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Characterization of *Aliivibrio fischeri* strains associated with disease outbreak in brill *Scophthalmus rhombus*

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ABSTRACT: Three bacterial isolates were recovered from a disease outbreak with high mortality affecting brill *Scophthalmus rhombus* (Linnaeus, 1758). Moribund fish showed no external signs of disease, but plentiful haemorrhages were observed in liver. On the basis of phenotypic and genotypic characterization, the isolates were identified as *Aliivibrio fischeri*. The phenotypic profile of the isolates was basically similar to that of the type strain of this species, although some discrepancies were observed, mainly in the BIOLOG GN profile. The main cellular fatty acids of strain a591 were also consistent with this species. The highest 16S rDNA sequence similarities were recorded with the type strain of *A. fischeri* (99.07%); other *Aliivibrio* species showed similarity values below 96%. The highest sequence similarities with *gyrB*, *rpoD* and *recA* genes were also recorded with *A. fischeri* type strain (99.31, 98.99 and 95.29% similarity, respectively). DNA–DNA hybridization assays confirmed that these isolates belong to *A. fischeri*; levels of DNA relatedness were 73.5 to 86.2% with isolate a591 (reciprocal values of 86.9 to 99.04%). Finally, a virulence evaluation of the isolates using Senegalese sole fry was also performed; significant mortalities (100% mortality within 5 d) were recorded by intraperitoneal injection, but only with high doses of bacteria (2×10^6 cfu g^{-1} body weight).

KEY WORDS: Fish pathogen · Diagnosis · Identification · Virulence

INTRODUCTION

The genus *Aliivibrio* (*Gammaproteobacteria*: *Vibrionaceae*) was created by Urbanczyk et al. (2007) after reclassification of a number of *Vibrio* species from the former *Vibrio fischeri* clade, based on phylogenetic and phenotypic differences. At present, the genus comprises 6 species: *A. fischeri*, *A. logei*, *A. salmonicida*, *A. wodanis*, *A. finisterrensis* and *A. sifiae*.

A. fischeri, the type species of the genus, can be found either free living in the marine environment or associated with a eukaryotic host; in fact, certain bioluminescent strains are symbiont in the light-emitting organs of certain squids and fishes (Farmer 2006, Ruby et al. 2005). These attributes make *A. fischeri* a useful model organism for examination of microbial bioluminescence, quorum sensing and bacterial–animal symbiosis (Dunn 2012). However, *A. fischeri* strains have also been found associated with disease outbreaks in shrimp *Penaeus monodon* (Fabricius, 1798) (Lavilla-Pitogo et al. 1998) and in marine fish in Spain — namely in sea bream *Sparus aurata* Linnaeus, 1758 and turbot *Scophthalmus maximus* Linnaeus, 1758 cultures — although its pathogenicity is not clear (Lamas et al. 1990, Balebona et al. 1998, Buller 2004).

To our knowledge, the present paper reports the first description and characterization of *A. fischeri*

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strains associated with a disease outbreak in brill *Scophthalmus rhombus*.

MATERIALS AND METHODS

Bacterial isolation

Mortalities occurred in adult-sized fish (1.7 to 2.8 kg) stocked at 3.5 kg m^{-2} in a recirculating system with fiberglass tanks. Samples for bacterial isolation were taken from liver and kidney of moribund fish, and cultured on *Flexibacter maritimus* medium (FMM) (Pazos et al. 1996) at 20°C for 24 to 96 h. For long-term preservation, strains were frozen at −80°C in sterile seawater supplemented with 20% (v/v) glycerol.

Phenotypic characterization

Phenotypic characterization was performed according to Bernardet et al. (1990) and Avendaño-Herrera et al. (2004). The Gram reaction was determined according to the KOH method proposed by Buck (1982) and by the Gram-staining method. Temperature tolerance was tested by checking growth on Marine Agar (Difco) at 4, 15, 25, 30, 35, 40 and 45°C for 10 d, and tolerance of salinity was tested with growth on basal medium (neopeptone 4 g l^{-1}; yeast extract 1 g l^{-1}; agar 15 g l^{-1}) supplemented with 0, 3, 6, 8, 10 and 12% (w/v) NaCl. Growth on thiosulfate–citrate–bile salts–sucrose (TCBS) agar (Difco) was also tested. All tests were incubated aerobically at 20°C. Commercial miniaturized API 20E and API 20NE galleries (bioMerieux), and Biolog GN2 Micro-plates were also utilized according to the manufacturer's instructions, but sterile sea water was used as a diluent and 20°C as the incubation temperature. The type strain of *Aliivibrio fischeri* (CECT 524^T) was characterized together with the isolates under study, with the same methodology.

