellular products into muscle tissue. (A) Panoramic view of tissue damage in a region close to the injection site (arrow). SP, spine; S, skin. Bar = 100 μm. (B) Detail of the area in panel A surrounded by a box, showing necrosis and liquefaction of tissues. Bar = 50 μm. (C) Intact muscle fibers from a control fish. Bar = 25 μm. (D) Muscle fibers affected by extracellular products. Tissue degeneration and invasion of connective tissue cells are evident. Bar = 25 μm. (E) Macrophage infiltration (arrows) in areas affected by extracellular products. Bar = 10 μm.

TABLE 2. Hydrophobicities of *V. alginolyticus* strains, abilities to grow in skin mucus, gill mucus, and intestinal mucus, and adhesion to fish cell lines and sea bream mucus

Strain	Growth in:			Salt agglutination test (M)	Agglutination of:			Adhesion to:				
	Skin mucus	Gill mucus	Intestinal mucus		Horse erythrocytes	Sea bream erythrocytes	Yeast cells	CHSE-214 cells	EPC cells	Skin mucus	Gill mucus	Intestinal mucus
CAN	+	+	+	1	−	−	−	+	−	+	+	+
AO35	+	+	+	1	−	−	+	+	−	+	+	+
DP1-HE-4	+	+	+	2	−	−	+	+	−	+	+	+

liquefaction, and digestion of the cells (Fig. 1B). This degradation could have been due to the hydrolytic action of the extracellular products and to the effects of the host response to the extracellular products. Thus, it was possible to detect the presence of eosinophilic granulocytes and macrophages in the affected areas (Fig. 1E). Extensive destruction of muscle fibers and invasion of the necrotic zone by connective tissue cells were also observed (Fig. 1D). Necrosis due to *V. alginolyticus*

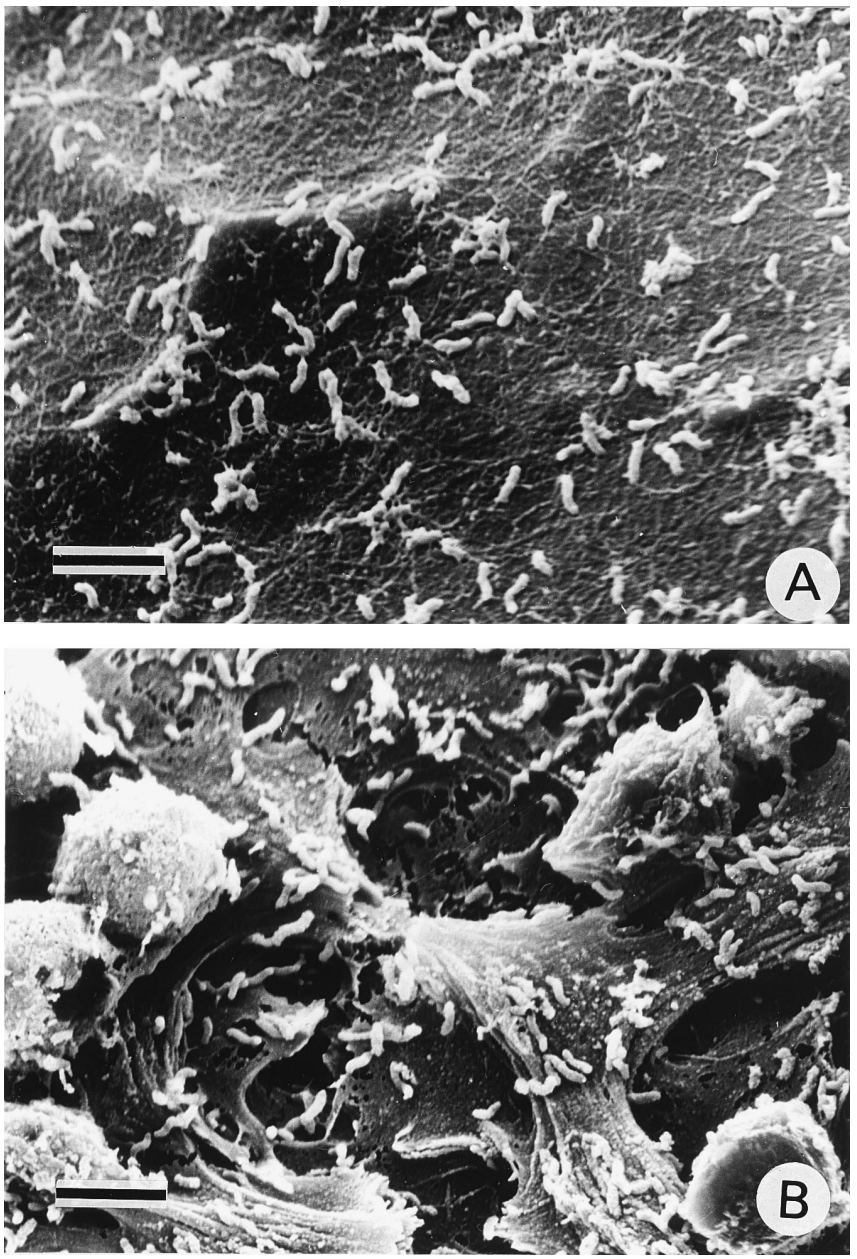

FIG. 2. Scanning electron photomicrograph showing adhesion of *V. alginolyticus* cells to mucus on the sea bream skin surface (A) and sea bream epidermal cells (B). Bars = 50 μm.

was also reported by Sedano et al. (48), who studied strains of *V. alginolyticus* isolated from diseased larvae of gilt-head sea bream and detected a strong necrotizing effect in the mucous epithelia of the anterior intestines of healthy larvae.

