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Screening and characterisation of potentially pathogenic bacteria associated with Atlantic cod *Gadus morhua* larvae: bath challenge trials using a multidish system

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ABSTRACT: In intensive aquaculture systems, high concentrations of nutrients and high densities of fish larvae provide favorable conditions for opportunistic pathogenic bacteria to flourish. We screened potentially pathogenic bacterial strains isolated from moribund Atlantic cod *Gadus morhua* larvae, pollack *Pollachius pollachius*, coalfish *Pollachius virens*, Atlantic halibut *Hippoglossus hippoglossus*, rotifers, algae and water samples from different hatcheries. Three identical challenge experiments tested a total of 53 strains. A multidish system was used: cod eggs were placed in single wells, together with 2 ml of sterile seawater, and exposed to the bacterial cultures. Final bacterial concentrations in the wells were 10^6 and 10^4 CFU ml^{-1}. Eggs and larvae not exposed to bacteria were used as unchallenged controls. Challenged controls were exposed to *Vibrio anguillarum* strain 610. Eggs were challenged approximately 48 h prior to hatching and mortality was recorded daily throughout the yolk-sac period. In spite of the high challenge dose of 10^6 CFU ml^{-1}, only 5 bacterial strains tested caused higher mortality than the unchallenged controls. Four of these strains were identified by 16S rDNA and gyrase B gene (*GyrB*) sequencing as resembling *V. anguillarum* and 1 strain resembled *Carnobacterium* sp. Most of the larvae exposed to these strains died within 10 d of challenge. Serotyping of the strains resembling *V. anguillarum* gave inconclusive results. This indicates differences in serology compared to the serotypes O1, O2 and O3, associated with disease. Three bacterial strains seemed to have a slower infection rate, indicating a longer incubation period. The remaining 45 strains did not seem to have a negative effect on larval survival, suggesting that these are not primary pathogens.

KEY WORDS: Screening · Cod larvae · Bath challenge · Opportunistic bacteria · Vibriosis · *Vibrio anguillarum*

INTRODUCTION

Atlantic cod *Gadhus morhua* L. aquaculture has been expanding rapidly in Norway since 2000 (Svåsand et al. 2004). In 2006, about 10 384 tonnes of farmed cod (full life cycle) were slaughtered and 214 licenses for cod farming to 104 companies were operational (Directorate of Fisheries, Bergen, Norway. Available at: www.fiskeridir.no/fiskeridir/kystsone_og_havbruk/statistikk/statistikk_for_akvakultur/torsk). Of these, 60 to 80 ongrowth farms and 15 to 20 hatcheries were in operation (Kongsvik 2007).

The production of juveniles has been a bottleneck in cod farming. Cod larvae have a relatively short yolk-sac stage and they start to consume live feed, i.e. rotifers and *Artemia* spp., around 3 to 4 d post-hatching. Rotifers and *Artemia* are filter-feeding organisms, capable of concentrating bacteria, and thus are a potential source of pathogenic bacteria in the larval rearing system. Larvae also ingest bacteria by drinking water. High larval densities

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and suboptimal rearing conditions may provide a good environment for opportunistic bacterial pathogens to flourish (Vadstein et al. 2004), and high rates of mortality are still common. Commercial enrichment diets for rotifers may enhance the growth of pathogens including *Vibrio anguillarum* (Korsnes et al. 2006). Adult cod are frequently subject to classical vibriosis caused by *V. anguillarum* (reviewed by Samuelsen et al. 2006). *V. anguillarum* is also known to cause high mortality in a variety of fish species (reviewed by Thompson et al. 2004a).

The epiflora of cod eggs seems to be dominated by members of the *Cytophaga/Flavobacterium/Flexibacter* group, while *Vibrio* spp. are not frequent (Hansen & Olafsen 1989). In the past overgrowth of eggs was regarded as a problem; however, disinfection has reduced this (reviewed by Olafsen 2001).

While the composition of the intestinal bacterial flora associated with yolk-sac larvae of fish generally resembles the egg epibiota, a shift in the intestinal microbiota from a generally non-fermentative to a fermentative flora dominated by the *Vibrio/Aeromonas* group coincides with the onset of exogenous feeding (Vadstein et al. 2004). During first feeding, yolk-sac larvae in general are subject to a massive inflow of bacteria from the live feed organisms *Brachionus plicatilis* and *Artemia* spp., resulting in a shift in intestinal bacterial flora (Brunvold et al. 2007), and suspected pathogens such as *V. anguillarum* may accumulate in the live feed (Korsnes et al. 2006). The mucosal surfaces of fish serve as a substrate for bacterial adhesion (Spangaard et al. 2000) and thereby a potential portal of entry. Uptake of intact antigens from bacteria in the intestine of 4 to 6 d old yolk-sac larvae of cod has been demonstrated, and it has been suggested that this uptake may play a role in immune development, or in nutrition (Olafsen & Hansen 1992).

Whether the bacteria are primary causes of mortality or secondary pathogens that take advantage of weakened larvae is disputed. An important step would be to test whether any of the bacterial strains found has the ability to cause disease. Knowledge concerning sources and species of bacteria most commonly associated with cod larvae at different life stages is essential. It is also important to know the amount of bacteria needed to induce infection. The objectives of this study were to screen a large number of bacterial strains associated with diseased cod larvae, other marine cold-water species and live feed cultures, and to test the virulence of the strains in a challenge model using cod yolk-sac larvae.

MATERIALS AND METHODS

Broodstock, eggs and larvae. Eggs were collected at the Sagafjord commercial cod hatchery, in the county of Hordaland, Norway (59° 45′ N, 5° 29′ E). The broodstock originated from the Bømlo and Halsnøy area in the same county.

All eggs were taken from the same group. They were disinfected immediately after fertilisation in glutardialdehyde 300 ppm for 10 min, and kept in black conical 150 l tanks at 6.5 to 7.0°C. Eggs were transported from the hatchery to the Institute of Marine Research in boxes filled with ice, with the eggs stored in plastic bags. The transfer took about 2 to 3 h.

Bacteria. A total of 117 bacterial isolates were collected: 85 isolates were isolated from dead or moribund cod *Gadus morhua* larvae, while 3 were isolated from pollack *Pollachius pollachius,* 2 from coalfish *Pollachius virens* and 13 from Atlantic halibut *Hippoglossus hippoglossus* fry, 3 from water samples, 4 from rotifer, *Brachionus plicatilis* and 3 from algal cultures of *Tetraselmis* sp. In addition, 1 strain was isolated from adult cod and 3 strains were isolated from salmon *Salmo salar*. To reduce the number of strains used, the growth patterns of all strains were compared on different growth media: marine agar (MA), thiosulphate citrate bile sucrose agar (TCBS) (Merck), Cytophaga medium (CA) (Whitman 2004) and blood agar (nutrient blood agar [Oxoid] supplemented with 5% sheep blood and 1.5% NaCl). Comparison of 16S rDNA sequences was also performed. All strains with similar growth patterns and similarities in 16S rDNA sequences greater than 97% (Hagström et al. 2000, 2002) were eliminated from the challenge trials (data not shown). A total of 53 different bacterial strains were selected for use in the 3 identical challenge experiments (see Table 1).

The challenge experiment included an unchallenged control (eggs and larvae not exposed to bacteria) and two challenged control groups (eggs and larvae challenged with *Vibrio anguillarum* strain 610, challenge dose 10^6 and 10^4 CFU ml^{-1}). *V. anguillarum* strain 610 is known to cause high mortality in various fish species such as Atlantic cod *Gadus morhua* (Samuelsen & Bergh 2004, Vik-Mo et al. 2005, Seljestokken et al. 2006) and Atlantic halibut *Hippoglossus hippoglossus* (Samuelsen et al. 1997) and is commonly used in our laboratory during challenge experiments.

