

## *Vibrio* spp. isolated from salmonids with shallow skin lesions and reared at low temperature

E Benediktsdóttir,<sup>1</sup> S Helgason<sup>2</sup> and H Sigurjónsdóttir<sup>2</sup>

1 Institute of Biology, Microbiology Laboratory, University of Iceland, Reykjavík, Iceland

2 Institute for Experimental Pathology, Fish Disease Laboratory, University of Iceland, Keldur, Reykjavík, Iceland

### Abstract

In Iceland the rearing temperature of salmonid fish, mostly Atlantic salmon, is generally below 10°C. Infections most often characterized by shallow skin lesions that may cause considerable mortality have been endemic in the country since rearing in salt water started. A variety of *Vibrio* spp. has been isolated from the diseased fish. In the present study, a total of 58 strains isolated from salmonid fish and 21 reference strains were subjected to a numerical taxonomy study. The results show that two phenons are most common in the diseased fish; one of them includes *V. marinus*, whereas the other does not include a reference strain but has similarities to *V. logei*. Challenge tests showed that a representative strain of the phenon that included *V. marinus* is pathogenic for Atlantic salmon with an LD<sub>50</sub> lower than  $3.5 \times 10^3$ .

### Introduction

Vibrios are ubiquitous in marine and estuarine water and are part of the normal flora of the intestines of marine fish. Fish and shellfish infections caused by bacteria of the genus *Vibrio* are well known in aquaculture around the world. *Vibrio anguillarum*, *V. ordalii*, *V. salmonicida*, *V. alginolyticus*, *V. vulnificus*, *V. harveyi* and *V. damsela* are presently recognized as marine fish or shellfish pathogens (Lavilla-Pitogo, Baticados, Cruz-Lacierda & de la Pena 1990; Hjeltnes & Roberts 1993; Austin & Austin 1993). The optimum growth

temperature of different *Vibrio* species varies widely, and the seasonal pattern of diseases caused by them is well-known.

In Iceland the rearing phase of salmonids, mainly Atlantic salmon, *Salmo salar* L., in salt water is generally at 10°C or below, either in cages or in shore-based tanks. *V. anguillarum* is not a problem in farmed fish, and *V. salmonicida* has been isolated from only two cold water vibriosis outbreaks. However, infections caused by vibrio bacteria have been fairly common, generally characterized by shallow skin lesions, and can lead to considerable mortality. A variety of *Vibrio* spp. has been isolated from kidneys and skin lesions of farmed salmonids over the years. A preliminary study indicated that two main groups of bacteria were most frequently detected (Benediktsdóttir, Helgason & Sigurjónsdóttir 1991). In Norway, two groups of *Vibrio* spp. are also frequently isolated from Atlantic salmon with similar clinical signs, called 'winter ulcer' (Lunder 1992; Lunder, Evensen, Holstad & Håstein 1995).

The aim of the present study was to clarify the taxonomy of *Vibrio* spp. isolated from diseased salmonids in Iceland and to evaluate their importance in pathogenesis.

### Materials and methods

#### Fish

Newly dead or moribund fish from 25 disease cases, 22 cases involving Atlantic salmon and three cases rainbow trout, *Oncorhynchus mykiss* (Walbaum), were brought to the laboratory for disease diagnosis. An additional three groups of apparently healthy Atlantic salmon, each of different origin, sent for

**Correspondence** Eva Benediktsdóttir, Institute of Biology, Microbiology Laboratory, University of Iceland, Ármúli 1A, IS-108 Reykjavík, Iceland

routine health monitoring were also included in the study. The postmortem examination included macroscopic observation for external and internal disease signs; microscopic examination of wet mount preparations from gills, skin mucus and skin lesions, and bacterial cultivation of material from skin lesions and kidneys on blood agar with and without extra salt added for cultivation of *Vibrio* spp. and *Aeromonas* spp., respectively. Additional screening for *Cytophaga* spp., *Renibacterium salmoninarum* and viruses was carried out when considered necessary.

On five occasions strains were isolated from diseased Atlantic salmon on a single farm by a pathologist and sent to the laboratory on culture plates for identification.

### Bacterial strains

#### Isolation and selection

Fifty-eight bacterial strains, isolated from samples with either pure or mixed bacterial cultures, and 21 reference strains, were included in the study. Samples came from 12 fish farms, five land-based and seven cage farms, during a 6 year period. Strains were isolated from skin lesions and/or kidneys of fish from 30 disease outbreaks, and also from kidneys of apparently healthy fish sent on three occasions for routine health monitoring. Samples from skin lesions and kidneys of four to six fish from each case were inoculated onto blood agar (5% horse blood) supplemented with 1.5% NaCl (BAs), incubated at 15°C, and examined daily for 7 days. Representative colonies were selected for purity plating and those presumptively diagnosed as *Vibrio* sp. selected for further identification. Strains isolated from the kidney were preferred to ones isolated from skin lesions of the same fish for further identification; the strains included in the study were also selected based on diversity of geographical origin and in biochemical reactions that resulted from the preliminary identification.

Representatives of species that are known as psychrotrophic or psychrophilic bacteria, or pathogens or opportunistic pathogens of fish were chosen for reference.

