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Vibrios Associated with Red Tides Caused by *Mesodinium rubrum*

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Vibrios were isolated from red tides caused by *Mesodinium rubrum* and also throughout the year in the Ria de Pontevedra, Spain. The isolates were grouped into 14 phena by numerical taxonomy. Strains associated with red tides were restricted to four phena: phena I and II were *Vibrio alginolyticus*, and phena III and IV were *Vibrio tubiashii* and *Vibrio anguillarum*, respectively. *V. anguillarum*-like strains (phena V through XI) predominated throughout the year outside the red tide areas. Cytotoxicity assays conducted in different poikilothermic and homoiothermic cell lines showed that cytotoxin production was not necessarily associated with the species selected during the red tides.

Mesodinium rubrum is one of the most interesting organisms that causes red tides all over the world (1, 8, 15, 17, 19, 21, 23, 25) because it has characteristics of both photoplankton and zooplankton. Blooms produced by this fast-swimming primary producer are commonly observed in nearshore areas, in regions of ocean upwelling, and in fjords and sheltered bays (18). Galician rias (Northwest Spain), a very important environment for marine aquaculture of fish and shellfish, are subjected to continuous studies of plankton, blooms of *M. rubrum* being observed with an annual frequency (9, 14, 19).

Species of the genus *Vibrio* are often found in estuarine and seawater environments and have an important role in the decomposition of organic matter, especially of zooplankton (13).

Marine vibrios include a great diversity of pathogenic species such as *V. parahaemolyticus*, *V. alginolyticus*, and *V. cholerae* O1 and non-O1, causing diseases in homoiothermic animals (6, 10); *V. anguillarum*, *V. tubiashii*, *V. carchariae*, and *V. ordalii*, pathogens for fish and shellfish (5, 28); and *V. vulnificus* and *V. damsela*, infecting both poikilothermic and homoiothermic animals (3, 11, 26). The pathogenicity for fish of classic environmental species like *V. splendidus* has been recently reported (B. Lupiani, A. Ledo, C. P. Dopazo, A. Baya, A. E. Toranzo, and J. L. Barja, 1989. Abstr. 4th Eur. Assoc. Fish Pathol. Int. Conf. Santiago de Compostela, Spain, p. 103, 1989). We previously described the selection of the genus *Vibrio* during two red tides caused by *M. rubrum* (22); a massive accumulation of organisms belonging to these *Vibrio* species in a localized area could present the risk of human infection or epizootics in mariculture facilities.

In this work, we compared by methods of numerical taxonomy the *Vibrio* species selected during the red tides with those isolated from a seasonal sampling. In addition, the cytotoxic activity, a well known virulence factor in multiple bacterial pathogens, was assayed as a measure of the potential risk of the strains for damage to human health and marine aquaculture.

Sample collection and clustering of strains. In the summer of 1984, two red tides caused by *M. rubrum* occurred in Ría de Pontevedra (Northwest Spain). Samples of blooms and surrounding water were taken daily. In addition, samples of subsurface water (depth, 5 m), bottom sediment, phytoplankton, and zooplankton were obtained from four places of the estuary at different seasons of the year. Upon collection, serial dilutions of the samples were inoculated onto thiosulfate-citrate-bile salts-sucrose (TCBS) medium (Oxoid), which is the best medium for the enumeration and isolation of vibrios (4). The numbers of vibrios in the two red tides (1×10^5 and 1.2×10^6) were greater than, by two or three logarithms, those obtained in the surrounding water (2.2×10^3 and 7×10^3, respectively). The numbers of vibrios in the surrounding waters of these red tides were similar to those obtained throughout the seasonal sampling.

The presumptive vibrios isolated from TCBS medium were characterized by using 90 morphological, physiological, and biochemical tests. The assays were performed by replica plating as previously described (22). Working cultures were maintained in Trypticase soy agar with 2% NaCl added. Stock cultures were kept frozen at −70°C in Trypticase soy broth supplemented with 15% glycerol. The *Vibrio* strains isolated from all samples were subjected to numerical taxonomy. Similarities among them were calculated by using the simple matching (S_{SM}) coefficient, and the dendogram was performed by means of the unweighted pair-group method with arithmetic averages (24).

