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Characterization of strains related to brown ring disease outbreaks in southwestern Spain

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ABSTRACT: Several characteristics, including biochemical, serological, drug resistance and plasmid profiles, of strains isolated from clams in southwestern Spain affected with brown ring disease have been comparatively studied. On the basis of 36 standard physiological and biochemical tests, all strains isolated were included in the genus *Vibrio* and further divided into 6 groups. The groups were differentiated on the basis of only 8 phenotypic traits: growth at 35 °C, arginine dihydrolase, gelatinase production, Voges-Proskauer and ONPG tests, and acid production from sucrose, amygdalin and mannitol. Applying these characteristics, the strains resembled *Vibrio pelagius* and *V. splendidus* species. However, the isolates of each group showed no cross-reactions with the antisera raised against several reference strains of different species of *Vibrio*, including *V. anguillarum, V. tubiashii, V. damsela, V. pelagius, V. splendidus* and the unclassified *Vibrio* P1. Although only 57.7 % of the strains tested harbored one or more plasmids, the majority of the plasmid-containing strains (93.3 %) carried a large plasmid band of 34.4 MDa. A high number of isolates, regardless of their taxonomic group, were resistant to ampicillin and erythromycin. However, all the *Vibrio* strains were sensitive to chloramphenicol, tetracycline, gentamicin, nitrofurantoin, nalidixic acid and trimethoprim sulphamethoxazole. No correlation between plasmid content and drug resistance was observed.

INTRODUCTION

Since summer 1988 several epizootic outbreaks causing high mortalities of manila clams *Tapes philippinarum* have appeared in the clam beds of Cadiz (southwestern Spain). The symptoms associated with these outbreaks are characterized by the presence of a brown deposit of organic matter on the inner surface of the shell, between the pallial line and the shell margins. The populations affected with brown ring disease (BRD) generally showed low development, shell deformities and mortality rates over 60 %. This disease was first reported in clam beds of Landeda (Finisterre, France) by Paillard et al. (1989), and has been extensively studied in France (Flassch 1989, Paillard & Maes 1990, Maes et al. 1992) and in Spain (Castro et al. 1990).

Paillard & Maes (1990) proposed an infectious origin of the disease, the causative agent being identified as a *Vibrio* strain called P1. However, studies carried out in our laboratory indicate the presence of a varied microflora associated with this disease composed mainly of *Vibrio* species.

In this study we have compared the biochemical and serological characteristics of the different strains isolated from affected clams in southwestern Spain. In addition, drug resistance and plasmid profiles of these strains were also analyzed.

MATERIALS AND METHODS

Sample processing. Samples of manila clams *Tapes philippinarum* affected with BRD symptoms were collected in different zones of the Cadiz Bay (Spain). In all the samplings, 100 specimens or more were collected and the incidence of the condition was recorded. Later, the clams with symptoms of BDR were microbiologically analyzed following the scheme described in Fig. 1. All the general and selective media employed for bacterial isolation were supplied by Difco. For long-term preservation, cultures were frozen at –80 °C in a

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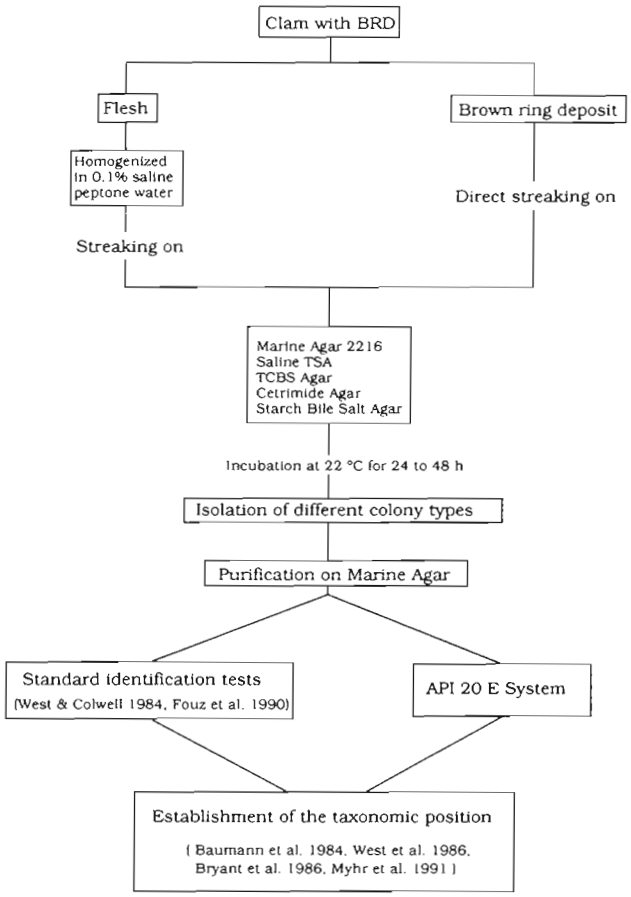