#### Reference strains

*Vibrio anguillarum* NCIMB 6<sup>T</sup>, *V. anguillarum* NCIMB 2129, *V. damsela* NCIMB 2184<sup>T</sup>, *V. alginolyticus* NCIMB 1903<sup>T</sup>, *V. fischeri* NCIMB 1281<sup>T</sup>,

*V. fluvialis* NCIMB 2249<sup>T</sup>, *V. harveyi* NCIMB 1280<sup>T</sup>, *V. logei* NCIMB 2252<sup>T</sup>, *V. logei* NCIMB 1143, *V. marinus* NCIMB 1144<sup>T</sup>, *V. ordalii* NCIMB 2167<sup>T</sup>, *V. parahaemolyticus* NCIMB 1902<sup>T</sup>, *V. salmonicida* NCIMB 2262<sup>T</sup>, *V. splendidus* NCIMB 1<sup>T</sup>, *V. splendidus* NCIMB 2251, *V. vulnificus* NCIMB 2046<sup>T</sup>, *Photobacterium phosphoreum* NCIMB 1282<sup>T</sup> and *P. phosphoreum* NCIMB 844 were obtained from the National Collections of Industrial and Marine Bacteria Ltd in Scotland. *V. iliopiscarius*, PS1, originally from J. Raa at the Norwegian College of Fishery Science, Tromsø, Norway, was kindly supplied by Linda Verdonck at the University of Ghent, Belgium. Two strains, VI 478/88 and VI 441/88, which represent two groups of vibrio bacteria isolated from diseased Atlantic salmon in Norway (Lunder 1992), were kindly supplied by Egil Myhr at the Veterinary Institute in Oslo, Norway.

#### Preservation

Stock cultures were maintained at 4°C or at room temperature on MB agar, and reinoculated every 1 or 2 months. All long-term storage of bacterial strains, including the reference strains, was at –80°C in Marine broth 2216 (Difco) (MB) supplemented with 10% glycerol.

### Biochemical and physiological tests

#### Presumptive identification

Colony morphology was observed on BAs after 2 days of incubation at 15°C. Gram staining and the oxidase test were carried out according to standard procedures. Oxidative and fermentative degradation of glucose, gas production and motility were tested in MOF medium (Difco) or ZOF medium (Lemos, Toranzo & Barja 1985), and read every day for 1 week. Sensitivity to the vibriostatic agent O129 was tested with the disc diffusion method using 10 and 150 µg discs (Oxoid) on BAs. Cells that were Gram negative rods, oxidase positive, oxidative and fermentative and sensitive to 150 µg of O129 were presumptively identified as *Vibrio* spp.

#### Further identification

Young cultures were used for the biochemical tests and incubation was at 15°C, except when testing for growth at different temperatures. MB was used

as a base in all test media (Hansen & Sørheim 1991) except for growth tests at different salt concentrations, and Oxoid agar no. 1 was used in all agar media at 1.2% concentration, unwashed. Tests for the production of acids from carbohydrates and amino acid decarboxylase and dihydrolase tests, nitrate reductase, Voges-Proskauer, methyl red test and production of indole from tryptophane were performed and read according to Hansen & Sørheim (1991), as were tests for enzymatic degradation of starch, chitin, gelatin, DNA, Tween 20 and Tween 80, but these were carried out on Petri plates, four strains on each plate. If no growth was observed after 3 days, plates were re-inoculated with a heavier load of bacteria.

The salt requirement was tested using nutrient broth (Difco) supplemented with agar, with or without NaCl in given concentrations. Luminescence and growth at different temperatures were tested on MB agar. As soon as growth was visible on the plates, luminescence was observed after 5 min in the dark, but growth at different temperatures and with different salt concentrations was examined after 1 week's incubation.

#### Numerical taxonomy analysis

The results of the phenotypic tests were coded as binary responses and subjected to numerical taxonomy analysis using the program NTSYS-pc 1.80 (Applied Biostatistics Inc., Exeter Software, USA). The  $S_{SM}$  coefficient was used, and clustering of strains was carried out by the UPGMA method.

Test reproducibility was evaluated by duplication of seven strains and the corresponding error was calculated according to Sneath & Johnson (1972).

#### Preparation of antisera and antigenicity testing

Antibody was obtained by multiple immunizations of rabbits with washed and formaldehyde-inactivated whole cells of strain K2, as a representative of phenon 1, and strain K8 as a representative of phenon 2. Cells were grown in MB for 3 days. Strain K2 was incubated at 15°C, and K8 at room temperature (22–24°C). Cells were suspended in PBS to OD<sub>590</sub> 0.3 in 1/40 dilution. Formaldehyde was added to 0.35% strength and the suspension kept at 4°C. The rabbits were injected intravenously with 0.5 ml of a 1:2 dilution of the bacterial suspension in distilled water, and booster injections were administered on days 5, 8, 12, 26 and 29.

Four boosters were administered 6 months later at 2, 4 and 3 day intervals, respectively. Blood was collected 21 days after the last booster. The sera were diluted in PBS, and agglutination tests were performed with young bacterial cells by slide agglutination.