All bacteria were stored at –80°C in a 20% glycerol/marine broth (MB) (Difco 2216) stock. They were incubated at 15°C and grown on petri dishes with Difco 2216 marine agar (MA) for 48 h. Colonies of the bacteria were transferred to Erlenmeyer flasks with 50 ml of MB and shaken at 80 rpm in a shaking incubator (INFORS AG CH-4103) for 48 h at 7°C. The bacterial cultures (30 ml) were harvested by centrifugation (Heraeus Sepathec Megafuge 1.0 R) at 2772 × *g* for 10 min at 4°C, washed twice in 30 ml phosphate-

buffered saline (PBS) and suspended in 30 ml PBS. The cell concentration was determined by counting, using a Hawksley counting chamber.

The *Vibrio anguillarum* strain 610 was originally isolated from cod suffering from vibriosis in the Parisvatnet research facility of the Institute of Marine Research (Øygarden municipality, Western Norway). *Moritella viscosa* was provided by Helene Mikkelsen at the Norwegian Institute of Fisheries and Aquaculture Research in Tromsø, and the strains F95B/98 and F95C/98 were provided by Anne Berit Olsen from the National Veterinary Institute in Bergen. Strains HI 21030 to HI 21069 were provided by Egil Karlsbakk at the Institute of Marine Research. These strains were all characterised by the above named researchers, except for the API results and the growth studies on TCBS and CA medium for HI 21030 to HI 21069.

Challenge experiments. Three identical challenge experiments were preformed. All eggs were exposed to bacteria approximately 48 h before hatching on Day 10 or Day 11 after fertilisation.

Eggs were randomly taken and transferred to 24-well polystyrene dishes (Nunc) by autoclaved Pasteur pipettes. The protocol was modified from Bergh et al. (1992, 1997). All eggs hatched within 10 h, and 7841 of 7848 eggs hatched successfully. The eggs were put separately into individual wells containing 2.0 ml of sterile 80% (28‰) seawater. The seawater was aerated just before use to minimize the possibility of contamination. Exactly 100 µl of bacterial suspension was added to each well. Final bacterial concentrations in the wells were 10^6 and 10^4 CFU ml^{-1}, respectively, for high- and low-challenge doses. Final volume in each well was 2.1 ml. Three plates (72 wells) were used for each bacterial concentration, thus 6 plates (144 wells) were used for each bacterial strain. Three plates with unchallenged larvae were used as negative controls. The eggs and larvae were incubated in darkness in a climate-controlled room at 7°C. All eggs and larvae were inspected each day, for up to 18 d, and mortality was registered.

API 20E (Biomérieux) tests were performed according to the manufacturer's manual, with the following modifications: bacterial cultures were dissolved in sterile physiological saline (9 g NaCl dissolved in 1 l distilled water). The McFarland 2 standard was used as reference to culture density. The API strips were incubated at 15°C for 48h.

Gram staining was performed with the Diagnostica (Merck Gram) staining set.

Immunostaining of bacterial smears. To test some of the *Vibrio anguillarum* strains for serotype, bacterial smears of HI 21412, HI 21413, HI 21414 and HI 21429 were stained with specific absorbed polyclonal antiserum against serotypes O2α, O2β and O1. All antisera were produced according to the method of Oeding (1957) and absorbed by the method of Knappskog et al. (1993). The antiserum against serotype O2α was absorbed against O2β and O1, the antiserum against O2β was absorbed against O1 and the antiserum against O1 was absorbed against O2β. All 3 antisera were diluted in tris-hydroxymethyl-aminomethane (Tris)-buffered saline (TBS) with 2.5% bovine serum albumin (BSA). To prevent non-specific antibody binding, sections were blocked by using 5% BSA in TRIS-buffered formaldehyde for 20 min. Avidin-biotin-alkaline phosphatase complex (ABComplex/AP) reaction kit and New Fuchsin Substrate system (Dako) were used according to the manufacturer's manual. During the staining procedures, the different bacterial strains were kept separate to prevent cross-contamination. During staining both positive and negative controls were used. Known O2α (strain HI 610), O2β (strain HI 618) and O1 (strain HI 644) serotypes isolates were used as positive controls. The O2α and O2β were used as negative controls during staining procedures; the O1 antiserum, O2β and O1 isolates were used when staining with O2α antiserum, and O2α and O1 isolates were used as negative controls when the O2β antiserum was used. All incubations were performed at room temperature (20°C) in a humidity chamber.

Mono-Va agglutination kit against *Vibrio anguillarum*. Mono-Va tests (Bionor Laboratories) were used to identify the isolates *V. anguillarum* 610, HI 21412, HI 21413, HI 21414 and HI 21429. The test was done according to the manufacturer's manual.

DNA isolation. Genomic bacterial DNA was isolated from 1 ml of a liquid culture harvested at the end of the exponential growth phase, using the purification kit DNeasy® 96 tissue kit (Qiagen). The protocol for Gram negative bacteria was used.

Polymerase chain reaction (PCR) amplification of 16S rDNA genes. Universal primers, 27f and 1492r (*Escherichia coli* numbering), were used for 16S rDNA analyses. The mix contained 2.0 µl PCR buffer (10 ×), 1.2 µl $MgCl_2$ (25 mM), 3.2 µl dNTP (1.25 mM each, Promega), 1.0 µl 27f forward primer (10 µm), 1.0 µl 1492r reverse primer (10 µm), 0.2 µl Taq polymerase (5 U $µl^{-1}$, Promega), 7.4 µl nuclease-free water (Eppendorf) and 4 µl template (approx. 50 ng $µl^{-1}$). The amplification was performed in an automated thermal cycler (Perkin Elmer, Gene Amp, PCR system 9700) and the cycles were as follows: initial denaturation at 95°C for 5 min, then 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 11 min. All PCR reactions were performed in 50 µl reaction tubes.

PCR amplification of *GyrB* gene. Primers used for amplification of the *GyrB* gene were GyrB-1 (forward)

and GyrB-2 (reverse) (Yamamoto & Harayama 1995). The mix contained 2.0 µl PCR buffer (10x), 1.5 µl $MgCl_2$ (25 mM), 4.0 µl dNTP (1.25 mM each, Promega), 1.0 µl GyrB-1 primer (10 µM), 1.0 µl GyrB-2 primer (10 µM), 0.5 µl Taq polymerase (5 U μl^{-1}) (Promega), 6 µl nuclease-free water (Eppendorf) and 4 µl template (approx. 50 ng μl^{-1}). The amplification cycle was as follows: initial denaturation at 95°C for 5 min, then 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 2 min and extension/hold for 7 min. The amplification was performed in an automated thermal cycler (Gene Amp PCR system 9700, Perkin Elmer).

Sequencing of 16S rDNA and *GyrB* genes. All PCR products were prepared for sequencing by using a Pre-Sequencing Kit (USB). One cycle of 37°C for 15 min and in addition, another 15 min at 80°C were run. Primers used for sequencing 16S rDNA genes were the same as for the PCR amplification described in the paragraph above. The pre-sequencing mix contained 1µl Big Dye mix (2.5×) (Big Dye version 3.1, Applied Biosystems), 1.5 µl sequence buffer (5.0×), 2.0 µl primer (10 µM) and 2.5 µl RNAse free water (Eppendorf) and 3 µl of template were used. The amplification cycles were as follows: initial denaturation at 96°C for 1 min then 25 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and extension at 60°C for 4 min.

Primers used for sequencing *GyrB* genes were different from the ones used for PCR amplification: GyrB-1s (forward) and GyrB-2s (reverse) (Yamamoto & Harayama 1995). The same pre-sequencing mix and amplification cycles as used for 16S rDNA genes were used on the sequencing of the *GyrB* genes as well. The amplification was performed in an automated thermal cycler (Gene Amp PCR system 9700, Perkin Elmer). The sequence analysis was performed by the sequence laboratory at the University of Bergen, using an ABI 3700 sequencing analyzer (Applied Biosystems). The 16S rRNA and *GyrB* encoding gene sequences were searched for nucleotide-nucleotide matches in the BLAST database at the NCBI (www.ncbi.nlm.nih.gov/BLAST/) to establish tentative strain identity (Altschul et al. 1990).

