

Characterization of *Vibrio viscosus* and *Vibrio wodanis* isolated at different geographical locations: a proposal for reclassification of *Vibrio viscosus* as *Moritella viscosa* comb. nov.

Eva Benediktsdóttir,¹ Linda Verdonck,² Cathrin Spröer,³ Sigurður Helgason⁴ and Jean Swings²

Author for correspondence: Eva Benediktsdóttir. Tel: +354 568 84 47. Fax: +354 568 84 57.
e-mail: eben@rhi.hi.is

¹ Institute of Biology, Microbiology Laboratory, University of Iceland, Ármúli 1A, IS-108 Reykjavík, Iceland

² Laboratory of Microbiology, University of Ghent, Ledeganckstraat 35, B-9000 Ghent, Belgium

³ Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany

⁴ Institute for Experimental Pathology, Fish Disease Laboratory, Keldur, University of Iceland, IS-112 Reykjavík, Iceland

***Vibrio viscosus* and *Vibrio wodanis* are recently described species of psychrotropic bacteria that have been found associated with a disease called 'winter ulcer', affecting salmonid fish reared in saline water in Norway, Iceland and recently in Scotland. *V. viscosus* and *V. wodanis* strains initially isolated from fish in Iceland and Norway were subjected to characterization using biochemical tests, SDS-PAGE of whole-cell proteins and a novel DNA fingerprinting method, amplified fragment length polymorphism (AFLP). The *V. viscosus* strains isolated from diseased fish grouped into homogeneous subgroups according to geographical origin and challenge experiments revealed that representatives of these groups are virulent. The results revealed that the *V. wodanis* strains are heterogeneous genotypically and phenotypically. Sequencing of almost complete 16S rRNA genes of *V. viscosus* and *V. wodanis* revealed that *V. viscosus* showed a 99.1% sequence similarity to *Moritella marina* and *V. wodanis* showed a 98.8% sequence similarity to *Vibrio logei* CIP 103204. A reclassification of *Vibrio viscosus* as *Moritella viscosa* comb. nov. is proposed.**

Keywords: *Moritella*, *Vibrio*, psychrophiles, fish pathogens

INTRODUCTION

Skin ulceration is one of the most frequently recorded disease conditions in farmed fish and can be produced by microbiological as well as mechanical means. A disease called 'winter ulcer', which affects salmonid fish reared at cold temperatures, is characterized by shallow skin lesions and often by diffuse or petechial haemorrhaging in internal organs. Winter ulcer has been observed for many years in Norway (Lunder, 1992; Lunder *et al.*, 1995) and Iceland (Benediktsdóttir *et al.*, 1991, 1998) and recently in Scotland (Bruno *et al.*, 1998; Laidler *et al.*, 1999). Two groups of psychrotropic vibrio bacteria are most often isolated from the lesions or the internal organs, either one of them or a mixed culture of both species. The two groups have

recently been described as two new species, *Vibrio viscosus* and *Vibrio wodanis* (Lunder *et al.*, 2000). Two strains of *V. viscosus* have been tested for pathogenicity in Norway and Iceland, respectively, and both were shown to be pathogenic for Atlantic salmon parr, but strains of *V. wodanis* that have been tested for pathogenicity have not been shown to be virulent to Atlantic salmon (Lunder *et al.*, 1995; Benediktsdóttir *et al.*, 1998).

