Phenotypic and genotypic characterization of *Vibrio viscosus* sp. nov. and *Vibrio wodanis* sp. nov. isolated from Atlantic salmon (*Salmo salar*) with 'winter ulcer'

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Two groups of *Vibrio* strains isolated from Atlantic salmon with 'winter ulcer' were characterized phenotypically and genotypically. The data obtained indicated that each of the two groups represented a new species in the genus *Vibrio*. The names *Vibrio viscosus* sp. nov. [type strain NVI 88/478^T (= NCIMB 13584^T)] and *Vibrio wodanis* sp. nov. [type strain NVI 88/441^T (= NCIMB 13582^T)] are proposed for the new species. *V. viscosus* strains exhibited a similar total DNA RFLP pattern and a similar plasmid DNA profile. DNA relatedness (hydroxyapatite method) of the *V. viscosus* type strain to nine other *V. viscosus* strains was 81–93% at 60 °C. Divergence within related sequences was 0.0–1.5% and relatedness at 75 °C was 74–100%. *V. wodanis* strains exhibited marked heterogeneity on the basis of RFLP analysis and plasmid profiles. DNA relatedness of the *V. wodanis* type strain to 10 other *V. wodanis* strains was 66–94% at 60 °C. Divergence within related sequences was 0.0–1.5% and relatedness at 75 °C was 55–97%. Relatedness between *V. viscosus* and *V. wodanis* type strains was approximately 20%. Among other *Vibrio* species, the closest relative of *V. viscosus* was *Vibrio marinus* (ATCC 15381^T) (43% relatedness at 60 °C) and that of *V. wodanis* was *Vibrio logei* (ATCC 15382) (57% relatedness at 60 °C). These same pairs were the closest phenotypic relatives. DNA sequence analysis of the 16S rRNA gene of *V. viscosus* indicated an intimate relationship to *V. marinus*. A total evaluation of the results, however, supports *V. viscosus* to be a separate species in the genus *Vibrio*. The analysis of the sequence of the 16S rRNA gene of *V. wodanis* supports that *V. logei* (ATCC 15382) was the most related species. Ability to degrade casein, oxidative production of acid from trehalose and production of lysine decarboxylase are important biochemical tests that will differentiate between *V. viscosus*, *V. wodanis*, *V. marinus* (ATCC 15381^T) and *V. logei* (ATCC 15382).

Keywords: *Vibrio viscosus*, *Vibrio wodanis*, salmon

INTRODUCTION

'Winter ulcer' is a disease affecting sea-farmed Atlantic salmon (*Salmo salar*) (Lunder *et al.*, 1995). The disease

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Abbreviations: AMPPD, 3-(2'-spiroadamantane)-4-methoxy-4-(3"-phosphoryloxy)-phenyl-1,2-dioethane; HMO, hypothetical mean organism.

The EMBL accession numbers for the 16S rDNA sequences of *Vibrio viscosus* 88/478^T and *Vibrio wodanis* 88/441^T are AJ132226 and AJ132227, respectively.

is characterized by skin ulcers confined to scale-covered parts of the body surface, most frequently along the sides of the fish. Winter ulcer occurs mainly during the winter season and is seen in farms with net pens in the sea and in land-located farms with sea water. The disease is observed all along the Norwegian coast from Finnmark county in the north to the Swedish border in the south-east. The mortality is limited, but the disease has economic significance due to lowered quality of the fish.

In a bacteriological examination of affected Atlantic salmon suffering from winter ulcer, two main groups

of bacteria were isolated (Lunder *et al.*, 1995). A limited characterization indicated that both groups belonged to the genus *Vibrio*, the groups being termed *Vibrio* sp. 1 and *Vibrio* sp. 2, respectively. Experimental infection with *Vibrio* sp. 1 induced a disease similar to winter ulcer in Atlantic salmon, while inoculation with *Vibrio* sp. 2 had no effect. Cohabitation experiments also showed that winter ulcer can be transmitted from diseased to healthy individuals. There are several fish-pathogenic bacteria that are members of the genus *Vibrio*, such as *Vibrio alginolyticus*, *Vibrio anguillarum*, *Vibrio carchariae*, *Vibrio damsela*, *Vibrio ordalii*, *Vibrio parahaemolyticus*, *Vibrio salmonicida* and *Vibrio vulnificus* biotype 2 (Colwell & Grimes, 1994; Egidius *et al.*, 1986).

The aim of this study was to obtain information about phenotypic and genotypic properties of the bacterial strains isolated from Atlantic salmon with winter ulcer. We also wanted to determine the relationship between these strains and other fish-pathogenic and psychrophilic species of the genus *Vibrio*.

METHODS

Bacterial strains. Eleven and 31 strains of *Vibrio* sp. 1 and *Vibrio* sp. 2, respectively, isolated in a previous investigation from Atlantic salmon with winter ulcer in eight different fish farms, were selected randomly for the present study (Lunder *et al.*, 1995). Furthermore, nine strains of *Vibrio* sp. 1 and four strains of *Vibrio* sp. 2 were randomly selected from the stock collection of bacteria at the National Veterinary Institute, Oslo, Norway. The strains were isolated from fish at numerous locations along the Norwegian coast during the winter season in the period from 1988 to 1990. One *Vibrio* sp. 1 strain (89/2148) was isolated from plaice (*Pleuronectes platessa*), the other strains were isolated from Atlantic salmon. One strain of *V. salmonicida* was isolated from a salmon with winter ulcer. The type and reference strains used in this study are listed in Table 1.

Vibrio logei ATCC 15382 and *V. logei* NCMB 1143 originated from the same strain, PS 207, isolated from the skin of Pacific cod *Gadus macrocephalus*. All strains included in the study were examined in the tests presented below.

The strains were stored as frozen cultures at -80°C in brain-heart infusion broth (BHIB) (Difco) containing 2% NaCl and 30% glycerol and maintained on brain-heart infusion agar (BHIA) (Difco) slants containing 2% NaCl at 4°C . All the *Vibrio* sp. 1 and *Vibrio* sp. 2 strains have been investigated in all the methods used in the study.

Physiological and biochemical characterization. Cells from a 2-d-old culture on heart infusion agar (Difco) containing 2% NaCl and 5% bovine blood (BA) were used in all tests. Unless otherwise noted the final NaCl content of the test media was adjusted to 2%. All the test media, except the media used to determine the temperature range for growth, were incubated at 15°C . The tests were read every other day for 14 d. Each strain was examined for 85 different characters as listed in Table 2. The tests were performed as described by West & Colwell (1984) with the following exceptions. Colony morphology and haemolysis were observed on BA. Production of DNases and caseinases was assayed from supernatants of BHIB cultures grown at 15°C under constant shaking for 3 d. The supernatants were inoculated onto agar

plates containing 0.3 g DNA l^{-1} and $9.18 \times 10^{-5}\text{ g}$ toluidine blue l^{-1} and then onto sodium caseinate (5 g l^{-1}) plates before incubation at 37°C overnight (Lachica *et al.*, 1971; Sandvik & Hagen, 1968). The ability to grow at different temperatures was tested on BA at 4, 15, 22, 25 and 30°C . Acid production from carbohydrates was determined in a medium containing 0.7% of the actual sugar, 6 ml 0.2% phenol red solution l^{-1} , 2.7 g nutrient broth (Difco) l^{-1} , 5 g proteose peptone (Difco) l^{-1} , 20 g NaCl l^{-1} , 0.3 g $\text{K}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O l}^{-1}$ and 10 ml mineral salt solution l^{-1} containing (g l^{-1}) $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (20), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (5.7), $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (0.5), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.25), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.25), Na_2MoO_4 (0.25) and $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (0.5) as described by Ford *et al.* (1958). Oxidative production of acid was determined for L-arabinose, cellobiose, dextrin, D-galactose, D-glucose, glycerol, lactose, D-mannitol, D-mannose, melibiose, raffinose, L-rhamnose, salicin, D-sorbitol, sucrose and trehalose, while fermentative production of acid and gas was determined for D-glucose. API ZYM kits (BioMérieux) testing for butyrate esterase, myristate lipase, leucine arylamidase, cystine arylamidase, trypsinase, acid phosphatase, naphthol phosphohydrolase, α -galactosidase, β -galactosidase, β -glucuronidase and acetyl β -glucosaminidase were used according to the manufacturer's instructions and read after incubation overnight at 22°C . Susceptibility to antimicrobial agents was determined by the disc diffusion method using vibriostatic agent O/129 (2,4-diamino-6,7-diisopropylpteridine) (150 μg), sulphamethizol (240 μg), ampicillin (33 μg), penicillin (5 μg), oxytetracycline (80 μg) and oxolinic acid (10 μg) (Neo-Sensitabs; Rosco). Müller-Hinton agar (Difco) containing 2% lysed horse blood was used for disc diffusion tests. The growth of *Vibrio marinus* was, however, inadequate on Müller-Hinton plates and BA was used for disc diffusion tests with this organism.

Numerical analysis. The statistical analysis was performed using SAS (versions 6.04 and 6.08) running under DOS and Windows on a 80386 PC. Uniformly positive or negative results were deleted from the data matrix. The similarity coefficients were calculated using simple matching $S_{ij} = C_{ij}/C_{ij} + D_{ij}$, where S_{ij} is the similarity coefficient, C_{ij} is the concordant result and D_{ij} is the discordant result between strains i and j . Clustering was done by unweighted mean linkage (Mardia *et al.*, 1979). The hypothetical mean organism (HMO) was calculated the following way. In each cluster the test results were coded 0 (present) and 1 (not present). The most common value observed (the mode) was calculated for each character using all the individuals belonging to the specific cluster. Thus, if more than 50% of the individuals in a cluster had a character present, the mean individual for this cluster was set to present. This process was repeated for all the characters recorded giving a mean organism.

Ultrastructural studies. Cells grown in Luria broth (Miller, 1972) with 1.5% NaCl for 48 h at 10°C were both negatively stained with 1.5% phosphotungstic acid and photographed under a Philips model CM12 electron microscope (80 kV) and under a JEOL model JSM 6400 scanning electron microscope (10 kV). For SEM the cells were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer and dried in a Balzers critical point dryer. Specimens for SEM were coated with Au/Pd in a Polaron SEM coating unit E5000 (1 kV/20 μA).

Western blotting. Antigen preparations used were either whole cells or Proteinase-K-treated cells. The cells were cultured in BHIB for 2 d at 15°C , harvested and washed twice in PBS, pH 7.2. The concentration of bacteria was

adjusted with PBS to $OD_{520} = 0.3$, the solution then being divided in slants of 1.5 ml in Eppendorf tubes. After centrifugation the supernatants were discarded and the pellets kept at $-22\text{ }^{\circ}\text{C}$ until use. Before use in SDS-PAGE the pellet was solubilized in 150 μl sample buffer (1 ml 0.5 M Tris/HCl, pH 6.8, 0.8 ml glycerol, 1.6 ml 10% SDS, 0.4 ml β -mercaptoethanol, 0.2 ml 0.2% bromophenol blue, 4.0 ml distilled water) and then boiled for 5 min in a water bath. Cells were treated with Proteinase K after boiling. To each tube 10–15 μl Proteinase K (0.0025 g Proteinase K in 1 ml 0.05 M Tris/HCl, pH 8.6) was added, the tubes being kept in a water bath for 1 h before use in SDS-PAGE. SDS-PAGE was performed in 4% stacking gel and 12% separating gel according to the method described by Laemmli (1970). Molecular mass markers, including Standard Low and Precoloured Standard markers (Bio-Rad) were run for each gel. Electrophoresis was performed at 200 V for 1 h (Bio-Rad Protean R II Dual Slab Cell). The gels were either stained with 0.2% Coomassie brilliant blue or transferred electrophoretically at 200 V for 1 h and immobilized onto 0.45 μm reinforced nitrocellulose sheets (Schleicher & Schuell) in a semi-dry system as described by Kyhse-Andersen (1984). The sheets were washed twice in PBS and non-specific binding was blocked by incubation with 1% BSA (A 9647; Sigma) for 1 h at room temperature, followed by three washings in PBS with 0.5% Tween (PBS-T). The membranes were incubated with two different polyclonal rabbit antisera (diluted 1:100 in PBS-T) overnight at $4\text{ }^{\circ}\text{C}$. These antisera, designated antiserum 1 and antiserum 2, had been raised against formalin-killed whole cells of strains 88/478^T and 88/441^T, respectively (Lunder *et al.*, 1995). The filters that had been incubated with serum were washed three times in PBS-T and incubated for 5 h at room temperature in peroxidase-labelled donkey anti-rabbit Ig (Amersham) diluted 1:2000 in PBS-T. The filters were then washed three times in PBS-T, once in PBS and rinsed once briefly in 0.05 M sodium acetate, pH 5.0. Finally the blots were developed in a substrate solution containing 3-amino-9-ethyl-carbazole (AEC; Sigma) (40 mg AEC dissolved in 5 ml *N,N*-dimethylformamide) in 0.05 M sodium acetate, pH 5.0, and H_2O_2 . The reaction was stopped by rinsing in tap water. The blots were read sufficiently using visual observation only.