Statistical analyses. Since the survival and mortality data were not normally distributed, non-parametric tests were used. A number of 2 × 2 contingency tables (performed using Statistica v 7.0, StatSoft) were used to examine rates of mortality in the treatment and control groups. Since multiple independent tests were used, the significance level was adjusted using the Bonferroni correction and set at $p < 0.00094$ (i.e. $p = 0.05/53$ number of tests performed) (Rice 1989). Additionally, Yates correction was used because there was only 1 degree of freedom (df).

Table 1. Characterisation of the bacterial strains used. Source = the bacterial strain's origin of isolation (salmon *Salmo salar*, cod *Gadus morhua*, pollack *Pollachius pollachius*, coalfish *Pollachius virens*, halibut *Hippoglossus hippoglossus*, Tetra. sp. = *Tetraselmis* sp. culture, Rot. culture = rotifer culture) Long fil. = long filamentous, Cocc. rod = coccoid rod shape, + = positive, – = negative, w = weak, gr = growth, TCBS = Thiosulphate citrate bile sucrose agar (Yel. colon. = yellow colonies, Gr. colon. = green colonies), CA = cytophaga agar, Hemolytic = haemolytic growth on blood agar 1.5 % NaCl, N.D = not determined, 16S rDNA, *GyrB* = closest sequence match for these 2 genes by BLAST search, No seq. obtained = no sequence was obtained for the strains

Strain	Source	Gram	Shape	Motility	Oxidase	Hemolytic	TCBS	CA	16S rDNA	*GyrB*
Vibrio anguillarum 610	Control	–	Cocc. rod	+	+	+	Yel. colon.	+	*Vibrio anguillarum*	*Vibrio anguillarum*
Marinomonas sp.	Cod	–	Rod	+	–	–	–	+	*Marinomonas* sp.	*Marinomonas* sp.
Moritella viscosa	Salmon	–	Rod	+	+	+	N.D	N.D	*Moritella viscosa*	
F95B/98	Salmon	–	Long fil.	–	+	No gr	N.D	N.D	*Tenacibaculum* sp.	*Tenacibaculum* sp.
F95C/98	Salmon	–	Long fil.	–	+	No gr	N.D	N.D	*Tenacibaculum* sp.	*Tenacibaculum* sp.
HI 21030	Cod larvae	–	Rod	+	+	–	w gr	+ (w)	*Marinobacter* sp.	
HI 21031	Cod larvae	–	Rod	+	+	–	–	+	*Pseudoalteromonas* sp.	
HI 21037	Cod larvae	–	Rod	+	+	–	–	+	*Pseudoalteromonas* sp.	
HI 21039	Cod larvae	–	Rod	+	+	–	Gr. colon.	+	*Vibrio logei*	
HI 21040	Cod larvae	–	Rod	+	+	–	Gr. colon.	–	*Vibrio logei*	
HI 21041	Cod larvae	–	Rod	+	+	–	w gr/ w Yel.	–	*Psychromonas* sp.	
HI 21047	Cod larvae	–	Rod	+	+	–	Gr. colon.	+	*Pseudoalteromonas* sp.	
HI 21050	Tetra. sp.	–	Rod	w	–	+ (w)	w gr/Gr. colon.	–	*Carnobacterium* sp.	

Table 1 (continued)

Strain	Source	Gram	Shape	Motility	Oxidase	Hemolytic	TCBS	CA	16S rDNA	*GyrB*
HI 21052	Cod larvae	–	Rod	+	+	–	Gr. colon.	+	*Vibrio logei*	
HI 21056	Cod larvae	–	Rod	+	+	–	Gr. colon.	+	*Vibrio* cf. *splendidus*	
HI 21059	Cod larvae	–	Rod	+	–	–	–	+	*Marinomonas* sp.	
HI 21061	Pollack	–	Rod	+	+	+	Gr. colon.	+	*Marinomonas* sp.	
HI 21063	Pollack	–	Rod	+	+ (w)	–	–	+	*Photobacterium* cf. *iliopiscarium*	
HI 21064	Pollack	–	Rod	+	+ (w)	–	Gr. colon.	+	*Vibrio wodanis*	
HI 21065	Cod larvae	–	Cocc. rod	+	+	–	Yel. colon.	+ (w)	*Shewanella–sairae/marinintestina*	
HI 21066	Coalfish	–	Rod	+	+	+	Gr. colon.	+	*Vibrio splendidus*	
HI 21068	Coalfish	–	Cocc. rod	+	–	–	w gr	+	*Photobacterium* cf. *iliopiscarium*	
HI 21069	Cod larvae	–	Rod	+	–	–	w gr	+	*Marinomonas* sp.	
HI 21400	Rot. culture	–	Rod	+	+	–	w gr	+	*Vibrio* sp.	*Vibrio parahaemolyticus* (86%)
HI 21402	Cod larvae	–	Rod	w	+	–	Gr. colon.	+	*Vibrio fisheri* or *Vibrio logei* (99%)	*Vibrio fisheri* (88%)
HI 21404	Cod larvae	–	Rod	+	+	+	–	+ (w)	*Vibrio* sp.	*Aeromonas salmonicida* ssp. *salmonicida* (100%)
HI 21405	Cod larvae	–	Rod	+	+	–	–	+ (w)	*Marinomonas* sp. (96%)	*Marinomonas vaga* (80%)
HI 21407	Cod larvae	–	Rod	w	+	–	Yel. colon.	+ (w)	*Vibrio* sp.	*Vibrio parahaemolyticus* (86%)
HI 21408	Cod larvae	–	Rod	+	+	–	Yel. colon.	+ (w)	*V. anguillarum* strain 010610–3 (100%)	*Vibrio logei* (94%)
HI 21410	Cod larvae	–	Rod	+	+	++	Gr. colon.	+	*Vibrio splendidus*	*Vibrio splendidus* (100%)
HI 21412	Cod larvae	–	Rod	+	+	+ (w)	Yel. colon.	+	*V. anguillarum* O2a (99%)	*V. anguillarum* (99%)
HI 21413	Cod larvae	–	Rod	+	+	+ (w)	Yel. colon.	+	*V. anguillarum* O2a (99%)	*V. anguillarum* NCMB 6 (98%)
HI 21414	Cod larvae	–	Rod	+	+	+ (w)	Yel. colon.	+	*V. anguillarum* (99%)	*V. anguillarum* (98%)
HI 21417	Cod larvae	–	Rod	+	–	–	–	+	*Rhodococcus* sp. (99%)	*Rhodococcus erythropolis* (99%)
HI 21424	Cod larvae	–	Cocc. rod	+	+	+	Yel./White colon.	+	*Vibrio wodanis* (99%)	*Vibrio splendidus* (91%)
HI 21427	Cod larvae	–	Rod	+	+	–	–	+	*Vibrio gallicus* (99%)	*Vibrio parahaemolyticus* (86%)
HI 21429	Cod larvae	–	Rod	+	+	+ (w)	Yel. colon./Yel. agar	+	*V. anguillarum* (99%)	*V. anguillarum* NCMB 6 (98%)
HI 21430	Cod larvae	–	Rod	+	+	–	–	+	*Vibrio gallicus* (97%)	*V. anguillarum* (98%)
HI 21433	Cod larvae	–	Rod	+	+	–	–	+	*Vibrio gallicus* (99%)	*Vibrio parahaemolyticus* (86%)
HI 22001	Cod larvae	–	Rod	+	+	–	–	+	*V. anguillarum*	*Pseudoalteromonas rubra* (98%)
HI22002	Cod larvae	–	Rod	+	–	–	w gr/Gr.	+	*Vibrio* sp.	No seq. obtained
HI 22019	Halibut	–	Rod	+	+	+	w Yel.	+	*Pseudoalteromonas nigrifaceis* (99%)	*Vibrio splendidus* LT 06 or LP1 (99%)
HI 22022	Halibut	–	Rod	+	+	–	–	+	*Bacillus herbersteinensis* type strain D–1,5a (96%)	No seq. obtained
HI 22025	Halibut	–	Rod	+	+	No gr	Yel. colon./Yel. agar	+	*Tenacibaculum ovolyticum* (96%)	*Vibrio splendidus* LT 06 or LP1 (99%)
HI 22027	Halibut	–	Rod	+	+	+	Gr. colon.	+	*Vibrio* sp. Da2 or PMV19 (98%)	*Vibrio splendidus* LP1 or LT 06 (99%)
HI 22029	Halibut	–	Rod	+	+	+	–	+	*Pseudoalteromonas haloplanktis* (98%)	*Pseudoalteromonas carrageenovora* (90%)
HI 22032	Halibut	–	Rod	+	+	+	Yel. colon./Yel. agar	+	*Vibrio splendidus* (98%)	*Vibrio splendidus* LP1 or LT 06 (98%)
HI 22034	Halibut	–	Rod	+	+	–	–	+	*Pseudoalteromonas* sp. EH–2–1 (99%)	*Pseudoalteromonas haloplanktis* (99%)
HI 22042	Halibut	–	Rod	+	+	–	–	+	*Pseudoalteromonas* sp.	*Pseudoalteromonas carrageenovora* (91%)
HI 22044	Halibut	–	Rod	+	+	+	Gr. colon.	+	*Vibrio* sp.	*V. splendidus* LT06 or PMV18 (99%)
HI 22051	Halibut	–	Rod	+	+	No gr	–	+ (w)	*Tenacibaculum* sp.	No seq. obtained
HI 22054	Halibut	–	Rod	+	+	No gr	–	–	*Pseudoalteromonas* sp. (99%)	No seq. obtained
HI 22077	Rot. culture	–	Rod	+	+	+	–	+	*Pseudoalteromonas* sp. (99%)	*Pseudoalteromonas haloplanktis* (98%)

