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Pathogenicity of *Vibrio splendidus* strains associated with turbot larvae, *Scophthalmus maximus*

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Abstract : Turbot larvae were challenged with eight strains of *Vibrio splendidus* isolated from diseased larvae, plus a ninth strain pathogenic to scallop larvae (A515; Nicolas et al. 1996). Six strains caused heavy mortality but the scallop pathogen and the other two strains did not. All the strains shared a large number of phenotypic traits, and an attempt was made to relate virulence to genotype and phenotype. Five of the six pathogenic strains were very similar, as shown by RAPD fingerprinting and phenotypic characteristics. The relatedness of the other strains was intermediate between the main pathogenic group and *V. splendidus* ATCC 33125, but the DNA–DNA homology between the pathogenic group and the reference strain was still high (78% of reassociation rate). The non-pathogenic isolates may be a useful tool for determining the possible virulence factors, as all the isolates differed by few characteristics.

INTRODUCTION

Most bacteria isolated from turbot larvae belong to *Vibrionaceae* (Nicolas et al. 1989; Munro et al. 1993, 1994; Keskin et al. 1994; Blanch et al. 1997). A strain tentatively identified as an *Aeromonas* sp. was isolated in larvae on the verge of high mortality (Gatesoupe 1990), and the mortalities were reproduced by experimental infection of larvae on days 5–8 after hatching (Gatesoupe 1991). The reproducibility of the infection allowed the development of a challenge test to assess the quality of turbot larvae from their resistance to the pathogen, re-named '*Vibrio* P' (Gatesoupe 1995). The strain was characterized as being very similar to *Vibrio splendidus* A515, a pathogen of scallop larvae (*Pecten maximus*; Nicolas et al. 1996). Turbot larvae have also been infected experimentally with *V.* anguillarum (Skiftesvik and Bergh 1993; Munro et al. 1995), *Aeromonas salmonicida* (Bergh et al. 1997) and *A. caviae* (Ringø and Vadstein 1998), and the intestinal route of septicaemia of *V. anguillarun* has been described in juveniles (Grisez et al. 1996). However, some variability in the virulence of closely-related strains was observed when the survival of seabass larvae, *Dicentrarchus labrax*, challenged with '*Vibrio* P', was significantly lower than the survival of larvae challenged with a strain characterized by the same API 20 E profile (Gatesoupe et al. 1997). The aim of the present experiment was to relate pathogenicity to the phenotypes and genotypes of eight isolates from turbot larvae.

MATERIALS AND METHODS

Bacteria
Over a period of 3 years, bacteria were isolated from turbot on days 7–8 after hatching, and eight strains with slightly different phenotypes were selected from larvae with a low survival

rate on day 10 (Table 1). Strain VS4 was not isolated from the larvae but from the bath in which the turbot were challenged on day 8 after hatching with another isolate (VS2). The strains were stored under liquid nitrogen on Tryptic Soy Broth (TSB, Merck) enriched with NaCl (13 g l^{-1}) and glycerol (20% v/v). *Vibrio splendidus* ATCC 33125 and A515 (Nicolas et al. 1996) were used as comparison controls.

Table 1 Origin of the eight isolates from turbot larvae, compared with *Vibrio splendidus* ATCC 33125 and A515 (Nicolas *et al.* 1996)

Code	Sampling date	Origin	Survival rate on day 10 (%)	Prior test of virulence
AH5	6 Apr. 1992	Turbot, 7 day old, starved	0	Not tested
VS1	9 July 1992	Turbot, 7 day old	0	Virulent
VS4	13 Dec. 1993	Challenge medium	Not applicable	Not tested
VS5	9 Mar. 1994	Turbot, 8 day old	4	Virulent
VS6	2 May 1994	Turbot, 8 day old	11	Virulent
VS9	26 Apr. 1995	Turbot, 8 day old	2	Not tested
VS10	14 June 1995	Turbot, 8 day old	3	Not tested
VS11	28 June 1995	Turbot, 8 day old	6	Virulent