Substrate gel electrophoresis. Substrate gel electrophoresis with gelatin as substrate was performed as described by Heussen & Drowdle (1980) and modified by Rockey *et al.* (1988). Gelatin was copolymerized at 0.01% to a 12% polyacrylamide gel. To supernatants of BHIB shaking cultures, incubated for 3 d, equal amounts of tracking dye, 5% SDS, 2% sucrose and 8 μg phenol red ml^{-1} were added. After electrophoresis, the gels were washed with 2.5% Triton X-100 (Sigma) in water for 30 min, incubated in 0.1 M glycine/HCl, pH 8.3, for 2 h and stained with Coomassie brilliant blue.

Plasmid profiles. All strains of *Vibrio* sp. 1 and *Vibrio* sp. 2 were subjected to plasmid profiling. The bacteria were grown in shaking cultures using BHIB (Difco), with a final NaCl content of 2%, and incubated at $15\text{ }^{\circ}\text{C}$ for 24 h. Plasmid isolation was performed by three different methods as described by Kado & Liu (1981), He *et al.* (1990) and a rapid small-scale modification of the alkaline lysis technique of Birnboim & Doly (1979) as described by Maniatis *et al.* (1982).

Agarose gel electrophoresis was performed in 1% agarose gels with Tris/borate/EDTA buffer (89 mM Tris, 89 mM

boric acid, 2.5 mM EDTA, pH 8.0) at 120 V for 3 h at room temperature in a vertical gel apparatus. The agarose gels were stained in ethidium bromide and destained in distilled water before photographs of the gels were taken under UV light. *Escherichia coli* V517 (Macrina *et al.*, 1978) with plasmids of eight different sizes and *E. coli* K-12 strains containing plasmids pDK9 and S-a (Bukhari *et al.*, 1977), respectively, were used as standards.

Plasmid DNA from 15 selected *Vibrio* sp. 1 strains and nine selected *Vibrio* sp. 2 strains were subjected to digestion with *Hind*III (Life Technologies). The digests were electrophoresed in a 0.7% agarose gel in a horizontal gel apparatus. λ DNA/*Hind*III fragments and Φ X174RF DNA/*Hae*III fragments (Pharmacia) were used as standards.

Total DNA isolation and purification. Isolation and purification of total bacterial DNA for colony hybridization and for restriction endonuclease digestion were carried out as described by Hull *et al.* (1981). Bacterial cells were harvested from 0.5 l BHIB broth cultures containing 2% NaCl, incubated with shaking for 2 d at $15\text{ }^{\circ}\text{C}$.

The cells were resuspended in a solution of 2 ml 25% sucrose, 1 M Tris, 0.5 M EDTA, pH 8.0, and lysed with lysozyme (Sigma). To this solution were added 0.1 mg Proteinase K (Sigma), 400 μl 0.5 M EDTA and 250 μl 10% *N*-lauroylsarcosine. The solution was then kept at $0\text{ }^{\circ}\text{C}$ for 10 min and at $50\text{ }^{\circ}\text{C}$ for 18 h. DNA was purified in a CsCl gradient containing PMSF. The DNA was dialysed in TE buffer, pH 8.0.

Colony hybridization. All the strains listed in Table 1, including type strains of genus *Vibrio*, except the type strains for *Vibrio anguillarum*, *Vibrio cholerae*, *Vibrio costicola*, *Vibrio gazogenes*, *Vibrio mytili*, *Vibrio navarrensis*, *Vibrio nigrapulchritudo* and *Vibrio vulnificus*, were inoculated onto nitrocellulose membranes (Schleicher & Schuell) placed on the surface of blood agar plates with a final NaCl content of 2% and incubated at $15\text{ }^{\circ}\text{C}$ overnight. The membranes were then soaked in 10% SDS for 3 min, denatured in 0.5 M NaOH for 5 min and neutralized in 1.5 M NaCl and 0.5 M Tris/HCl twice for 5 min. The membranes were then air-dried and baked under vacuum at $80\text{ }^{\circ}\text{C}$ for 2 h and deproteinized at $68\text{ }^{\circ}\text{C}$ for 1 h in a solution of $2\times\text{SSC}$ ($1\times\text{SSC} = 0.15\text{ M NaCl}$ and $0.015\text{ M sodium citrate}$), 0.1% SDS and 100 mg Proteinase K ml^{-1} (Maniatis *et al.*, 1982). Total bacterial DNAs from *Vibrio* sp. 1 strains 88/478^T and 90/4271-1b and *Vibrio* sp. 2 strains 88/441^T and 90/4271-3a were used as probes. The DNAs were digested with *Hae*III (Life Technologies), 1 μg being labelled with digoxigenin-11-dUTP using random-primed labelling (Boehringer Mannheim) in accordance with the manufacturer's instructions.

Hybridization and immunological detection with alkaline phosphatase-conjugated antibodies against digoxigenin were performed as recommended by the manufacturer (Boehringer Mannheim). Prehybridization and hybridization were performed at $68\text{ }^{\circ}\text{C}$ (selected after initial comparison of various temperatures) in $5\times\text{SSC}$, 0.1% *N*-lauroylsarcosine, 0.02% SDS and 1% blocking reagent, a purified fraction of dry milk powder (Boehringer Mannheim). The membranes were then washed twice with $2\times\text{SSC}$ and 0.1% SDS at room temperature and twice with $0.1\times\text{SSC}$ and 0.1% SDS at $68\text{ }^{\circ}\text{C}$ for 1 h. The blots were allowed to develop with 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate for 15–60 min. Two identical membranes were always assayed in parallel.

Table 1. Reference strains, type strains and new isolates used in this study

Species	Strain*
<i>Aeromonas salmonicida</i>	ATCC 14174 (NCMB 833)
' <i>Vibrio abalonicus</i> ' (Pienta & Tang, 1996)	ATCC 27390
' <i>Vibrio adaptus</i> ' (Sneath & Skerman, 1966)	ATCC 19263 (NCMB 1052)
<i>Vibrio aestuarianus</i>	ATCC 35048 ^T (NCMB 2236 ^T)
<i>Vibrio alginolyticus</i>	ATCC 17749 ^T (NCMB 1903 ^T)
' <i>Vibrio algosus</i> ' (ZoBell & Upham, 1944)	ATCC 14390 (NCMB 2073)
<i>Vibrio anguillarum</i>	ATCC 19264 ^T (NCMB 6 ^T); ATCC 14181 (NCMB 829)
<i>Vibrio campbellii</i>	ATCC 25920 ^T
<i>Vibrio carchariae</i>	ATCC 35084 ^T
<i>Vibrio cholerae</i>	ATCC 14035 ^T (NCTC 8021 ^T); NVI Ft 863
<i>Vibrio cincinnatiensis</i>	ATCC 35912 ^T (NCTC 12012 ^T)
<i>Vibrio costicola</i>	ATCC 33508 ^T (NCMB 701 ^T)
' <i>Vibrio cuneatus</i> ' (Davis & Park, 1962)	ATCC 6972
' <i>Vibrio cyclosites</i> ' (Lessel, 1962)	ATCC 14635 (NCIB 2581)
<i>Vibrio damsela</i> (<i>Photobacterium damsela</i> subsp. <i>damsela</i>)	ATCC 33539 ^T (NCMB 2184 ^T , CDC 2588-80 ^T)
<i>Vibrio diazotrophicus</i>	ATCC 33466 ^T (NCMB 2169 ^T)
<i>Vibrio fischeri</i>	ATCC 7744 ^T (NCMB 1281 ^T)
<i>Vibrio fluvialis</i>	ATCC 33809 ^T (NCMB 2249 ^T , NCTC 11327 ^T)
<i>Vibrio furnissii</i>	ATCC 35016 ^T (CDC B3215 ^T)
<i>Vibrio gazogenes</i>	ATCC 29988 ^T
<i>Vibrio harveyi</i>	ATCC 14126 ^T (NCMB 1280 ^T)
<i>Vibrio hollisae</i>	ATCC 33564 ^T
' <i>Vibrio hyperoptica</i> ' (Baumann <i>et al.</i> , 1971)	CDC 9100-79
<i>Vibrio ichthyenteri</i>	ATCC 700023 ^T (IFO 15847 ^T)
<i>Vibrio iliopiscarius</i> (<i>Photobacterium iliopiscarium</i>)	ATCC 51760 ^T
<i>Vibrio logei</i>	ATCC 29985 ^T ; ATCC 15382 (NCMB 1143)
' <i>Vibrio marinofulvus</i> ' (ZoBell & Upham, 1944)	ATCC 14395 (NCMB 2076)
<i>Vibrio marinus</i> (<i>Moritella marina</i>)	ATCC 15381 ^T (NCMB 1144 ^T)
<i>Vibrio mediterranei</i>	ATCC 43341 ^T (CECT 621, NCTC 11946 ^T)
<i>Vibrio metschnikovii</i>	NCTC 8443 ^T (ATCC 7708 ^T)
<i>Vibrio mimicus</i>	ATCC 33653 ^T (CDC 1721-77 ^T)
<i>Vibrio mytili</i>	CECT 632 ^T
<i>Vibrio natriegens</i>	ATCC 14048 ^T
<i>Vibrio navarrensis</i>	CIP 103381 ^T
' <i>Vibrio neocistes</i> ' (Lessel, 1962)	ATCC 14636 (NCIB 2582)
' <i>Vibrio neptuna</i> ' (Baumann <i>et al.</i> , 1971)	CDC 9102-79
<i>Vibrio nereis</i>	ATCC 25917 ^T (NCMB 1897 ^T)
<i>Vibrio nigrapulchritudo</i>	ATCC 27043 ^T
<i>Vibrio ordalii</i>	ATCC 33509 ^T (NCMB 2167 ^T)
<i>Vibrio orientalis</i>	ATCC 33934 ^T (NCMB 2195 ^T)
<i>Vibrio parahaemolyticus</i>	ATCC 17802 ^T (EB 101 ^T)
<i>Vibrio pelagius</i> I (<i>Listonella pelagia</i>)	ATCC 25916 ^T (NCMB 1900 ^T)
<i>Vibrio pelagius</i> II (<i>Listonella pelagia</i>)	NCMB 2253
<i>Vibrio penaeicida</i>	ATCC 51842
<i>Vibrio proteolyticus</i>	ATCC 15338 ^T (NCMB 1326 ^T)
' <i>Vibrio psychroerythrus</i> ' (D'Aoust & Kushner, 1972)	ATCC 27364 (NRC 1004)
<i>Vibrio salmonicida</i>	ATCC 43839 ^T (NCMB 2262 ^T); NCMB 2245; NVI 90/1667-10c (isolated from fish farm 'e')†
<i>Vibrio scopthalmi</i>	CECT 4638 ^T
<i>Vibrio splendidus</i> I	ATCC 33125 ^T (NCMB 1 ^T)
<i>Vibrio splendidus</i> II	NCMB 2251
<i>Vibrio tubiashii</i>	ATCC 19109 ^T (NCMB 1340 ^T)
' <i>Vibrio tyrosinaticus</i> ' (Sneath & Skerman, 1966)	ATCC 19378 (NCIB 9045)
<i>Vibrio vulnificus</i>	ATCC 27562 ^T ; ATCC 33147 (ES-7601)
<i>Yersinia ruckeri</i>	ATCC 29473 ^T (CDC 2396-61 ^T)

Table 1 (cont.)