#### Challenge tests

PBS supplemented with 0.1% peptone and 1.5% NaCl (P-PBS-S) was used for all suspensions and dilutions of bacteria in the challenge tests and for the viable counts. A bacterial suspension in 1 ml P-PBS-S was inoculated onto two blood agar plates with a Pasteur pipette, excess liquid removed, and the plates dried. After 19 h incubation at 15°C, the growth was harvested with 3 ml of ice-cold P-PBS-S, serial 10-fold dilutions were prepared and kept on ice, and the fish challenged within 1 h. A viable count was made by inoculating double 10 µl of appropriate dilutions on blood agar that were spread and dried and incubated at 15°C for 3 days.

Atlantic salmon parr of ~10 g originating from a smolt-producing facility with no known history of disease were transferred to the experimental facility 4 days before challenge. The fish were kept in fresh water at 11°C.

Representatives of phenon 1 and 2, K58 and K59 (M 147/92–1 and 2, respectively) were used for challenge, but these strains had been used in an experimental vaccine (see 'Discussion'). Initially the strains were passed serially three (K59) or four (K58) times through fish by inoculating four to six fish each time with 10<sup>7</sup>–10<sup>10</sup> colony-forming units (CFU) per fish, and a colony growing from the lowest dose was selected for the next challenge step. Preliminary experiments using K59 showed that injections of a dose of 1.7 × 10<sup>8</sup> CFU or lower did not affect the fish. The experiments using K59 were terminated and an attempt to determine LD<sub>50</sub> was made using K58 only. Ten fish were inoculated with each dilution of bacteria (four groups) and ten fish with P-PBS-S for control. The fish were injected intramuscularly near the lateral line with 0.05 ml, such that the dose per fish was from 3.5 × 10<sup>3</sup> to 3.5 × 10<sup>6</sup> CFU. The fish were observed daily during the experiment, and from 29 out of the 36 fish that died samples were taken aseptically from kidney, liver, spleen and from muscle lesions at the injection site. The experiment was terminated 5 weeks later and an attempt was made to isolate the bacteria on BAs medium from all surviving fish, including the

control group. Phenon 1 bacteria were recognized by their typical colony appearance, haemolytic zone, and agglutination in phenon 1 sera.

## Results

### Biochemical, physiological and serological tests

All strains were sensitive to 150 µg O129 and all gave negative results in the production of acid from salicin, rhamnose and inositol, so these tests were deleted from the programme, and dendrograms were made based on the remaining 40 tests. The error due to lack of reproducibility with duplicate strains was acceptable, at 3.2%. The dendrogram based on the  $S_{SM}$  coefficient is presented in Fig. 1. Levels of 0.85 similarity were selected for phenon definition. Two main clusters appeared, including 19 and 15 Icelandic strains, and five smaller clusters that included one to six Icelandic strains. The validity of clusters was confirmed by a dendrogram based on the  $S_j$  coefficient. The biochemical reactions of the strains in the different phenon and the reference strains are shown in Table 1.

Phenon 1 consisted of 19 Icelandic strains, the Norwegian strain VI 478/88 and *V. marinus* NCIMB 1144. The main cluster of strains within phenon 1 isolated from diseased fish was resistant to 10 µg O129. These strains were arginine dihydrolase (ADH) and ornithine decarboxylase (ODC) negative; produced lysine decarboxylase (LDC); did not produce acid from any carbohydrate tested except *N*-acetyl-glucosamine (NAG), mannose, ribose, maltose and mannitol; produced amylase, gelatinase, chitinase, DNase and lipases; and showed a strong zone of beta-haemolysis with, on average, three times the diameter of the colony. Four strains differed in four tests from the other Icelandic strains in this phenon; they were lysine decarboxylase, mannose, maltose and chitinase negative, and showed a weak haemolysis on blood agar. These four strains were the only strains of phenon 1 that were isolated from fish farms in North Iceland and might represent another biotype of this phenon. All the Icelandic strains of phenon 1 grew at 4°C and 21°C, but none was able to grow at 25°C. On primary isolation, colonies became visible after 2–4 days of incubation on BAs at 15°C. The bacteria were easily lost if not subcultured within a few days. The colonies were transparent to yellowish on BAs, and some of the strains produced a yellowish colour that diffused into the agar. The colonies were very

viscous after 2–3 days' growth, such that a thread formed when they were picked up from the agar. This viscosity was more prominent on BAs than on Marine agar. The diameter of colonies varied from a pinpoint up to 1 mm after 2 days' growth on BAs at 15°C. All the Icelandic strains and the Norwegian strain agglutinated in a 1/32 dilution of sera prepared against the K2 strain from this cluster. *V. marinus* differed from other strains in this phenon in that it was amylase negative, non-haemolytic, showed a small zone around the 10 µg disc of O129, did not grow at 21°C, and did not agglutinate, not even with undiluted antiserum against K2.

Phenon 2 included 15 Icelandic strains and the Norwegian strain VI 441/88. All were ADH, LDC and ODC negative, indole positive, and produced acid from *N*-acetyl-glucosamine, mannose, sucrose (13 of 16 strains), trehalose, glycerol, ribose and maltose. They produced chitinase but no amylase, and haemolysis was seen around the colonies after 2–3 days of incubation. The colonies were yellow and typically 1–2 mm in diameter after 2 days of incubation on BAs at 15°C, but pinpoint colonies were also frequently seen. All the strains in this phenon agglutinated with sera prepared against K8, but in different dilutions: K8 agglutinated at dilution 1/128, 12 strains at dilution 1/64, and three strains at dilution 1/16 or lower. The type strain that was most closely related to this phenon was *V. logei* NCIMB 2252. It differed from phenon 2 in being LDC and ODC positive, indole negative, trehalose and sucrose negative, cellobiose positive, chitinase negative, luminous, and in not agglutinating in the K8 antisera.