Analysis of fatty acid methyl esters

Preparation of fatty acid methyl esters (FAMEs) from strain a591, grown at 20°C on Marine Agar plates, was performed according to the instructions of the Microbial Identification System (MIDI) as described by Sasser (1990). FAMEs were analyzed by gas chromatography in an Agilent 6850 system, using the MIDI operating system and the aerobic bacteria library TSBA6 (MIDI 2008).

Phylogenetic analysis

Template DNA from pure cultures was prepared by boiling bacterial colonies for 5 min in distilled water followed by centrifugation at 16 168 × *g* for 2 min to sediment the cell debris. The concentration and purity of genomic DNA was calculated from measurements of absorbance at 260 (A_{260}) and 280 nm (A_{280}), recorded using a NanoDrop 1000 spectrophotometer. Partial 16S rRNA gene sequences were obtained using the universal primers 20F and 1500R, capable of amplifying nearly full-length 16S rDNA (Weisburg et al. 1991). Sequencing of the housekeeping genes *gyrB*, *rpoD* and *recA* was performed using the primers proposed by Santos & Ochman (2004), Yamamoto & Harayama (1998) and Islam et al. (2013), respectively. PCRs were performed basically as indicated in the literature in each case in a total reaction volume of 25 µl, using the commercial kit MyTaq^{TM} DNA Polymerase (Bioline) which includes all necessary reagents except the primers and DNA. PCR products were purified with the commercial kit Illustra ExoProStar 1-step (GE Healthcare) following the manufacturer's instructions. Direct sequencing of purified PCR products was performed by Secugen (Madrid). The sequences were analyzed using Chromas LITE and BioEdit programs and subjected to BLAST (https://blast.ncbi.nlm.nih.gov) and EzTaxon (www.ezbiocloud.net/eztaxon) searches to retrieve the most closely related sequences. Sequence similarities were calculated using SIAS software (http://imed.med.ucm.es/Tools/sias). DNA sequences were aligned with others from related species using Clustal Omega software, and phylogenetic trees were constructed according to the neighbor-joining method (Saitou & Nei 1987) by using the program MEGA. The accuracy of the resulting tree was measured by bootstrap resampling of 1000 replicates.

Identification by PCR

A PCR assay using a pair of specific primers targeting the *luxA* gene of *A. fischeri* was carried out as previously described (Buller 2004), but using a higher annealing temperature (60°C). Template DNA was extracted as mentioned previously and 100 ng of DNA were used for each strain. PCRs were

performed using the commercial kit MyTaq™ DNA Polymerase. DNA from the *A. fischeri* type strain was included as a positive control and distilled water as negative control. PCR products were electrophoresed on a 2% agarose Tris-borate-EDTA buffer gel stained with SYBR Safe DNA Gel Stain (Invitrogen). A 100 bp DNA ladder H3RTU (Nippon Genetics) was included as a molecular weight marker.

Molecular fingerprinting

Template DNA was extracted as mentioned previously and 100 ng of DNA were used for each strain. Repetitive extragenic palindromic (REP)-PCR and enterobacteria repetitive intergenic consensus (ERIC)-PCR analysis were performed as previously described (Rodríguez et al. 2006) using the commercial kit MyTaq™ DNA Polymerase, in a Veriti 96 well thermal cycler (Applied Biosystems). PCR products were electrophoresed as mentioned above. Similarity between isolates and the *A. fischeri* type strain was estimated using the Dice similarity coefficient (S_D) (Dice 1945) with the software DendroUPGMA (http://genomes.urv.cat/UPGMA).