New isolate†	Fish farm origin‡	New isolate†	Fish farm origin‡
<i>Vibrio</i> sp. 1 88/478 ^T	a	<i>Vibrio</i> sp. 2 90/551-2	t
<i>Vibrio</i> sp. 1 89/1407-1b	f	<i>Vibrio</i> sp. 2 90/685-1	m
<i>Vibrio</i> sp. 1 89/1902	b	<i>Vibrio</i> sp. 2 90/685-2	m
<i>Vibrio</i> sp. 1 89/2067	i	<i>Vibrio</i> sp. 2 90/685-3	m
<i>Vibrio</i> sp. 1 89/2148§	h	<i>Vibrio</i> sp. 2 90/765-1	o
<i>Vibrio</i> sp. 1 90/793-1	c	<i>Vibrio</i> sp. 2 90/765-2	o
<i>Vibrio</i> sp. 1 90/793-2	c	<i>Vibrio</i> sp. 2 90/1027-2a	j
<i>Vibrio</i> sp. 1 90/793-3	c	<i>Vibrio</i> sp. 2 90/1027-4a	j
<i>Vibrio</i> sp. 1 90/1027-3b	j	<i>Vibrio</i> sp. 2 90/1027-6	j
<i>Vibrio</i> sp. 1 90/1097-1	c	<i>Vibrio</i> sp. 2 90/1027-8	j
<i>Vibrio</i> sp. 1 90/1097-2	c	<i>Vibrio</i> sp. 2 90/1162-10	d
<i>Vibrio</i> sp. 1 90/1097-3	c	<i>Vibrio</i> sp. 2 90/1162-12	d
<i>Vibrio</i> sp. 1 90/1162-10	d	<i>Vibrio</i> sp. 2 90/1207-11	k
<i>Vibrio</i> sp. 1 90/1207-16b	k	<i>Vibrio</i> sp. 2 90/1207-14	k
<i>Vibrio</i> sp. 1 90/1667-12	e	<i>Vibrio</i> sp. 2 90/1207-16a	k
<i>Vibrio</i> sp. 1 90/1667-14	e	<i>Vibrio</i> sp. 2 90/1667-13	e
<i>Vibrio</i> sp. 1 90/4271-1b	g	<i>Vibrio</i> sp. 2 90/1667-21	e
<i>Vibrio</i> sp. 1 90/4271-2b	g	<i>Vibrio</i> sp. 2 90/2024-1	r
<i>Vibrio</i> sp. 1 90/4271-3b	g	<i>Vibrio</i> sp. 2 90/2024-2	r
<i>Vibrio</i> sp. 1 90/4271-5b	g	<i>Vibrio</i> sp. 2 90/2024-3	r
<i>Vibrio</i> sp. 2 88/441 ^T	q	<i>Vibrio</i> sp. 2 90/2024-4	r
<i>Vibrio</i> sp. 2 89/11	p	<i>Vibrio</i> sp. 2 90/2024-5	r
<i>Vibrio</i> sp. 2 89/1829	s	<i>Vibrio</i> sp. 2 90/2024-6	r
<i>Vibrio</i> sp. 2 89/5532	n	<i>Vibrio</i> sp. 2 90/2024-7	r
<i>Vibrio</i> sp. 2 90/325-1	l	<i>Vibrio</i> sp. 2 90/2024-8	r
<i>Vibrio</i> sp. 2 90/325-2	l	<i>Vibrio</i> sp. 2 90/4271-2a	g
<i>Vibrio</i> sp. 2 90/325-3	l	<i>Vibrio</i> sp. 2 90/4271-3a	g
<i>Vibrio</i> sp. 2 90/551-1	t		

* ATCC, American Type Culture Collection, Manassas, VA, USA; CDC, Centers for Disease Control and Prevention, Atlanta, GA, USA; CECT, Colección Española de Cultivos Tipo, Valencia, Spain; CIP, Collection of the Institut Pasteur, Paris, France; IFO, Institute for Fermentation Osaka, Osaka, Japan; NCMB, National Collection of Marine Bacteria, Aberdeen, UK; NCTC, National Collection of Type Cultures, London, UK; NVI, National Veterinary Institute, Oslo, Norway.

† NVI bacterial stock collection at the National Veterinary Institute, Oslo, Norway. All strains studied from this collection originate from Norway. The first two digits from left indicate the year of isolation. The next three or four digits after the slash represent the journal number and the digit after the hyphen represents isolates from different fish in the same farm isolated during the same disease outbreak. Lower-case letters to the right in the designation after the hyphen indicate different isolates from the same fish.

‡ Lower-case letters indicate different farms with Atlantic salmon in net pens located along the coast of Norway, except farms 'g' and 'k' which are land-located with an intake of sea water from depths of 50–60 m.

§ Isolated from a plaice (*Pleuronectes platessa*) which was a wild-capture stock fish kept in net pens for 5 months before development of winter ulcer.

Agarose gel electrophoresis of restriction-endonuclease-digested total DNA. Based on results from phenotypical characterization and colony hybridization, total DNA from eight *Vibrio* isolates from Atlantic salmon with winter ulcer and 14 reference strains of the genus *Vibrio* (strains listed as target strains in Table 7) were selected for restriction endonuclease digestion and electrophoresis. *Aeromonas salmonicida* subsp. *salmonicida* and *Yersinia ruckeri* were included as controls.

Total DNA (4 µg) of each isolate was digested with *Hae*III or *Hind*III (the enzymes were selected after initial comparison of various enzymes) before electrophoresis in a 0.7% agarose gel. DNA was stained with ethidium bromide and photographed under UV light.

Southern hybridization. *Hind*III-digested DNAs from agarose gels were transferred to GeneScreen nylon membranes (DuPont) by Southern blotting (Southern, 1975). The nylon membranes were air-dried and the DNA was fixed to the membranes by UV light. *Hind*III-digested total DNAs from the strains listed in Table 7 were used as probes. Probe DNA was labelled with digoxigenin-11-dUTP (Boehringer Mannheim) according to the instructions of the respective manufacturers. Detection with 3-(2'-spiroadamantane)-4-methoxy-4-(3''-phosphoryloxy)-phenyl-1,2-dioethane (AMPPD) and luminescence following hybridization with digoxigenin-labelled probe DNA were performed as recommended by the manufacturer (Boehringer Mannheim). Prehybridization and hybridization were performed at 42 °C

Table 2. Physiological and biochemical characterization of 20 strains of *Vibrio* sp. 1, 35 strains of *Vibrio* sp. 2, one strain of *V. salmonicida* isolated from Atlantic salmon with winter ulcer, and 16 type and reference strains of *Vibrio*

1, *Vibrio* sp. 1; 2, *Vibrio* sp. 2; 3, *V. marinus* NCMB 1144^T; 4, *V. ordalii* NCMB 2167^T; 5, *V. logei* ATCC 15382; 6, *V. logei* NCMB 1143; 7, *V. logei* ATCC 29985^T; 8, *V. salmonicida* NCMB 2262^T; 9, *V. salmonicida* NCMB 2245; 10, *V. salmonicida* 90/1667-10c (isolated from Atlantic salmon with winter ulcer); 11, *V. pelagius* I NCMB 1900^T; 12, *V. pelagius* II NCMB 2253; 13, *V. splendidus* I NCMB 1^T; 14, *V. splendidus* II NCMB 2251; 15, *V. damsela* NCMB 2184^T; 16, *V. anguillarum* ATCC 14181; 17, *V. orientalis* NCMB 2195^T; 18, *V. tubiashii* NCMB 1340^T; 19, *V. fischeri* NCMB 1281^T. +, All strains positive; –, all strains negative. Numerical values indicate percentage positive strains. All strains were positive for catalase, oxidase, motility, growth at 15 °C, growth in 1, 2, 3 and 4% NaCl, susceptibility to vibriostatic agent, oxytetracycline and oxolinic acid, fermentation of D-glucose, oxidative production of acid from D-galactose and D-glucose, production of alkaline phosphatase and caprylate esterase. All strains were negative for Gram stain, production of ornithine decarboxylase, oxidative production of acid from inositol, D-xylose, production of chymotrypsinase, α -glucosidase, β -glucosidase, α -mannosidase and α -fucosidase.

Character	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Colony colour:																			
White	–	–	–	–	–	–	–	–	–	–	–	+	+	+	–	–	+	+	–
Yellow	–	+	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
Grey	+	–	+	+	–	–	–	+	+	+	+	–	–	+	+	+	–	+	+
Green	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–
Colony opacity:																			
Translucent	+	–	+	+	–	–	–	+	+	+	+	–	–	–	+	–	–	–	+
Opaque	–	+	–	–	+	+	+	–	–	–	–	+	+	+	–	+	+	+	–
Degradation of:																			
Starch	95	+	–	–	–	–	–	–	–	–	–	+	+	+	–	+	+	+	–
Alginate	–	23	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–	–	–
Gelatin	+	+	+	+	–	–	–	–	–	–	–	+	+	+	–	+	+	+	–
Casein	+	–	–	+	–	–	–	–	–	–	–	–	+	–	–	+	+	+	+
DNA	+	89	–	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	+
Tween 80	+	+	+	–	+	+	+	–	–	–	+	+	+	+	–	+	+	+	–
Lecithin	+	3	+	+	+	+	+	–	–	–	+	–	+	+	–	+	+	+	+
Urea	+	20	+	–	+	+	–	–	–	–	–	–	–	–	+	–	–	–	–
Aesculin	–	3	+	–	+	+	+	–	–	–	+	–	+	–	–	–	–	–	+
Haemolysis of bovine erythrocytes	+	94	+	+	–	–	–	–	–	–	–	–	+	+	+	+	–	+	+
Luminescence	–	–	–	–	–	–	+	–	–	–	–	–	+	–	–	–	+	–	–
Methyl red test	5	+	–	–	+	+	+	–	–	–	–	+	+	+	+	–	+	+	–
Voges-Proskauer	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–	+
Production of:																			
Gas from 0.7% glucose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–
Arginine dihydrolase	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+	+	+	+	–
Lysine decarboxylase	+	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	+
Indole	–	89	–	–	–	–	–	–	–	–	–	+	+	+	–	–	+	+	–
Susceptibility to:																			
Ampicillin	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	+	+	+
Penicillin	+	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	+	–
Sulphamethizol	+	+	+	+	+	+	+	+	+	+	–	+	+	–	+	–	–	+	+
Oxidative production of acid from:																			
0.7% L-Arabinose	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–
0.7% Cellobiose	–	–	–	–	+	+	+	–	–	–	–	–	+	+	–	–	+	+	–
0.7% Dextrin	+	+	–	–	+	+	+	–	–	–	+	+	+	+	+	–	+	+	–
0.7% Glycerol	–	94	+	–	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–
0.7% Lactose	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
0.7% D-Mannitol	–	34	–	+	–	–	+	+	+	+	+	+	+	+	–	+	+	+	+
0.7% D-Mannose	–	+	+	–	+	+	+	+	+	+	+	+	+	–	+	+	+	+	–
0.7% Melibiose	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–	+	–
0.7% Raffinose	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–
0.7% L-Rhamnose	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–

Table 2 (cont.)