Phenotyping

The phenotypes of the eight strains and A515 were compared using 142 characteristics. Inocula were prepared from 24 h cultures on Plate Count Agar (PCA, AES Laboratoire) diluted in aged half-strength sea water (18 psu, practical salinity unit) with the pH adjusted to 7.8. Cells were harvested in aged half-strength sea water, and inoculated into API 20 E and API ZYM strips according to the manufacturer's instruction (bioMérieux). Cells were also harvested in API 50 CHE medium, enriched with saturated NaCl solution to reach a final concentration of 18 g l^{-1}, before inoculation into API 50 CH strips. The use of 47 substrates as sole carbon and energy sources was observed according to the method of Baumann and Baumann 1981: b-alanine; L-aalanine; DL-a-amino-*n*-butyric acid, sodium salt; L-(+)-arabinose; arbutin; L-(+)-arginine hydrochloride; L-aspartic acid, monosodium salt; betaine; D-(+)-cellobiose; citric acid, trisodium salt; ethanol; D-fructose; fumaric acid, disodium salt; D-galactose; D-galacturonic acid, sodium salt; D-gluconic acid, potassium salt; D-(+)-glucosamine hydrochloride; D-(+)- glucose; L-glutamic acid, monosodium salt; glycerol; glycine; L-histidine; DL-b-hydroxybutyric acid, sodium salt; DL-isoleucine; L-leucine; L-lysine; maltose; D-mannitol; D-(+)-mannose; D-(+)-melibiose; meso-erythritol; myo-inositol; Lornithine; L-proline; propionic acid, sodium salt; L-(+)- rhamnose; salicin; DL-serine; sodium acetate; sodium pyruvate; D-(–)-sorbitol; succinic acid, disodium salt; sucrose; D-(+)-trehalose; L-tyrosine; L-valine; D-(+)-xylose. The production of exoenzymes was deduced from the appearance of clear zones on special agars (caseinase on calcium caseinate agar, phospholipase on egg yolk agar, lipase on tributyrin agar, and amylase on starch azure agar; Gatesoupe *et al*. 1997). Additional tests were Kovacs's oxidase and motility. Flagellum characteristics of strains VS4 and VS10 were determined by electron microscopy (Lambert *et al*. 1998).

Table 2 Survival rates of turbot larvae at day 7 after hatching (means standard errors; superscripts indicate significant differences among survival rates of the challenge test; $P < 0.001$). The medium was infected on day 2

Strain	Inoculum concentration on day 2 (cfu ml^{-1})	Survival rate on day 7 (%)
A515	3×10^6	$92^{ab} \pm 2$
AH5	2×10^6	$97^a \pm 1$
VS1	2×10^5	$30^c \pm 2$
VS4	3×10^6	$89^b \pm 2$
VS5	3×10^6	0^e
VS6	8×10^5	$1^e \pm 0.4$
VS9	5×10^6	$1^e \pm 0.6$
VS10	2×10^6	$14^d \pm 2$
VS11	5×10^6	$0.4^e \pm 0.4$
Not infected	0	$94^{ab} \pm 3$

Genotyping

DNA/DNA hybridization :DNA of strains VS6, A515 and *Vibrio splendidus* ATCC 33125 were extracted, labelled and hybridized according to the method described by Lambert *et al*. (1998).

RAPD analysis. DNA of the 10 strains was extracted as for normal PCR (Lambert *et al*. 1998), then analysed by Random Amplified Polymorphic DNA (RAPD). The RAPD reaction was performed with the Ready-To-Go kit (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Two short oligonucleotide primers of arbitrary sequences were successively tested (primer 3, 5'-d[GTAGACCCGT]-3' and primer 6, 5'-d[CCCGTCAGCA]-3'). Amplification was performed according to the recommendations of the manufacturer (1 cycle at 95 °C for 5 min followed by 45 cycles at 95 °C for 1 min, 36 °C for 1 min and 72 °C for 2 min). The products were analysed by electrophoresis on agarose gel (1%) in TAE buffer containing 0.5 mg ml^{-1} ethidium bromide. The base pair (bp) ladder was phage l, digested by ECoRI and Hind III.

