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Characterization by numerical taxonomy and ribotyping of *Vibrio splendidus* biovar I and *Vibrio scophthalmi* strains associated with turbot cultures

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R. FARTO, M. MONTES, M.J. PÉREZ, T.P. NIETO, J.L. LARSEN AND K. PEDERSEN. 1999. Twelve *Vibrio* strains were examined phenotypically in 91 biochemical characters and genotypically by ribotyping. Ten were isolated from sea water and two from diseased turbot (*Scophthalmus maximus*). All isolates originated from one experimental system located in Ría de Vigo (Galicia, north-west Spain). Different type strains were used for comparative purposes. The taxonomic position was analysed with the NTSYST-pc and similarities among strains were calculated by the Simple Matching coefficient (S_{SM}). rRNA gene restriction patterns were performed with the *Hin*dIII enzyme. The S_{SM} coefficient separated the 12 *Vibrio* strains into two groups which included strains that showed a S_{SM} coefficient quite similar to *V. splendidus* biovar I (ATCC 33125) and *V. scophthalmi* (CECT 4638). None of 91 phenotypical characters were specific in distinguishing both species. The ribotyping confirmed the taxonomic classification of strains. The pathogenicity of each strain was evaluated; 10 environmental strains were avirulent and two, isolated from diseased turbot, were virulent. Different biotypes and ribotypes were found among the avirulent isolates. This work showed ribotyping to be a valuable tool for identification and confirmed the necessity of extending the ribotype database within closely related *Vibrio* species in order to clarify the taxonomic position.

INTRODUCTION

The taxonomy of the genus *Vibrio* is not yet clear. An increasing number of isolates from diseased fish and the environment must therefore be allocated to the approved list of species using biochemical and serological methods. This process is difficult when the species compared are very similar in their biochemical and physiological behaviour, and molecular biological techniques may therefore be of great value.

rRNA gene restriction pattern analysis has been suggested as a potential tool for the subtyping of *Vibrio* species (Koblavi 1996) and for distinguishing between clinical and environmental isolates of *V. anguillarum* (Rehnstam *et al.* 1989; Pedersen and Larsen 1993), *V. damsela* (Pedersen *et al.* 1997) and different species of *Aeromonas* (Kuijper *et al.* 1989; Moyer *et al.* 1992a,b; Pedersen *et al.* 1996).

The first description of *V. splendidus* was recorded by Reichelt *et al.* (1976) but it was first classified as *Photobacter splendidum* by Beijerink (1900). *Vibrio splendidus* has been included in several numerical taxonomic studies of *Vibrio* species (Hada *et al.* 1984; Bryant *et al.* 1986a; West *et al.* 1986,b; Cerdà-Cuéllar *et al.* 1997; Austin *et al.* 1997) and in some studies, this species has been designated as *V. anguillarum*-like or *V. anguillarum*-related (VAR) (Bryant *et al.* 1986a,b; Toranzo and Barja 1990; Myhr *et al.* 1991b; Pazos *et al.* 1993; Angulo *et al.* 1994a,b).

Vibrio splendidus is commonly considered to be an environmental organism with no pathogenic importance (Baticados *et al.* 1990; Paillard and Maes 1990; Myhr *et al.* 1991b; Castro *et al.* 1992), but several studies have reported that it is involved in infections in turbot, rainbow trout (*Oncorhynchus mykiss*) (Myhr *et al.* 1991b; Pazos *et al.* 1993; Angulo *et al.*

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1994a,b) and in shellfish (Jeffries 1982). Also, other authors (Lupiani *et al*. 1989; Bloch *et al*. 1991; Angulo *et al*. 1994a,b) have demonstrated an association between *V. splendidus* and other infective agents.

Vibrio scophthalmi is a recently described species isolated from the gastrointestinal tracts of juvenile turbot (Cerdà-Cuéllar *et al*. 1997). These authors demonstrated that isolates of this *Vibrio* species constitute a genotypically well-defined new group with a phenotype clearly different from related species.

The aim of the present study was to identify *Vibrio* strains, 10 of which were isolated from water and two from diseased turbot. The efficacy of phenotypic characteristics, numerical taxonomy and the ribotyping (rRNA) pattern as methods of classification was investigated.