DNA–DNA hybridization

For DNA–DNA hybridization assays, DNA was extracted with the kit NucleoSpin Tissue (Macherey-Nagel) and the concentration and purity of each sample were determined by measuring the A_{260} and A_{260}/A_{280} ratio, respectively. DNA–DNA hybridization assays were performed by the plate method proposed by Ziemke et al. (1998), combining the hydroxyapatite method with non-radioactive detection of released DNA. The hybridization temperature (T_m) was 60°C.

Pathogenicity assays

To investigate the pathogenicity of the isolates, experimental infections were performed by intraperitoneal injection of 3 different doses (2×10^4, 2×10^5, and 2×10^6 cfu g^{-1} body weight) of isolate a591 in Senegalese sole *Solea senegalensis* (Kaup, 1858) with weights between 4.5 and 5.5 g. Groups of 10 fish were used for each dose. This assay was performed in duplicate. Bacteria were growth in FMM at 20°C for 24 h, and bacterial concentration was estimated by absorbance of bacterial cultures at 600 nm wavelength. After recovery by centrifugation (16 168 × *g*, 2 min), bacteria were washed in phosphate buffered saline (PBS) and finally re-suspended in PBS. Doses were confirmed with total viable counts after spreading 0.1 ml volumes of each dose over the surface of duplicate plates of FMM. A control group (challenged with PBS only) of 10 fish was included in each virulence assay. After bacterial challenge, experimental and control fish were kept without feeding in 18 l tanks at 18 to 20°C in continually flowing seawater, and mortalities were recorded daily for a 10 d period. Dead fish were removed and subjected to bacteriological examination as previously indicated; recovered strains were identified by specific PCR.

RESULTS

Bacterial isolation

During July 2013, a epizootic outbreak with a high mortality rate (near 100%) occurred in a marine farm located in southwestern Spain, affecting brill *Scophthalmus rhombus* adult cultures in water temperatures of 18 to 20°C. Affected fish showed no external signs of disease. Internally, however, plentiful haemorrhages were observed, mainly in the liver (Fig. 1). Numerous pale yellow colonies appeared in the culture medium from liver and kidney samples, almost in pure culture. Three isolates (a589, a590 and a591) were selected for identification.

Phenotypic characterization

Colonies of the isolates under study were pale yellow in color, luminescent, not adherent to agar, and consisted of Gram-negative, fermentative rods. All isolates were cytochrome oxidase and catalase positive. Growth was observed at 4 to 35°C, but not at 40°C. All strains grew in 3 to 6% NaCl, but none in 0% or 8 to 12% NaCl. All isolates were able to grow in TCBS, displaying green colonies. The arginine dihydrolase test was negative, but all isolates were positive for lysine and ornithine decarboxylase. Hydrolysis of Tween-20 and Tween-80 were positive, but starch and casein were not hydrolysed. In API 20E galleries, positive results were recorded for nitrate reduction and acid production from glucose, mannitol, amygdalin; the ornithine decarboxylase test was also generally positive. In API 20NE galleries, only nitrate reduction, glucose fermentation, esculin hydrolysis, and the β-galactosidase test gave

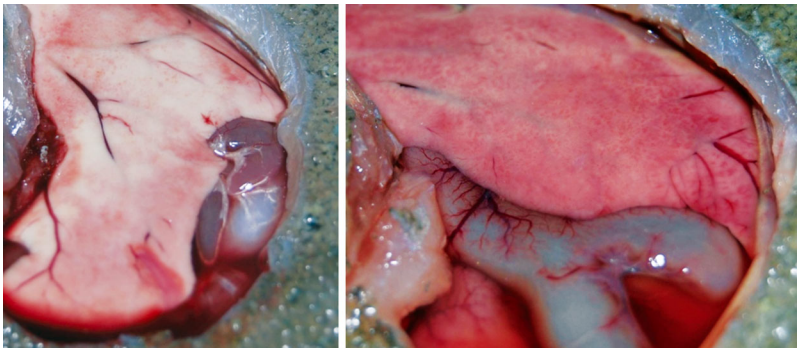

Fig. 1. Red-tinged fluid in the abdomen and haemorrhages in liver, the main symptoms observed in diseased brill *Scophthalmus rhombus*