Plasmid search. The same electrophoresis procedure was used to search for plasmids in strains VS4 and VS11 after extraction with the QIAprep Spin Miniprep kit (Qiagen, Courtabouef, France).

Sequencing of the 16S rDNA gene. The 16S rDNA genes of strains A515 and VS6 were sequenced with the method described by Anderson *et al*. (1992) and modified by Ruimy *et al*. (1994). Nine DNA primers were used in the sequencing reactions. These primers corresponded to the following positions in the *Escherichia coli* sequence: S2, 99–119; S4, 342–356; S6, 518– 534; S8, 684–702; S10, 906–925; S12, 1099–1114; S15, 1384– 1400 and S17, 1493–1509.

Phylogenetic analysis. The sequences were analysed with Phylo–win software (Galtier *et al*. 1996; Villeurbanne, France). The data described below were obtained by alignment of the different sequences and phylogenetic analysis. SSU rRNA sequences were aligned by the

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Megalign program (Multiple and Pairwise Sequence Alignment, DNAstar). The domains used for deriving phylogenies were restricted to the parts of sequences with reliable homologies and few undetermined nucleotides.

The phylogenetic trees were constructed using the 'neighbour- joining' algorithm developed by Saitou and Nei (1987). The maximum likelihood algorithm used in Phylo–win was the same as in fastDNAml (a faster DNA maximum likelihood phylogeny estimator; Olsen *et al*. 1994). The maximum parsimony algorithm was that of DNApars (DNA Parsimony Program), a part of the PHYLogeny Inference Package (PHYLIP; Felsenstein 1996, Washington, USA). Bootstrap analysis (500 replications) was performed to check each topology for robustness.

The nucleotide sequences used in this study have the following EMBL database accession numbers: *V. mediterranei* X74710, *V. orientalis* Z21731, *V pectenicida* Y13830, *V. splendidus* Z31657, *V. tapetis* Y08430, *Vibrio* 2P44 U57919, A515 AJ132986, VS6 AJ132988.

Challenge test

One hundred batches of 50 newly-hatched turbot larvae were introduced into Nalgene square bottles filled with 500 ml sea water and kept for 1 week at 15 °C without renewing the medium. Cell suspensions of fresh cultures of the eight strains and A515 were introduced, on day 2 after hatching, into 10 bottles per strain, to reach a final concentration between 2.10^5 and 5.10^6 cfu ml^{-1} (Table 2). In 10 further bottles, no bacteria were added to serve as a non-infected control group. The dead were counted on day 7 after hatching, and the survival rates were compared by ANOVA. The effects of the individual treatments were assessed by an *a priori* test (Sokal and Rohlf 1969).

Fig. 1 Dendogram constructed with phenotypic characterization. Similarity rate (%) was computed on the basis of 19 variable characters (Table 3) using the coefficient of Sokal and Michener (1958). The strains were clustered according to variance
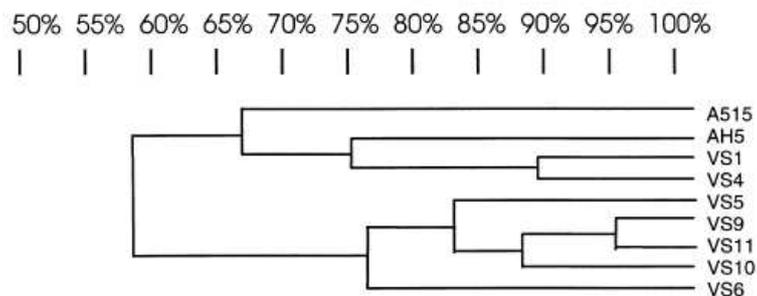
RESULTS

Differences among strains were observed for 19 of the 142 characteristics tested (Table 3), discriminating two main clusters (Fig. 1). The first group comprised A515 and the oldest turbot isolates (AH5, VS1 and VS4). The most recent isolates, VS5–VS11, were closely related. One polar flagellum was observed on VS4 and VS10 cells.