The present work is based on a strain collection isolated mostly from fish suffering from winter ulcer in Iceland and Norway. Two fingerprinting techniques were used to compare strains of *V. viscosus* and *V. wodanis* isolates from different geographical locations: SDS-PAGE of whole-cell soluble proteins and amplified fragment length polymorphism (AFLP) (Vos *et al.*, 1995; Janssen *et al.*, 1996), in addition to biochemical and physiological tests. For characterization and epidemiological purposes, the AFLP technique has been used to differentiate strains of bacteria, including *Aeromonas* (Huys *et al.*, 1996), *Vibrio vul-*

The EMBL accession numbers for the 16S rDNA sequences of *Moritella viscosa* and *Vibrio wodanis* are Y17574 and Y17575, respectively.

nificus (Arias *et al.*, 1997) and *Bacillus* (Keim *et al.*, 1997). The AFLP technique depends on the selective amplification of restriction fragments of whole chromosomal DNA and discriminates between bacterial strains below the species level. SDS-PAGE depends on the products of the whole chromosome and groups strains to species and sometimes subspecies levels. Both methods have been proposed to provide a reliable way to measure genomic relatedness that would be comparable to DNA–DNA hybridization (Kersters, 1985; Huys *et al.*, 1996).

DNA hybridization has shown that the closest known relatives to *V. viscosus* and *V. wodanis* are *Vibrio marinus* and *Vibrio logei*, respectively (Lunder *et al.*, 2000). Sequencing of the 16S rRNA of *V. marinus* (Kita-Tsukamoto *et al.*, 1993; Gauthier *et al.*, 1995) has confirmed earlier studies based on 5S rRNA sequencing (MacDonell & Colwell, 1985) and DNA–DNA hybridization (Steven, 1990) that indicated that *V. marinus* does not belong to the *Vibrio* genus. Accordingly, a new genus, *Moritella*, has been proposed to accommodate this species (Urakawa *et al.*, 1998). Hereafter this species will be referred to as *Moritella marina*.

In this study, genotypic and phenotypic variability of strains of *V. viscosus* and *V. wodanis* isolated at different geographic locations were investigated and the sequences of almost complete 16S rRNA genes of *V. viscosus* and *V. wodanis* were determined.

METHODS

Bacterial strains. A total of 68 fish strains and 3 reference strains were included in the study. Of the fish strains, 37 were isolated in Iceland and 29 in Norway, mostly from diseased Atlantic salmon. The reference strains *M. marina* NCIMB 1144^T (ATCC 15381^T), *V. logei* NCIMB 1143 (ATCC 15382) and *V. logei* NCIMB 2252^T (ATCC 29985^T) were obtained from the National Collections of Industrial and Marine Bacteria, Aberdeen, UK. Strains 95/373-2988, 95/373-2989, 95/378, 95/403, 95/449, 95/453, 95/371, 95/461 and 95/880 were kindly supplied by S. Høie and strains NVI 88/478^T and NVI 88/441^T by E. Myhr, both at the National Veterinary Institute in Oslo, Norway. Strains 94/1793-1, 94/1793-2, 95/240, 95/313, 95/325 and 95/326, which originally came from the National Veterinary Institute in Oslo, were kindly given by K. Pedersen, Royal Veterinary and Agricultural University, Copenhagen, Denmark. Strains 138/94, 153/94, 157/94, 270/95, 271/95, 287/95, 288/95, 289/95, 290/95, 291/95, 292/95 and 293/95 were kindly supplied by E. Greger, ALPharma, Bellevue, WA, USA. Strains labelled K were isolated at the Fish Disease Laboratory, Keldur, Reykjavik, Iceland; strains labelled F were isolated at the Biology Institute, Reykjavik, Iceland. A list of strains included in this study and their origin is presented in Table 1.

The strains were maintained on Marine agar 2216 (Difco) at 4 °C and reinoculated every 1 or 2 months. Long-term storage was at –80 °C in Marine broth 2216 supplemented with 10% glycerol.

16S rDNA sequencing. Two strains were used, *V. viscosus* 88/478^T and *V. wodanis* 88/441^T. Extraction of genomic

DNA and amplification of the 16S rDNA was carried out as described by Rainey *et al.* (1992). PCR products were purified using the Prep-A-Gene kit and sequenced directly using the *Taq* DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems). Purified sequence reactions were electrophoresed on a 6% (w/w) polyacrylamide gel for 12 h using an Applied Biosystems model 373A Automated DNA Sequencer.