MATERIALS AND METHODS

Bacterial strains

Twelve isolates were investigated, together with some other selected reference strains: *V. aestuarianus* (ATCC 35048), *V. alginolyticus* (ATCC 17749), *V. anguillarum* (775, RG-111, ATCC 43307, serotypes O1, O2 and O3, respectively), *V. natriegens* (ATCC 18084), *V. scophthalmi* (CECT 4633), *V. splendidus* biovar I and biovar II (ATCC 33125, NCIMB 2251, respectively), *V. pelagius* biovar I and II (NCIMB 1900, NCIMB 2253, respectively), *V. proteolyticus* (ATCC 15338), *V. tubiashii* (NCIMB 1340) and *V. vulnificus* biotipo I (ATCC 27562). The source of the tested isolates and the closest type strains are listed in Table 1.

Table 1 Designations and sources of studied strains included in the numerical taxonomy and ribotyping analysis

Species and strain	Source	Donor*
Vibrio splendidus biovar *I*		
1·103	Sea water†	T.P. Nieto
1·105	Sea water	T.P. Nieto
2·14	Aea water	T.P. Nieto
2·21	Sea water	T.P. Nieto
16N	Diseased turbot†	L. Angulo
43N	Diseased turbot	L. Angulo
ATCC 33125	Marine fish	ATCC
Vibrio scophthalmi		
1·104	Sea water	T.P. Nieto
1·106	Sea water	T.P. Nieto
1·109	Sea water	T.P. Nieto
1·111	Sea water	T.P. Nieto
1·113	Sea water	T.P. Nieto
1·114	Sea water	T.P. Nieto
CECT 4638	Turbot	CECT

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†Sea water and diseased *Scophthalmus maximus* from one experimental system located in the Ría de Vigo (Galicia, northwest Spain).

Phenotypic characterization

Strains were examined in 91 tests. Gram-staining, cell morphology, motility, and susceptibility to the vibriostatic agent, O/129 (150 μg), with discs (Oxoid) were done by standard methods. Other tests included: physiology and resistance to physical and chemical factors (the ability to grow at different temperatures, pH, final NaCl concentrations, tolerance to brilliant green, crystal violet, neutral red, salmonella-shigella (SS) agar, thiosulphate-citrate-bile salts (TCBS) agar), biochemical tests (Thornley's arginine: ADH, Moeller's lysine: LDC, ornithine: ODC, histidine: HDC, haemolysis of sheep erythrocytes, nitrate reduction, methyl red, Voges-Proskauer, catalase, oxidase, hydrogen sulphide production from sodium thiosulphate, indole production, citrate (Simmons), fermentative or oxidative metabolism of glucose), degradation of organic compounds (casein, cellulose, chitin, chondroitin, DNA, esculin, lecithin, starch, Tween-20, Tween-40, Tween-80 and urea) and growth on acetate, L-β-alanine, DL-alanine, alginate, L-arginine, aspartate, citrate, formate, glycine, inulin, L-lysine, malonate, L-phenylalanine, L-proline, propanol, pyruvate, L-serine, succinate, L-tartrate, L-tryptophan, uracil as sole carbon sources. These were performed using previously described methods (Kaper *et al*. 1983). Acid production from carbohydrates (D-amygdalin, L-arabinose, arbutin, butanol, D-cellobiose, ethanol, D-fructose, D-galactose, glycerol, myo-inositol, lactose, D-mannitol, D-mannose, D-melibiose, D-raffinose, L-rhamnose, D-ribose, salicin, D-sorbitol, sucrose and D-trehalose) was tested as described by Esteve (1995). Each strain was spot-inoculated (Denley multipoint inoculator) and incubated at 22 °C for 48 h, except for chitinase, lipase and hydrolysis of cellulose when they were incubated for 5 d, and growth on as sole carbon source when they were incubated for 7 d. In the tests using methyl red, urea, arginine dihydrolase, ornithine, lysine and histidine decarboxylases, they were inoculated in tubes. In addition, API 20 NE strips (BioMérieux, SA) were used for nitrate reduction, hydrolysis of gelatine and β-galactosidase activity.

Coding of data and computer analysis

The phenotypic characters were analysed using the NTSYS-pc numerical taxonomy and multivariate analysis sys-

tem (Rolf 1994) on an ASUS-Pentium II computer. The results of the 91 tests were scored as 1 for positive results, 0 for negative results and 9 for non-comparable data. Similarities among strains were calculated by the Simple Matching coefficient (Sokal and Michener 1958).