The phylogenetic position of VS6 was very close to that of *V. splendidus* ATCC 33125, whereas strain A515 was slightly more distant (Fig. 2). However, similar rates of reassociation of 79 and 78% were observed when DNA–DNA homology was tested between the probe *V. splendidus* ATCC 33125 and strains A515 and VS6, respectively. Three different RAPD band patterns were obtained with primer 3 (Fig. 3, upper gel). The simplest of these characterized *V. splendidus* ATCC 33125, A515 and VS1 with one common band at approximately 900 bp. Strain AH5 shared the same common band with the previous group,

but three other bands appeared. One of these bands (830 bp) was common to AH5 and the pattern of the remaining group VS4– VS11.
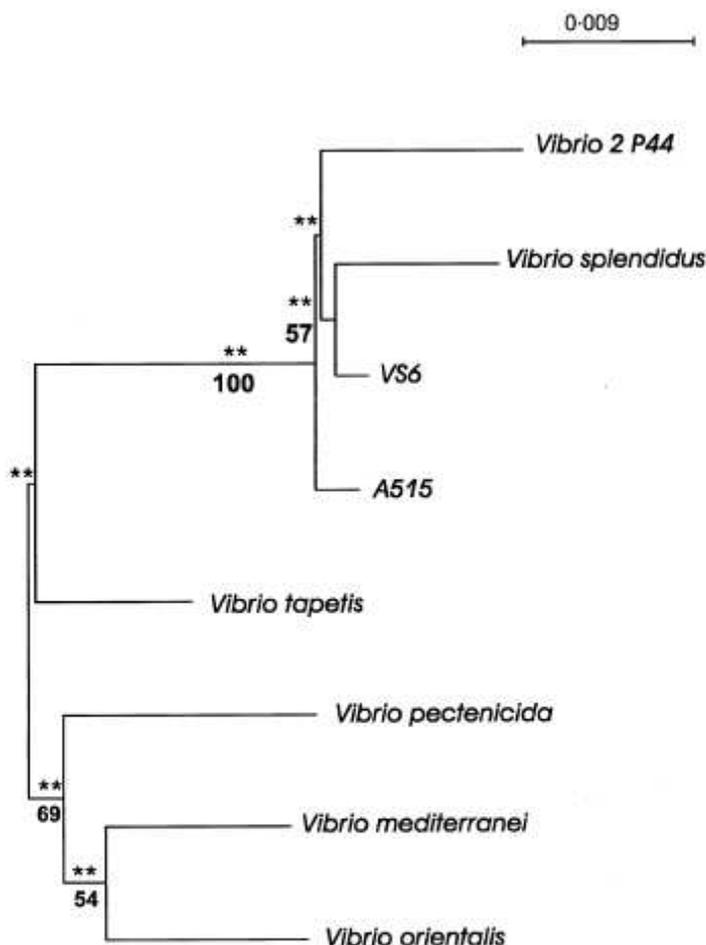

Fig. 2 Phylogenetic positions of strains VS6 and A515 with regard to reference strains. The unrooted tree was obtained with the neighbour–joining method using 1123 sites. The branches retrieved with a maximum-likelihood algorithm are marked with two asterisks. The bootstrap values obtained by maximum parsimony analysis are shown above the branch only when they are greater than 50% (500 replicates)