The 86 isolates were grouped into 14 phena by their similarities (Fig. 1). The characteristics of all of the phena are shown in Table 1. Interestingly, only four phena comprised all of the isolates from the red tides (I through IV). This can be related to the creation by the bloom of a "phycosphere" under the influence of which the microbial activity could be altered and a selective stimulation of certain bacteria would be possible (2). Phena I and II are clearly differentiated from the others and contain strains belonging to *V. alginolyticus*. The differential characteristics of these strains are growth at high temperatures, no hydrolysis of Tween 80, and utilization of leucine, citrulline, sorbitol, sucrose, and hydroxybutyric acid as the sole carbon source. Phena III and IV are composed of *V. tubiashii* and *V. anguillarum* strains, respectively.

Phena V to XI are homogeneous groups composed of closely related vibrios. These phena of environmental vibrios differ from *V. anguillarum* and *V. tubiashii* in the Voges-Proskauer reaction, the hydrolysis of elastin, acid production from arabinose, the ability to degrade xanthine, the utilization of melibiose, and growth at 4°C. These findings are similar to those obtained by Bryant et al. (7) and

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TABLE 1. Characteristics of the phena of the *Vibrio isolates*[a]

Test	% of positive strains in phenon:													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Indole	75	83	83	41	0	0	0	0	0	33	0	0	0	0
Voges-Proskauer	75	33	0	0	0	0	0	0	0	0	0	0	0	50
ZOF	100	100	100	67	100	100	100	100	100	100	100	100	100	100
Production of H_2S	0	0	0	0	0	0	0	0	0	0	0	0	0	100
Hemolysis of:														
Erythrocytes														
Sheep	25	100	83	100	100	100	100	100	100	90	100	75	0	0
Human	100	83	100	92	100	86	100	100	100	0	67	25	0	0
Salt tolerance														
0%	0	0	0	100	0	0	0	0	0	0	0	0	0	50
0.5%	100	17	17	100	100	71	56	0	0	100	67	75	50	100
2%	100	100	100	100	100	100	100	100	100	100	100	100	100	100
6%	100	100	100	100	100	100	93	67	50	100	67	75	50	50
8%	100	50	67	58	0	14	0	0	0	90	0	0	0	50
10%	100	17	0	0	0	0	0	0	0	0	0	0	0	0
Growth at:														
37°C	100	17	17	75	0	0	0	0	0	0	0	75	0	50
42°C	100	17	0	42	0	0	0	0	0	0	0	0	0	0
Resistance to:														
Ampicillin	100	17	83	100	100	0	22	100	0	0	0	50	0	100
Streptomycin	100	100	100	100	0	0	0	0	0	0	0	0	0	0
Novobiocin	100	100	100	100	0	0	0	50	0	10	0	50	0	100
Polymyxin	100	17	0	100	0	0	11	0	0	0	0	25	0	100
Tetracycline	75	100	100	100	100	100	100	100	100	100	100	100	100	100
Penicillin	50	100	100	100	100	7	33	100	25	0	0	75	0	100
Chloramphenicol	0	0	17	100	0	0	0	0	0	0	0	0	0	0
Nitrofurantoin	100	100	100	100	0	0	0	0	0	0	0	0	0	0
Crystal violet	100	83	67	100	0	0	0	0	0	0	0	0	0	50
0/129	0	0	0	0	0	79	89	100	0	40	67	0	0	50
Hydrolysis of:														
Gelatin	100	50	100	92	100	100	100	100	100	90	100	75	50	100