Ribotyping

The method described by Pedersen and Larsen (1993) was used. Chromosomal DNA was isolated and digested with *Hin*dIII (Promega, Madison, WI, USA), electrophoresed in 0·8% agarose (SeaKem GTG, Rockland, ME, USA) gels in Tris-acetate buffer (Tris 40 mmol l^{-1}, sodium acetate 5 mmol l^{-1}, EDTA 1 mmol l^{-1}) transferred to nylon hybridization membranes (Hybond-N; Amersham, UK), and fixed to the membranes by incubation at 80 °C for 1 h. Hybridization was carried out with a digoxigenin-labelled probe complementary to 16S and 23S rRNA of *Escherichia coli*, and the hybridized fragments were visualized with alkaline phosphatase-labelled antidigoxigenin and nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Boehringer Mannheim).

Virulence assays

The virulence for fish was studied in turbot (60 g) kept in aerated sea water at 18 °C and salinity of 30‰. Turbot were inoculated intraperitoneally with 0·1 ml of a bacterial suspension obtained during exponential growth, at a dose of 10^7 cfu $fish^{-1}$. Groups of four fish were used for each experiment. A control group was injected with 0·1 ml nutrient broth. Bacterial isolates were considered to be avirulent strains (Santos *et al*. 1991) if no inoculated fish was dead after 1 week. This assay were performed for all the strains except 16N and 43N recorded by Angulo *et al*. (1994a,b).

RESULTS

Phenotypic characterization

A total of 91 phenotypic features were investigated for the 12 isolates and reference strains. The isolates were compared with different species represented by their type strains (data not shown), and they were found to group with the type strains of *Vibrio splendidus* biovar I (ATCC 33125) and *V. scophthalmi* (CECT 4638). Similarity values derived from the S_{SM} coefficient are shown in Table 2. The average probability (P) of an erroneous test result was 0·023 calculated from the pooled variance $s^2 = 0{\cdot}029$ of all characters scored for the duplicate cultures. Then, the isolates were separated into two groups. One group included the type strain of *V. splendidus* and 16N, 43N, 2·14 and 2·21. This group shared an S_{SM} higher than 82%. The two strains isolated from turbot showed a more homogeneous phenotype, and with the type strain, than environmental strains. The strains 1·103 and 1·105 were included in this group as they showed a higher homology with *V. splendidus* than *V. scophthalmi*. Eighteen new properties were evaluated for these species that had not been described before.

Table 2 Percentages of similarities between experimental strains and type strains using S_{SM}

Experimental isolates	Type strains	
	Vibrio splendidus biovar I ATCC 33125	*Vibrio scophthalmi* CECT 4638
1·103	69	68
1·105	75	69
2·14	83	72
2·21	84	72
16N	91	76
43N	97	75
1·113	69	73
1·106	72	75
1·114	73	76
1·109	74	77
1·104	76	82
1·111	77	83

The other group included the type strain of *V. scophthalmi* and isolates 1·104, 1·106, 1·109, 1·111, 1·113 and 1·114. This group shared an S_{SM} higher than 72%, the lowest value being for strain 1·113. Sixty-two properties were reported for this species for the first time. The results of the phenotypic study are presented in Tables 3 and 4, respectively.

Ribotyping

On the basis of ribotyping, two different groups were found when the patterns of the isolates were compared with those of the type strains. All the isolates belonging to each group showed a ribotype pattern with a remarkable homology to the type strain of *V. splendidus* and *V. scophthalmi*, respectively. None of the strains had ribotypes identical to any of the type strains, but all the strains shared several bands. The profiles were arbitrarily designated as 1 to 6 in *V. splendidus* and 7 to 10 in *V. scophthalmi*.

The first group was separated into six ribotypes; all the strains shared multiple bands with the type strain of *V. splendidus*, profile 2. Profile 1 was found in two isolates from diseased turbot (16N and 43N) and they shared 84 of 91 phenotypical properties (Fig. 1). Each strain isolated from sea water (1·103, 2·14, 2·21 and 1·105) showed a unique ribotype banding pattern (profile 3, 4, 5 and 6, respectively), although they shared most bands (Fig. 2).