Sequences were manually aligned with published sequences of the γ -proteobacteria included in the Ribosomal Database Project (Maidak *et al.*, 1997). Pairwise evolutionary distances were computed using the correction of Jukes & Cantor (1969). Phylogenetic analyses were carried out as described by DeSoete (1983). Bootstrap values, based on the analyses of 1000 trees of 796 polymorphic sites, were calculated using the programs NJFIND and NJBOOT.

AFLP

Extraction of genomic DNA. Cells were harvested from Marine agar and washed in 500 μ l RS buffer (150 mM NaCl, 10 mM EDTA, pH 8.0). After centrifugation the cells were re-suspended in 100 μ l TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 7.6) and 15 μ l RNase (10 mg ml⁻¹) was added to the suspensions. Lysis of the cells using Sarkosyl/guanidium thiocyanate (Sigma) and further extraction of genomic DNA was as described by Pitcher *et al.* (1989). The DNA was finally dissolved in 100 μ l TE buffer (10 mM Tris/HCl, 0.1 mM EDTA, pH 8.0). The DNA concentration was determined by measuring A_{260} on a spectrophotometer (1 OD₂₆₀ unit = 50 μ g DNA ml⁻¹). The integrity of the DNA was checked by electrophoresis in a submerged horizontal agarose gel (0.8–1.0%, w/v) using an electrophoresis buffer (40 mM Tris/acetate, 1 mM EDTA, pH 8.0) containing 0.5 μ g ethidium bromide ml⁻¹. DNA preparations were stored at –20 °C.

Oligonucleotide adaptors and primers. The sequences of the *Hind*III and *Taq*I adaptors and primers used in this study are given in detail by Janssen *et al.* (1996).

Template preparation. Template preparation was as described by Vos *et al.* (1995) and Janssen *et al.* (1996). Thus, in this study, 1 μ g DNA was digested with 10 units each of *Hind*III and *Taq*I in a final volume of 30 μ l. Adaptors were added to a final concentration of 0.04 and 0.4 μ M for *Hind*III and *Taq*I adaptors, respectively, and ligated to the restriction fragments using T4 ligase. The DNA was subsequently precipitated with 1.25 M ammonium acetate and 50% (v/v) 2-propanol. Template DNAs were stored at –20 °C.

AFLP reactions. AFLP reactions employed two oligonucleotide primers: H01, corresponding to the *Hind*III ends, and T01, corresponding to the *Taq*I ends. The *Hind*III primer was radioactively end-labelled using [γ -³³P] ATP and T4 polynucleotide kinase (Vos *et al.*, 1995). Selective amplification and PCR reactions were performed on a PE 9600 thermocycler (Perkin Elmer) as described by Janssen *et al.* (1996).

Gel analysis. Prior to gel loading and electrophoresis, mixtures were heated for 3 min at 95 °C and then rapidly cooled on ice to prevent nucleic acids from reannealing. Each sample (1.8–2.0 μ l) was loaded on a 5% denaturing sequencing polyacrylamide gel (obtained as premixed SequaGel solutions from National Diagnostics). To 100 ml casting solution, 0.8 ml 10% ammonium persulfate and 40 μ l TEMED were added and gels were cast using a SequiGen 38 \times 50 cm gel apparatus (Bio-Rad). TBE (100 mM Tris/