Casein	100	67	100	92	100	93	100	100	100	80	33	75	50	50
Elastin	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tween 80	0	0	0	82	33	36	44	50	0	80	0	50	50	100
Lecithin	0	40	100	50	100	100	89	100	75	100	100	0	50	0
Starch	100	100	100	100	100	100	89	50	50	100	100	75	0	50
Cellulose	50	0	60	62.5	0	0	0	0	0	0	0	0	0	0
Esculin	50	50	67	17	0	0	0	0	0	0	0	0	0	0
Alginate	0	0	83	58	67	100	0	100	50	100	0	0	0	0
Chitin	75	50	25	58	100	93	100	100	100	89	33	100	100	50
Xanthine	0	17	20	0	0	0	0	0	0	0	0	0	0	0
Tyrosine	75	0	50	60	50	57	44	0	100	100	67	25	0	50
Urea	0	0	50	0	0	0	0	0	0	0	0	0	0	0
DNA	75	83	100	100	100	100	100	100	100	100	100	100	50	100
β-Galactosidase	0	67	83	83	50	86	56	50	25	100	0	100	0	50
ADH	0	0	83	100	50	100	100	100	100	20	33	75	0	0
LDC	100	80	40	0	0	0	0	0	0	0	0	75	50	0
ODC	100	80	17	0	0	0	0	0	0	0	0	0	0	0
Acid from:														
Melibiose	25	0	0	17	0	0	0	0	0	0	0	0	0	0
Sucrose	50	83	83	58	33	43	11	0	0	90	100	100	100	50
Lactose	25	0	0	0	0	0	0	0	0	0	0	0	0	0
Maltose	100	100	100	100	100	100	100	100	100	90	100	100	50	0
Trehalose	100	100	100	100	100	100	100	100	100	100	100	75	50	50
Mannose	25	67	100	100	100	100	100	100	75	100	100	100	50	100
Galactose	25	67	83	100	100	100	89	100	50	80	100	100	100	0
Fructose	83	100	100	100	100	100	100	100	100	100	100	100	100	50
Mannitol	83	100	100	100	100	100	100	100	100	100	100	100	50	0
Sole carbon source														
Alanine	100	83	100	83	17	71	11	50	25	70	0	0	0	0
Leucine	100	17	0	25	0	0	0	0	0	0	0	0	0	0
Arginine	100	33	17	0	0	0	0	0	0	0	0	0	0	0
Citrulline	100	100	0	25	17	7	0	0	0	60	67	0	50	0
Gluconate	25	83	83	75	17	71	0	50	0	60	100	0	0	0
Glucuronate	0	33	0	58	0	7	22	100	0	10	67	0	0	0
Mannitol	100	100	50	67	0	0	0	0	0	0	0	0	0	0

Continued on following page

TABLE 1—*Continued*

Test	% of positive strains in phenon:													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Sorbitol	100	100	0	0	0	7	0	0	0	0	0	0	0	0
Glycerol	100	83	100	100	83	86	44	100	25	90	67	0	0	0
Ethanol	100	50	0	25	0	0	0	0	0	0	0	0	0	0
Xylose	25	17	0	25	0	0	0	0	0	0	0	0	0	0
Arabinose	100	50	0	0	0	0	0	0	0	20	0	0	0	0
Cellobiose	100	83	100	100	83	100	89	100	25	100	67	0	0	50
Sucrose	100	83	33	100	0	0	0	0	0	0	0	0	0	0
Melibiose	0	0	0	8	0	0	0	0	0	10	33	0	0	0
Hydroxybutyrate	100	100	0	42	0	0	0	0	0	0	0	0	0	0
Casamino Acids	100	100	83	100	100	100	100	100	100	100	100	100	0	50
Growth on:														
CLED agar	0	0	0	100	0	0	0	0	0	0	0	75	0	50
SS agar	100	17	0	0	0	0	0	0	0	0	0	0	0	0
MacConkey agar	100	17	0	25	17	0	0	0	0	50	0	75	0	5

[a] All of the strains were gram negative and oxidase and catalase positive, reduced nitrate, and produced acid but not gas from glucose. No isolates were bioluminescent; none produced acid from arabinose, myoinositol, sorbitol, or salicin; none grew on adenine. Abbreviations: ZOF, see reference 16a; ADH, arginine dihydrolase; LDC, lysine decarboxylase; ODC, ornithine decarboxylase; CLED, cysteine-lactose-electrolyte-deficient medium; SS, Salmonella-Shigella.