Table 3 Phenotypic characteristics of *Vibrio splendidus* biovar I studied strains

Tests*	Experimental isolates						Type strain	% Strains that share their result with the type strain
	1·103	1·105	2·14	2·21	16N	43N	ATCC 33125	
ADH	−	+	+	+	+	+	+	83†
Haemolysis	−	+ (*β*)	+ (*β*)	−	−	−	−	67
Indole	−	+	+	+	+	+	+	83
Growth:								
7% NaCl	+	+	−	−	−	−	−	67
Brilliant green	+	+	−	−	−	−	−	67
Citrate (Simmons)	+	+	+	−	−	−	−	50
Crystal violet	−	+	+	−	−	−	−	67
Hydrolysis of:								
Casein	+	+	+	+	−	−	−	33
Chitin	−	−	+	+	−	−	+	33
Cellulose	+	−	−	−	−	−	−	83
DNA	+	−	−	−	−	−	−	83
Esculin	−	−	+	−	+	+	+	50
Gelatine	+	+	+	+	−	−	+	67
Lecithin	−	−	−	+	+	+	+	50
Starch	−	+	+	+	+	+	+	83
Tween-20	−	+	+	+	−	+	+	67
Tween-40	−	+	−	+	−	−	−	67
Tween-80	−	−	−	+	−	−	−	83
Acid from:								
D-amygdalin	+	−	−	−	−	−	−	83
Arbutin	−	−	−	−	+	−	+	17
D-galactose	+	+	+	+	−	−	−	33
Glycerol	+	+	+	−	−	−	−	50
D-melibiose	+	+	−	−	−	−	−	67
myo-inositol	+	−	−	−	−	−	−	83
Sorbitol	+	−	−	−	−	−	−	83
Sucrose	+	+	−	−	+	+	+	67
D-trehalose	+	−	+	+	+	+	+	83
Use as sole carbon source:								
Acetate	+	+	−	+	+	−	−	33
β-alanine	−	+	−	+	+	+	+	67
L-arginine	+	+	+	−	+	+	+	83
L-aspartate	+	+	−	−	+	−	−	50
Inulin	+	+	−	−	+	+	+	67
L-lysine	+	+	−	−	+	−	−	50
Malonate	+	+	−	−	+	+	+	67
L-phenylalanine	+	−	−	−	+	−	−	67
Propanol	+	+	−	−	+	+	+	67
L-serine	+	+	+	+	+	−	−	17
L-tartrate	+	−	+	+	+	+	+	83
L-tryptophan	+	+	−	−	−	−	−	67
Uracil	+	−	−	−	+	+	+	50

*Tests that were positive for all of the strains: rod-shaped, motility, oxidase, catalase, O/129 sensitivity (150 μg), growth at 4, 10 and 28 °C, pH: 9 and 10, growth on: TCBS, 0·5% NaCl, NO_2 production, neutral red, methyl red, fermentative in O/F test; acid from D-cellobiose, D-fructose, D-mannitol, D-mannose, D-ribose; use as sole carbon source: DL-alanine, alginate, citrate, *β*-galactosidase, glycine, L-proline, pyruvate, succinate. Tests that were negative for all of the strains: Gram-staining, growth at 37 and 44 °C, pH 4·5, 10% NaCl, Agar SS, HDC, LDC, ODC, H_2S production, Voges-Proskauer, haemolysis α; acid from: L-arabinose, butanol, ethanol, lactose, D-raffinose, rhamnose, salicin; hydrolysis of: chondroitin, urea; use as sole carbon source of formate.
†The results were considered as: +, positive for 90–100%, v, variable for 89–11%, −, negative for $\leqslant$10%.