RESULTS

Bacterial characterisation

Further characterisation of the bacterial strains used in the challenge experiments showed that all bacterial strains were Gram-negative. Most of the strains were short rod-shaped bacteria except for F95B/98 and F95/C98, which were long filamentous bacteria. These 2 strains were also the only non-motile strains. Additionally, HI 21050, HI 21402 and HI 21407 proved to be only weakly motile. The majority of the strains were found to be oxidase-positive, except for the strains *Marinomonas* sp., HI 21050, HI 21059, HI 21068, HI 21069, HI 21017 and HI 22002 (Table 1 shows an overview of all details). In certain cases, the API results were inconclusive (Table 2). HI 21050, which resembled *Carnobacterium* sp., did not produce any positive results in the API tests. API tests of the strains HI 21412, HI 21413, HI 21414 and HI 21429 revealed similar phenotypic characterisation as is known for other *Vibrio anguillarum* strains, referring to the ALO test (A: arginine decarboxylase; L: lysine decarboxylase; O: ornithine decarboxylase,): A+/L-/O- (Alsina & Blanch 1994) (see Table 2 for more details). This was confirmed by the 16S rDNA and *GyrB* sequence analysis, which showed 98 to 99% similarity to various *V. anguillarum* (Table 1) gene sequences. Some of the sequencing analyses produced inconclusive results when the 2 genes were compared. The 16S rDNA sequence of HI 21404 showed similarities to *Vibrio* sp. while the *GyrB* sequence was 100% similar to *Aeromonas salmonicida* subsp. *salmonicida*. The 16S rDNA sequence for strain HI 21408 was 100% similar to *V. anguillarum*, while the *Gyr*B sequence was 94% similar to *Vibrio logei*. HI 22022 was similar to *V. anguillarum* when the 16S rDNA sequence was BLASTed, but the *GyrB* sequence showed most similarity to *Pseudoalteromonas rubra* (98%). The 16S rDNA and the *GyrB* sequence of HI 22019 were similar to *Pseudoalteromonas nigrificans* and *Vibrio splendidus*, respectively. The last strain to show differences was HI 22025. The 16S rDNA sequence was 96% similar to *Tenacibaculum ovolyticum* while the *GyrB* gene sequence was almost identical (99%) to *V. splendidus*. The bacterial strain HI 22022 came out as 96% similar to the Gram-positive bacteria *Bacillus herbersteinensis* isolated from a medieval wall painting in the chapel of Castle Herberstein, Styria (Austria) (Wieser et al. 2005). Unfortunately, no *GyrB* gene sequence was obtained for this strain. No *GyrB* sequences were obtained for HI 22019, HI 22015 and 22054.

Table 2. API 20E results for all strains used. ONPG: β-galactosidase; ADH: arginine dihydrolase; LDC: lysine decarboxylase; ODC: ornithine decarboxylase; CIT: citrate utilization; H_2S: H_2S production; URE: urease; TDA: tryptophane deaminase; IND: indole production; VP: acetoin production; GEL: galatinase; GLU: glucose; MAN: mannitol; INO: inositol; SOR: sorbitol; RHA: rhamnose; SAC: saccharose; MEL: melibiose; AMY: amygdalin; ARA: arabinose; *V. ang* 610: *Vibrio anguillarum* strain 610; +/–: used when results differed between API runs; N.D: not determined

Strain	ONPG	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA
V. ang 610	+	+	–	–	–/+	–	–	–	–	–	+	+	–/+	–	+	–	+/–	–	–	–
Marinomonas sp.	+	–	–	+	–	–	–	–	–	–	–	+/–	+/–	+	+	–	+	+/–	+/–	+/–
Moritella viscosa	N.D	–	+	–	N.D	N.D	N.D	N.D	N.D	N.D	+	+	–	N.D	N.D	N.D	–	N.D	N.D	N.D
F95B/98	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
F95C/98	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
HI 21030	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	+	–	–
HI 21031	–	–	–	–	+/–	–	+/–	–		+	+	–	+	–	–	–	–	–	–	–
HI 21037	–	–	–	–	+	–	–	–	–	+	–	+	–	–	–	–/+	–	+	–	+
HI 21039	–	–	+	–	–	–	–	–	–	+/–	–	+/–	+	–	–	–	–	–	+/–	–
HI 21040	–	–	+	–	+/–	–	–	–	–	+/–	–	+/–	+	–	–	–	–	–	+/–	–
HI 21041	+	–	+	–	–	–	–	–	–	+/–	–	–	+	–	–	–	–	–	+/–	–
HI 21047	–	–	+	–	–	–	+	–	–	+/–	–	+/–	+/–	–	–	–	–	–	+	–
HI 21050	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Table 2 (continued)