Fouz et al. (12). Therefore, these phena can be considered to consist of the same species of vibrios described by these authors, i.e., *V. pelagius* and *V. splendidus* and other environmental *Vibrio* spp. This group of vibrios could be termed *V. anguillarum*-like organisms. Phena XII and XIII correspond to *V. fisherii*, and phenum XIV is composed of unidentified vibrios.

Although all of the *Vibrio* species selected during the red tides are autochthonous flora in the estuary, they are usually present in a very low proportion. It is noteworthy that the selected species are opportunistic pathogens of homoiothermic (*V. alginolyticus*) or poikilothermic (*V. tubiashii* and *V. anguillarum*) organisms. The high concentrations of these microorganisms in a localized area could be the origin or the cause of increased toxic effects of some red tides. In addition, the species in the environmental group of *V. anguillarum*-like organisms represent a potential risk in marine aquaculture, since, as previously described, some strains belonging to this group are pathogenic for several fish species (Lupiani et al., Abstr. 4th Eur. Assoc. Fish Pathol. Int. Conf. Santiago de Compostela, Spain, p. 103, 1989).

Cytotoxic activities. Fifty-six representative strains belonging to the different phena were selected to evaluate their cytotoxic effects in in vitro assays, using poikilothermic and homoiothermic cell lines: CHSE-214 (chinook salmon embryo), FHM (fathead minnow peduncle), and L-929 (mouse lung fibroblast). By using the procedures of Toranzo et al. (27), filtered supernatants from bacterial cultures in Trypticase soy broth supplemented with 1% NaCl were added to cell monolayers and incubated at 18°C (fish cell lines) or 37°C (homoiothermic cell line). The method of Bradford (6) was used to measure the protein content of the extracellular