Table 4 Phenotypic characteristics of *Vibrio scophthalmi* studied strains

Tests*	Experimental isolates						Type strain	% Strains that share their result with the type strain
	1·104	1·106	1·109	1·111	1·113	1·114	CECT 4638	
ADH	+	+	+	−	−	−	−	50†
LDC	−	−	−	−	+	−	−	83
Haemolysis	−	+ (*β*)	−	−	−	−	−	83
Indole	−	−	−	−	+	−	−	83
Growth:								
4 °C	+	−	−	−	+	+	+	50
37 °C	−	+	−	−	+	+	−	50
44 °C	+	+	−	−	−	+	−	50
pH 4·5	−	+	−	−	−	+	−	67
7% Na Cl	+	+	+	+	+	+	−	0
Brilliant green	+	+	+	+	+	+	−	0
Crystal violet	−	−	−	−	+	+	−	67
Hydrolysis of:								
Chitin	−	−	−	−	−	−	+	0
Tween-20	+	−	−	−	+	+	−	50
Tween-40	+	−	−	−	−	−	−	83
Urea	−	+	−	−	−	−	−	83
Acid from:								
D-amygdalin	−	−	+	−	−	−	−	83
L-arabinose	−	−	−	−	+	−	−	83
D-cellobiose	−	−	+	−	−	−	−	83
D-galactose	−	−	−	−	+	−	+	17
Glycerol	−	−	−	−	+	−	−	83
D-mannitol	−	−	+	−	+	−	−	67
D-melibiose	−	−	+	−	−	−	−	83
D-raffinose	−	−	+	−	+	−	−	67
Salicin	−	−	+	−	−	−	−	83
D-sorbitol	−	−	+	−	−	+	−	67
D-trehalose	+	+	+	+	−	+	−	17
Use as sole carbon source:								
B-alanine	−	+	+	+	+	+	−	17
β-galactosidase	−	−	−	+	−	−	−	83
Glycine	+	+	+	+	+	+	−	0
Formate	−	+	−	−	+	+	+	50
Inulin	+	+	+	+	+	+	−	0
Malonate	+	+	+	+	+	+	−	0
L-Phenylalanine	−	+	−	+	+	+	−	33
Pyruvate	+	+	−	−	+	+	−	33
L-Serine	+	+	+	+	+	+	−	0
L-Tartrate	−	+	−	+	+	+	−	33
L-Tryptophan	+	+	−	−	+	+	−	33
Uracil	−	+	−	−	+	+	−	50

*Tests that were positive for all of the strains: rod-shaped, motility, catalase, oxidase, O/129 sensitivity (150 μg), growth at 10 and 28 °C, pH: 10 and 9, growth on: TCBS, 0·5% NaCl, NO_2 production, neutral red, methyl red, fermentative in O/F test; acid from D-mannose, D-fructose, D-ribose, sucrose; hydrolysis of esculin; use as sole carbon source: acetate, DL-alanine, alginate, L-arginine, L-aspartate, citrate, L-lysine, L-proline, propanol, succinate. Tests that were negative for all of the strains: Gram-staining, growth at 10% NaCl, SS agar, HDC and ODC, H_2S production, Voges-Proskauer, haemolysis α, citrate (Simmons); acid from: arbutin, butanol, ethanol, myo-inositol, lactose, rhamnose; degradation of casein, cellulose, chondroitin, DNA, gelatine, lecithin, starch, Tween-80.

†The results were considered as: +, positive for 90–100%, v, variable for 89–11%, −, negative for $\leqslant$10%.

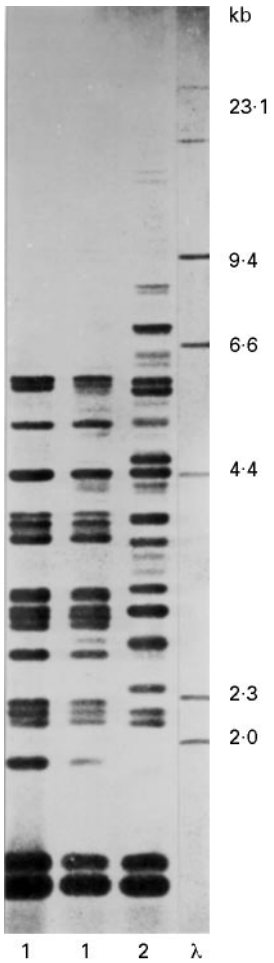

Fig. 1 Ribotype pattern of *Vibrio splendidus* biotype I. Lanes 1: the ribotype pattern of virulent strains 16 N and 43N, respectively. This pattern is shown in gel 1 and 2, but in the latter, lower fragments were lost when they were transferred to a nylon hybridization membrane. Lane 2: Ribotype pattern of type strain ATCC 33125. Lanes 3, 4, 5 and 6: ribotype pattern of environmental strains (1.103, 2.14, 2.21 and 1.105, respectively). The fragment sizes of the digoxigenin-labelled λ marker digested with Hind III shown in kb