Fig. 1. Sample processing and bacterial identification procedure used in this study. TCBS: thiosulfate-citrate-bile-sucrose; TSA: tryptic soy agar

basal medium composed of 0.4 % peptone (Difco), 0.1 % yeast extract (Difco), 2 % NaCl and 15 % (v/v) glycerol.

Biochemical and serological characterization. All strains were identified using 36 standard morphological, physiological and biochemical tests following the procedures of West & Colwell (1984) and Fouz et al. (1990). In parallel, the commercial miniaturized API 20-E System (BioMerieux) was also employed using half strength seawater as diluent (Kent 1982).

The taxonomic position of the bacterial isolates was determined following mainly the criteria of Baumann et al. (1984), Bryant et al. (1986), West et al. (1986) and Myhr et al. (1991).

In order to examine the possible serological relationships among the *Vibrio* isolated in the present study and other related vibrios, slide agglutination tests were conducted as described by Sørensen & Larsen (1986) and Toranzo et al. (1987a), using rabbit antisera raised against the following reference species: *Vibrio anguillarum* O1, strain R-82 isolated from turbot; *V. anguillarum* O2, ATCC 19264; *V. anguillarum* O3, strain 13A5 isolated from water; *V. tubiashii* EX1 isolated from oysters; *V. pelagius* ATCC 25916; *V. pelagius* RG-165 isolated from turbot; *V pelagius* SG-12 isolated from salmon; *V. splendidus* ATCC 25914; *V. damsela* RG-191 isolated from turbot; and *Vibrio* P1 isolated from clams. To obtain these antisera, rabbits were injected intravenously with formalin killed cells twice weekly in consecutive doses of 0.2, 0.4, 0.8 and 1 ml (10^9 cells ml^{-1}). Rabbits were bled from the ear vein 1 wk after the last injection. The blood was allowed to clot and the sera were collected and stored at −30 °C until used.

Reactions were performed using as antigens both the whole cells and the correspondent thermostable 'O' antigens, which were obtained by heating the bacterial suspensions (10 % v/v in PBS) at 100 °C for 1 h (Toranzo et al. 1987a). A strong agglutination occurring before 10 s was registered as a positive test.

Patterns of drug resistance. Antimicrobial resistance patterns of the isolates were determined by the disc diffusion method (Barry & Thornsberry 1991) on Mueller Hinton agar (BioMerieux) supplemented with 1.5 % NaCl, using the following antimicrobial substances (µg $disc^{-1}$): ampicillin (10), streptomycin (10), gentamicin (10), tobramycin (10), amikacin (30), kanamycin (30), chloramphenicol (30), tetracycline (30), erythromycin (15), nitrofurantoin (300), trimethoprim-sulphamethoxazole (25), and nalidixic acid (30). All the antibiotic discs were supplied by BioMerieux.

Plasmid detection. The isolation of plasmid DNA was carried out using the method of Kado & Liu (1981) as modified by Toranzo et al. (1983). Cultures in exponential growth phase were centrifuged for 3 min at 12 000 × *g*. Cell pellets were resuspended in 100 mM Tris-acetate buffer (40 mM Tris, 2 mM sodium EDTA adjusted to pH 7.9 with glacial acetic acid) and 200 l of lysis solution (SDS 3 % in 50 mM Tris, pH 12.4) and incubated at 55 to 60 °C for 30 min, after which the plasmid DNA was extracted with an equal volume of phenol-chloroform solution (1:1, v/v). The emulsion was separated by centrifugation for 10 min at 12 000 × *g* at 4 °C. The aqueous phase was removed and mixed with tracking dye solution (bromocresol purple 0.25 %-glycerol 50 % in Tris-acetate buffer). DNA samples (20 µl) were separated by horizontal electrophoresis in an agarose 0.7 % gel (type I; Sigma) in Tris-acetate buffer at 75 V for about 3 h. The gels were stained in ethidium bromide solution 0.5 µg ml^{-1}, and photographed through a UV transilluminator with Kodak Imagelink film and 23A Wratten filter.