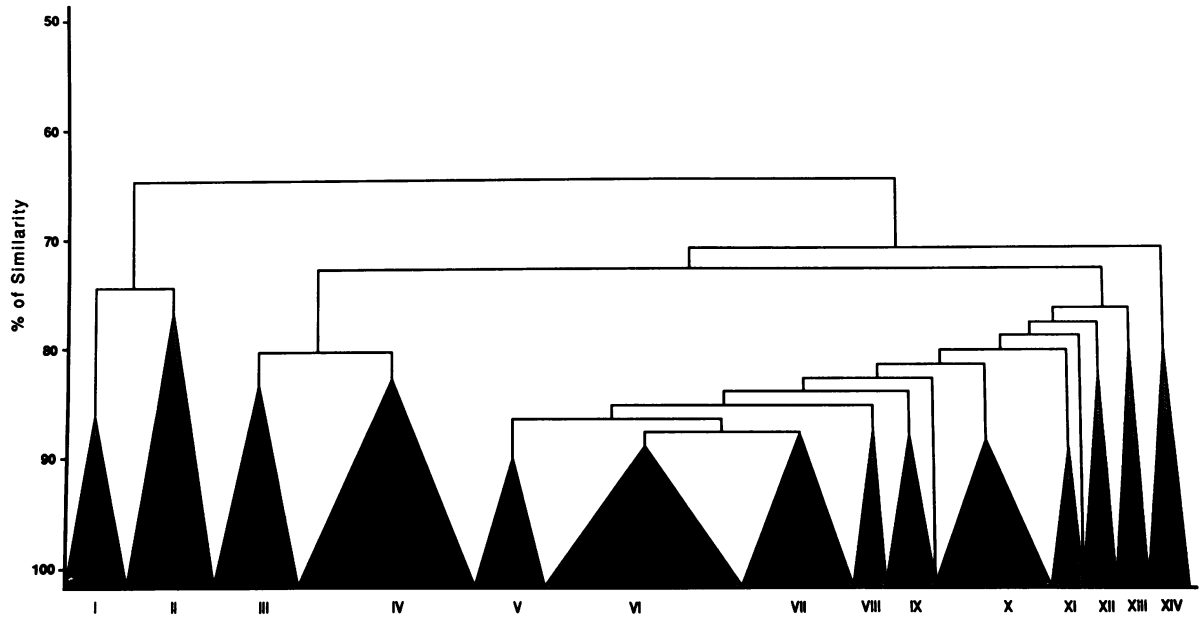

FIG. 1. Simplified dendogram showing the clustering in phena (I through XIV) of the *Vibrio* isolates by the unweighted pair-group method.

TABLE 2. Cytotoxicity patterns shown by the *Vibrio* strains to the different cell lines

Origin of samples	Species or medium	Phena	Cytotoxicity to cell line:		
			CHSE-214	FHM	L-929
Red tide	*V. alginolyticus*	I, II	−	−	+
	V. tubiashii	III	−	+	+
	V. anguillarum	IV	+	+	+
			−	+	+
Seasonal sampling	*V. anguillarum*-like	V to XI	+	−	−
			+	+	+
	V. fisherii	XII, XIII	+	+	+
	Vibrio spp.	XIV	+	+	+
Control	*V. cholerae* non-O1 LA 4808		+	+	+
	V. anguillarum R82		+	+	+
	Escherichia coli K-12 185		−	−	−
	TSB-1 medium		−	−	−

products. The minimal amount of protein needed to produce a cytotoxic effect was determined by inoculating serial dilutions of the positive samples. Total or partial destruction of monolayers within a 3-day period was scored as a positive cytotoxic effect. Heat treatment of positive samples at 80°C caused the loss of the cytotoxic activity.

Of the 56 strains subjected to this assay, 24 (42.8%) showed cytotoxic activity against some of the cell lines tested, producing degenerative changes such as vacuolization, rounding, dendritic elongation, and finally cell detachment. Isolates positive for this activity were detected in all of the phena. However, while the *V. anguillarum* strains produced degenerative changes with protein concentrations ranging from 20 to 25 μg/ml, higher amounts of protein (80 to 100 μg/ml) were necessary to obtain similar effects in the other species. The groups also differed in the spectrum of activities against the different cell lines (Table 2). Although it has been reported that it is not possible to establish a relationship between this characteristic and the virulence of the species for fish (27, 28), other authors (16) found that cytotoxicity could cause damage in the skin of fish and play a role in the attachment to the host cells. In *Vibrio* species that are pathogenic for homoiotherms like *V. alginolyticus*, *V. parahaemolyticus*, and *V. cholerae*, this relationship has been described (29).

Isolates of phena I and II of *V. alginolyticus* only displayed cytotoxic activity against the homoiothermic cell line L-929 (Table 2). Interestingly, this species is a well recognized opportunistic pathogen for humans (20), and this could be the reason for its lack of activity against the fish cell lines. It is known that bivalve shellfish can be heavily contaminated with *Vibrio* species (10); this possibility is of great importance in areas of massive production of mussels and other shellfish such as the Galician rias where many of these products are caught locally, increasing the potential for human contamination.

On the other hand, whereas strains belonging to phenon III of *V. tubiashii* displayed cytotoxic activity against FHM and L-929 cell lines, the isolates of *V. anguillarum* (phenon IV) showed this activity against all of the cell lines tested (Table 2). Moreover, the presence of this putative virulence factor in strains of environmental species such as *V. pelagius* or *V. splendidus* supports the idea that these species can be considered opportunistic pathogens for fish.

The results reported here suggest the importance of classic nontoxic red tides (caused by zooplankton and similar organisms) since the bacterial flora associated with them may represent a risk for human health and marine aquaculture.

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