Table 1. Differences in carbon compound utilization between *Aliivibrio fischeri* strains, including both the isolates obtained in this work from brill *Scophthalmus rhombus* (a589, a590 and a591) and the type strain of *A. fischeri* (CECT 524^T), determined using the BIOLOG GN system

Characteristic	Positive strains
Dextrin	All except a591
Maltose	a589
D-psicose	a589, a590
Cis-aconitic acid	a589
Sebacic acid	a591
Bromosuccinic acid	a589, CECT 524^T
L-asparagine	a589
L-aspartic acid	a589
L-leucine	a589
L-proline	a589
Phenyethylamine	All except a591

clear positive results. Malate assimilation was also generally positive.

Similar results were displayed by the type strain of *A. fischeri*, except for the following tests: ornithine decarboxylase (in both tube and API 20E), growth at 4°C and at 35°C, urease (in both API 20E and API 20NE), Voges-Proskauer (API 20E) and malate assimilation (API 20NE).

Data obtained from GN MicroPlates (Biolog) showed differences between the strains characterized in this work. All the brill isolates utilized the following carbon sources: N-acetyl-D-glucosamine, D-fructose, D-galactose, gentiobiose, α-D-glucose, D-mannose, inosine, uridine, thymidine, glycerol and glucose-6-phosphate. Variable results were found for 11 carbon sources (Table 1). All the isolates were negative for the remaining 73 tests. The *A. fischeri* type strain can be clearly differentiated from the brill isolates with the following tests: N-acetyl-D-glucosamine, D-fructose, D-galactose, gentiobiose, α-D-glucose, D-mannose, inosine, uridine, thymidine, glycerol and glucose-6-phosphate.

Analysis of FAMEs

The FAME analyses showed that the major cellular fatty acids (>5% of the total) in strain a591 were iso-$C_{12:0}$ (8.34%), iso-$C_{12:0}$ 3OH (6.04%), iso-$C_{14:0}$ (8.03%), iso-$C_{16:0}$ (13.79%) and sum in features 2 (5.72%), 3 (48.10%) and 8 (9.22%). These results agreed with the FAME profile published for the *A. fischeri* type strain (Yoshizawa et al. 2010).

Phylogenetic analysis

Almost complete 16S rDNA sequences were obtained from strains a589, a590 and a591 and used for BLAST and EzTaxon homology searches to retrieve the most closely related species. Based on sequence analysis, the isolates were included in the genus *Aliivibrio*, within the family *Vibrionaceae*. The highest sequence similarity for isolate a591 (1431 bp) was recorded with *A. fischeri* ATCC 7744^T (99.07% similarity), followed by the type strains of *A. finisterrensis* (95.67%), *A. sifiae* (94.96%) and *A. wodanis* (94.89%). Sequence similarities between the 3 isolates under study were 100%. The phylogenetic tree derived from these sequences illustrates the position of the isolates recovered from brill, clearly grouped with the *A. fischeri* type strain and separated from other *Aliivibrio* and *Vibrio* species (Fig. 2). Phylogenetic analysis based on *gyrB* gene sequences confirmed the clustering with high bootstrap values of the 3 isolates with *A. fischeri* (Fig. 3). The closest type strains were that of *A. fischeri* (99.31% similarity), *A. wodanis* (85.02%), *A. salmonicida* (84.51%), *A. logei* (83.74%), *A. sifiae* (80.2%) and *A. finisterrensis* (79.17%). Partial *rpoD* and *recA* gene sequences were also obtained for the 3 isolates. The highest sequence similarities for isolate a591 were obtained with *A. fischeri* type strain (98.99 and 95.29% similarity, respectively). Sequences from other *Aliivibrio* species were not available in the GenBank database. The GenBank/EMBL/DDBJ accession numbers for the sequences obtained in this work are LT616977 to LT616988.