Reference plasmids from *Escherichia coli* V517 (containing 6 plasmids ranging from 35.8 to 1.4 megadaltons, MDa), *E. coli* R40a (96 MDa) and *V. anguillarum* 775 (47 MDa) were included as markers in the agarose gel electrophoresis.

RESULTS

Biochemical and serological characterization

In total 189 strains isolated from clams affected with BRD were examined following the scheme specified in Fig. 1. All the isolates were included in the genus *Vibrio*, on the basis of the following characteristics: morphology and Gram stain (curved Gram-negative rod-shaped bacteria), motile, oxidase-positive, glucose-fermenting, and sensitive to the vibriostatic agent O/129 (150 μg).

Each strain was examined for 36 additional physiological and biochemical characters which were compared with those exhibited by the *Vibrio* P1 reported as the causative agent of BRD in France (Paillard & Maes 1990) (Table 1). All our strains possessed 27 common characteristics among themselves and with the *Vibrio* P1. However, our *Vibrio* isolates can be separated in 6 groups on the basis of 8 phenotypic traits: growth at

Table 1. Comparison of biochemical characteristics of *Vibrio* strains isolated from clams in Spain affected with brown ring disease with those of the French isolate *Vibrio* P1. n: No. of strains tested within each group; +: positive reaction; −: negative reaction; V: variable reaction among isolates, with no. of strains showing positive reaction in parentheses

Test	*Vibrio* groups						*Vibrio* P1
	1 (n = 35)	2 (n = 49)	3 (n = 14)	4 (n = 21)	5 (n = 34)	6 (n = 34)	
Common characteristics							
Growth in:							
0 % NaCl	−	−	−	−	−	−	−
3 % NaCl	+	+	+	+	+	+	+
10 % NaCl	−	−	−	−	−	−	−
Growth at:							
15 °C	+	+	+	+	+	+	+
22 °C	+	+	+	+	+	+	+
Growth on TCBS	+	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	+	+
Urease	−	−	−	−	−	−	−
H_2S production	−	−	−	−	−	−	−
Motility	+	+	+	+	+	+	+
Indole	+	+	+	+	+	+	+
Lysine decarboxylase	−	−	−	−	−	−	−
Ornithine decarboxylase	−	−	−	−	−	−	−
Tryptophane deamynase	−	−	−	−	−	−	−
Gas from glucose	−	−	−	−	−	−	−
Acid from:							
Glucose	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+
Inositol	−	−	−	−	−	−	−
Sorbitol	−	−	−	−	−	−	−
Rhamnose	−	−	−	−	−	−	−
Melobiose	−	−	−	−	−	−	−
Arabinose	−	−	−	−	−	−	−
Utilization of citrate	−	−	−	−	−	−	−
Amylase	+	+	+	+	+	+	+
Lipase	+	+	+	+	+	+	+
Differential characteristics							
Growth at 4 °C	−	−	−	−	−	−	+
Growth at 35 °C	+	−	+	−	+	+	−
ONPG	V(21)	−	V(10)	V(14)	−	−	+
Voges-Proskauer	V(28)	−	−	−	V(20)	−	−
Arginine dihydrolase	−	−	−	−	+	+	−
Acid from:							
Sucrose	+	+	−	−	+	−	−
Amygdalin	+	+	V(8)	+	−	+	−
Mannitol	+	+	+	+	V(19)	+	−
Gelatinase	+	+	V(12)	−	+	+	+

35 °C, ONPG, Voges-Proskauer, arginine dihydrolase, acid production from sucrose, amygdalin and mannitol, and gelatinase activity (Table 1).

Group 1 contained strains that resemble those described by West et al. (1986) as unidentified marine *Vibrio* sp. and included in the phenons 19 and 34 (*Vibrio* sp.) by Bryant et al. (1986). The differential characteristics of the strains of this group are: positive growth at 35 °C, variable reactions for ONPG and Voges-Proskauer tests, production of acid from sucrose, amygdalin and mannitol, production of gelatinase, and negative reaction for arginine dihydrolase. The strains belonging to Groups 2 and 4 do not grow at 35 °C, and are negative for the tests Voges-Proskauer and arginine dihydrolase, but they produce acid from amygdalin and mannitol. The differentiation of both groups is based on the gelatinase activity and the acid production from sucrose. None of the 433 strains studied by West et al. (1986) coincide with the biochemical profiles of the strains of these groups, but are very similar to the phenon 50 (*Vibrio* sp.) proposed by Bryant et al. (1986).