Table 1. Bacterial strains used in the study and their origin

Species	Designation	Origin	Place of isolation
<i>V. viscosus</i> (<i>M. viscosa</i>)	K6 (= 137/89-2); K7 (= 311/89-1); K9 (= 427/89-1); K2 (= 80/90-4); K3 (= 79/90-3); K23 (= 407/90); K30 (= 78/90); K34 (= 14/90); K38 (= 21/90); K27 (= 73/91); K58 (= 147/92-1); F70; K49 (= 11/94-2); K54 (= 65/94-4)	Atlantic salmon	South-west Iceland, 64° N
	K35 (= 32/90); K37 (= 79/89-6)	Rainbow trout	South-west Iceland, 64° N
	K11 (= 451/89-1); K25 (= 537/90-2); K47 (= 136/93-1); K56 (= 195/94-1)	Atlantic salmon	North Iceland, 66° N
	F57	Lumpsucker	South-west Iceland, 64° N
	95/449 (= 3039)	Atlantic salmon	Norway, 58–59° N
	138/94; 288/95 (= AL 266); 289/95 (= AL 262); 290/95 (= AL 265); 292/95 (= AL 267); 293/95; 88/478 ^T ; 94/1793-2; 95/371 (= 2987); 95/373 (= 2988); 95/378 (= 2991); 95/403 (= 3041); 95/453 (= 3040); 95/461 (= 3049); 95/880 (= 3135)	Atlantic salmon	Norway, 62–64° N
	153/94; 270/95; 271/95	Atlantic salmon	Norway, 67–68° N
	K8 (= 419/89-4); K16 (= 396/90-3); K26 (= 42/91); K29 (= 78/90); K32 (= 80/90-1); K48 (= 11/94-1); K59 (= 147/92-2)	Atlantic salmon	South-west Iceland, 64° N
	K16 (= 396/90-3)	Rainbow trout	South-west Iceland, 64° N
	K4 (= 312/90); K12 (= 451/89-2); K13 (= 525/89-3); K14 (= 525/89-4); K24 (= 537/90-1); K28 (= 77/90); K31 (= 114/90-1); K57 (= 195/94-2)	Atlantic salmon	North Iceland, 66° N
F31	Haddock	South-west Iceland, 64° N	
95/240	Atlantic salmon	Norway, 59–60° N	
88/441 ^T	Atlantic salmon	Norway, 61–62° N	
157-94; 287/95 (= AL 263); 291/95 (= AL 264); 94/1793-1; 95/313; 95/325; 95/326	Atlantic salmon	Norway, 62–64° N	
<i>V. wodanis</i>			

HCl, 100 mM boric acid, 2 mM EDTA, pH 8.3) was used as the electrophoresis buffer. Gels were run at a constant power of 110 W for approximately 150 min. After electrophoresis, gels were transferred to 3MM Whatman chromatography paper and vacuum dried on a gel dryer (model 583; Bio-Rad) for 50 min at 80 °C. A sheet of Hyperfilm-MP (Amersham) was exposed to the dried gel. Exposure times varied between 19 and 24 h, depending on the measured radioactivity of the gel. Films were developed using a Fuji RGII X-ray Film Processor. Autoradiograms were scanned by a RayVen RSU1 densitoscanner (X-Ray Scanner Corporation). Digitized optical densities were saved as TIFF files and further processed using GelCompar 3.1 software (Applied Maths). Digital images were normalized and combined according to the methods described by Vauterin & Vauterin (1992). A similarity matrix was created using the Dice similarity coefficient, S_D (Sneath & Sokal, 1973). For band comparison, a band position tolerance value of 0.8% was allowed to compensate for misalignment of homologous bands due to technical imperfections. The unweighted pair group methods using average linkages (UPGMA) was used to cluster the patterns (Vauterin & Vauterin, 1992).

SDS-PAGE. Bacterial growth was harvested from one or two Marine agar plates and washed three times in 1 ml phosphate

buffer with 2% NaCl, pH 7.3, while keeping all material on ice. The pellet was resuspended in 0.9 ml STB buffer consisting of 0.75% (w/v) Tris/HCl, 5% (v/v) mercapto-ethanol and 5% (w/v) glycerol to an OD_{590} of 0.8–1.1 and mixed before adding 100 µl 20% SDS. The sample was heated at 95 °C for 10 min, cooled and centrifuged at 10000 r.p.m. for 5 min. The supernatant was kept frozen until used. The samples were run overnight in a 1.5 mm 5% (w/v) acrylamide stacking gel, pH 6.8, and a 12% acrylamide separating gel, pH 8.8, at 6 mA per gel using Hoefer SE600 electrophoresis equipment. After fixation in 3% (w/v) TCA, gels were stained with Coomassie blue, destained and dried. For correction of gel to gel variation, four samples of *Psychrobacter immobilis* LMG 1125 were run on each gel. The gels were scanned, normalization was performed and clustering of the strains was made by using the UPGMA method with GelCompar software (Vauterin & Vauterin, 1992).