Character	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
0.7 % Salicin	—	—	—	—	—	+	—	—	—	—	+	—	—	—	—	—	—	—	+
0.7 % D-Sorbitol	—	6	—	—	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—
0.7 % Sucrose	—	83	—	+	+	+	—	—	—	—	+	+	—	—	—	+	+	+	+
0.7 % Trehalose	—	+	—	—	+	+	+	+	+	+	+	+	+	+	—	+	+	+	—
Growth in NaCl:																			
0.5 %	—	97	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5 %	—	97	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7 %	—	60	—	+	—	—	—	—	—	—	+	+	—	+	—	+	+	+	+
Growth at:																			
4 °C	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—	—	+	—	—
10 °C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
22 °C	+	91	+	+	+	+	+	—	—	—	+	+	+	+	+	+	+	+	+
25 °C	+	83	—	+	+	+	—	—	—	—	+	+	+	+	+	+	+	+	+
30 °C	—	—	—	+	—	+	—	—	—	—	+	+	+	+	+	+	+	+	+
API ZYM tests:																			
Butyrate esterase	+	83	+	+	+	+	+	+	—	+	+	+	+	+	+	+	+	+	—
Myristate lipase	+	3	—	—	—	—	—	—	—	—	—	—	+	—	—	—	+	—	—
Leucine arylamidase	+	+	+	+	+	+	+	—	—	—	—	+	+	+	+	+	+	+	+
Valine arylamidase	5	86	—	—	—	—	—	—	—	—	—	—	—	+	—	+	+	+	—
Cystine arylamidase	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	—	+	—
Trypsinase	—	—	—	—	—	—	—	—	—	—	—	+	+	+	—	—	—	—	—
Acid phosphatase	+	69	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—	+
Naphthol phosphohydrolase	+	97	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
α-Galactosidase	—	—	—	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—
β-Galactosidase	—	—	—	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—
β-Glucuronidase	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Acetyl β-glucosaminidase	—	77	—	+	—	—	—	—	—	—	—	—	+	—	+	+	+	+	+

in 50 % formamide, 5 × SSC, 0.1 % *N*-lauroylsarcosine, 0.02 % SDS and 2 % blocking reagent. The membranes were then washed in 5 × SSC and 0.1 % SDS at 65 °C. Luminescence was detected after exposure of X-ray films (Kodak XRP) to the membranes for 15 min–12 h. The membranes were washed in distilled water and the labelled DNA was eluted from the filters in 0.2 M sodium hydroxide and 0.1 % SDS at 37 °C.

Hybridization by the hydroxyapatite method. Strains were grown on BHIA plates with a final NaCl content of 2 %. Lysis of cells with subsequent purification of DNA and DNA hybridization assays using hydroxyapatite were carried out as described previously (Brenner *et al.*, 1982). DNAs from *Vibrio* sp. 1 strain 88/478^T and *Vibrio* sp. 2 strain 88/441^T were labelled with ³²P by the nick-translation method using a commercial system (Life Technologies).

Determination of G + C content. The G + C content (mol %) was determined for DNAs from *Vibrio* sp. 1 strain 88/478^T and *Vibrio* sp. 2 strain 88/441^T by the thermal denaturation method (Mandel *et al.*, 1970).

16S rDNA sequencing. Two primers, V1 [5'-AGAGTTTG-ATCATGGCTCAGA-3'; positions 8–28 (*E. coli* 16S rRNA numbering)] and V3 (5'-GGTTACCTTGTTACGACTTC-3'; 1509–1490) were used for amplification of the 16S rRNA gene of *Vibrio* sp. 1 (Phenon 1) 88/478^T and *Vibrio* sp. 2 (Phenon 2) 88/441^T. Total DNA (5 µl) was added to 45 µl of

a mix of PCR reagents containing 1 × PCR buffer (F-511 for Dynazyme DNA polymerase; Finnzymes) with 0.7 units of *Taq* polymerase (F-501L Dynazyme DNA polymerase; Finnzymes), 10 pmol each primer and 200 µM each dNTP (F-560L dNTP mix; Finnzymes). PCR was performed in a thermal cycler and the temperature profile was: denaturation at 95 °C for 60 s, then 30 cycles at 95 °C for 60 s, 60 °C for 30 s and 72 °C for 60 s. The PCR products were purified on a Centricon-100 (Perkin Elmer) column and directly sequenced on a Perkin Elmer ABI Prism 377 automatic sequencer in both directions by using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase (Perkin Elmer). In addition to primers V1 and V3, forward primers V5 (5'-GTCCACAC-TCTACGGGAGGC-3'; 331–351), 538C (5'-GTAGTCC-ACGCCGTAAACG-3'; 799–817) (Pettersson *et al.*, 1996) and V6 (5'-GGGGAYGACGTCAAGTC-3'; 1183–1199), and reverse primers 3 (5'-ACTGCTGCCTCCCGT-3'; 357–343) (Dewhirst *et al.*, 1992), 5 (5'-CTACCAGGGTATCT-AATC-3'; 802–785) (Dewhirst *et al.*, 1992) and V4 (5'-GTTTATCACCGGCAGTCTC-3'; 1171–1153) were used. Primers V1 and V3–V6 were designed in this study. Nucleotide sequences were analysed using the GCG Sequence Analysis Software Package (version 8) from the Genetics Computer Group (Madison, WI, USA).

16S rDNA analysis. The 16S rDNA sequences of the two test strains were aligned using CLUSTAL W software (Thompson

et al., 1994). The phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987). Bootstrap confidence values were obtained with 1000 resamplings.

RESULTS

Physiological and biochemical characterization

All the strains isolated from winter ulcers were facultative anaerobic, Gram-negative rods, which were susceptible to vibriostatic agent, growing only in media supplemented with NaCl. The optimum temperature for growth was below 30 °C. The results from physiological and biochemical characterization are listed in Table 2. Sixty characters were used to calculate the similarity coefficients based on numerical taxonomy, while a further 25 characters were identical for all strains (see legend to Table 2).

Clustering

The isolates were divided into clusters using unweighted average linkage clustering. *Vibrio* sp. 1 and *Vibrio* sp. 2 isolates were divided into two different clusters, Phenon 1 and Phenon 2, respectively (Table 3), no type strains being included in these clusters.

For bacteria clustered in Phenon 1 long cells could be seen after 1 d incubation in liquid media. When exponential-phase cultures were grown on solid media, isolates of Phenon 1 formed grey, translucent, rounded colonies up to 0.5 mm in diameter after 1 d incubation at 15 or 22 °C. The colonies were viscous and adhered to the medium, forming long threads when removed from the agar surface. Haemolysis was only visible as a moderate transparency of the agar. After 2 d incubation the colony diameter was 1–2 mm and β -haemolysis was detected underneath the colonies. All the strains included in Phenon 1 survived freezing. Only three of the isolates in Phenon 1 varied from the HMO, each with regard to one character. *V. marinus* NCMB 1144^T and *V. ordalii* NCMB 2167^T were the type strains most closely related to Phenon 1, differing from the HMO of this cluster with regard to 12 and 14 characters, respectively. Among actual characters, Phenon 1 differed from both *V. marinus* NCMB 1144^T and *V. ordalii* NCMB 2167^T in being sensitive to penicillin, not growing in 5% NaCl, and in producing lysine decarboxylase, myristate lipase and acid from dextrin.

Exponential-phase bacteria clustered in Phenon 2 showed colonies 1–2 mm in diameter after 1 d incubation at 15 or 22 °C, and 2–3 mm after 2 d incubation. The colonies were round and yellow, with an opaque centre and a butyrous consistence. If present, β -haemolysis only occurred when the bacteria were grown on media containing 2% NaCl. A haemolytic zone extending into the surrounding medium was seen after 2 d incubation. For isolates clustered in Phenon 2 only four isolates represented the HMO, while up to seven characters differed from the HMO.

Vibrio pelagius biotype II NCMB 2253 and *V. logei* ATCC 15382 were the reference strains most closely related to Phenon 2. *V. pelagius* biotype II NCMB 2253 consistently differed from Phenon 2 having white colonies and growing at 30 °C and not at 4 °C. All the other characters that differed between the HMO of Phenon 2 and *V. pelagius* biotype II NCMB 2253 varied in the Phenon 2 isolates. The single isolate of Phenon 2 that was most closely related to *V. pelagius* biotype II NCMB 2253 differed from this strain in nine characters, while the single isolate of Phenon 2 that was most closely related to *V. logei* ATCC 15382 differed from this strain in 10 characters, including its ability to hydrolyse starch and gelatin, and its inability to produce acid from cellobiose and lactose. Other tests that differed showed variable results within the Phenon 2 isolates.

Ultrastructural studies

All bacterial cells were pleomorphic, short or long, straight or curved rods. Strains in Phenon 1 had one single polar flagellum. The scanning electron micrograph of strain 88/478^T shows that the flagellum has a diameter that is twice as large as seen in the transmission electron micrograph (Fig. 1a and b). This difference is probably caused by the two separate techniques used for electron microscopy. Preparation of bacterial cells for SEM is more gentle to the cells than preparation for TEM, possibly leaving the sheath surrounding the flagellum intact. Bacteria belonging to Phenon 2 had multiple (often 5–6) polar flagellae. As with *Vibrio* sp. 1 strain 88/478^T, the *Vibrio* sp. 2 strain 88/441^T probably has an intact sheath around the flagella in the scanning electron micrograph while the sheath has disappeared in the transmission electron micrograph (Fig. 1c and d).

Whole-cell protein gel electrophoresis and Western blotting

Based on protein electrophoretic patterns, the strains isolated from winter ulcer could be divided into two groups, equivalent to Phenon 1 and Phenon 2, respectively (Fig. 2a). Variation in the protein electrophoresis patterns was limited both among the *Vibrio* sp. 1 and *Vibrio* sp. 2 strains. The protein patterns for the *Vibrio* sp. 1 strains were most similar to the pattern for *V. marinus* NCMB 1144^T, but differentiated from this strain by several bands. The *Vibrio* sp. 2 strains had patterns very similar to *V. logei* ATCC 15382. *V. pelagius* biotype II NCMB 2253 did not, however, have many protein bands in common with the *Vibrio* sp. 2 strains.

In Western blotting antiserum 1 and antiserum 2 reacted with whole-cell preparations only, not with Proteinase-K-treated cells. Both antisera reacted with all the tested *Vibrio* species (Fig. 2b and c). Antiserum 1 gave the strongest reaction with the *Vibrio* sp. 1 strains and *V. marinus* (Fig. 2b). The reactions for the

Fig. 1. (a) Scanning electron micrograph of *Vibrio* sp. 1 strain 88/478^T (Phenon 1) and (b) transmission electron micrograph of negatively stained bacterial cells of *Vibrio* sp. 1 strain 88/478^T. (c) Scanning electron micrograph of *Vibrio* sp. 2 strain 88/441^T (Phenon 2) and (d) transmission electron micrograph of negatively stained bacterial cells of *Vibrio* sp. 2 strain 88/441^T. Bar, 1 µm (all micrographs are at the same magnification). The arrow in (b) indicates what may be remnants of the sheath surrounding the flagellum in (a).

Vibrio sp. 1 strains were identical. Electrophoretic mobility of the bands differentiated between the *Vibrio* sp. 1 strains and *V. marinus*. Antiserum 2 gave the strongest reaction with the *Vibrio* sp. 2 strains, *V. ordalii* NCMB 2167^T, *V. anguillarum* ATCC 14181, *V. logei* ATCC 15382, *V. pelagius* II NCMB 2253, *V. salmonicida* NCMB 2262^T and *V. logei* ATCC 29985^T (Fig. 2c). The reactions for the *Vibrio* sp. 2 strains were identical (Fig. 2c), while all the *Vibrio* reference strains showed moderately different profiles.

Substrate gel electrophoresis

Vibrio sp. 1 strains had identical proteinase profiles, but these profiles were different from the *Vibrio* sp. 2 strains (Fig. 3). The profile of *V. ordalii* was similar to the profile of the *Vibrio* sp. 1 strains, with only minor differences in electrophoretic mobility (Fig. 3). The *Vibrio* sp. 2 strains had identical proteinase profiles, these profiles being similar to *V. logei* strains ATCC 15382 and *V. logei* ATCC 29985^T (Fig. 3).