Only 14 strains could be isolated in Group 3, whose differential characteristics resemble other strains belonging to *Vibrio pelagius*, according to the classifications of West et al. (1986) and Bryant et al. (1986). Groups 5 and 6 comprised strains with very similar biochemical characteristics, and their differentiation from other groups is the presence of the enzyme arginine dehydrolase. Group 5 corresponds with the phenons 12 and 13 of Bryant et al. (1986), which are *Vibrio* sp., and Group 6 resembles the phenon 25, classified as *V. splendidus* I.

On the other hand, on the basis of the biochemical characterization of 264 strains isolated from diseased and healthy fish carried out by Myhr et al. (1991), our isolates can be included into the following species: Groups 1 and 2 as *Vibrio pelagius* bv. II, Groups 3 and 4 could be assigned to either *V. pelagius* bv. II or *V. splendidus* bv. II, and Groups 5 and 6 to *V. splendidus* bv. I. Interestingly, the Group 5 strains also share some characteristics with *V. tubiashii* and *V. anguillarum*. Although the French isolate *Vibrio* P1 seems to be very similar to our Group 3, the most important differential traits are the capacity of the P1 strain to grow at 4 °C, and its unability to support 35 °C and to produce acid from mannitol (Table 1).

In another phase of this study, strains of each group were compared serologically with reference *Vibrio* strains isolated from fish and shellfish (Table 2). Each strain showed an antigenic homogeneity only with the antiserum obtained from itself. However, none of the isolates presented a cross-reaction with the antisera for other strains. On the basis of these findings, it can be deduced that although there are biochemical similarities among the strains isolated in the present study with the species *V. pelagius*-*V. splendidus*-*V. tubiashii*-*V. anguillarum*, serological analyses demon-

Table 2. Slide agglutination reactions using as antigens both the whole cells and the somatic O antigens. n: No. of strains tested within each group; ++: strong agglutination within 10 s; −: negative agglutination

O antigens	Rabbit antisera									
	R-82	19264	13 A5	EX 1	25916	RG-165	SG-12	25914	RG-191	P1
***Vibrio* groups**										
Group 1 (n = 7)	−	−	−	−	−	−	−	−	−	−
Group 2 (n = 10)	−	−	−	−	−	−	−	−	−	−
Group 3 (n = 3)	−	−	−	−	−	−	−	−	−	−
Group 4 (n = 6)	−	−	−	−	−	−	−	−	−	−
Group 5 (n = 10)	−	−	−	−	−	−	−	−	−	−
Group 6 (n = 7)	−	−	−	−	−	−	−	−	−	−
Reference strains										
V. anguillarum R-82 (O1)[a]	++	−	−	−	−	−	−	−	−	−
V. anguillarum ATCC 19264 (O2)[a]	−	++	−	−	−	−	−	−	−	−
V. anguillarum 13A5 (O3)[a]	−	−	++	−	−	−	−	−	−	−
V. tubiashii EX 1	−	−	−	++	−	−	−	−	−	−
V. pelagius ATCC 25916	−	−	−	−	++	−	−	−	−	−
V. pelagius RG-165	−	−	−	−	−	++	−	−	−	−
V. pelagius SG-12	−	−	−	−	−	−	++	−	−	−
V. splendidus ATCC 25914	−	−	−	−	−	−	−	++	−	−
V. damsela RG-191	−	−	−	−	−	−	−	−	++	−
Vibrio P1	−	−	−	−	−	−	−	−	−	++

[a] Serotype

strate that antigenic identities do not exist among the groups.

Drug resistance patterns

Determination of resistance patterns to 12 antibiotics showed that of the 100 strains tested, 84 % were resistant to at least 1 antimicrobial drug (Table 3). All groups showed resistance, in different degrees, to ampicillin and erythromycin, and were susceptible to gentamicin, chloramphenicol, tetracycline, nitrofurantoin, trimethoprim-sulphametoxazole and nalidixic acid. The resistance to streptomycin, tobramycin, amikacin and kanamycin was variable among the groups. Total resistance of Groups 2, 3 and 6 was observed only for ampicillin, which may be used as an additional tool to discriminate among the *Vibrio* groups.

Our results showed higher percentages of resistance in the present isolates than in other species belonging to the same genus and isolated from several sources (Kelch & Lee 1978, Beringer & Hirsch 1984, Myhr et al. 1991). Resistance patterns seem to be independent of the taxonomic inclusion of the strains in different *Vibrio* groups.