The LD_{50}s of the extracellular products ranged from 5.8 to 15.5 μg of protein/g of fish body weight when extracellular products were inoculated intramuscularly and from 4.3 to 200 μg of protein/g of fish body weight when intraperitoneal inoculation was used.

The results which we obtained show that extracellular products of *V. alginolyticus* play a crucial role in virulence during infections of *S. aurata* and that these products cause massive tissue damage in the host and enable the bacteria to enter the host cells. Similar results have been reported by several other authors for other fish and shellfish pathogens (26, 38). A 34-kDa protease that is toxic for grouper (*Epinephelus malabaricus*) has recently been purified from the extracellular products of a *V. alginolyticus* strain isolated from an outbreak of vibriosis (29).

Adhesion of *V. alginolyticus* strains. Bacterial adhesion to host surfaces has been described as one of the initial steps in microbial pathogenesis (6). It has been suggested that hydrophobicity is a determining factor in the adhesive process and in the survival of pathogens in cells (16, 54). The strains assayed exhibited weak hydrophobicity in the salt agglutination test; bacterial aggregation was observed only in the presence of 1 to 2 M ammonium sulfate (Table 2). The fact that the strains exhibited weak or intermediate hydrophobicity and the fact that they were able to attach to cells and mucus of gilt-head sea bream (Table 2) suggest that hydrophobicity is not an important factor in the adhesion to host cells of these strains. Santos et al. (46) reported previously that hydrophobic interactions are not essential in the colonization of host tissues by fish pathogens, including *Yersinia ruckeri*, *Aeromonas* spp., and *V. anguillarum*.

The strains of *V. alginolyticus* assayed did not agglutinate horse or sea bream erythrocytes, while yeast cells were agglutinated by only two of the three strains tested. Several authors have proposed that adhesion to erythrocytes or yeast cells could be used as a test to determine the ability of bacterial strains to adhere to host cells. The *V. alginolyticus* strains tested were positive for adhesion to skin, gill, and intestinal mucus from gilt-head sea bream and CHSE-214 cells but not to EPC cells (Table 2). Scanning electron microscope studies performed with gilt-head sea bream tissues confirmed that *V. alginolyticus* strains are able to adhere to mucus on the skin surface of sea bream (Fig. 2A). Moreover, adhesion to sea bream epidermal cells was also observed (Fig. 2B). The fact that the strains adhered to mucus, CHSE-214 cells, and (in some cases) yeast cells but not to erythrocytes or EPC cells indicated that there are different types of adhesins in this bacterial species.

The abilities of different *Vibrio* species to adhere to skin mucus were studied previously by Balebona et al. (3) and Bordas et al. (9), who observed that only the most pathogenic strains of *V. alginolyticus* and *V. anguillarum* possessed this ability. Similarly, Chen and Hanna (14) reported that only two of the 11 *Vibrio* species which they tested, *V. alginolyticus* and *V. anguillarum*, attached to cells and tissues from giant tiger prawn.

Ability to use mucus as a sole nutrient source. All of the strains of *V. alginolyticus* assayed grew on agarose plates supplemented with mucus suspensions after three passages on this medium (Table 2). No growth was detected on control plates containing agarose without mucus. These results revealed the presence of enzymatic activities in *V. alginolyticus* strains able to hydrolyze the mucus from sea bream. It has been demonstrated that in some fish mucus acts as an active defense barrier against microorganisms, and several bactericidal activities have been detected (19). In contrast, Garcia et al. (21) demonstrated that *V. anguillarum* was able to grow in intestinal mucus of salmon, suggesting that this mucus is an excellent medium for recovery from starvation and proliferation of *V. anguillarum*. Our results suggest that sea bream mucus is probably a nutrient source for invading *Vibrio* strains, including *V. alginolyticus* strains.

In short, the results obtained in this study show that the strains of *V. alginolyticus* assayed should be considered pathogenic for gilt-head sea bream. We suggest that the sequence of events during infection by this bacterial species starts with adhesion of bacterial cells to sea bream mucus, proliferation on the mucosal surface as a result of hydrolytic activities and the ability to use the mucus as a sole nutrient source, and colonization of the underlying epithelium. Subsequently, other hydrolytic enzymes secreted by *V. alginolyticus* could be responsible for the ulcers and tissue damage, when the bacteria invade other host tissues and cause disease symptoms in the fish. Moreover, survival and proliferation of the pathogen in the host should be enhanced by the ability of the microorganism to obtain iron from chelated sources, as we demonstrated for *V. alginolyticus* in this study.

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