Phenon 3, which was close to phenon 2, consisted of three strains that were non-luminescent, and ADH and ODC negative, but differed in the production of LDC; they produced acid from sucrose and lactose but not from cellobiose and were autoagglutinating.

Phenon 4, which included the type strain of *V. logei*, consisted of three Icelandic strains that differed from the type strain in being non-luminous, produced acid from mannitol, and were chitinase producers.

Phenon 5 consisted of five strains including *V. logei* NCIMB 1143. These were autoagglutinating and were able to grow at higher temperature and salt concentrations than strains in phenon 4.

Phenon 6 consisted of only one Icelandic strain and the reference strain of *V. iliopiscarius*, PS1.

Phenon 7 included six Icelandic strains that

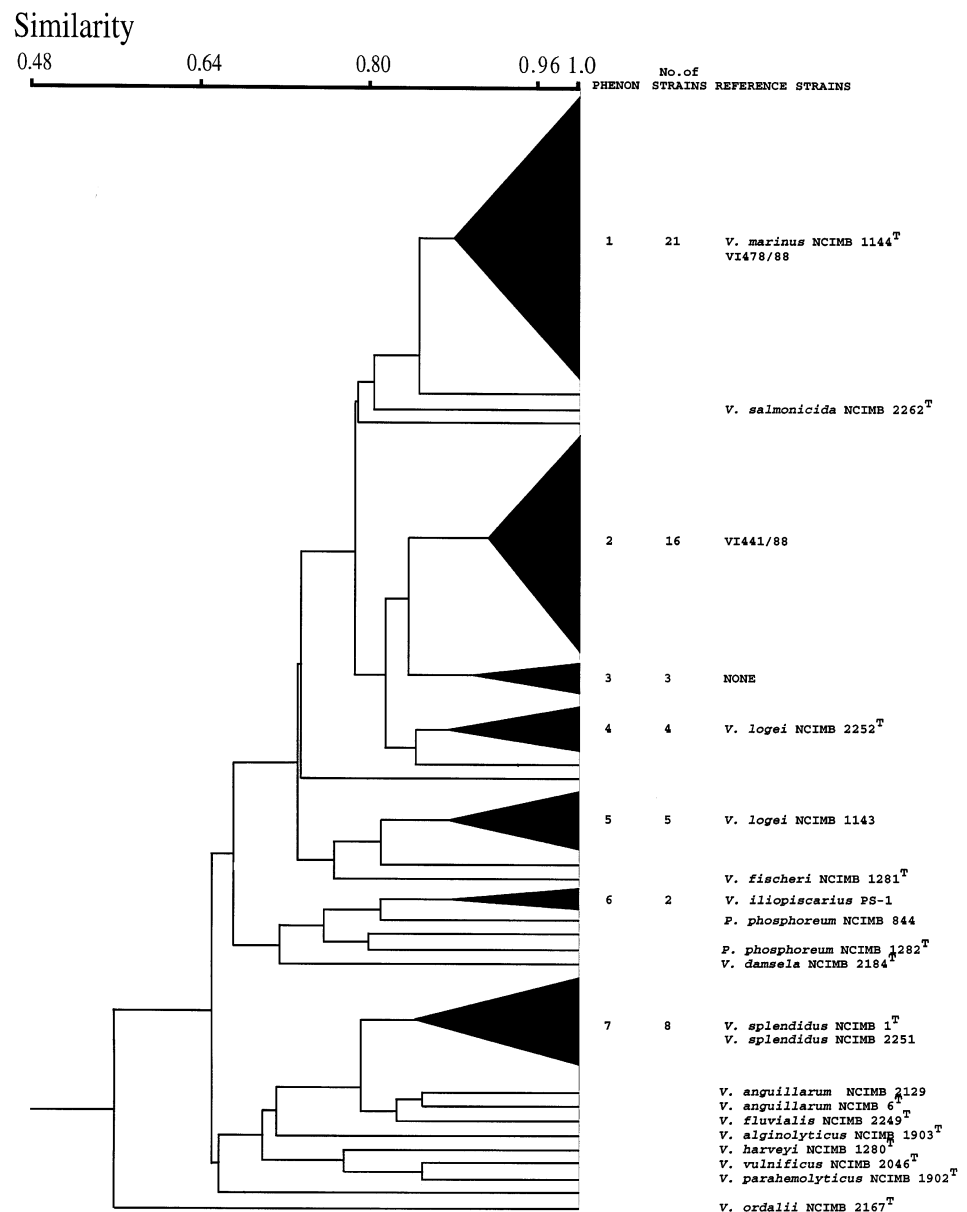


Figure 1 Simplified dendrogram showing relationships between strains and phena.

clustered with the type strain of *V. splendidus* at a 0.88 similarity level, and with *V. splendidus* NCIMB 2251 at a 0.84 similarity level, shown in Fig. 1 as one phenon. The Icelandic strains differed from the type strain in being chitinase positive, none of them were luminous, and five were able to grow on media with only a 0.5% salt concentration.