Plasmid content of selected strains involved in BRD

Twenty-six strains in the 6 groups of vibrios implicated in BRD were examined for plasmids, as described above. Only 57.7 % of the strains harbored 1 or more plasmids ranging from 1.1 to 34.4 MDa (Table 4). Interestingly, practically all the strains carrying plasmids contained the 34.4 MDa band. This plasmid was harbored alone or in combination with low molecular weight (< 10 MDa) plasmids.

DISCUSSION

Vibriosis is one of the most threatening diseases in fish and shellfish cultures in marine waters. Among the great number of species recognized within the genus *Vibrio*, only 8 have been described as important pathogens for aquatic animals: *V anguillarum, V. ordalii, V. tubiashii, V. damsela, V. vulnificus, V. salmonicida, V. carchariae* and *V. cholerae* non-O1. However, vibrioses caused by *V. anguillarum* and *V. tubiashii* are the most serious bacterial infections limiting the production of marine fish and shellfish over the world (Toranzo & Barja 1990).

On the other hand, there are vibrios in the estuarine environment taxonomically and serologically related to *Vibrio anguillarum* and *V. tubiashii* (Bryant et al. 1986, Fouz et al. 1990) which, although usually considered strains without pathogenic significance, have recently been associated with diseases in marine fish, molluscs and crustaceans (Lupiani et al. 1989, Masumura et al. 1989, Baticados et al. 1990, Fouz et al. 1990, Lavilla-Pitogo et al. 1990, Toranzo et al. 1990, Myhr et al. 1991). These vibrios correspond mainly to different biotypes of *V. splendidus* and *V. pelagius* and can be compiled under the general designation of *V. anguillarum*-related (VAR) organisms (Larsen 1983, 1985, Fouz et al. 1990, Myhr et al. 1991). Table 5 presents important differential phenotypic traits among these VAR species and the most substantiated pathogenic vibrios for poikilothermic animals which can be useful for diagnostic purposes. Although in the present work all the isolates from diseased clams belonged to different groups of *V. pelagius* and *V. splendidus* (Table 1), in some cases a clear distinction between these 2 species is difficult because of the number of variable reactions existing within the strains of each species (Table 5).

Table 3. Drug resistance patterns (% of resistance) of the *Vibrio* strains isolated from clams affected with brown ring disease. n: No. of strains tested

Chemotherapeutic agents	Group 1 (n = 20)	Group 2 (n = 20)	Group 3 (n = 10)	Group 4 (n = 10)	Group 5 (n = 20)	Group 6 (n = 20)
Ampicillin	60	100	100	60	80	100
Streptomycin	20	15	0	30	0	20
Gentamicin	0	0	0	0	0	0
Tobramycin	20	30	0	30	0	0
Amikacin	0	30	0	60	0	0
Kanamycin	0	15	50	30	20	0
Chloramphenicol	0	0	0	0	0	0
Tetracycline	0	0	0	0	0	0
Erythromycin	60	60	50	30	40	20
Nitrofurantoin	0	0	0	0	0	0
Nalidixic acid	0	0	0	0	0	0
Trimethoprim-sulphamethoxazole	0	0	0	0	0	0

Table 4. Plasmid profiles and antimicrobial susceptibility of several *Vibrio* strains involved in brown ring disease. Ap: Ampicillin; Sm: streptomycin; NN: tobramycin; E: erythromycin; An: amikacin; Km: kanamycin; –: sensitive strains

Strain	Plasmid profiles		Antimicrobial resistance patterns
	No.	Sizes (MDa)	
Group 1			
V 519	0	–	–
V 543	0	–	Ap, Sm, NN, E
V 556	2	34.4, 5.1	Ap, E
V 558	0	–	Ap, E
Cl-8G	1	34.4	–
Group 2			
V 526	0	–	Ap, Sm, NN, An, E
V 538	0	–	Ap
V 540	0	–	Ap, E
V 542	1	34.4	Ap
V 555	1	34.4	Ap, Km, E
V 557	2	34.4, 6.0	Ap, E
V 559	1	34.4	Ap, NN, An
Group 3			
V 525	2	34.4, 7.4	Ap, E
V 568	2	34.4, 15.0	Ap, Km
Group 4			
V 528	0	–	NN, AN
AM 112	6	34.4, 15.0, 2.9, 2.4, 2.0, 1.1	Ap
Group 5			
V 521	0	–	Ap
V 541	1	34.4	Ap
V 548	1	34.4	Ap, E
V 551	1	34.4	Ap, Km
Cl-16G	0	–	E
Group 6			
V 529	0	–	Ap
V 539	1	7.4	Ap, Sm
V 549	1	34.4	Ap
V 560	1	34.4	Ap
AM 113	0	–	Ap, E