Fig. 2. SDS-PAGE of whole-cell preparations of *Vibrio* sp. 1 (Phenon 1), *Vibrio* sp. 2 (Phenon 2) and different *Vibrio* type and reference strains. (a) Coomassie-brilliant-blue-stained gels. (b) Western blot developed with antiserum raised against *Vibrio* sp. 1 (Phenon 1) 88/478^T. (c) Western blot developed with antiserum raised against *Vibrio* sp. 2 (Phenon 2) 88/441^T. Lanes: A, *V. ordalii* NCMB 2167^T; B, *V. anguillarum* ATCC 14181; C, *V. marinus* NCMB 1144^T; D, *Vibrio* sp. 1 (Phenon 1) 88/478^T; E, *Vibrio* sp. 1 (Phenon 1) 90/4271-1b; F, *Vibrio* sp. 2 (Phenon 2) 90/325-1; G, *Vibrio* sp. 2 (Phenon 2) 90/685-2; H, *V. logei* ATCC 15382; I, *V. pelagius* II NCMB 2253; J, *V. salmonicida* NCMB 2262^T; K, *V. logei* ATCC 29985^T; L, *V. splendidus* I NCMB 1^T; M, *V. splendidus* II NCMB 2251; N, *V. pelagius* I; NCMB 1900^T; O, *V. orientalis* NCMB 2195^T.

Plasmid profiles

The results of plasmid profiling presented in Tables 4 and 5 are based on repeated plasmid isolations with the three different plasmid isolation methods. The plasmid profiles of Phenon 1 isolates showed relatively limited variation, but no plasmids were common to all the isolates (Table 4). All Phenon 1 isolates harboured four or more plasmids of various sizes. Except for one plasmid, 3.9 MDa, which was missing in two of three

isolates from the same origin (Table 4, fish farm c), isolates from the same farm had identical plasmid profiles.

Preparation of plasmid DNA by the alkaline lysis method (Birnboim & Doly, 1979), produced more plasmid bands than the other methods employed; however, similar results were obtained. The additional plasmid bands observed when the alkaline lysis method was used were open circular plasmids and

Fig. 3. Substrate gel electrophoresis of culture supernatants from *Vibrio* sp. 1 (Phenon 1), *Vibrio* sp. 2 (Phenon 2) and different *Vibrio* type and reference strains. Gelatin was copolymerized in the gel. Lanes: A, *V. ordalii* NCMB 2167^T; B, *V. anguillarum* ATCC 14181; C, *V. logei* ATCC 15382; D, *Vibrio* sp. 2 (Phenon 2) 90/325-1; E, *Vibrio* sp. 2 (Phenon 2) 90/685-2; F, *Vibrio* sp. 1 (Phenon 1) 88/478^T; G, *Vibrio* sp. 1 (Phenon 1) 90/4271-1b; H, *V. marinus* NCMB 1144^T; I, *V. pelagius* II NCMB 2253; J, *V. salmonicida* NCMB 2262^T; K, *V. logei* ATCC 29985^T; L, *V. splendidus* I NCMB 1^T; M, *V. splendidus* II NCMB 2251; N, *V. pelagius* I, NCMB 1900^T; O, *V. orientalis* NCMB 2195^T.

to some extent linearized plasmids that produced one or two extra bands from the same plasmid on the agarose gel in addition to the supercoiled plasmid band. Following *Hind*III digestion of the plasmid DNA, two large fragments of 9.4 and 5.4 kb were only detected in samples from strains with plasmids of 8.0 and 5.5 MDa.

The plasmid profiles within Phenon 2 were heterogeneous (Table 5). Nine isolates had no plasmids. Another group of nine isolates (the first nine isolates in Table 5) shared varying numbers of small plasmids. None of these isolates had exactly the same plasmid profile.

Colony hybridization

Colony hybridization using multiprimed *Hae*III-digested total DNA probes from representatives of Phenon 1 and Phenon 2, sorted the collection of strains isolated from Atlantic salmon suffering from winter ulcer in two groups. These two groups contain the same strains as Phenon 1 and 2 (Table 6). All Phenon 1 isolates were positive on all the membranes used when hybridized with both Phenon 1 probes. Four of the Phenon 2 isolates showed only a weak colour reaction against the two Phenon 2 probes in both parallel membranes used for each probe, or only showed hybridization on one of the two membranes.

The probes from Phenon 1 showed hybridization with *V. anguillarum* on one of four membranes and weak hybridization with *V. marinus* on two of four membranes, but not with other reference strains (Table 6).

The probes from Phenon 2 showed weak hybridization with all *V. salmonicida* and *V. logei* strains, but not with other reference strains.

Total DNA agarose gel electrophoresis

Electrophoresis of both *Hae*III- and *Hind*III-digested total DNA (Fig. 4a and b, respectively) showed that the three isolates from Phenon 1 were similar, but that the NVI 90/4271-1b isolate was not identical to the other two (Fig. 4a and b; lanes B, C and D). Phenon 1 strains differed from all the other *Vibrio* strains tested. The Phenon 2 isolates showed a relatively low degree of similarity (Fig. 4a and b, lanes I–L) and they differed from both the *V. salmonicida* (lanes G and H) and the *V. logei* isolates (lanes M and N). The two *V. salmonicida* isolates were identical (Fig. 4a and b, lanes G and H). There was no obvious similarity between the rest of the isolates based on the restriction-enzyme-digested total DNA. The two *V. logei* strains, ATCC

Table 4. Plasmid profiles of *V. viscosus* (Phenon 1) isolates

No. of isolates	Origin (fish farms)	Plasmid (MDa)							
10	a,b,c,d,e,f	8.0	5.5	3.9	3.7	3.0	2.2		
2	c	8.0	5.5		3.7	3.0	2.2		
4	g			3.9	3.7	3.0	2.2		
2	h,i	8.0	5.5		3.7	3.3	2.8	2.25	
1	j	8.0	5.5		3.7		2.8	2.4	
1	k	8.0	7.3	6.1	5.5	4.2	3.5	3.1	2.4

Table 5. Plasmid profiles of *V. wodanis* (Phenon 2) isolates

No. of isolates	Origin (fish farms)	Plasmid (MDa)									
1	l	64.0	50.0	39.0	11.2		5.6				1.08
1	l	64.0			11.2				1.6	1.5	1.4
1	m				11.2			3.2			1.2
1	n	64.0			11.2	9.8	4.9			1.4	1.2
1	o	64.0			11.2					1.4	1.08
1	p				11.2					1.4	1.08
1	d	64.0			11.2			3.0	1.5	1.4	1.08
1	k				11.2		4.3	4.05	1.5	1.4	
1	k						4.3	4.05	1.5	1.4	
1	o				11.2		6.5				
1	l						6.3				
1	m					8.7	8.5				
1	d		42.0	28.0							
1	k					18.5					
1	q	77.0	48.0				9.8	5.6			
2	r,s					9.8					
3	r,e			23.0	11.7						
1	r	70.0			15.0		7.0				
1	r				12.5						
3	r,j			30.0							
1	j			30.0				6.3			
9	e,g,j,m,r,t	No plasmids									

15382 and ATCC 29985^T, were dissimilar (Fig. 4a and b, lanes M and N).

Southern blot hybridization

The elution of labelled DNA was easy and efficient with the AMPPD luminescence development (Fig. 5). Certain bands hybridized in all *Vibrio* strains independent of the probe used. However, the intensity of these bands varied according to the probe.

When strain 88/478^T from Phenon 1 was used as probe, all three strains from Phenon 1 showed hybridization that involved the majority of the fragments (Fig. 5a, lanes B, C and D). Although strain 90/4271-1b (Fig. 5a, lane B) showed weaker hybridization signals than the two other Phenon 1 strains, results were similar when 90/4271-1b was used as probe (data not shown). If the time of exposure was prolonged during development with AMPPD and luminescence, hybridization with strain 88/478^T as probe was visible in all bands in 90/4271-1b in contrast to *V. marinus* NCMB 1144^T. The Southern hybridization showed, however, that *V. marinus* was the reference strain most closely related to Phenon 1 isolates (Fig. 5a and b, lanes A, B, C and D), but to a lesser extent than, for instance *V. anguillarum* and *V. ordalii*, which showed similarity with each other (data not shown).

When DNA from 90/4271-1b (Phenon 1) was used as probe, one strong band, a 5.4 kb fragment, was missing in the 90/4271-1b digest compared to the two other strains in Phenon 1 (data not shown). When DNA from 88/478^T was used as probe, strong signals from bands of both 5.4 and 9.4 kb were observed only in the lanes for 88/478^T and 90/1097-1 from Phenon 1 and not in 90/4271-1b (Fig. 5a, lanes B, C and D). Similarly

a 2.0 kb fragment band gave strong signals only in 89/5532 (Phenon 2) when 89/5532 was used as probe (Fig. 5c, lane K).

Phenon 2 isolates also gave signals from the majority of bands when strains from Phenon 2 were used as probes (Fig. 5c, lanes I, L and K). Similar hybridization was also observed in *V. logei* ATCC 15382 (Fig. 5c, lane N), though fewer fragments hybridized in *V. logei* ATCC 29985^T or *V. salmonicida* (Fig. 5c, lanes M, G and H). Using DNA from *V. logei* ATCC 15382 as probe revealed strong hybridization only with homologous DNA, not with Phenon 2 strains (Fig. 5d, lane N).

When the *V. salmonicida* type strain was used as probe, hybridization was obtained with both *V. salmonicida* strains and *V. logei* ATCC 29985^T. *V. logei* ATCC 29985^T likewise hybridized as strongly with the two *V. salmonicida* strains as with the homologous DNA but not with the other *V. logei* strain, ATCC 15382 (data not shown).

The Phenon 2 isolate 90/4271-3a showed weak hybridization with the Phenon 2 target DNA, including homologous DNA and hybridization signals were only observed after prolonged exposure. A summary of the results from Southern hybridization is given in Table 7.

DNA base composition

The G+C content of the DNA of Phenon 1 strain 88/478^T was 42.5 mol%, whereas that of the DNA of Phenon 2 strain 88/441^T was 40.0 mol%. These values lie well within the accepted range of 38–51 mol% for members of the genus *Vibrio* (Baumann & Schubert, 1984).

Table 6. Data from colony hybridization

Digoxigenin-labelled multiprimed *Hae*III-digested total DNA from *V. viscosus* 88/478^T and 90/4271-1b, and from *V. wodanis* 88/441^T and 90/4721-3a were used as probes. +, Hybridization; (+), weak hybridization in both parallel blots; ±, positive and negative on the parallel blots; −, no hybridization.

Target DNA (no. of strains)	Probe DNA			
	<i>V. viscosus</i> (Phenon 1)		<i>V. wodanis</i> (Phenon 2)	
	88/478 ^T	90/4271-1b	88/441 ^T	90/4721-3a
<i>V. viscosus</i> (20)	+	+	−	−
<i>V. wodanis</i> (33)	−	−	+ *	+ *
<i>V. marinus</i> ATCC 15381 ^T (1)	−	(+)	−	−
<i>V. anguillarum</i> ATCC 14181 (1)	−	±	−	−
<i>V. salmonicida</i> ATCC 43839 ^T , NCMB 2245, NVI 90/1667-10c (3)	−	−	(+)	(+)
<i>V. logei</i> ATCC 29985 ^T , ATCC 15382 (2)	−	−	(+)	(+)
Reference strains listed in Table 1 (28)	−	−	−	−
<i>A. salmonicida</i> subsp. <i>salmonicida</i> (1)	−	−	−	−

* Four *V. wodanis* isolates showed weak hybridization or varied between the parallel blots.