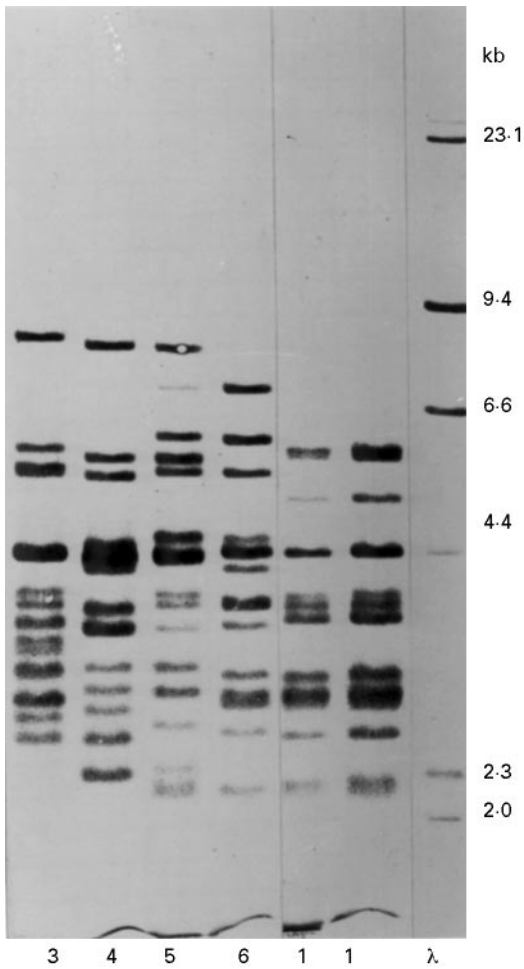

Fig. 2 Ribotype pattern of *Vibrio splendidus* biotype I

The second group was separated into four ribotypes; profile 8 was found in four isolates from sea water (1·104, 1·106, 1·111 and 1·114). The profiles 7, 9 and 10 were found in CECT 4638, 1·113 and 1·109, respectively (Fig. 3).

The S_{SM} values of isolates 1·103 and 1·105 with *V. splendidus* were lower than 82% and made it difficult to allocate these isolates taxonomically, but the ribotyping patterns allowed these isolates to be assigned to *V. splendidus*.

Virulence

The virulence assay demonstrated that 10 strains isolated from sea water were avirulent. However, the strains isolated from turbot (16N and 43N) were virulent as they had LD_{50} values $1{\cdot}2 \times 10^4$ cfu turbot^{-1} (Angulo *et al.* 1994a) and $1{\cdot}6 \times 10^4$ cfu turbot^{-1} (Angulo *et al.* 1994b), respectively.

DISCUSSION

Among the strains grouped with *V. splendidus*, all isolates gave the same results as the type strain with respect to growth at pH 10 and 4·5, growth in 10% NaCl, neutral red, H_2S and nitrite production, acid from D-cellobiose, hydrolysis of chondroitin and urea, and use of formate and pyruvate as sole carbon source. This is in agreement with results reported by other authors (Hada *et al.* 1984; Bryant *et al.* 1986a,b; West *et al.* 1986; Myhr and Larsen 1991a; Alsina and Blanch 1994; Holt *et al.* 1994; Cerdà-Cuéllar *et al.* 1997). When isolates 1·103 and 1·105 were included in this group, 40 variable results were found among *V. splendidus* strains. From these, indol, hydrolysis of DNA, starch, acid from D-melibiose, myo-inositol and D-sorbitol have been reported as 100%