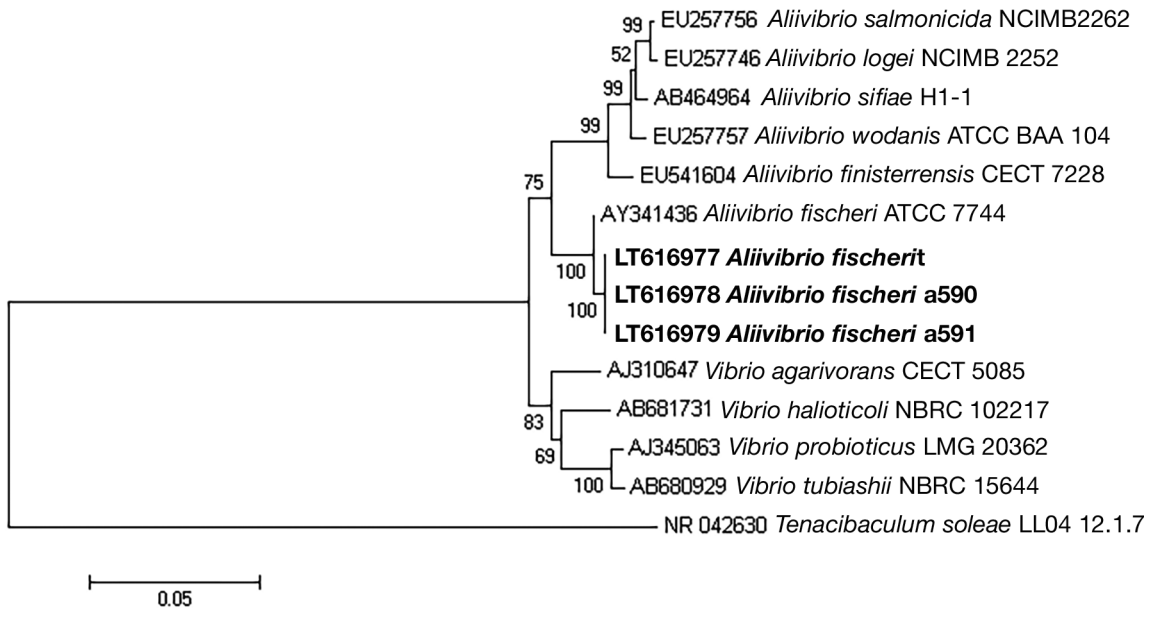

Fig. 2. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships between the isolates under study and the type strains of related members of *Aliivibrio* and *Vibrio*. The *Tenacibaculum soleae* sequence was used as an outgroup. Numbers at the nodes indicate the levels of bootstrap based on 1000 replicates. Sequences from related species were obtained from GenBank database; their accession numbers are indicated before the species name