The differences in patterns were more defined with primer 6 (Fig. 3, lower gel), confirming the similarity of *V. splendidus* ATCC 33125 and A515, but showing a new band in strain VS1. Strain AH5 was still intermediate between the two other groups, but VS4 appeared also with a different intermediary fingerprint. Plasmids were not observed in VS4 or in VS11. The survival of turbot larvae challenged with strains VS5, VS6, VS9 and VS11 was very low, and strains VS10 and VS1 were also clearly pathogenic (Table 2). The survival rates obtained with the other isolates were not significantly different from the control group without infection.
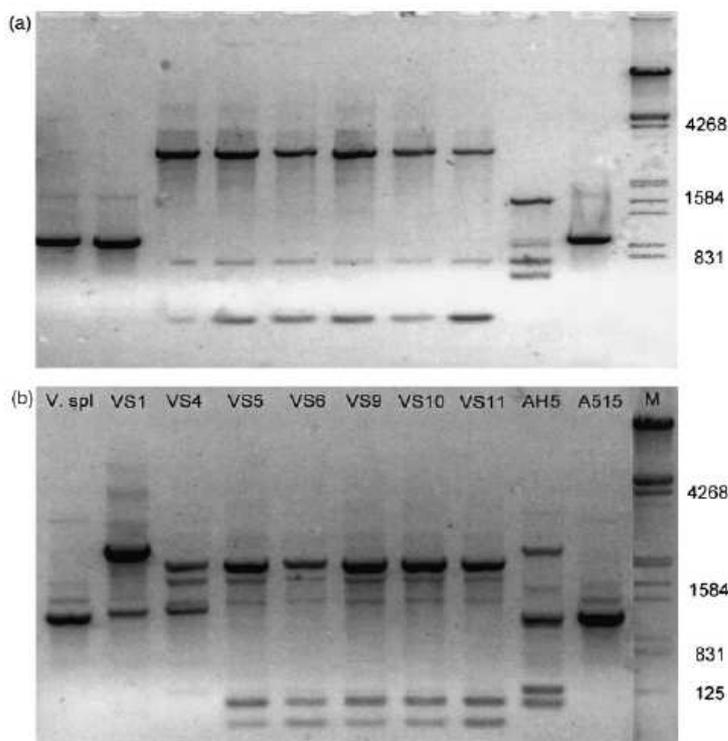
Fig. 3 RAPD fingerprints of the 10 strains obtained with primers 3 and 6 (upper (b) and lower (a) gel, respectively; V.spl: *V. splendidus* ATCC 33125; M: ladder, with numerical marks in bp on the right side of the photographs)

DISCUSSION

The phenotypic and genotypic characterizations indicated that all the strains are closely related. The phylogenetic analysis showed that even strain VS6 may be considered as a *V. splendidus* biovar, though relatively distant from A515, considering both phenotype and RAPD profile.

The two methods were complementary in differentiating the relatedness between strains. The group VS5–VS11 is genetically homogeneous, in spite of some phenotypic differences, and it comprises most of the pathogenic isolates. As recommended by Paffetti *et al*. (1995), the use of several RAPD primers may remove typing ambiguity, and the non-pathogenic strain VS4 was thus differentiated from the pathogenic group with primer 6. Strain VS1 was also pathogenic, though phenotypically and genetically nearer to *V. splendidus* ATCC 33125 than to the group VS5–VS11. Biovars of *V. splendidus* have been shown to be pathogenic to many marine animals, including fish (Miranda and Rojas 1996; Austin *et al*. 1997; Santos *et al*. 1997; Balebona *et al*. 1998), shrimps (Baticados *et al*. 1990; Chen Bisheng *et al*. 1995; Leano *et al*. 1998) and bivalves (Nicolas *et al*. 1996; Austin *et al*. 1997; Sugumar *et al*. 1998). The virulence of bacteria may vary with the host, as observed by Bergh *et al*. (1994) in an interspecific comparison between larvae of turbot, halibut (*Hippoglossus hippoglossus*) and cod (*Gadus morhua*), but the strain of *V. splendidus* tested in that study was not pathogenic to the three species. The present experiment is not sufficient to compare the virulence of the two biovars VS1 and VS5–VS11, particularly as the inoculum of VS1 was the least concentrated (2.10^5 cfu ml^{-1}), whereas the 50% lethal dose was estimated to be 10^6 cfu ml^{-1} in a previous experiment (Gatesoupe 1994).