Hybridization with the hydroxyapatite method

Labelled DNA from Phenon 1 strain 88/478^T was 81–93 % related (89 % mean relatedness) to unlabelled DNAs from nine other Phenon 1 strains in reactions done at 60 °C (Table 8). Divergence within related nucleotide sequences was 0.0–1.5 %. In reactions done at 75 °C, relatedness was 74–100 % (95 % mean relatedness). Labelled DNA from Phenon 2 strain 88/441^T was 66–94 % related (77 % mean relatedness) to 10 other Phenon 2 strains (Table 8). Divergence within related nucleotide sequences was 0.0–1.5 %. In reactions done at 75 °C, relatedness was 55–97 % (76 % mean relatedness). In reciprocal reactions with labelled DNA from 88/478^T and 88/441^T, relatedness between Phenon 1 and Phenon 2 strains at 60 °C was 5–22 %. Relatedness of Phenon 1 to other *Vibrio* and *Vibrio*-like species was 0–43 %, with *V. marinus* its closest relative. Relatedness of Phenon 2 to other *Vibrio* and *Vibrio*-like species was 2–57 %, with *V. logei* its closest relative. In 75 °C reactions, relatedness of Phenon 2 to *V. logei* was 38 %.

16S rDNA analysis

The results from 16S rDNA analysis are summarized in Fig. 6. The 16S rDNA sequence from *Vibrio* sp. 1 strain 88/478^T was nearly identical to the 16S rDNA sequence from *Moritella* HAR08 and *Moritella* HAR13 (Urakawa *et al.*, 1998). There was only one single base at position 101 (*E. coli* 16S rRNA numbering) that separated the sequence of *Vibrio* sp. 1 strain 88/478^T from the two *Moritella* sequences. *Moritella* SC27 had 2 bp (positions 101 and 472) that separated its 16S rDNA sequence from the sequence of

Vibrio sp. 1 strain 88/478^T. Other *Moritella* strains from the study of Urakawa *et al.* (1998) had four (SC25), five (SC22 and DSK1) and 10 (ODA02) bp that separate them from the sequence of *Vibrio* sp. 1 strain 88/478^T. *V. marinus*, proposed by Urakawa *et al.* (1998) to be transferred to a new genus as *Moritella marina* (see Discussion), has 12 bp that separates it from the *Vibrio* sp. 1 strain 88/478^T sequence (99.3 % of the basepairs are identical). The basepairs in the *Moritella* strains, including *M. marina*, that deviate from the *Vibrio* sp. 1 strain 88/478^T sequence are often common and are located between positions 380 and 476.

The 16S rDNA sequence from *Vibrio* sp. 2 strain 88/441^T was relatively well separated from its closest neighbours with the 16S rDNA sequence from *V. salmonicida* as the most similar with 98.2 % identical bases. *V. logei* ATCC 15382 has 98.1 % identical bases, while *Vibrio fischeri* had 96.0 % in common with *Vibrio* sp. 2 strain 88/441^T.

DISCUSSION

On the basis of phenotypic characterization, colony hybridization, Southern blot hybridization and hydroxyapatite hybridization, it can be concluded that the isolates in the two groups *Vibrio* sp. 1 and *Vibrio* sp. 2 from the study of Lunder *et al.* (1995) represent two new species. Phenotypic characterization showed that the bacteria share properties common to isolates of the genus *Vibrio*, according to the definitions given by Baumann & Schubert (1984). The species names *Vibrio viscosus* sp. nov. and *Vibrio wodanis* sp. nov. are proposed for the *Vibrio* sp. 1 (Phenon 1) and *Vibrio* sp. 2 (Phenon 2) strains, respectively.

Fig. 4. Agarose gel electrophoresis of restriction enzyme digests of total DNA from different *Vibrio* reference strains and isolates from fish with winter ulcer. Ethidium-bromide-stained gels after electrophoresis of (a) *Hae*III-digested and (b) *Hind*III-digested total DNAs. Lanes: X, λ DNA/*Hind*III fragments and Φ X174 RF DNA/*Hae*III fragments; A, *V. marinus* NCMB 1144^T; B, *V. viscosus* (Phenon 1) 90/4271-1b; C, *V. viscosus* (Phenon 1) 90/1097-1; D, *V. viscosus* (Phenon 1) 88/478^T; E, *V. anguillarum* ATCC 14181; F, *V. ordalii* NCMB 2167^T; G, *V. salmonicida* 90/1667-10c; H, *V. salmonicida* NCMB 2262^T; I, *V. wodanis* (Phenon 2) 90/1667-13; J, *V. wodanis* (Phenon 2) 90/4271-3a; K, *V. wodanis* (Phenon 2) 89/5532; L, *V. wodanis* (Phenon 2) 88/441^T; M, *V. logei* ATCC 29985^T; N, *V. logei* ATCC 15382.

Numerical taxonomy based on physiological and biochemical characters has often been applied for differentiation of members of the *Vibrionaceae*, and simple matching (S_{SM}) and clustering using unweighted average linkage give the best result (Bryant *et al.*, 1986). In this study clustering was performed using a program based on a given number of clusters. Whole-cell protein electrophoresis patterns are valuable when comparing bacterial isolates (Jackman, 1987). In this study antisera reacted only with untreated whole-cell preparations, not with Proteinase-K-digested samples. This finding indicates that the antibodies react with

protein epitopes of the cells. Other workers have shown that while substrate gel electrophoresis with gelatin as substrate is able to separate some *Vibrio* species, it is unable to differentiate between closely related species such as *V. anguillarum* and *V. ordalii* (Nottage & Birkbeck, 1987). In this investigation, several different proteinase profiles were detected, indicating that the method is useful for comparison of isolates.

Physiological and biochemical characterization of the *V. viscosus* strains revealed little variability, supporting the theory that these strains represent one species. This was in agreement with the results from whole-cell protein electrophoresis, Western blotting and substrate gel electrophoresis, in which the strains were also very similar. The fact that *V. viscosus* strains had a restricted salt tolerance range not growing in 0.5 or 5% NaCl might explain why infection with these bacteria is limited to fish kept in sea water. Whole-cell protein electrophoresis confirmed that *V. marinus* was the species most closely resembling *V. viscosus*. In gelatin gel electrophoresis *V. marinus* did not show proteolytic activity, despite being gelatinolytic in the biochemical characterization. This might have been due to the specific conditions used in this assay. *V. ordalii* showed a protease profile quite similar to *V. viscosus* strains.

V. wodanis strains showed more variation when characterized biochemically, though results from whole-cell protein electrophoresis, Western blotting and substrate gel electrophoresis indicated that these strains are homologous and also comprise one single species. The *V. wodanis* strains in this investigation proved to be relatively closely related to the ATCC 15382 strain of *V. logei* and the *V. pelagius* biotype II strain NCMB 2253, isolated from sea water (Baumann *et al.*, 1971; P. Baumann *et al.*, 1980).

The limited variation among *V. viscosus* strains compared to the *V. wodanis* strains might indicate that *V. viscosus* is clinically more important and adapted to the host. The important fish pathogen *A. salmonicida* subsp. *salmonicida* also shows limited variation in phenotypical features (McCarthy & Roberts, 1980). Challenge experiments indicate that the *V. viscosus* strains induce winter ulcer, while *V. wodanis* does not (Lunder *et al.*, 1995). *V. viscosus* strains are able to produce several extracellular products that constitute possible virulence factors, being haemolytic, lecithinolytic, caseinolytic and gelatinolytic. *V. wodanis* strains also produced potential virulence factors, being haemolytic and hydrolysing gelatin, amylase and lipase. Eight of the *V. wodanis* strains produced alginase, a property differentiating the usually non-pathogenic *Vibrio splendidus* (*V. anguillarum*-like bacteria) from the fish-pathogenic *V. anguillarum* (Myhr *et al.*, 1991). *V. viscosus* and *V. wodanis* are both able to grow at low temperatures and they are isolated from fish during a period with low water temperatures (Lunder *et al.*, 1995).

Colony hybridization was convenient for the selection of reference strains for further studies. The interpret-

Fig. 5. Demonstration of Southern blots of total DNAs from different *Vibrio* reference strains and isolates from fish with winter ulcer hybridized with the following total DNA probes: (a) *V. viscosus* 88/478^T (Phenon 1); (b) *V. marinus* NCMB 1144^T; (c) *V. wodanis* 89/5532 (Phenon 2); (d) *V. logei* ATCC 15382. The membranes were hybridized with digoxigenin-labelled probes and developed using AMPPD and luminescence. The target and probe DNAs were digested with *Hind*III. Lanes: X, λ DNA/*Hind*III fragments and Φ X174 RF DNA/*Hae*III fragments; A, *V. marinus* NCMB 1144^T; B, *V. viscosus* (Phenon 1) 90/4271-1b; C, *V. viscosus* (Phenon 1) 90/1097-1; D, *V. viscosus* (Phenon 1) 88/478^T; E, *V. anguillarum* ATCC 14181; F, *V. ordalii* NCMB 2167^T; G, *V. salmonicida* 90/1667-10c; H, *V. salmonicida* NCMB 2262^T; I, *V. wodanis* (Phenon 2) 90/1667-13; J, *V. wodanis* (Phenon 2) 90/4271-3a; K, *V. wodanis* (Phenon 2) 89/5532; L, *V. wodanis* (Phenon 2) 88/441^T; M, *V. logei* ATCC 29985^T; N, *V. logei* ATCC 15382. The arrowheads indicate the lanes containing target DNA of the same strain as the probe DNA.

ation of colony hybridization was relatively simple and the negative results that occurred with *V. wodanis* probes on some *V. wodanis* strains were most probably due to technical problems, i.e. colonies loosening from the membranes during the procedure. Occasional hybridization across the species level observed between *V. anguillarum* and *V. viscosus*, and between *V. wodanis*, *V. salmonicida* and *V. logei* could probably have been avoided by more stringent washing. The intention of this assay was, however, to reveal reference strains closely related to Phenon 1 and Phenon 2 strains.

Gel electrophoresis of restriction-endonuclease-digested total DNA confirmed the limited variation between the *V. viscosus* strains revealed by phenotypic characterization. *V. wodanis* strains showed, however, large variation in the length of restriction enzyme fragments between the isolates.

The relationship between the strains was elucidated in more detail in Southern hybridization than with colony hybridization. Hybridization was expressed as luminescence from AMPPD. The intensity was, however, sensitive to the amount of target DNA of each strain. Exposure of ethidium-bromide-stained agarose gels to UV light revealed the amount of DNA in each lane.

When DNA from strain 90/4271-1b (*V. viscosus*) was used as probe, the homologous DNA showed less hybridization than the two other *V. viscosus* strains because the amount of DNA in the agarose gel was relatively small for this strain. The amount of DNA for strain 90/4271-3a (*V. wodanis*) was relatively even less, the hybridization being visible only after prolonged development. The other *V. wodanis* strains showed strong hybridization signals using 90/4271-3a (*V. wodanis*) as probe. The hybridization signals were obtained from the majority of bands in *V. wodanis*

Table 7. Data from Southern hybridization using total DNA as probe and target

+, Hybridization in most fragments; (+), hybridization restricted to a majority of the fragments; (–), hybridization in a minority of the fragments; –, no hybridization or limited to a few fragments.