Biochemical, physiological and serological tests. The ability to utilize four different sugars as sole organic source of carbon with and without 2 mg Casamino acids per 100 ml was tested in a basal medium according to Lee *et al.* (1981). Bacteriological Peptone L37 (Oxoid) supplemented with synthetic seawater (Colwell & Morita, 1964) was used for

testing the minimal amount of peptone required for growth. Nutritional requirements of the strains for growth on amino acids as sole source of carbon and nitrogen were performed in synthetic seawater supplemented with 1% amino acid and 1.5% agar as described by Colwell & Morita (1964). All other biological and serological tests were performed as described previously (Benediktsdóttir *et al.*, 1998). Thermometers used to control temperatures in incubators when testing growth at 21 and 25 °C were calibrated according to measurement standard no. G50 075 traceable to the International Temperature Standard (ITS-90).

Virulence. Atlantic salmon parr of approximately 10 g, originating from a smolt-producing facility with no known disease history, were transferred to the experimental facility at least 4 d before challenge. The fish were kept in fresh water at 11 °C. Pathological changes in fish were examined after inoculation with the three strains K56, F57 and 88/478^T, but LD₅₀ was determined for K56 and F57, which were passed serially four times through fish to enhance the virulence. Cultivation of bacteria, determination of viable count and performance of the challenge were carried out as described previously for strain K58 (Benediktsdóttir *et al.*, 1998).

RESULTS

16S rDNA sequence studies

To determine the relationship of *V. viscosus* and *V. wodanis* to other members of the genus *Vibrio* the almost complete 16S rRNA sequences were compared to the *Vibrio* group and the *Pseudoalteromonas* group within the γ -proteobacteria (Maidak *et al.*, 1997). A tree constructed from the matrix of sequence similarities (Fig. 1) demonstrates that *V. viscosus* clusters within the *Pseudoalteromonas* group, whereas *V. wodanis* clusters within the *Vibrio fischeri* assemblage.

As seen in Table 2, *Vibrio viscosus* shows the highest 16S rRNA gene sequence similarity of 99% to the sequence of *M. marina*.

V. wodanis shows the highest 16S rRNA gene sequence similarity of 98.8% to the sequence of *V. logei* CIP 103204 (NCIMB 1143) and forms a distinct cluster with *V. logei* and *Vibrio salmonicida* (Fig. 1). Within this cluster *V. wodanis* represents a distinct branch.

AFLP

Numerical analysis of the AFLP banding patterns allowed the delineation of two main clusters at 40% similarity. All *V. viscosus* strains ($n = 30$) included in this study grouped together in one cluster and the *V. wodanis* strains, except for K59, formed another cluster (Fig. 2). Reference strains of *M. marina* NCIMB 1144^T, *V. logei* NCIMB 2252^T and *V. logei* NCIMB 1143 were included, but none of them clustered with the *V. viscosus* or *V. wodanis* strains.