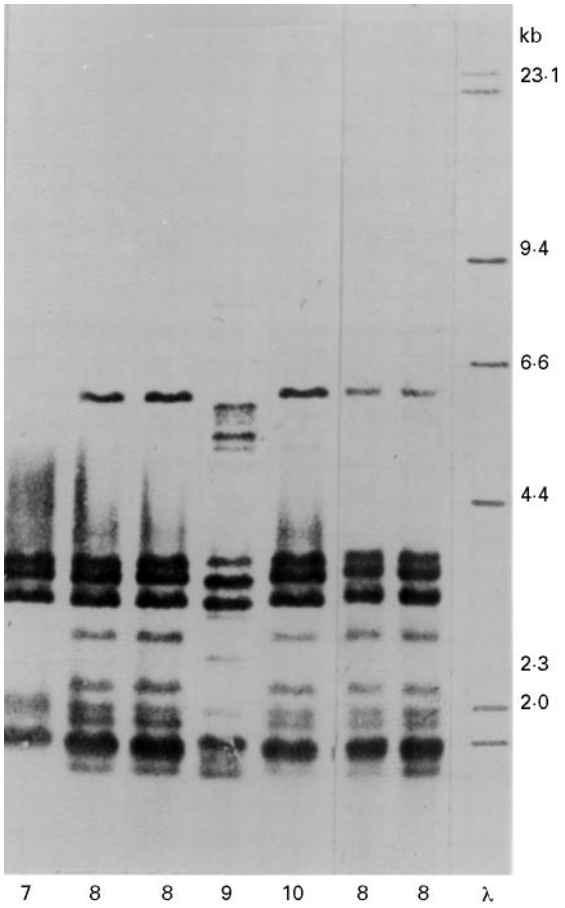

Fig. 3 Ribotype pattern of *Vibrio scophtalmi* strains. Lane 7: the ribotype pattern of type strain CECT 4638; Lane 8: the ribotype pattern of strains 1.106, 1.114, 1.111 and 1.104, respectively; Lanes 9 and 10: the ribotype pattern of 1.113 and 1.109 respectively; λ, the fragment sizes of the digoxigenin-labelled λ phage marker digested with HindIII shown in kilobases. (Each number indicates one ribotype pattern)

positive or negative for *V. splendidus* by several authors (Hada *et al.* 1984; Bryant *et al.* 1986a,b; West *et al.* 1986; Myhr *et al.* 1991a; Alsina *et al.* 1994; Holt *et al.* 1994; Cerdà-Cuéllar *et al.* 1997).

All isolates showed the same results as the type strain in growth at 4, 10, 28 and 37 °C, Voges-Proskauer, LDC, ODC, acid from D-mannitol, D-mannose, lactose, salicin. and use as sole carbon source of DL-alanine, L-proline and succinate, but discrepancies in these characters have been reported by other authors (Hada *et al.* 1984; Bryant *et al.* 1986a,b; West *et al.* 1986; Myhr *et al.* 1991a; Alsina *et al.* 1994; Holt *et al.* 1994; Cerdà-Cuéllar *et al.* 1997). Results were also obtained that were in agreement with those of the type strain but were opposite to those previously recorded by other authors (Hada *et al.* 1984; Bryant *et al.* 1986a,b; West *et al.* 1986; Myhr *et al.* 1991a; Alsina *et al.* 1994; Holt *et al.* 1994; Cerdà-Cuéllar *et al.* 1997), for example hydrolysis of Tween-40, -80 and DNA, and use as sole carbon source of β-alanine, malonate and glycine. Heterogeneity among *V. splendidus* isolates was recorded previously by Austin *et al.* (1997) and these results confirmed the existence of different biotypes among *V. splendidus* isolates.

The characteristics ODC, citrate (Simmons), production of amylase, gelatinase and acid production from sucrose, were described as differential for *V. scophthalmi* and our results are in agreement with those proposed for this species by Cerdà-Cuéllar *et al.* (1997). However, we obtained variable phenotypic results in the tests ADH, LDC, indole, utilization of D-mannitol, and growth at 4, 35 and 44 °C, that were reported as 100% positive or negative by these authors. Properties that were unable to separate closely-related species (utilization of D-melibiose, D-raffinose, D-sorbitol, D-trehalose, hydrolysis of urea, and use as sole carbon source of β-galactosidase and L-phenylalanine) were also variable in our results. Other characters (growth at 7% NaCl, Brilliant green, hydrolysis of chitin, and use as sole carbon source of glycine, inulin, malonate and L-serine) were demonstrated for the type strain and not for the present isolates. These results showed different biotypes among *V. scophthalmi* isolates. Interestingly, this is the first description of *V. scophthalmi* isolated from the water of experimental turbot cultures.

Discrepancies between laboratories can be generated by different suppliers of media and methodology (Bryant *et al.* 1986a). For instance, the citrate test for *V. splendidus* was described as positive by Cerdà-Cuéllar *et al.* (1997) and discrepancies were reported for this species by Alsina *et al.* 1994. For this species, gelatinase was recorded as positive (Hada *et al.* 1984; Bryant *et al.* 1986a,b; Myhr *et al.* 1991a; Alsina *et al.* 1994; Holt *et al.* 1994; Cerdà-Cuéllar *et al.* 1997) and variable (West *et al.* 1986). Therefore, experimental protocols must be standardized among laboratories in order to intercalibrate differential tests for distinguishing closely-related *Vibrio* species.