Target DNA	Probe DNA												
	1	3	4	5	7	8	9	10	11	13	14	15	21
1. <i>V. viscosus</i> 88/478 ^T	+	+	–	–	–	–	–	–	–	(–)	–	–	–
2. <i>V. viscosus</i> 90/1097-1	+	+	–	–	–	–	–	–	–	(–)	–	–	–
3. <i>V. viscosus</i> 90/4271-1b	+	+	–	–	–	–	–	–	–	–	–	–	–
4. <i>V. wodanis</i> 88/441 ^T	–	–	+	+	+	+	(–)	(–)	–	–	–	–	–
5. <i>V. wodanis</i> 89/5532	–	–	(+)	+	+	+	(–)	–	–	–	–	–	–
6. <i>V. wodanis</i> 90/325-1	–	–	–	–	+	–	–	–	–	–	–	–	–
7. <i>V. wodanis</i> 90/4271-3a*	–	–	(–)	(+)	(+)	(+)	–	–	–	–	–	–	–
8. <i>V. wodanis</i> 90/1667-13	–	–	(+)	+	+	+	(–)	–	–	–	–	–	–
9. <i>V. logei</i> ATCC 15382	–	–	(–)	(+)	(+)	(+)	+	–	–	–	–	–	–
10. <i>V. logei</i> ATCC 29985 ^T	–	–	(–)	(–)	(–)	(–)	(–)	+	(+)	–	–	–	–
11. <i>V. salmonicida</i> NCMB 2262 ^T	–	–	(–)	(–)	(–)	(–)	(–)	+	+	–	–	–	–
12. <i>V. salmonicida</i> 90/1667-10c	–	–	(–)	(–)	–	(–)	(–)	+	+	–	–	–	–
13. <i>V. marinus</i> NCMB 1144 ^T	(–)	(–)	–	–	–	–	–	–	–	+	–	–	–
14. <i>V. anguillarum</i> ATCC 14181	–	–	–	–	–	–	–	–	–	–	+	(+)	–
15. <i>V. ordalii</i> NCMB 2167 ^T	–	–	–	–	–	–	–	–	–	–	(+)	+	–
16. <i>V. orientalis</i> NCMB 2195 ^T	–	–	–	–	–	–	–	–	–	–	–	–	–
17. <i>V. damsela</i> NCMB 2184 ^T	–	–	–	–	–	–	–	–	–	–	–	–	–
18. <i>V. vulnificus</i> ATCC 33147	–	–	–	–	–	–	–	–	–	–	–	–	–
19. <i>V. aestuarianus</i> NCMB 2236 ^T	–	–	–	–	–	–	–	–	–	–	–	–	–
20. <i>V. splendidus</i> II NCMB 2251	–	–	–	–	–	–	–	–	–	–	–	–	–
21. <i>V. pelagius</i> II NCMB 2253	–	–	–	–	–	–	–	–	–	–	–	–	+
22. <i>V. pelagius</i> I NCMB 1900 ^T	–	–	–	–	–	–	–	–	–	–	–	–	–
23. <i>Y. ruckeri</i> ATCC 29473 ^T	–	–	–	–	–	–	–	–	–	–	–	–	–
24. <i>A. salmonicida</i> ATCC 14174	–	–	–	–	–	–	–	–	–	–	–	–	–

* The amount of target DNA was relatively low.

isolates despite the fact that the RFLP patterns between the same isolates showed large variation, indicating that these strains have common sequences, but have been subject to genetic divergence.

The definition of a genetic species (genomospecies) is a group of strains whose DNAs are 70 % or more related under optimal reassociation conditions and in which the related DNA sequences have diverged 5 % or less (Wayne *et al.*, 1987). A genomospecies can be easily differentiated from all other species in its genus. All of the *V. viscosus* (Phenon 1) strains were in a closely related hybridization group, conforming to this definition. They were easily differentiated from the type strains of all of the described *Vibrio* species as well as from a number of *Vibrio*-like species that have not been validly described (non-validly described species are put in quotation marks in Table 8). One of the *V. wodanis* strains (90/685) was just less than 70 % related to the labelled *V. wodanis* strain, although divergence of its related sequences was well under 5 %. This strain is maintained in *V. wodanis* because it fits all other criteria of the species description and because there are several strains within *V. wodanis* whose relatedness to the type strain is just over 72 %. *V. wodanis* is easily

differentiated from all *Vibrio* and *Vibrio*-like species on the basis of DNA relatedness (Table 8).

The polynucleotide sequence relationship between *V. ordalii* and *V. anguillarum* strains has been estimated by Schiewe *et al.* (1981) to be 53–69 %. In the present investigation *V. anguillarum* was most closely related to *V. ordalii*. The difference between these strains might serve as a reference level for differentiation between species in Southern blot hybridization. There was a higher level of hybridization between *V. salmonicida* and *V. logei* strain ATCC 29985^T, than between *V. anguillarum* and *V. ordalii*. This might suggest that *V. salmonicida* and *V. logei* should be amalgamated into one species. This is in accordance with data from other workers (J. J. Farmer, personal communication).

V. logei ATCC 15382, however, did not strongly hybridize with either *V. logei* ATCC 29985^T or *V. salmonicida*. This strain is more closely related to *V. wodanis* based on hybridization with various *V. wodanis* probes. When *V. logei* ATCC 15382 was used as probe, however, hybridization with *V. wodanis* was very limited. The hydroxyapatite hybridization shows conclusively that *V. logei* and *V. wodanis* are separate

Table 8. DNA relatedness of *Vibrio* strains to Phenon 1 strain 88/478^T and Phenon 2 strain 88/441^T

³²P-labelled DNA was reacted with unlabelled DNA from the same strain (homologous reaction) and from a series of other strains (heterologous reactions). Each reaction was done at least twice. The mean association value in homologous reactions was 64% before normalization. Control reactions, in which labelled DNA was incubated in the absence of unlabelled DNA, showed 1.7–4.9% binding to hydroxyapatite. These control values were subtracted before normalization.

Source of unlabelled DNA*	Source of labelled DNA†					
	Phenon 1 88/478 ^T			Phenon 2 88/441 ^T		
	RBR at 60 °C	D	RBR at 75 °C	RBR at 60 °C	D	RBR at 75 °C
Phenon 1						
88/478 ^T	100	0.0	100	22		
89/1407-1b	93	1.5	100	17		
89/1902	90	1.0	100	9		
90/793	86	1.0	99			
90/1027-3b	93	1.0	94			
90/1097-1	86	0.5	97			
90/1162-6	90	0.0	98	12		
90/1667-12	92	0.5	94			
90/4271-1b	91	1.5	88			
90/4271-5b	81	1.5	74			
Phenon 2						
88/441 ^T	16			100	0.0	100
90/325	15			88	0.5	97
90/325-2	11			94	0.5	95
90/325-3	5			72	0.5	59
90/551-2	14			77	0.0	77
90/685	5			66	1.5	55
90/765	10			72	1.0	76
90/667-13	8			77	0.5	72
90/2024-7	5			83	0.5	79
90/2024-8	12			72	1.0	81
90/4271-3a	9			72	1.0	68
' <i>V. abalonicus</i> ' ATCC 27390	6			18		
' <i>V. adaptus</i> ' ATCC 19263	4			14‡		
<i>V. aestuarianus</i> ATCC 35048 ^T (NCMB 2236 ^T)	12			14		
<i>V. alginolyticus</i> ATCC 17749 ^T (NCMB 1903 ^T)	6			13		
' <i>V. algosus</i> ' ATCC 14390	5			15‡		
<i>V. anguillarum</i> ATCC 19264 ^T	10			14		
<i>V. campbellii</i> ATCC 25920 ^T	6			11		
<i>V. carchariae</i> ATCC 35084 ^T	7			11		
<i>V. cholerae</i> ATCC 14035 ^T	5			9		
<i>V. cincinnatiensis</i> ATCC 35912 ^T	4			12‡		
<i>V. costicola</i> ATCC 33508 ^T	4			8		
' <i>V. cuneatus</i> ' ATCC 6972	1			3‡		
' <i>V. cyclosites</i> ' ATCC 14635	0			2‡		
<i>V. damsela</i> ATCC 33539 ^T (NCMB 2184 ^T)	7			12		
<i>V. diazotrophicus</i> ATCC 33466 ^T (NCMB 2169 ^T)	8			12		
<i>V. fischeri</i> ATCC 7744 ^T (NCMB 1281 ^T)	8			21		
<i>V. fluvialis</i> ATCC 33809 ^T (NCMB 2249 ^T)	6			10		
<i>V. furnissii</i> ATCC 35016 ^T	6			9		
<i>V. gazogenes</i> ATCC 29988 ^T	6			9		
<i>V. harveyi</i> ATCC 14126 ^T	5			11		
<i>V. hollisae</i> ATCC 33564 ^T	5			12		
' <i>V. hyperoptica</i> ' CDC 9100-79	3			3		

Table 8 (cont.)

Source of unlabelled DNA*	Source of labelled DNA†					
	Phenon 1 88/478 ^T			Phenon 2 88/441 ^T		
	RBR at 60 °C	D	RBR at 75 °C	RBR at 60 °C	D	RBR at 75 °C
<i>V. ichthyenteri</i> ATCC 700023 ^T (IFO 15847 ^T)	6			18		
<i>V. iliopiscarius</i> ATCC 51760 ^T	10			23		
<i>V. logei</i> ATCC 29985 ^T	14			57‡		38‡
' <i>V. marinus</i> ' ATCC 14395	5			8‡		
<i>V. marinus</i> ATCC 15381 ^T (NCMB 1144 ^T)	43			9		
<i>V. mediterranei</i> ATCC 43341 ^T	5			19‡		
<i>V. metschnikovii</i> NCTC 8443 ^T (ATCC 7708 ^T)	5			6		
<i>V. mimicus</i> ATCC 33653 ^T	4			15‡		
<i>V. mytili</i> CECT 632 ^T	4			14‡		
<i>V. natriegens</i> ATCC 14048 ^T	7			14		
<i>V. navarrensis</i> CIP 103381 ^T	3			7‡		
' <i>V. neocistes</i> ' ATCC 14636	0			2‡		
' <i>V. neptuna</i> ' CDC 9102-79	5			15‡		
<i>V. nereis</i> ATCC 25917 ^T (NCMB 1897 ^T)	13			16		
<i>V. nigripulchritudo</i> ATCC 27043 ^T	5			8		
<i>V. ordalii</i> ATCC 33509 ^T (NCMB 2167 ^T)	4			4		
<i>V. orientalis</i> ATCC 33934 ^T (NCMB 2195 ^T)	6			10		
<i>V. parahaemolyticus</i> ATCC 17802 ^T	6			16‡		
<i>V. pelagius</i> ATCC 25916 ^T (NCMB 1900 ^T)	7			17‡		
<i>V. penaeicida</i> ATCC 51842	5			16		
<i>V. proteolyticus</i> ATCC 15338 ^T (NCMB 1326 ^T)	4			9		
' <i>V. psychroerythrus</i> ' ATCC 27364	5			6‡		
<i>V. salmonicida</i> ATCC 43839 ^T (NCMB 2262 ^T)	8			27		
<i>V. scophthalmi</i> CECT 4638 ^T	4			20		
<i>V. splendidus</i> ATCC 33125 ^T (NCMB 1 ^T)	5			13		
<i>V. tubiashii</i> ATCC 19109 ^T (NCMB 1340 ^T)	3			10		
' <i>V. tyrosinaticus</i> ' ATCC 19378	4			6‡		
<i>V. vulnificus</i> ATCC 27562 ^T	2			6		

* Strains obtained from two different sources have the second source in parentheses.

† RBR, relative binding ratio, expressed as a percentage; D, level of divergence, expressed as a percentage. A blank space indicates that the reaction was not done.

‡ These strains were tested against labelled DNA from Phenon 2 strain 90/551-2.

species. Phenotypically, *V. wodanis* and *V. logei* ATCC 15382 may be differentiated by degradation of starch and gelatin and by oxidative production of acid from cellobiose and lactose. The large number of bands hybridizing across the species level between the different vibrios included in the Southern hybridizations in this study indicates, however, that these strains have a genetic relationship within the genus level.

Southern hybridization of the *V. viscosus* isolates tested showed that *V. marinus* is the *Vibrio* species that is most closely related to *V. viscosus*. The cross-hybridization was, however, limited.

The DNA–DNA hybridization results show that *V. viscosus* and *V. marinus* are only 43 % related at the

total DNA level. In addition, phenotypical tests such as lysine decarboxylase production, degradation of casein and growth at 25 °C separates the two species. However, comparison of the 16S rDNA sequence of *V. viscosus* 88/478^T and *V. wodanis* 88/441^T with those of other *Vibrio* species showed that *V. viscosus* is close to *V. marinus* (12 bp difference) and very close to two other strains (1 bp difference) proposed to be members of a new genus *Moritella* (Urakawa *et al.*, 1998) containing also the former *V. marinus* as the type species (*M. marina*). Because this nomenclature was not yet accepted during the preparation of this manuscript, we prefer to continue to use the objective synonym *Vibrio marinus* and to create two new *Vibrio* species.