None of the *Vibrio* strains analyzed in our study were serologically related with the classic pathogenic serotypes of *V. anguillarum* or *V. tubiashii*. However, we cannot rule out that some of the *V. splendidus* bv. I positive for arginine dehydrolase and utilization of sucrose (Group 5) could belong to the environmental serotypes (from O4 to O10) of *V. anguillarum* (Sørensen & Larsen, 1986). In fact, it was found recently that some *V. splendidus* associated with diseases in marine fish in northwestern Spain should be assigned to the serotypes O4 and O5 of *V. anguillarum* (Pazos et al. 1992).

There are several studies of pathogenic vibrios regarding the possible correlation between the presence of plasmid and virulence properties. Although for some species, such as *Vibrio ordalii* and *V. salmonicida*, it was reported that the majority of the isolates harbor a 23 and 21 MDa plasmid respectively (Schiewe & Crosa 1981, Wiik et al. 1989a), their role with pathogenicity was not demonstrated. At present, only in the serotype O1 of *V. anguillarum* was the presence of a plasmid of 47 MDa clearly associated with the virulence of these strains (Crosa 1984, Toranzo et al. 1987b, Wiik et al. 1989b, Conchas et al. 1991). This plasmid mediates a very efficient iron-sequestering system which allows bacteria to grow at the low concentrations of available iron in the host tissue (Walter et al. 1983, Tolmasky et al. 1985, Wolf & Crosa 1986). In the present study, regardless of taxonomic group or drug resistance pattern, a plasmid of 34.4 MDa was found in the majority of the plasmid-containing isolates (93.3%) (Table 4). These findings support the fact that these *V. pelagius-V. splendidus* groups must be closely related phylogenetically and can represent phenotypic variants of a major and unique group.

Since it is not possible to establish a clear association of a particular *Vibrio* group with the BRD, other factors may be involved such as: (1) physiological conditions of the clams seeded, (2) seed density in the clam bed, and (3) influence of environmental factors, such as insolation, tide, and type of clam bed. These stress conditions

Table 5. Main differential taxonomic characteristics among important pathogenic *Vibrio* species for poikilothermic animals and the groups of *V. splendidus* and *V. pelagius*. V: Variable character among the strains; R: resistant strains; S: sensitive strains; (y): yellow colonies; (g): green colonies; NT: not tested

Characteristics	*V. anguillarum*	*V. tubiashii*	*V. ordalii*	*V. damsela*	*V. salmonicida*	*V. vulnificus*	*V. splendidus*	*V. pelagius*
Growth on TCBS	+(y)	+(y)	+(y)	+(g)	−	+(g)	+(y/g)	+(y/g)
Gas from glucose	−	−	−	+	−	−	−	−
Arginine dihydrolase	+	+	−	+	−	−	V	−
Lysine decarboxylase	−	−	−	−	−	+	−	−
Ornithine decarboxylase	−	−	−	−	−	−	−	−
Acid from:								
Sucrose	+	+	+	−	−	−	V	V
Mannitol	+	+	−	−	NT	−	+	V
Gelatinase	+	+	+	−	−	+	+	V
Urease	−	−	−	+	−	−	−	−
Amylase	+	+	−	+	−	+	+	V
Lipase	+	+	−	−	−	+	+	+
Susceptibility to ampicillin	R	S	S	R	NT	R	R/S	R/S

can favor the selection and subsequent proliferation of the *V. splendidus* and *V. pelagius* groups which become dominant among the marine microflora of both water and molluscs. In addition, some of these dominant *Vibrio* strains can possess the pathogenic potential needed to produce mortality in shellfish previously weakened by stressing factors (Baticados et al. 1990, Lavilla-Pitogo et al. 1990). In fact, we have previously demonstrated that the microflora of healthy clams is composed of a varied bacterial population that includes: *V. harveyi, V. alginolyticus, V. splendidus, V. pelagius, V. nereis, V. fluvialis, V. costicola, Aeromonas* sp. and *Pseudomonas* sp. (Castro et al. 1990).

Further studies on the virulence factors and pathogenicity for molluscs of the VAR microorganisms are being conducted to assess their possible implication in brown ring disease.

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