Seven strains did not cluster with any other strain at the similarity level chosen for definition of phenon. Two strains were most closely related to the reference

strains of *V. marinus* and *V. salmonicida*. They were slow growing and one of them did not grow at 21°C or on nutrient broth agar supplemented with 3% NaCl. Three of the strains had similarities to *V. logei* in that they were ADH negative and LDC positive, they differed in ODC production, were amylase negative, and one was luminous. One strain clustered with the type strain of *P. phosphoreum* at a 0.8 similarity level, and one strain clustered far from the other Icelandic strains and was the only

**Table 1** Results of the biochemical and physiological tests on the strains included in phenons 1–7 and reference strains. +, 100% of strains positive; –, 100% of strains negative. Numerical values are the percentage of positive strains. The following test results were positive for all strains: sensitivity for 150 µg O129; growth in nutrient broth agar supplemented with 3% NaCl; and acid production from *N*-acetyl-glucosamine. The following test results were negative for all strains: acid production from rhamnose salicin and inositol

	Phenon 1 <sup>a</sup>	Phenon 2	Phenon 3	Phenon 4 <sup>a</sup>	Phenon 5 <sup>a</sup>	Phenon 6 <sup>a</sup>	Phenon 7 <sup>a</sup>	NCIMB 1144 <i>V. marinus</i>	NCIMB 2262 <i>V. salmonicida</i>	NCIMB 2252 <i>V. logei</i>	NCIMB 1143 <i>V. logei</i>	NCIMB 1281 <i>V. fischeri</i>	PS1 <i>V. illipiscarius</i>	NCIMB 844 <i>P. phosphoreum</i>	NCIMB 1282 <i>P. phosphoreum</i>	NCIMB 2184 <i>V. damsela</i>	NCIMB 1 <i>V. splendidus</i>	NCIMB 2251 <i>V. splendidus</i>	NCIMB 2129 <i>V. anguillarum</i>	NCIMB 6 <i>V. anguillarum</i>	NCIMB 2249 <i>V. fluvialis</i>	NCIMB 1903 <i>V. alginolyticus</i>	NCIMB 1280 <i>V. harveyi</i>	NCIMB 2046 <i>V. vulnificus</i>	NCIMB 1902 <i>V. parahaemolyticus</i>	NCIMB 2167 <i>V. ordalii</i>	
Number of strains	20	16	3	3	4	1	6																				
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gas production	-	-	-	-	-	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Luminescence	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sensit. f. O129 10 µg	-	+	+	+	+	-	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 0% NaCl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Growth at 0.5% NaCl	-	-	-	-	-	+	83	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 6% NaCl	-	6	-	-	75	+	+	-	-	-	+	-	-	+	+	-	+	+	+	+	+	+	-	-	+	+	
Growth at 8% NaCl	-	6	-	-	75	-	+	-	-	-	+	-	-	+	+	-	+	+	+	+	+	+	-	-	+	-	
Growth at 4°C	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	-	-	-	-	-	-	-
Growth at 21°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 26°C	-	6	-	-	75	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 30°C	-	-	-	-	50	-	83	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 37°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	+	+	+	+	+	+
ADH	-	-	-	-	-	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
LDC	80	-	33	+	+	+	-	+	-	+	+	+	+	+	-	-	-	-	-	-	-	+	+	+	+	+	-
ODC	-	-	-	67	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-
Nitrate reduction	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Indole production	5	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	
Voges-Proskauer	-	-	-	-	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Methyl Red	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Arabinose	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	+	-	
Cellobiose	5	-	-	+	+	-	+	-	-	+	+	+	-	-	-	+	+	+	+	+	+	-	-	+	-	-	-
Glycerol	5	+	+	+	+	-	33	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	-	-	+	33	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-
Mannitol	10	19	67	+	+	-	+	-	-	-	-	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+
Mannose	75	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	65	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Melibiose	-	13	-	-	-	67	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	+	
Ribose	95	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Sorbitol	-	-	-	-	-	-	17	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Sucrose	-	80	+	-	50	-	17	-	-	-	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
Trehalose	-	+	67	67	+	-	+	-	-	-	+	-	-	-	-	-	+	+	-	+	+	-	+	+	+	+	+
Degr. of starch	95	6	-	-	-	-	50	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	
Degr. of gelatin	+	+	-	+	25	-	+	+	+	+	-	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	-
Degr. of DNA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Degr. of Tween 20	+	+	67	67	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-
Degr. of Tween 80	+	+	-	67	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Degr. of chitin	70	94	67	+	75	+	+	+	-	-	-	+	+	+	+	-	+	+	+	+	-	-	+	+	+	+	-

<sup>a</sup>The test results of the reference strains that are shown in separate columns are not included.

strain included in the study besides *V. fluvialis* NCIMB 2249 that was able to grow in media from 0% up to 8% NaCl, and was the only strain that did not produce acid from *N*-acetyl-glucosamine.