The species that should be included in the genus

Fig. 6. Phylogenetic tree showing the relationship between *Vibrio* sp. 1 strain 88/478^T (*V. viscosus*) and *Vibrio* sp. 2 strain 88/441^T (*V. wodanis*) and related organisms based on complete 16S rDNA sequences. The scale bar represents 1 nt substitution per 100 nt. Bootstrap confidence values obtained from the neighbour-joining tree are given at the branch points. The reference organisms used for analysis of the 16S rDNAs and their accession numbers are given in the tree.

Photobacterium present a problem that still has not been satisfactorily resolved (L. Baumann *et al.*, 1980; Baumann *et al.*, 1983; Gauthier *et al.*, 1995; MacDonell & Colwell, 1985; Reichelt *et al.*, 1976; Smith *et al.*, 1991). There is agreement that the genus contains *Photobacterium phosphoreum*, *Photobacterium angustum* and *Photobacterium leiognathi*. Some workers believe that species formerly in the genus *Vibrio*, *V. fischeri*, *V. damsela* and *V. logei* should be contained within the genus *Photobacterium*. Previous DNA relatedness studies indicated that the 'traditional' *Photobacterium* species are no more related to *V. fischeri*, than to any other *Vibrio* species (L. Baumann *et al.*, 1980; Baumann *et al.*, 1983; Reichelt *et al.*, 1976). The nearest relative of Phenon 1 is *V. marinus*. The nearest relative of Phenon 2 is *V. logei*, which some consider to be a *Photobacterium*. We have placed both of these two new species in the genus *Vibrio* without prejudice, pending a final disposition of the status of the genus *Photobacterium*.

V. viscosus strains proved to be very similar genotypically, no matter which method was used. All *V. viscosus* isolates showed several plasmids and similar plasmid profiles were found among isolates from different fish farms, and indeed within the same fish farm. Investigations of *Hind*III-digested plasmid DNA confirmed that plasmids of similar size also had common restriction fragment lengths. The 3.9 MDa plasmid that was lacking in two out of three strains

from the same fish farm, might indicate that this plasmid represents a conformational variant of another plasmid, i.e. the 3.7 MDa plasmid.

The plasmid profile of the isolate from plaice was identical to the plasmid profile found in an isolate from Atlantic salmon. These two fish were caught at locations distant to each other. The limited variation in plasmid profiles of *V. viscosus* is parallel to the variation seen in the psychrophilic fish pathogen *V. salmonicida* (Sørum *et al.*, 1990).

The single 5.4 kb band, or the two strong bands at 5.4 and 9.4 kb, depending on which probe was used, that differentiated between 90/4271-1b and the two other strains in *V. viscosus*, have molecular masses identical to fragments obtained from *Hind*III digestion of plasmids in 88/478^T and 90/1097-1. This indicates that both bands might represent fragments of plasmids which were present in multiple copies in 88/478^T and 90/1097-1, but not in 90/4271-1b. The limited variation between the *V. viscosus* isolates observed in the RFLP profiles seems to be in accordance with Southern hybridization data and plasmid profiling.

Within *V. wodanis* isolates, strong signals were detected from a 2.0 kb fragment in 89/5532 using DNA from this strain as probe, but not when using DNA from 88/441^T, 90/1667-13a or 90/4271-3a as probes. This fragment also corresponds to a fragment obtained by *Hind*III digestion of 89/5532 plasmids.

The explanation for the limited genetic divergence among isolates of the fish pathogen *A. salmonicida* subsp. *salmonicida* isolates has been suggested to be recent dispersion of the organism in fish farming or minimal evolutionary divergence (McCarthy & Roberts, 1980). Such limited genetic variation might also be an expression of the great success of *A. salmonicida* subsp. *salmonicida* as a fish pathogen and its close adaptation to the host. Similarly, the phenotypically and genetically homologous *V. viscosus* has proven to be able to induce disease in Atlantic salmon, in contrast to the more genetically variable *V. wodanis* (Lunder *et al.*, 1995).

However, one of the *V. viscosus* isolates originates from a plaice with skin ulcer, suggesting that these bacteria may cause skin lesions among marine fish in general. The plaice isolate (89/2148) was phenotypically and genotypically very similar to the Atlantic salmon isolates, indicating that *V. viscosus* is not restricted to causing disease in Atlantic salmon only. Similarly, the homogeneous *V. salmonicida* also causes disease in cod (*Gadus morhua*) in addition to its main salmonid host, Atlantic salmon (Sørum *et al.*, 1990).

The genetic variation between the *V. wodanis* isolates was observed in plasmid profiling, total DNA restriction enzyme fragment gel electrophoresis and Southern blotting. Nevertheless, the overall hybridization results, including the results from hydroxyapatite hybridization and phylogenetic analysis, support the view that the Phenon 2 strains comprise one species. The phenotypic characterization of these isolates also showed larger variation than found in strains of *V. viscosus*. However, several isolates from different fish farms possessed small plasmids in common, although none of these isolates had identical plasmid profiles. The two different isolates (90/4271-3a and 90/551-1) used in inoculation experiments (Lunder *et al.*, 1995) did not have plasmids. The fact that *V. wodanis* and not *V. viscosus* were isolated in fish from two farms with winter ulcer, suggests that *V. wodanis* may participate as a secondary invader in the development of ulcers in salmon even without contribution from *V. viscosus* (Lunder *et al.*, 1995).

Description of *Vibrio viscosus* sp. nov.

Vibrio viscosus (vis.co'sus. L. masc. adj. *viscosus* viscous, because of its thread-forming, adherent colonies).

Conforms to the description of the family *Vibrionaceae* and of the genus *Vibrio* (Baumann & Schubert, 1984). Cells are Gram-negative, short or elongated, non-spore-forming rods that are motile by a single polar flagellum when grown on solid or in liquid media. Colonies on bovine blood agar containing 2% NaCl after 48 h incubation at 15 °C are round, translucent, grey, haemolytic, non-swarming and non-luminescent. Colonies are viscous and can form long threads when removed from the agar surface. Oxidase- and catalase-

positive. Produce acid, but not gas aerobically and anaerobically from D-glucose. Growth occurs at 4–25 °C, but not at 30 °C. NaCl is required for growth, at 1–4%. Negative reactions in tests for indole production and Voges–Proskauer. A majority of the strains (95%) have a negative methyl red reaction. Lysine is decarboxylated, but arginine dihydrolase and ornithine decarboxylase tests are negative. Gelatin, casein, DNA, Tween 80, urea and lecithin are hydrolysed. A majority of the strains hydrolyse starch. Alginate and aesculin are not hydrolysed. Acid is produced from dextrin and D-galactose, but not from L-arabinose, cellobiose, glycerol, inositol, lactose, D-mannitol, D-mannose, melibiose, raffinose, L-rhamnose, salicin, D-sorbitol, sucrose, trehalose nor D-xylose. Susceptible to 2,4-diamino-6,7-diisopropylpteridine (O/129 vibriostatic agent), penicillin, ampicillin, sulphamethizol, oxytetracycline and oxolinic acid. The enzymes alkaline phosphatase, acid phosphatase, butyrate esterase, caprylate esterase, myristate lipase, leucine arylamidase and naphthol phosphohydrolase are produced. A minority of the strains (5%) produce valine arylamidase. Chymotrypsinase, trypsinase, cystine arylamidase, α -fucosidase, α -galactosidase, β -galactosidase, β -glucoronidase, acetyl β -glucosaminidase, α -glucosidase, β -glucosidase and α -mannosidase are not produced. Isolated from and pathogenic for Atlantic salmon, plaice and perhaps other marine fish, causing an ulcerative skin disease called winter ulcer.

Description of the type strain of *Vibrio viscosus* sp. nov.

The type strain of *V. viscosus* is NVI 88/478^T, isolated in 1988 in Norway from an Atlantic salmon with winter ulcer. The characteristics of the type strain are identical to those given in the species description. The G+C content is 42.5 mol%. This strain has been deposited at the National Collection of Industrial and Marine Bacteria (Aberdeen, UK) as NCIMB 13584^T.

Description of *Vibrio wodanis* sp. nov.

Vibrio wodanis (wo.da'nis. M.L. gen. n. *wodanis* from Wodan, the Norse god of art, culture, war and the dead, because its closest relative, *Vibrio logei*, also a cold water vibrio, takes its name from another Norse God, Loge).

Conforms to the description of the family *Vibrionaceae* and of the genus *Vibrio* (Baumann & Schubert, 1984). Cells are Gram-negative, short or elongated, non-spore-forming rods that are motile by means of multiple polar flagella when grown on solid or in liquid media. Colonies on bovine blood agar containing 2% NaCl after 48 h incubation at 15 °C are round, opaque, yellow, non-swarming and non-luminescent. Oxidase- and catalase-positive. Produce acid, but not gas from D-glucose aerobically and anaerobically. Growth occurs at 4–25 °C, but not at 30 °C. NaCl is required

Table 9. Phenotypical differentiation between *V. viscosus*, *V. wodanis* and their closest relatives (Baumann *et al.*, 1971)

Phenotype	<i>V. viscosus</i>	<i>V. wodanis</i>	<i>V. marinus</i>	<i>V. logei*</i>	<i>V. salmonicida</i>
Haemolysis of bovine red blood cells	+	+†	+	—	—
Growth at 25 °C	+	+‡	—	—	—
Lysine decarboxylase production	+	—	—	+	—
Oxidative production of acid from trehalose	—	+	—	+	+
Degradation of casein	+	—	—	—	—

* ATCC 29985^T.

† 94 % of the strains produce haemolysis.

‡ 83 % of the strains grow.

for growth, at 1–4 %. A majority of strains produce indole. Positive methyl red reaction; negative Voges–Proskauer reaction. Negative tests for lysine decarboxylase, arginine dihydrolase and ornithine decarboxylase. Gelatin, starch and Tween 80 are hydrolysed. A majority of the strains hydrolyse DNA. A minority of strains hydrolyse alginate, lecithin, urea and aesculin. Casein is not hydrolysed. Acid is produced from dextrin, D-mannose, D-galactose and trehalose, but not from L-arabinose, cellobiose, inositol, lactose, melibiose, raffinose, L-rhamnose, salicin nor D-xylose. A majority of strains produce acid from glycerol and sucrose, and a minority of strains produce acid from D-mannitol and D-sorbitol. The enzymes alkaline phosphatase, caprylate esterase and leucine arylamidase are produced. Butyrate esterase, valine arylamidase, acid phosphatase, naphthol phosphohydrolase and acetyl β -glucosaminidase are produced by the majority of the strains. Myristate lipase is produced by one of the strains only. Chymotrypsinase, trypsinase, cystine arylamidase, α -fucosidase, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase and α -mannosidase were not produced. Susceptible to 2,4-diamino-6,7-diisopropylpteridine (O/129 vibriostatic agent), ampicillin, sulphamethizol, oxytetracycline and oxolinic acid, and resistant to penicillin. The majority of strains (94 %) are haemolytic when grown on bovine blood agar containing 2 % NaCl. Isolated from the lesions of Atlantic salmon with the ulcerative skin disease called winter ulcer.

Description of the type strain of *Vibrio wodanis* sp. nov.

The type strain of *V. wodanis* is NVI 88/441^T, isolated in 1988 in Norway from an Atlantic salmon with winter ulcer. It is housed in the bacterial culture collection of the National Veterinary Institute, Oslo, Norway. The characteristics of the type strain is as given for the species, except as follows. Hydrolysis of

DNA, but not alginate, lecithin, urea and aesculin, production of indole, oxidative production of acid from glycerol and sucrose, but not from D-mannitol and D-sorbitol. The type strain produces valine arylamidase, acid phosphatase, naphthol phosphohydrolase and acetyl β -glucosaminidase, but not butyrate esterase or myristate lipase. The type strain haemolyses bovine red blood cells. The G + C content is 40.0 mol %. This strain has been deposited at the National Collection of Industrial and Marine Bacteria (Aberdeen, UK) as NCIMB 13582^T.

Phenotypic differentiation between *V. viscosus*, *V. wodanis* and their closest relatives is shown in Table 9.

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