Strain	ONPG	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA
HI 21052	–	–	+	–	–	–	+	–	–	+/–	–	+/–	+	–	–	–	–	–	+/–	–
HI 21056	+	–	–	–	–	–	–	–	–	+/–	+	+	+	–	–	–	–	–	+	–
HI 21059	+	–	–	–	+	–	–	–	–	+	–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–
HI 21061	+	–	–	–	–	–	–	–	–	+/–	+	+	+	–	–	–	–	–	+	–
HI 21063	–	+	+	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–	–	–
HI 21064	+	–	+	–	–	–	–	–	–	+/–	–	+	–	–	–	–	–	–	+	–
HI 21065	–	–	+	–	–	–	+	–	+	–	–	+	+	–	–	–	+	–	+	–
HI 21066	+	–	–	–	–	–	–	–	–	+	+	+	+	–	–	–	+	+/–	–	–
HI 21068	+	+	–	–	+	–	–	–	–	+	–	+	+/–	+/–	+/–	–	+/–	+	+	+/–
HI 21069	+	–	–	–	+	–	–	–	–	+	–	+/–	+/–	+/–	+/–	+/–	–	+/–	+/–	+/–
HI 21400	–	–	–	–	–	–	–	+	–	–	–	+	+	–	–	–	+	–	–	+
HI 21402	–	–	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
HI 21404	+	–	–	–	–	–	–	–	–	+	+	+	+	–	–	–	–	–	+	–
HI 21405	+	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
HI 21407	–	–	–	–	–	–	–	–	–	–	–	+/–	+	–	–	–	+	–	–	+
HI 21408	+	–	+	–	–	–	–	–	–	–	+/–	+	–	–	–	+	–	–	–	–
HI 21410	+	–	–	–	–	–	–	–	–	–	+	+/–	+	–	–	–	–	+/–	+	–
HI 21412	+	+	–	–	+/–	–	–	–	–	+	+	+	+	–	+	–	+	–	+	+
HI 21413	+	+	–	–	+/–	–	–	–	–	+	+	+	+	–	+	–	+	–	+	–
HI 21414	–	+	–	–	+/–	–	–	–	–	+	+	+	+	–	+	–	+	–	+	–
HI 21417	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
HI 21424	–	–	–	–	–	–	–	–	–	–	+	+	+	–	–	–	+	–	+	–
HI 21427	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	+	–	+/–	+
HI 21429	+	+	–	–	–	–	–	–	–	+	+	+	+	–	+	–	+	–	+	–
HI 21430	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	+	–	+	+
HI 21433	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	+	–	+/–	+/–
HI 22001	–	–	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–
HI22002	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
HI 22019	+/–	–	–	–	–	–	–	–	–	+/–	+	+	+	–	–	–	–	+	–	–
HI 22022	+	–	–	–	–	–	–	–	–	+/–	+	–	–	–	–	–	–	–	–	–
HI 22025	+	–	–	–	–	–	–	–	–	–	+	+	+	–	–	–	+	–	+	–
HI 22027	+	–	–	–	–	–	–	–	–	+	+	+	+	–	–	–	+	+	+	–
HI 22029	+	–	–	–	–	–	–	–	–	+/–	+	+	+	–	–	–	+	–	–	+
HI 22032	+	–	–	–	–	–	–	–	–	+/–	+	–	+	–	–	–	+	–	+	–
HI 22034	+	–	–	–	+/–	–	–	–	–	+/–	+	+	+	–	–	–	+	–	+	–
HI 22042	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+/–	–	+/–
HI 22044	–	–	–	–	–	–	–	–	–	+	–	+	+	–	–	–	–	–	+	–
HI 22051	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–
HI 22054	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
HI 22077	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–

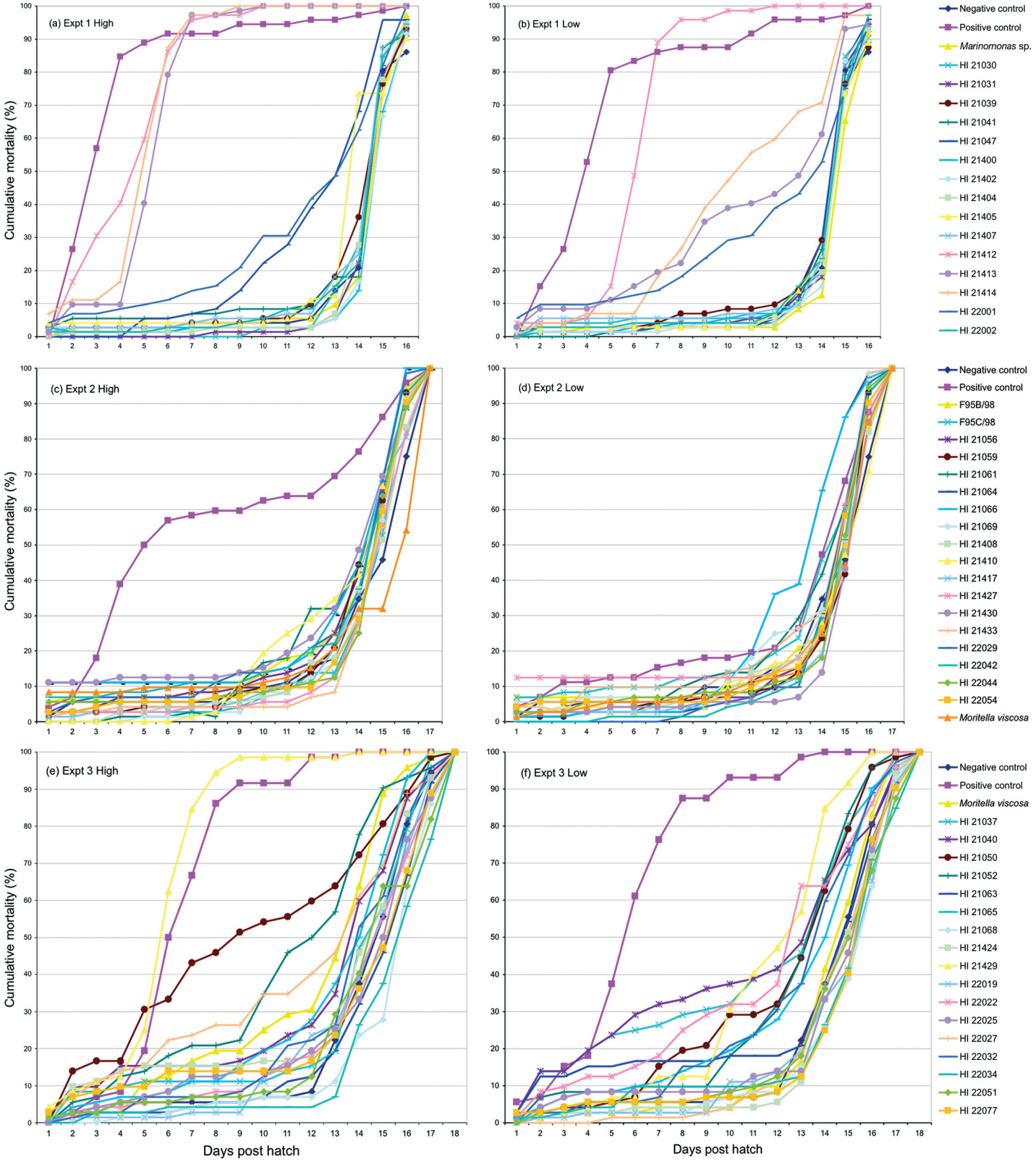

Fig. 1. *Gadus morhua*. Cumulative mortality (percentages) in (a,b) challenge Expt 1, (c,d) challenge Expt 2, (e,f) challenge Expt 3. Challenge dose is (a,c,e) 10^6 CFU ml^{-1} (high) or (b,d,f) 10^4 CFU ml^{-1} (low). Negative control is larvae not challenged with bacteria. Positive control is larvae challenged with *Vibrio anguillarum* strain 610. Remaining names refer to bacterial strains

Cumulative mortality

Each of the 3 experiments contained a 'negative' unchallenged control group and 2 'positive' challenged controls (i.e. low- and high-challenge doses. Large differences in survival rates were found between the unchallenged and the challenged control groups (Fig. 1). The differences in mortality rates in the control groups were significantly different throughout all 3 experiments, except for the lowest challenge dose, 10^4 CFU ml^{-1}, in Expt 2 (Table 3). Mortality rates in the unchallenged control groups were not significantly different from each other except at the end of the experiments on Day 15 ($p < 0.00094$, data not shown).

Only a few of the strains tested caused high mortality rates (significantly different from the negative control groups) early in the experiments (Tables 3, 4 & 5). The increase in mortality observed at the end of the experiments was due to the lack of feeding.