### Clinical findings

After characterization of the strains, a retrospective analysis of the cultivation results, origin of fish, and clinical examination of the fish in each case was made. Bacteria of phenon 1 and/or phenon 2 were isolated in 25 cases. Sixteen cases came from nine farms without other known underlying disease problems, and nine cases were from two farms with a history of *Aeromonas salmonicida* subsp. *achromogenes* infections. The 19 Icelandic strains in phenon 1 were isolated from 18 outbreaks. In seven outbreaks, they were the only or the dominating species isolated, and no other suspected pathogen or strains from phenon 2 were isolated from the fish. In seven outbreaks, strains of phenon 2 were isolated as the only or dominating species and no other suspected pathogens were found, except in one case where long slender rods were seen. In eight cases, mixed cultures of strains of phenon 1 and 2 were found; in two outbreaks phenon 1 bacteria were found with *A. salmonicida* subsp. *achromogenes*; and in one case all three species were found.

Macroscopic disease signs were similar for outbreaks where bacteria of phenon 1 or phenon 2, or both, were isolated. Excluding four cases where examination results are missing and the three cases where *A. salmonicida* subsp. *achromogenes* was also found, the clinical signs from 18 outbreaks were as follows. The external sign most frequently detected, on 16 occasions, was skin lesions. They were frequently large and in various parts of the body, especially on the flanks and extending to the mouth region. Eye damage, mostly traumatic or subcorneal haemorrhage, fin rot and petechial haemorrhage of the skin was seen in one to three cases. In the two cases where open skin lesions were not detected, eye damage, or diffuse or petechial haemorrhage on the skin or muscle was noted. In internal organs, a diffuse or petechial haemorrhage in liver, pyloric caeca, peritoneal or subperitoneal membranes, perivisceral fat and lower intestines was most frequently seen.

The 24 strains that were not included in phenon 1 or 2 were isolated from eight farms on 16 occasions. In eight cases they were isolated from a mixed culture along with *A. salmonicida* subsp. *achromogenes*, bac-

teria of phenon 1 and/or phenon 2, and generally in a minor amount on culture plates. In eight cases, no pathogen or suspected pathogen was isolated. In three of these eight cases, apparently healthy fish came in for routine control, in two cases the bacterial strains were sent to the laboratory on BAs culture plates for identification, and in one case examination results are missing. In the two remaining outbreaks, large lesions were seen on the flanks and the nose, and a mixture of bacteria belonging to phenon 4 or not belonging to any of the above-described phenon were isolated. In both these cases, mechanical damage was found to be the primary cause of lesions.

### Challenge tests

In all, 36 out of 40 fish infected with K58 died, and none of the control fish died. All fish that were infected with  $3.5 \times 10^6$  CFU died within 4 days, and all fish infected with  $3.5 \times 10^5$  CFU died within 9 days. Nine out of 10 fish that were infected with  $3.5 \times 10^4$  CFU died within 15 days, and seven out of 10 fish infected with  $3.5 \times 10^3$  CFU died, the last one 19 days post-infection.

No bacteria were isolated from the surviving fish. Bacteria were isolated from 26 out of 29 sampled fish that died during the experiment. Phenon 1 bacteria were isolated from 90% of muscle samples, 67% of kidney, 62% of liver and 34% of spleen samples. Two of the negative muscle samples were from fish that had large open lesions, and the 70% alcohol used for surface disinfection of skin might have killed the pathogen. The third muscle sample that was negative for phenon 1 bacteria yielded a heavy mixed flora of bacterial contaminants that might have overgrown the phenon 1 bacteria. Confluent growth of phenon 1 bacteria was observed in all positive muscle samples. Samples from the inner organs yielded fewer CFU. Thus, out of 44 positive samples from inner organs, 1–10 CFU were noted in 26 samples and 11–100 CFU in 10 samples; only eight samples yielded confluent growth of phenon 1 bacteria, all of them from fish that received the lower doses of  $3.5 \times 10^4$  and  $3.5 \times 10^3$  CFU.

All fish that died had haemorrhage and necrosis in the muscle at the injection site and petechial haemorrhage in the liver, pyloric caeca and the perivisceral fat. One fish had haemorrhage in the eyes and exophthalmia, and three fish had shallow skin lesions at the injection site.

## Discussion

The results presented here demonstrate that vibrio bacteria isolated from farmed salmonids in Iceland suffering a disease characterized by large superficial skin lesions fall mainly into two phenon, based on biochemical and physiological tests. This is consistent with findings in Norway, where two main groups of bacteria were isolated from Atlantic salmon suffering a disease known as 'winter ulcer' (Lunder 1992; Lunder *et al.* 1995). Reference strains of these groups, named *Vibrio* spp. groups 1 and 2, i.e. VI 478/88 and VI 441/88, respectively, were included in the present study; they clustered within phenon 1 and 2. The descriptions of clinical signs of 'winter ulcer' disease by the Norwegian authors are also comparable to what has been experienced in Iceland.

Virulence was demonstrated in a strain belonging to phenon 1, with an LD<sub>50</sub> of lower than  $3.5 \times 10^3$  CFU in Atlantic salmon parr when injected intramuscularly. A slightly higher dose of a *Vibrio* sp. group 1 strain isolated in Norway,  $1.4 \times 10^4$  CFU, killed 18% of the experimental fish, and a dose of 14 CFU was enough to induce skin ulceration at the inoculation site (Lunder *et al.* 1995). High colony counts on BAs from muscle samples indicate that the bacteria are capable of multiplication in muscle. In spite of extensive haemorrhaging, often few or no CFU were seen in internal organs, indicating that the clinical signs are caused by extracellular enzymes and/or toxins.