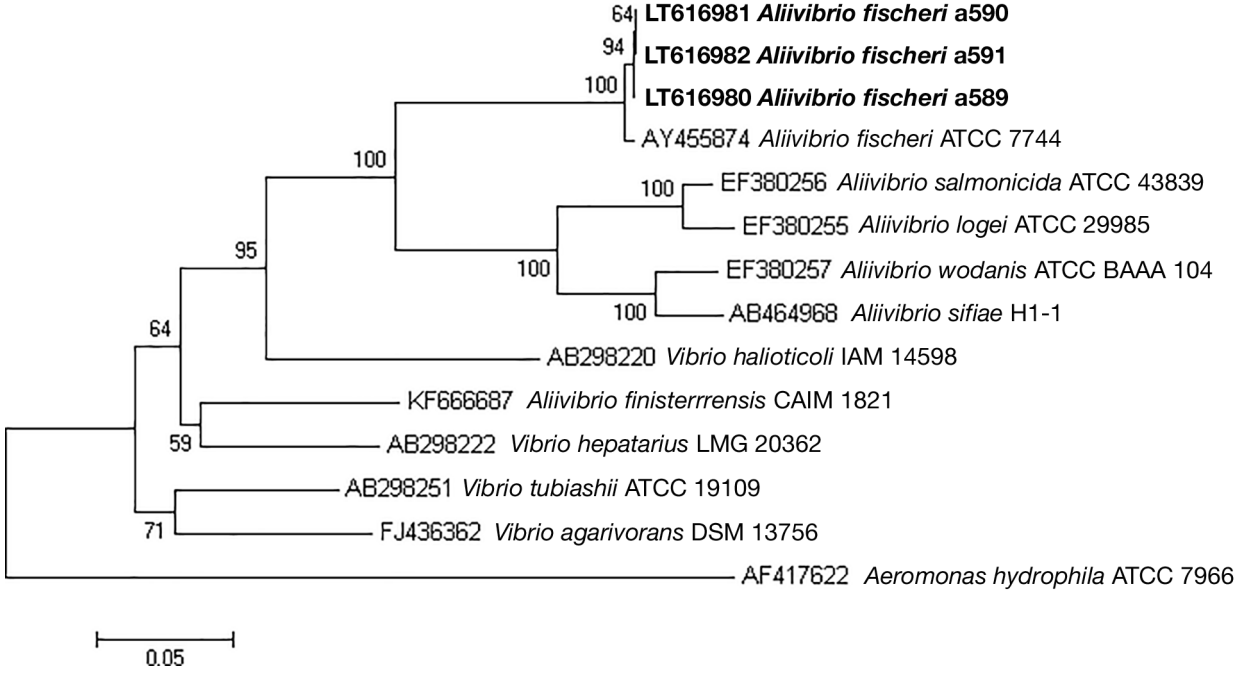

Fig. 3. Neighbor-joining phylogenetic tree based on *gyrB* gene sequences showing the relationships between the isolates under study and the type strains of related members of *Aliivibrio* and *Vibrio*. The *Aeromonas hydrophila* sequence was used as an outgroup. Numbers at the nodes indicate the levels of bootstrap based on 1000 replicates. Accession numbers are indicated before the species name

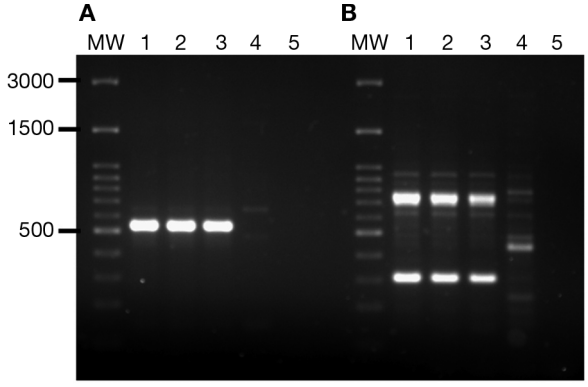

Fig. 4. (A) Repetitive extragenic palindromic (REP)-PCR and (B) enterobacteria repetitive intergenic consensus (ERIC)-PCR patterns of *Aliivibrio fischeri* strains. MW: 100 bp DNA Ladder H3 RTU molecular size marker (Nippon Genetics); lanes 1 to 5: a589, a590, a591, CECT 524 and negative control. Numbers on the left indicate the position of molecular size marker (in bp)

Identification by PCR and molecular fingerprinting

All brill isolates showed the expected 428 bp band, identical to that of the *A. fischeri* type strain, when the specific PCR protocol targeting the *luxA* gene of this species was employed (data not shown). No amplification product was detected in the negative control.

In REP-PCR and ERIC-PCR analysis, a unique profile was observed for all brill isolates, clearly different from that of *A. fischeri* type strain (Fig. 4). Dice coefficient between the 3 brill isolates was 1 for both REP-PCR and ERIC-PCR analysis. On the contrary, Dice coefficient between these isolates and the *A. fischeri* type strain was 0.333 for REP-PCR and 0.857 for ERIC-PCR. In ERIC-PCR, however, clear differences were observed between the brill isolates and the type strain with regard to the intensity of a number of bands.

DNA–DNA hybridization

DNA-DNA hybridization experiments with *A. fischeri* type strain were done in duplicate and confirmed the results obtained previously. Levels of DNA re-association between strain a591 and the type strain of *A. fischeri* were 73.5 to 86.2%. Reciprocal values were 86.9 to 99.04% with the 3 brill isolates. On the other hand, DNA–DNA hybridization values between strain a591 and the other isolates under study ranged from 91.7 to 100%.

Pathogenicity tests

Strain a591 was selected for experimental infection. Mortalities were observed only at the highest of the 3 doses employed (2×10^6 cfu g^{-1} body weight), and were 100% within the first 5 d after exposure to the pathogen. The inoculated strains were recovered from most of the dead fish. None of the control fish died during the assays.