Within the *V. viscosus* strains three subgroups could be distinguished at 75% relationship. One subgroup contained the isolates originating from Norway, while

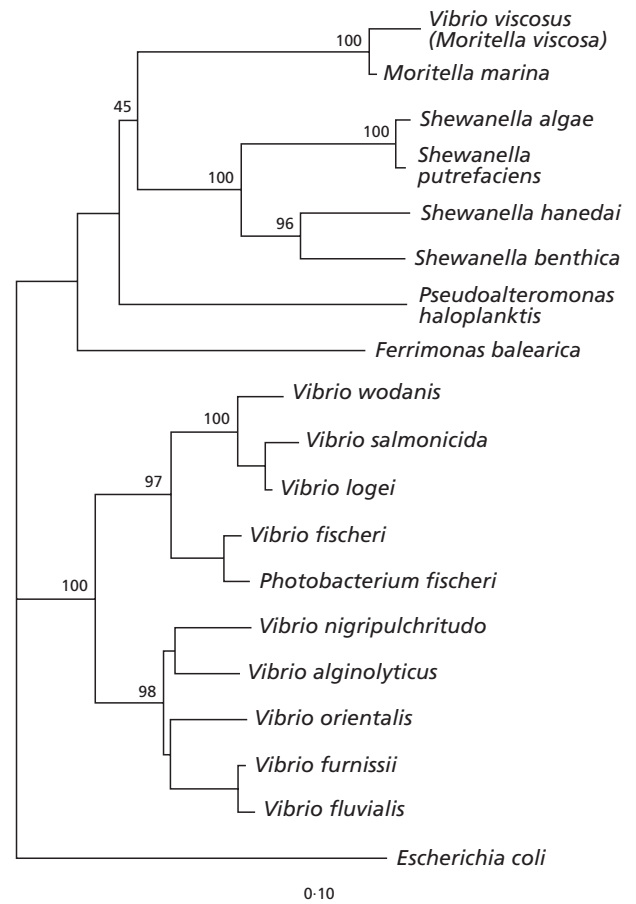


Fig. 1. Dendrogram derived from the almost complete 16S rRNA sequences of *V. viscosus* (*M. viscosa*), *V. wodanis* and *Vibrio* reference strains. The tree was constructed from a distance matrix by using the algorithm of DeSoete (1983). The distance matrix was derived from a similarity matrix (data not shown) by using the correction of Jukes & Cantor (1969). *E. coli* was used as the root organism. The numbers are the estimated confidence levels, expressed as percentages, for the positions of the branches, as determined by a bootstrap analysis. Bar, 10% difference between sequences, as determined by measuring the lengths of the horizontal lines connecting two species.

the isolates from Iceland constituted two subgroups and a single strain. Within each subgroup of *V. viscosus* the isolates shared almost identical banding patterns. All the Norwegian isolates were very homogeneous with AFLP genomic patterns, which are characterized by two or three heavy bands. One of the Icelandic subgroups contained strains isolated from salmonids in south-west Iceland, whereas the other subgroup contained four strains isolated from salmonids in north Iceland. F57, isolated from a healthy lumpsucker and clustered within the *V. viscosus* cluster, was not included in these homogeneous subgroups.

The *V. wodanis* strains made one heterogeneous cluster at 45% similarity, Icelandic and Norwegian strains clustering side by side. A strain isolated from a healthy haddock off the Icelandic coast and identified as *V.*

Table 2. Levels of 16S rRNA sequence similarity based on a comparison of almost complete 16S rRNA sequences between *V. viscosus* (*M. viscosa*), *V. wodanis* and related *Vibrio* species