The preliminary attempts at this laboratory to challenge Atlantic salmon parr with a strain that belonged to phenon 2 do not indicate that bacteria of this phenon are primary pathogens. Lunder *et al.* (1995) reported that intramuscular injection in fish of  $10^{11}$  CFU of *Vibrio* sp. group 2 did not result in any ulceration or mortality. In that study as well as this study, however, bacteria belonging to this phenon or group were isolated from outbreaks where no other suspect or known pathogenic or opportunistic microbe was found. The separate findings in Norway and Iceland might indicate that this phenon of bacteria is composed of opportunistic pathogens in farmed salmonid fish. It is also possible that these findings reflect the fact that bacteria belonging to this phenon are common saprophytes on fish, and an undetected pathogen or mechanical damage was responsible for the diseased fish detected in both countries. Strains of phenon 1, which are easily overgrown, sensitive in transport and difficult to cultivate, might have been present but not detected.

In Iceland skin lesions caused by vibrio infections have been an endemic problem since the rearing of Atlantic salmon in salt water started about 12 years ago. Since February 1993, strains of phenon 1 and 2 have been used in an experimental autogenous vaccine produced by a commercial company, A.L.Pharma, on one farm. Results up to now are promising (Laxdal, B. & Jónsson, G., pers. comm.) and support the present results that bacteria of at least one of these phenon are primary pathogens in salmonid fish in Iceland.

Phenon 1 included the type strain of *V. marinus*, which was the only strain in this phenon that did not agglutinate the antisera prepared against one of the Icelandic phenon 1 strains, and which differed in several tests from the other strains in the phenon. Phenon 2 included no type strain, and more antigenic heterogeneity was found than among the phenon 1 strains. *V. marinus* was originally isolated from sea water at 1200 m depth (Colwell & Morita 1964), and this species has never, to the authors' knowledge, been associated with a fish disease. Research to characterize the strains of phenon 1 and 2 and to study their taxonomic positions is in progress.

*Vibrio logei*, *V. splendidus*, *V. iliopiscarius*, unnamed small phenon or bacteria that did not belong to any phenon, were found in low numbers in mixed cultures from diseased fish, along with strains from phenon 1, phenon 2 or *A. salmonicida* subsp. *achromogenes*, in apparently healthy fish screened for routine health control, or in outbreaks where mechanical skin damage was the primary cause of disease. This finding indicates that these heterogeneous bacteria are a normal microbiota of fish and sea water, contaminating the samples rather than acting as opportunistic pathogens. Myhr, Larsen, Lillehaug, Gudding, Heum & Håstein (1991) found *V. splendidus* biovar I in samples from healthy salmonid fish and incidentally in low numbers from mixed cultures from the kidneys of salmonid fish suffering another major bacterial or viral disease. In the same study, *V. splendidus* was found in diseased turbot and sea bass in Norway. Two strains of *V. logei* have been reported from necrotic lesions on tanner crabs obtained from depths of over 1000 m (Bang, Baumann & Neilson 1978), but the type strain of *V. logei* originates from the gut of scallops from the Arctic (Bang *et al.* 1978), and *V. logei* NCIMB 1143 originates from the skin of Pacific cod (Colwell & Morita 1964). The newly described species *V. iliopiscarius* (Onarheim, Wiik, Burghardt &



Stackebrandt 1994), which has many phenotypical traits similar to *P. phosphoreum*, is indigenous to the gastrointestinal tract of fish in cold sea water. A representative of the species was found to be non-pathogenic for Atlantic salmon (Onarheim *et al.* 1994). The present findings are consistent with these reports that indicate that these groups are not pathogenic for salmonid fish, but they might be important pathogens for other fish or shellfish species.

The identification of bacteria of the genus *Vibrio* is complicated by the inter-species differences in optimum growth temperature, growth rate and salt requirement, and by the fact that the methods for biochemical classification are not standardized. Many of the tests used here are not included in Bergey's Manual, and no tests are normally performed at 15°C. In fact, the methodology is generally more applicable to human pathogenic bacteria or bacteria isolated in warm climates. Cultivable psychrotrophic marine bacteria are often slow growing on artificial media, and require various salts and growth factors in addition to prolonged incubation at a low temperature, i.e. 20°C or lower. Primary isolation of the vibrio bacteria from fish was made on agar media supplemented with blood, i.e. the media that best supported growth for these bacteria. Often it took 3 days at 15°C for bacteria of phenon 1 to grow to visible size, and they might be easily overlooked if blood agar is not used for primary isolation. During the first attempts in Iceland to identify phenon 1 bacteria, they were found to show no growth in many ordinary test media commonly used for vibrio bacteria. This problem was solved by using Marine broth 2216 as a base for the test media, which supports satisfactory growth of phenon 1 bacteria and is both a standardized medium and commercially available. However, a large inoculum was sometimes needed for the growth of phenon 1 bacteria, and utilization tests that are read as growth or no growth, commonly used for bacteria of the genus *Vibrio*, were not considered appropriate in this study. Tests were chosen that distinguished between psychrotrophic species of *Vibrio* and separated them from eventual *Aeromonas* spp. that might have passed through the presumptive identification as *Vibrio* spp. Incubation was at 15°C and was long enough to allow good growth so that a proper comparison of all strains tested could be ensured. All the Icelandic strains isolated in this study grew well at 15°C, but the reference strains isolated at warmer temperatures grew slowly at this

temperature and were far from their optimum. Some of the reference strains that are motile at their optimum temperature showed no motility at 15°C, so motility was not included in the numerical taxonomy analysis. Tests for urease production were also omitted from the present study. Some test strains were found to convert the control test media for urease production without urea to alkaline, probably because the amount of peptone in MB is too high for this test, and lower peptone amounts do not support the growth of either phenon 1 or phenon 2 bacteria. In a numerical taxonomy analysis, more than 50 characters should be included, but growth problems and the unreactivity of the phenon 1 strains limit the number of biochemical tests that can be used successfully. However, the results indicate that, when these problems are taken into account, these tests and methods can be used to identify and distinguish between strains of psychrotrophic vibrio bacteria.