DISCUSSION

This work reports the first isolation of *Aliivibrio fischeri* associated with disease in brill *Scophthalmus rhombus*, a candidate species for diversification of marine aquaculture in Spain. The main symptoms observed in the outbreak, which reached a mortality rate of nearly 100%, consisted of haemorrhages in the liver and the presence of haemorragic liquid in the peritoneal cavity; no external alterations were observed. *A. fischeri* has been previously described associated with disease in other cultured fish in Spain. It was recovered by Lamas et al. (1990) from turbot affected by skin papillomas and visceral tumors; in some cases, fish also showed similar symptoms to those observed in brill (liver with petechia, red tinged fluid in the abdomen). Bacteria were recovered from both external lesions and from internal organs, and mortality was 39% in a year. *A. fischeri* has also been described from sea bream (Balebona et al. 1998), although in this case neither symptomatology nor mortality data were indicated. Other *Aliivibrio* species have also been associated with disease in fish exhibiting a variety of symptoms. *A. wodanis* has been found in Atlantic salmon *Salmo salar* affected by the winter ulcer disease (Lunder et al. 2000), and *A. logei* and *A. salmonicida* strains cause the Hitra disease in salmonids (Egidius et al. 1986, Benediktsdottir et al. 1998). The symptoms observed in brill were quite similar to that observed with this septicemic disease, characterized by the presence of internal haemorrhages and large amounts of red-tinged fluid in the peritoneal cavity.

Identification of the isolates recovered from diseased brill was performed on the basis of phenotypic and genotypic characterization. Analysis of 16S rDNA sequences showed similarity of 99% between the isolates and the *A. fischeri* type strain; on the contrary, these values were always below 96% with other *Aliivibrio* species. These results place *A. fischeri* as a unique species, with similarity values above the limit of intraspecific variability (98.7%) proposed

by Stackebrandt & Ebers (2006). Phylogenetic analyses based on housekeeping genes (*gyrB*, *rpoD*, *recA*) confirmed the clustering of the 3 isolates with *A. fischeri* and their distinction from other known *Aliivibrio* species (similarity with this species was 99.31, 98.99, and 95.29%, respectively). On the other hand, results from the analysis of REP-PCR and ERIC-PCR showed that the isolates recovered from brill constitute a homogeneous group, with band profiles clearly different from that of the *A. fischeri* type strain.

Biochemical and physiological characteristics of the isolates, and the FAME profile, were found to basically agree with those reported for *A. fischeri* by other authors (Buller 2004, Yoshizawa et al. 2010) and with that of the type strain of this species, also characterized in this work. Discrepancies were observed for a number of tests, such as ornithine decarboxylase, urease, Voges-Proskauer and growth at 4 and 35°C; however, the largest differences were observed in Biolog carbon source utilization profiles, since brill isolates could be easily differentiated from *A. fischeri* type strain by 11 tests.

Despite differences in genotypic and phenotypic profiles, DNA–DNA hybridization experiments confirmed the identification of the isolates as *A. fischeri*. Levels of DNA re-association of strain a591 with the type strain of *A. fischeri* and with the other isolates were 73.5 to 86.2% (reciprocal values were 86.9 to 99.04%) and 91.7 to 100%, respectively; these values are above the threshold value for species delineation of 70% proposed by Wayne et al. (1987), proving that these strains all belong to a unique species.

Finally, experimental infection tests carried out by intraperitoneal injection clearly demonstrated the pathogenic potential of these isolates; high mortalities rates (100%) were observed, but only at the highest dose (2×10^6 cfu g^{-1} body weight), which could indicate that these strains act as opportunistic pathogens. In this way, Lamas et al. (1990) suggested that although *A. fischeri* did not play the primary role in the outbreak described in turbot, this bacterium, together with *Vibrio harveyi*, probably contributed to the development of fish mortality. On the other hand, the degree of virulence of the isolates obtained from brill match with that observed for *A. fischeri* strains recovered from sea bream by Balebona et al. (1998), which displayed LD_{50} values that ranged from 7×10^5 to 1.3×10^7 cfu g^{-1} body weight.

In summary, this work represents the first report of *A. fischeri* in brill, and allows the number of potential pathogens that affect this species to be extended.

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