The challenged control groups displayed some differences in terms of when the larvae started to die. In the first experiment the larva tended to die about 4 d earlier (between Days 3 and 4) than the larvae in Expts 2 and 3 (see Fig. 1). In the second experiment the cumulative mortality among the challenged control group was lower and significantly different ($p < 0.00094$) from that of the challenged control group in Expts 1 and 3 ($p < 0.00094$) (Fig. 1). On the other hand, the cumulative mortalities for all other bacterial strains tested in Expt 2 were in the same range as the negative control group (Fig. 1c,d) and not significantly different from each other. However, the Chi-square analysis

Table 3. Yates-corrected Chi-square (χ^2) values and p-values ($p < 0.00094$, Bonferroni correction) for individual 2×2 contingency tables of negative control vs. positive control groups for all 3 challenge experiments. 10^6 and 10^4 = challenge dose 10^6 and 10^4 CFU ml^{-1}. All significant p-values in **bold**. Expt = experiment, V. ang 610 = *Vibrio anguillarum* strain 610, Day = days post-hatch

Expt	Strain	Dose	Day 3		Day 6		Day 9		Day 12		Day 15	
			χ^2	p	χ^2	p	χ^2	p	χ^2	p	χ^2	p
1	V. ang 610	10^6	51.13	**0.0000**	114.33	**0.0000**	113.80	**0.0000**	113.80	**0.0000**	10.72	0.0011
1	V. ang 610	10^4	16.78	**0.0000**	95.68	**0.0000**	97.37	**0.0000**	113.80	**0.0000**	8.51	0.0035
2	V. ang 610	10^6	4.27	0.0388	41.89	**0.0000**	37.53	**0.0000**	33.57	**0.0000**	24.25	**0.0000**
2	V. ang 610	10^4	0.82	0.3657	1.35	0.2448	1.45	0.2283	0.42	0.5157	6.37	0.0116
3	V. ang 610	10^6	0.60	0.4383	33.27	**0.0000**	103.44	**0.0000**	114.33	**0.0000**	38.61	**0.0000**
3	V. ang 610	10^4	5.41	0.0200	47.53	**0.0000**	93.90	**0.0000**	100.02	**0.0000**	38.61	**0.0000**

Table 4. Yates-corrected Chi-square (χ^2) values and p-values ($p < 0.00094$, Bonferroni correction) for individual 2×2 contingency tables of negative control vs. all tested strains in challenge Expt 2 (only challenge dose 10^6 CFU ml^{-1}). All significant p-values in **bold**. Day = days post-hatch, V. ang 610 = *Vibrio anguillarum* strain 610

Strain	Day 3		Day 6		Day 9		Day 12		Day 15	
	χ^2	p	χ^2	p	χ^2	p	χ^2	p	χ^2	p
V. ang 610	4.27	0.0388	41.89	**0.0000**	37.53	**0.0000**	33.57	**0.0000**	24.25	**0.0000**
Moritella viscoca	0.11	0.7431	0.39	0.5304	0.08	0.7785	0.05	0.8168	2.37	0.1239
F95b/98	0.00	1.0000	0.00	1.0000	0.08	0.7785	0.19	0.6599	1.00	0.3173
F95c/98	0.17	0.6767	0.17	0.6767	0.97	0.3254	0.00	1.0000	0.44	0.5049
HI 21056	0.00	1.0000	0.00	1.0000	0.08	0.7785	0.00	1.0000	4.75	0.0293
HI 21059	0.17	0.6767	0.00	1.0000	0.00	1.0000	0.00	1.0000	3.38	0.0658
HI 21061	2.31	0.1282	0.83	0.3626	0.00	1.0000	4.66	0.0309	2.79	0.0948
HI 21064	0.82	0.3657	0.82	0.3657	0.00	1.0000	0.19	0.6599	6.37	0.0116
HI 21066	0.82	0.3657	0.82	0.3657	0.00	1.0000	0.19	0.6599	6.37	0.0116
HI 21069	2.31	0.1282	0.83	0.3626	1.90	0.1685	0.06	0.8096	1.36	0.2432
HI 21408	2.31	0.1282	0.00	1.0000	0.39	0.5304	0.00	1.0000	0.25	0.6169
HI 21410	2.31	0.1282	2.31	0.1282	0.00	1.0000	0.00	1.0000	5.53	0.0187
HI 21417	0.17	0.6767	0.00	1.0000	0.39	0.5304	0.24	0.6224	7.28	0.0070
HI 21427	0.17	0.6767	0.17	0.6767	0.97	0.3254	1.07	0.3016	1.33	0.2482
HI 21430	0.82	0.3657	1.35	0.2448	0.27	0.6055	1.11	0.2924	7.28	0.0070
HI 21433	0.17	0.6767	0.00	1.0000	0.97	0.3254	1.76	0.1849	2.79	0.0948
HI 22029	0.00	1.0000	0.00	1.0000	0.27	0.6055	0.42	0.5157	6.37	0.0116
HI 22042	0.00	1.0000	0.39	0.5304	0.00	1.0000	0.42	0.5157	4.04	0.0445
HI 22044	0.13	0.7160	0.13	0.7160	0.00	1.0000	0.24	0.6224	4.04	0.0445
HI 22054	0.00	1.0000	0.13	0.7160	0.09	0.7630	0.57	0.4497	2.26	0.1330

Table 5. Yates-corrected Chi-square (χ^2) values and p-values ($p < 0.00094$, Bonferroni correction) for individual 2×2 contingency tables of negative vs. control tested strains. Only strains found to cause mortality significantly different from the negative control are presented in the table. 10^6 and 10^4 = challenge doses 10^6 and 10^4 CFU ml^{-1}. All significant p-values in **bold**

Expt	Strain	Dose	Day 3		Day 6		Day 9		Day 12		Day 15	
			χ^2	p	χ^2	p	χ^2	p	χ^2	p	χ^2	p
1	HI 21412	10^6	20.70	**0.0000**	101.59	**0.0000**	121.02	**0.0000**	125.08	**0.0000**	13.37	**0.0003**
1	HI 21412	10^4	0.26	0.6121	40.33	**0.0000**	97.37	**0.0000**	113.80	**0.0000**	8.51	0.0035
1	HI 21413	10^6	3.31	0.0689	87.33	**0.0000**	124.79	**0.0000**	125.08	**0.0000**	13.37	**0.0003**
1	HI 21413	10^4	2.40	0.1212	7.36	0.0067	19.55	**0.0000**	25.52	**0.0000**	3.88	0.0489
1	HI 21414	10^6	4.27	0.0389	104.65	**0.0000**	128.67	**0.0000**	125.08	**0.0000**	13.37	**0.0003**
1	HI 21414	10^4	0.26	0.6121	1.57	0.2109	23.68	0.0000	45.61	**0.0000**	8.51	0.0035
1	HI 21047	10^6	0.00	1.0000	0.83	0.3626	3.04	0.0810	21.25	**0.0000**	6.67	0.0098
1	HI 21047	10^4	0.00	1.0000	0.00	1.0000	0.00	1.0000	0.13	0.7160	0.05	0.8285
1	HI 22001	10^6	1.57	0.2109	1.57	0.2095	7.68	0.0056	24.06	**0.0000**	0.00	0.0000
1	HI 22001	10^4	3.31	0.0689	5.27	0.0218	9.81	0.0017	21.25	**0.0000**	0.36	0.5476
3	HI 21429	10^6	2.69	0.1012	49.50	**0.0000**	121.21	**0.0000**	114.33	**0.0000**	38.61	**0.0000**
3	HI 21429	10^4	0.26	0.6121	0.00	1.0000	1.35	0.2448	25.23	**0.0000**	22.34	**0.0000**
3	HI 21050	10^6	6.41	0.0114	16.00	**0.0001**	34.92	**0.0000**	40.09	**0.0000**	9.23	0.0024
3	HI 21050	10^4	0.00	1.0000	0.00	1.0000	6.06	0.0138	11.05	0.0009	8.09	0.0045
3	HI 21052	10^6	0.60	0.4383	4.27	0.0388	7.03	0.0080	28.27	**0.0000**	20.25	**0.0000**
3	HI 21052	10^4	1.19	0.2751	0.39	0.5304	0.39	0.5304	9.98	0.0016	11.81	0.0006
3	HI 22027	10^6	2.69	0.1012	7.03	0.0080	10.14	0.0015	18.27	**0.0000**	2.40	0.1213
3	HI 22027	10^4	0.51	0.4764	0.83	0.3626	0.17	0.6767	0.08	0.7785	0.11	0.7383

confirmed that mortality rates of the high-dose challenged controls and the unchallenged control group were significantly different from each other (Table 4). The mortality rates of the positive controls in Expts 1 and 3 were not found to be significantly different (data not shown).