The high variability found in this study either for *V. splendidus* biovar I or for *V. scophthalmi* makes it difficult to propose phenotypic differential tests. Therefore, arginine dihydrolase and acid production from D-mannitol were proposed by Cerdà-Cuéllar *et al.* (1997) as positive and negative characters in *V. scophthalmi*. However, we found that only 50% and 67% of the strains were positive and negative in this character, respectively.

The production of indole has been proposed by Cerdà-Cuéllar *et al.* (1997) as non-variable and differential in both species. However, we found that 83% of the strains showed results in agreement with those of the type strain.

Other characters, such as citrate, and production of gelatinase and amylase, were proposed as positive for all *V. splendidus* strains (Bryant *et al.* 1986a; Myhr *et al.* 1991a; Holt *et al.*

1994; Cerdà-Cuéllar *et al*. 1997), but we obtained variable results.

Strains with a variable phenotype for one test between two species being compared invalidates this test as differential (Bryant *et al*. 1986a). In accordance with this, none of the 91 tests used in this study was specific for distinguishing *V. splendidus* biovar I from *V. scophthalmi*. However, numerical taxonomy and the S_{SM} coefficient allowed these two groups to be differentiated. Therefore, strain 1.103 was different from the *V. splendidus* type strain in tests such as ADH, indole, hydrolysis of starch, and utilization of inositol and sorbitol. From these tests, production of indole and amylase suggest that this strain approaches *V. scophthalmi,* but it was grouped with *V. splendidus* because it showed an S_{SM} coefficient slightly higher for *V. splendidus* than for *V. scophthalmi*.

On the basis of ribotyping, we have established two different groups which are in agreement with the phenotypic classification. One of them included the type strain of *V. splendidus* ATCC 33125 and the other, the type strain of *V. scophthalmi* CECT 4638. The strains 16N and 43N were isolated from epizootics among juvenile turbot during December 1991 and January 1992 (Angulo *et al*. 1994a), and February 1992 (Angulo *et al*. 1994b), respectively, and showed the same ribotyping pattern. The results of ribotyping and phenotyping could indicate that there has been a clonal spread of a single strain as in some cases, a strain may change slightly with time (Pedersen *et al*. 1996, 1997). Interestingly, both strains were virulent and showed biotypes different to the avirulent environmental strains.

Furthermore, the type strain and environmental strains showed closely related patterns in agreement with the S_{SM} values. Although in the 1.103 isolate the S_{SM} value was very similar to that of the *V. scophthalmi* and *V. splendidus* type strains, this ribotyping pattern was clearly different from *V. scophthalmi*.

The different rRNA gene restriction patterns found among isolates of one species have been previously reported for various others pathogens (Pedersen and Larsen 1993, 1997) and supports the concept of this technique as a tool for epidemiological investigations.

In the second group with *V. scophthalmi*, profile 8 included four isolates with a similar S_{SM} coefficient (data not shown). However, the isolate of profile 10 (1.109) showed an S_{SM} coefficient of 87% compared with the 1.111 strain of profile 8, but a lower value compared with the other strains (1.104, 1.106 and 1.114). Profile 9 (1.113) shared several bands with profile 8 whose S_{SM} coefficient was closer to strains of this ribotype than to the strain of profile 10. The ribotype patterns of the *V. scophthalmi* isolates indicated that they constituted a homogeneus group, which is in agreement with the conclusions reached by Cerdà-Cuéllar *et al*. (1997). In contrast, the phenotypic results in the present study showed considerable variation for 91 phenotypic tests in six isolates, with 38 variable characters, whereas only 11 were found from 97 tests carried out by Cerdà-Cuéllar *et al*. (1997).

From the phenotypical tests, it was possible to distinguish two closely-related species, *V. splendidus* biovar I and *V. scophthalmi*, using numerical taxonomy, but no single test was specifically differential. Ribotyping allowed classification of strains that were closely related to both species by studying their phenotypical characters, and this was of great value as a supplement to phenotypical characterization. This confirms the applicability of ribotyping as a differential tool for the investigation of *Vibrio* species.

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