Several of the phenon recovered in this study of psychrotrophic bacteria may correspond to as yet undescribed taxa. In the present study, bacteria that were found to infect or colonize the fish were more closely related to known psychrophilic or psychrotrophic vibrio bacteria, *V. marinus*, *V. salmonicida* and *V. logei*, than to fish pathogens known to infect or colonize fish in warmer waters, such as *V. anguillarum*, *V. harveyi* or *V. vulnificus*, or possible psychrotrophic biovars of these species. A numerical taxonomy study of Vibrionaceae isolated from the seasonally cold coastal waters of Newfoundland indicated that most of the regional Vibrionaceae strains studied, isolated at low temperatures, were different from previously described species belonging to this family (Martin-Kearley & Gow 1994). This finding is consistent with the present results and would explain the difference in the aetiology of vibriosis seen in warm and cold waters. In intensive aquaculture, the fish and shellfish are under stress conditions periodically or constantly, and the characterization of pathogens and opportunistic pathogens is of primary importance. A knowledge of the normal bacterial background in different kinds of environments is one of the keys to the successful monitoring of fish health in different environments and highlights the need for further studies on vibrio bacteria in Arctic or cold-temperate regions.

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## References

- Austin B. & Austin D.A. (1993) *Bacterial Fish Pathogens, Disease in Farmed and Wild Fish*, 2nd edn, pp. 265–307. Ellis Horwood, London.
- Bang S.S., Baumann P. & Neelson K.H. (1978) Phenotypic characterization of *Photobacterium loeji* (sp. nov.), a species related to *P. fischeri*. *Current Microbiology* **1**, 285–288.
- Benediktsdóttir E., Helgason S. & Sigurjónsdóttir H. (1991) Comparison of forty strains of *Vibrio* spp. isolated from kidneys of Atlantic salmon, *Salmo salar* L., with skin lesions. Abstract. In: *EAFP Conference Handbook Diseases of Fish and Shellfish*, Budapest, 125.
- Colwell R.R. & Morita R.Y. (1964) Reisolation and emendation of description of *Vibrio marinus* (Russell) Ford. *Journal of Bacteriology* **88**, 831–837.
- Hansen G.H. & Sørheim R. (1991) Improved method for phenotypical characterization of marine bacteria. *Journal of Microbiological Methods* **13**, 231–241.
- Hjeltnes B. & Roberts R.J. (1993) Vibriosis. In: *Bacterial Diseases of Fish* (ed. by V. Inglis, R.J. Roberts & N.R. Bromage), pp. 109–121. Blackwell Scientific Publications, Oxford.
- Lavilla-Pitogo C.R., Baticados M.C.L., Cruz-Lacierda E.R. & de la Pena L.D. (1990) Occurrence of luminous bacterial disease of *Penaeus monodon* larvae in the Philippines. *Aquaculture* **91**, 1–13.
- Lemos M.L., Toranzo A.E. & Barja J.L. (1985) Modified medium for the oxidation-fermentation test in the identification of marine bacteria. *Applied and Environmental Microbiology* **49**, 1541–1543.
- Lunder T. (1992) 'Winter ulcer' in Atlantic salmon. A study of pathological changes, transmissibility, and bacterial isolates. *Thesis for the degree of Doctor Scientiarum*. The Norwegian College of Veterinary Medicine, Oslo, Norway.
- Lunder T., Evensen Ø., Holstad G. & Håstein T. (1995) 'Winter ulcer' in the Atlantic salmon *Salmo salar*. Pathological and bacteriological investigations and transmission experiments. *Diseases of Aquatic Organisms* **23**, 39–49.
- Martin-Kearley J. & Gow J.A. (1994) Numerical taxonomy of *Vibrionaceae* from Newfoundland coastal waters. *Canadian Journal of Microbiology* **40**, 355–361.
- Myhr E., Larsen J.L., Lillehaug A., Gudding R., Heum M. & Håstein T. (1991) Characterization of *Vibrio anguillarum* and closely related species isolated from farmed fish in Norway. *Applied and Environmental Microbiology* **57**, 2750–2757.
- Onarheim A.M., Wiik R., Burghardt J. & Stackebrandt E. (1994) Characterization and identification of two *Vibrio* species indigenous to the intestine of fish in cold sea water; description of *Vibrio iliopiscarius* sp. nov. *Systematic and Applied Microbiology* **17**, 370–379.
- Sneath P.H.A. & Johnson R. (1972) The influence on numerical taxonomic similarities of errors in microbiological tests. *Journal of General Microbiology* **72**, 377–392.