As Fig. 1a,b,e,f shows, only the 5 strains HI 21412, HI 21413, HI 21414, HI 21429 and HI 21050 caused high mortality. Of these 5 strains, HI 21412 appeared to be the most virulent. Mortality caused by this strain was significantly different from the mortality rate found in the negative control group on Day 3 post-hatch (challenge dose 10^6 CFU ml^{-1}) and on Day 6 (challenge dose 10^4 CFU ml^{-1}). The mortality rates for the remaining 4 strains were found to be significantly different from that of the unchallenged control group from Day 6 onwards for the high-challenge dose and from Day 9 in the low-challenge dose group ($p < 0.00094$, Table 5). The low-challenge dose did not cause any increase in mortality rate in larval groups challenged with HI 21050. Compared to the positive control, these strains did not produce significantly different results. Strains HI 21052, HI 22001 and HI 22027, resembling *Vibrio logei*, *V. anguillarum* and *V. splendidus*, respectively, (see Table 1) led to cumulative mortality rates that were different from challenged control groups (Fig. 1a,b,e,f). However, the mortality rates for these strains were significantly different from the unchallenged control group ($p < 0.00094$, Table 5) only at the end of the experiment (i.e. from Day 12 onwards), indicating that they had a longer incubation period. The same strains were also significantly different from the positive control, indicating they are less virulent (data not shown). It should be noted that the low-challenge dose of these 3 strains did not appear to cause the same increase in mortality as was found in the high-challenge dose groups.

Cumulative mortality rates in the remaining 45 strains (see Fig. 1) did not differ significantly from the negative control groups ($p > 0.00094$, data not shown). The mortality rates for the same groups were, however, found to be significantly different from the positive control group ($p < 0.00094$, data not shown). This indicates that these 45 strains had no harmful effect on mortality rates.

Immunostaining of bacterial smears and Mono-Va testing

The 4 pathogenic strains HI 21412, HI 21413, HI 21414 and HI 21429, which were found to have similar 16S rDNA and *GyrB* sequences as *Vibrio anguillarum*, were tested for positive immunostaining with 3 different antisera against the *V. anguillarum* serotypes O2α, O2β and O1. Positive immunostaining was only found when they were stained with the antiserum against the O2α serotype. On the other hand, when tested with the Mono-Va agglutination kit for *V. anguillarum* strains, no positive reaction was found in any of the 4 strains.

DISCUSSION

The aim of this study was to evaluate the virulence of candidate pathogenic bacteria among bacterial isolates associated with diseased cod larvae, other marine cold-water fish and live fish cultures. Out of 53 bacterial strains tested, only the 5 strains HI 21412, HI 21413, HI 21414, HI 21429 and HI 21050 could be classified as primary pathogens, i.e. had a negative effect on cod larva survival. This indicated that most of the bacteria associated with and isolated from moribund cod *Gadus morhua* larvae, halibut *Hippoglossus hippoglossus*, coalfish *Pollachius virens* and pollack *Pollachius pollachius*, are not primary pathogens, i.e. they are probably not primary causes of disease. Similar results were found by Verner-Jeffreys et al. (2003) when testing virulence among bacterial strains isolated from halibut hatcheries.

Four strains in this high mortality group were shown to resemble *Vibrio anguillarum* by 16S rDNA and *GyrB* analyses. This confirms that vibriosis may also be a problem in the aquaculture of early life stages of cod. However, serotyping of these 4 strains, HI 21412, HI 21413, HI 21414 and HI 21429, did not provide any clear results. Serological testing with specific antisera against the *V. anguillarum* serotypes, O1, O2α and O2β, produced positive results only against the O2α serum. At the same time, no positive results were found by using a Mono-Va agglutination kit, which should have produced positive results for the O1, O2 and O3 serotypes. This can be explained by the possibility that these *V. anguilllarum* strains differ from serotypes known today, for which commercially produced antisera exist. A recent study by Mikkelsen et al. (2007) showed that bacteria isolated from diseased cod differ from O2α and O2β isolates serologically, biochemically and genotypically. These authors further indicate that these *V. anguillarum* isolates belong to a new sero-subtype. However, the 4 isolates used in the present study are biochemically and genotypically consistent with *V. anguillarum*. The ALO test gave A+/L-/O- as a classification of the *V. anguillarum* strains (see Table 2). However, further studies are needed to compare already known isolates and serotypes with these findings. Studies of this kind are probably essential if efficient vaccines are to be developed. Vaccines developed for cod do not provide sufficient protection; vibriosis is still a problem in cod farming even though vaccines for cod have been on the market for more than 10 yr (Samuelsen et al. 2006).

Strain HI 21050, resembling *Carnobacterium* sp., was isolated from a culture of the alga *Tetraselmis* sp. This alga is commonly used as a feed and enrichment in rotifer cultures (Muller-Feuga et al. 2003). Algal cultures are associated with bacterial populations. Population studies of algal cultures used as feed for scallop larvae have identified a variety of bacterial strains associated with the algal cultures (Sandaa et al. 2003, Nicolas et al. 2004), probably including opportunistic pathogens as well as commensal or mutualistic bacteria. In intensive aquaculture, cod larvae are offered rotifers, usually *Brachionus plicatilis*, and brine shrimp, mostly *Artemia franciscana,* as live feed (Svåsand et al. 2004, Reitan 2005). Recently, Korsnes et al. (2006) demonstrated the presence of bacteria with high sequence similarity to *Vibrio anguillarum* in rotifer cultures and the gut of cod larvae fed rotifers. Both rotifers and *Artemia* sp. are filter feeders capable of concentrating large amounts of bacteria, and live feed is a major source of bacterial influx to the gastrointestinal tract of fish (Nicolas et al. 1989, Skjermo & Vadstein 1993, Makridis et al. 2000a,b). The present results support the view that the composition of this influx influences larval survival. However, as most strains did not induce mortality, they could be viewed as secondary pathogens, i.e. opportunists that invade already stressed or weakened larvae.

Three strains, HI 21052, HI 22001 and HI 22027, resembling *Vibrio logei*, *V. anguillarum* and *V. splendidus*, respectively, caused mortality rates that were significantly different from the negative control from Day 9 post-hatching. These strains appeared to have a slower infection rate that could have been caused by non-optimal growth conditions for the specific bacterium, such as temperature, salinity and nutrients. Therefore, it cannot be ruled out that these bacterial strains might act like primary pathogens under different growth conditions. During laboratory studies fish are kept under controlled optimal conditions, which might make them more capable of dealing with an infection compared to fish kept in commercial farms. The bacterial strain HI 22001 is probably a less virulent strain of *V. anguillarum*. Differences in virulence among O1 isolates of *V. anguillarum* were reported by Pedersen et al. (1997), and it is likely that differences in virulence will occur among all *V. anguillarum* serotypes. Less virulent O2 strains of *V. anguillarum*, isolated from cod, have also been reported (Mikkelsen et al. 2007).