Valine aminopeptidase was correlated with a virulence factor because the pathogenic group VS5–VS11 produced this enzyme whereas the other isolates did not. Aminopeptidases produced by bacteria may cause tissue damage (Suido *et al*. 1986), but the absence of lesion in the infected larvae does not support the hypothesis in the present case (Baudin-Laurencin 1997, personal communication). Otherwise, the virulence determinants did not seem plasmid-

borne in strain VS11. The non-pathogenic strain VS4 is particularly close to the main pathogenic group, and this may serve as a tool to investigate virulence and the possible involvement of toxins further.

Table 3 Phenotypic differences among the isolates (w: weak reaction)

Strain	A515	AH5	VS1	VS4	VS5	VS6	VS9	VS10	VS11
API 20 E-tests									
Arginine dihydrolase	+	+	+	+	+	+	−	+	+
Sodium citrate utilization	−	−	+	−	−	−	−	−	−
Voges Proskauer	−	−	−	+	−	−	−	+	−
Gelatinase	+	+	+	+	−	+	+	−	+
Melibiose fermentation	+	+	+	+	−	−	−	−	−
API 50 CHE tests									
Glycerol	+	+	+	+	−	+	−	−	−
Lactose	+	+	−	−	−	−	−	−	−
L-fucose	+	−	−	−	−	−	−	−	−
API ZYM tests									
Lipase (C14)	+	+	+	+	−	+	+	+	+
Leucine arylamidase	+	w	w	w	+	+	+	+	+
Valine arylamidase	−	−	−	−	+	+	+	+	+
Trypsin	+	w	w	w	w	w	w	w	w
acid phosphatase	+	−	+	+	+	+	+	+	+
β-Galactosidase	+	−	−	−	+	+	+	+	+
α-Glucosidase	+	−	−	−	+	−	+	+	+
Naphtol-AS-BI-phosphohydrolase	+	−	−	−	+	+	+	+	+
Motility	+	+	+	+	+	−	+	+	+
Lipase (tributyrin agar)	+	−	+	+	−	+	+	+	+
Propionate utilization (single carbon source)	+	−	+	+	+	+	+	+	+

Positive tests without difference among strains: β-galactosidase, indole production, oxidase, NO₂ production, esculin, phospholipase, caseinase, amylase, alkaline phosphatase, esterase lipase (C8), acid production from D-glucose, D-mannose, amygdalin (API 20 E fermentation/oxidation), ribose, galactose, D-fructose, mannitol, N-acetyl-glucosamine, cellobiose, maltose, melibiose, trehalose, starch, glycogen, and gluconate; utilization as sole carbon source of acetate, L-α-alanine, DL-α-amino-n-butyrate, L-(+)-arginine, L-aspartate, D-(+)-cellobiose, citrate, D-fructose, fumarate, D-galactose, D-gluconate, D-(+)-glucosamine, D-(+)-glucose, L-glutamate, glycerol, glycine, maltose, D-(+)-mannose, D-mannitol, L-ornithine, L-proline, pyruvate, succinate, D-(+)-trehalose, and L-tyrosine.

Negative tests without difference among strains: lysine decarboxylase, ornithine decarboxylase, H₂S production, urease, tryptophan deaminase, NO₃-NO₂ reduction to N₂ gas, cystine arylamidase, α-chymotrypsin, α-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, no acid production from inositol, sorbitol, rhamnose, sucrose, D-arabinose, L-arabinose, erythritol, D-xylose, L-xylose, adonitol, β-methyl-D-xyloside, L-sorbose, dulcitol, α-methyl-D-mannoside, α-methyl-D-glucoside, amygdalin (API 50 CHE fermentation), arbutin, salicin, inulin, melezitose, D-raffinose, xylitol, β-gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, D-arabitol, 2-keto-gluconate, and 5-keto-gluconate; no utilization as sole carbon source of β-alanine, l-(+)-arabinose, arbutin, betaine, ethanol, D-galacturonate, L-histidine, DL-β-hydroxybutyrate, L-leucine, L-lysine, D-(+)-melibiose, meso-erythritol, DL-isoleucine, myo-inositol, L-(+)-rhamnose, salicin, DL-serine, D-(−)-sorbitol, sucrose, L-valine, and D-(+)-xylose.

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