None of the 3 different *Vibrio logei* strains tested had any negative effect on larval survival. This was not expected prior to the challenge experiments. *V. logei* is frequently isolated from moribund and dead larvae and it has been assumed to play a significant role in bacterial problems experienced in hatcheries. There are several possible explanations to why bath-challenge did not have any effect. A challenge dose of 10^6 CFU ml^{-1} may not be sufficient to cause disease. It is possible to grow *V. logei* in cultures up to 10^8 CFU ml^{-1} (Ø. Bergh pers. obs.), so a higher challenge dose

should be tested in repeated experiments. Additionally, cod larvae drink water from hatching onwards (Mangor-Jensen & Adoff 1987), thus bacteria will enter the gastrointestinal tract. Consequently, the intestine as a route of entry for pathogenic bacteria cannot be ruled out. Our results suggest that a route of entry other than bath-challenge alone is required or, alternatively, that a combination of both bath and oral exposure is needed. Experiments that deliver challenges via live feed have been performed on turbot *Scophthalmus maximus* larvae (Grisez et al. 1996, Planas et al. 2005) but to the best of our knowledge no such experiments have been performed on cod larvae. Another explanation for the lack of pathogenicity is the possibility of quorum sensing. *V. logei* strains were first described as symbiotic with *Vibrio fisheri* in squid (*Sepiola robusta* and *Sepiola affinis*) light organs (Fidopiastis et al. 1998). The 2 luminous bacteria are closely related, and with *V. logei* being symbiotic with a bacterium capable of quorum sensing (Dunlap 1999, Milton 2006), it is reasonable to believe that *V. logei* might possess some of the same abilities as its fellow organism. This leaves room for speculation about whether or not *V. logei* is an opportunist taking advantage of other bacteria, perhaps through mechanisms of quorum sensing. The work done by Fidopiastis et al. (1998) also confirms the difficulty of distinguishing 2 closely related coexisting bacteria by growth and genetic analyses. During a disease outbreak, finding and isolating the primary pathogen could thus be difficult if other agents are present at high densities, as *V. logei* often is. The role of *V. logei* in disease outbreaks in cod hatcheries still needs to be elucidated.

Moritella viscosa has been isolated from cultivated cod that display skin lesions similar to the ones seen on salmonids (Colquhoun et al. 2004), but the bacteria showed some phenotypical differences from the NCIMB 13584^T strain. The *M. viscosa* isolate used in these experiments did not have any negative effect on cod larvae. In a study performed by Gudmundsdóttir et al. (2006), a bath challenge with *M. viscosa* resulted in mortality only when the challenge dose was as high as 10^7 CFU ml^{-1}. Similar results were obtained by Björnsdóttir et al. (2004), in challenge experiments on turbot juveniles (50 g). No clinical signs were found on fish challenged with the lowest dose (10^6 CFU ml^{-1}) in either of these 2 experiments. On this basis, future experiments on cod larvae should include a higher challenge dose. However, in the present experiments we chose to use the same challenge dose for all strains tested. A challenge dose of 10^6 CFU ml^{-1} is frequently used in challenge experiments and is generally considered to be a high challenge dose (Bergh et al. 1992, Vik-Mo et al. 2005, Sandlund et al. 2006, Schrøder et al. 2006).

When comparing growth temperatures, Tunsjø et al. (2007) found that *Moritella viscosa* grew denser and had better motility at 4°C and with the addition of 3 to 4% NaCl, than at 15°C with 1% NaCl. Conditions for growth in the present experiments should be within the range of optimal growth for this bacterium. The same temperature was used both to grow the bacterial cultures and as the incubation temperature inside the air-conditioned room. This was done to keep conditions as close to the natural environment as possible, where the bacteria grow under the same conditions as the larvae. Changes in characteristics when bacterial strains have been grown at different temperatures have been observed (Ø. Bergh pers. obs).

The reason for the differences seen between the challenged control groups, especially in Expt 2, is not known. It could have been caused by inaccuracy when the dilutions of these particular bacterial suspensions were made. It is known that loss of flagella, for example during the washing procedure, makes the bacterium less pathogenic, as the flagella are very important as a source of virulence (Milton et al. 1996, O'Toole et al. 1996). Before challenge, all bacterial suspensions were examined to verify that the motile bacteria were still intact and motile after the washing procedure. Hence it is unlikely that the loss of flagella was the cause of differences in mortality. Reduction or loss of virulence induced by washing of bacterial suspensions should also be considered. However, given that all bacterial suspensions were grown under the same conditions and that this washing procedure is routinely used in our laboratory this is unlikely to have affected the results.

To keep unfed cod larvae alive for up to 14 d post-hatch shows that the multi-dish system is well adapted for studying bath challenge for cod larvae as well as for halibut (Bergh et al. 1992, 1997), turbot (Bergh et al. 1997, Hjelm et al. 2004) and great scallop larvae (Sandlund et al. 2006). Only at the end of the experiments, 15 d post-hatching were significant differences found between the unchallenged control groups in the 3 experiments. These differences might be caused by differences in the yolk-sac content among different larval groups.

To obtain a complete starvation induced mortality curve the larvae were kept alive until death by starvation. This was done to avoid losing important data, which has occurred in previous cod yolk-sac larvae experiments. To the best of our knowledge this is the first study to carry out such experiments on cod larvae. Furthermore, all 3 experiments were performed identically, which ensures the results are reliable. Similar future experiments using similar conditions can now be brought to an end at an earlier stage.

None of the bacterial strains were re-isolated from the larvae. Larvae possess a sterile digestive system

until hatching, when it is colonized by the egg flora (reviewed by Vine et al. 2006). Reisolation of bacteria from larvae is difficult, particularly due to the small size of the larvae; the need for exterior washing or disinfection results in a decreased number of viable bacteria. Concerning this matter we chose immunochemistry methods to verify the presence of *Vibrio anguillarum* (Engelsen et al. 2008). Given that sterile water and a high concentration of bacteria were added to each well, it is reasonable to believe that the dominant bacteria inside the wells were the bacteria used for challenge. Furthermore, the eggs were selected at random and most of the larvae lived until the point of starvation, indicating that the larvae did not die of factors other than the bacterial strains added.

The use of API 20E as a diagnostic tool for aquatic bacteria has been debated for decades, as reviewed by Popovic et al. (2007). According to Alsina & Blanch (1994), this could be a useful tool when adjustments or modifications to the manufacturer manual are being made and as a supplement to other methods of identification. In our laboratory we modified the API protocol to suit cold-water bacterial strains on the basis of previous experience. In spite of this we observed some inconclusive results among some of the bacterial strains tested, especially concerning the fermentation of sugars. This has occasionally been observed in our laboratory (authors' pers. obs.).

Analyses of 16S rDNA and *GyrB* gene sequences were primarily used as a preliminary stage of identification to limit the number of bacteria used in these challenge trials and for further characterisation. When the 16S rDNA and *GyrB* sequences were compared, some identifications were inconclusive (see Table 1). This may have been due to polymorphism and heterogeneity in the 16S rDNA gene (Dahllöf et al. 2000, Moreno et al. 2002), which would make it difficult to identify strains based on 1 gene only. Several other genes have been suggested as additional sources of information for identifying bacterial strains, for instance *recA* (Thompson et al. 2004b), *rpoB* (Dahllöf et al. 2000), G*yrB*, *fusA*, and *nifD* (Holmes et al. 2004). Another aspect is the limited number of *GyrB* sequences available in the GeneBank compared to 16S rDNA sequences. This is probably the cause of the low frequency of matches with *GyrB* found in the database. The average length of the fragments used in this study is in the range of 1250 to 1350 nucleotides for the 16S rDNA gene and 1100 to 1200 nucleotides for the *GyrB* gene, which is normally sufficient to obtain a match.

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