Strain (EMBL accession no.)	Percentage 16S rRNA sequence similarity to:	
	<i>V. viscosus</i> (<i>M. viscosa</i>)	<i>V. wodanis</i>
<i>Vibrio viscosus</i> (<i>Moritella viscosa</i>)	–	90·1
<i>Pseudoalteromonas haloplanktis</i> ATCC 14393 ^T (X67024)	90·4	90·1
<i>Shewanella algae</i> Bry (X81621)	91·5	89·1
<i>Shewanella putrefaciens</i> LMG 2268 ^T (X81623)	91·5	89·1
<i>Shewanella hanedai</i> CIP 103077 ^T (X82132)	91·0	89·7
<i>Shewanella benthica</i> ATCC 43992 (X82131)	92·0	89·9
<i>Moritella marina</i> MP-1 ^T , ATCC 15381 ^T , NCIMB 1144 ^T	99·0	90·7
<i>Ferrimonas balearica</i> DSM 9799 ^T (X93021)	90·9	89·4
<i>Vibrio wodanis</i>	90·1	–
<i>Vibrio orientalis</i> CIP 102891 ^T , ATCC 33934 ^T , NCIMB 2195 ^T (X74719)	89·1	94·8
<i>Vibrio furnissii</i> CIP 102972 ^T , ATCC 35016 ^T (X74704)	89·9	94·9
<i>Vibrio salmonicida</i> NCIMB 2262 ^T (X70643)	89·6	98·5
<i>Vibrio logei</i> CIP 103204, ATCC 15382, NCIMB 1143 (X74708)	89·8	98·8
<i>Vibrio fischeri</i> NCIMB 1281 ^T , ATCC 7744 ^T (X74702)	90·2	98·0
<i>Photobacterium fischeri</i> MJ-1 (Z21729)	90·0	97·1
<i>Vibrio nigripulchritudo</i> CIP 103195 ^T , ATCC 27043 ^T (X74717)	90·0	95·0
<i>Vibrio alginolyticus</i> CIP 75.03 ^T , ATCC 17749 ^T (X74690)	89·8	95·3
<i>Vibrio fluvialis</i> NCIMB 2249 ^T , ATCC 33809 ^T , NCTC 11327 ^T (X74703)	89·9	94·9
<i>Escherichia coli</i>	87·3	90·1

wodanis by biochemical tests and SDS-PAGE clustered among the *V. wodanis* strains isolated from salmonids.

SDS-PAGE

Numerical analysis of the SDS-PAGE data of 25 *V. viscosus* strains isolated from diseased fish showed that the *V. viscosus* strains clustered at 92·8% similarity, but 23 *V. wodanis* strains clustered at 88·3% similarity. *M. marina* NCIMB 1144^T showed an 82·5% relationship with the *V. viscosus* strains, and *V. logei* NCIMB 2252^T and *V. logei* NCIMB 1143 showed 84·5% similarity to the *V. wodanis* cluster (Fig. 3a, b). All *V. viscosus* strains isolated from salmonids in south-west Iceland and in Norway exhibited an identical SDS-PAGE protein pattern. Four strains isolated in north Iceland showed a pattern that only differed from the main cluster by one protein band, and a third pattern was shown by the strain isolated from a lumpsucker, F57. The main difference between clusters of *V. viscosus* was the presence of a major band of approximately 32 kDa in strains of the main cluster that was lacking in the strains originating in north Iceland and that instead had a broad, approximately 34 kDa band and, in F57, a broad band of 36 kDa. (Fig. 4a).

More strain to strain variability was seen among *V.*

wodanis strains, and subclusters that formed within the *V. wodanis* cluster were not dependent on the origin of the strains. The main difference within the *V. wodanis* cluster was the presence or absence of a major protein band between 66 and 90 kDa. (Fig. 4b).

Biochemical tests and growth requirements

Strains of *V. viscosus* and *M. marina* produced acid from glucose, ribose and *N*-acetylglucosamine. They did not produce acid from the following carbohydrates: arabinose, cellobiose, glycerol, inositol, lactose, mannitol, melibiose, rhamnose, salicin, sorbitol, sucrose and trehalose. Salmonid strains of *V. viscosus* originating from south-west Iceland produced acid from mannose and maltose while strains originating from north Iceland and Norway and *M. marina* were negative. All *V. viscosus* strains and *M. marina* were negative in arginine dihydrolase and ornithine decarboxylase production, and all except the four strains isolated in north Iceland produced lysine decarboxylase.

V. viscosus and *M. marina* degraded gelatin, DNA, Tween 20 and Tween 80. *V. viscosus* strains degraded starch, and 50% of Norwegian strains and 70% of

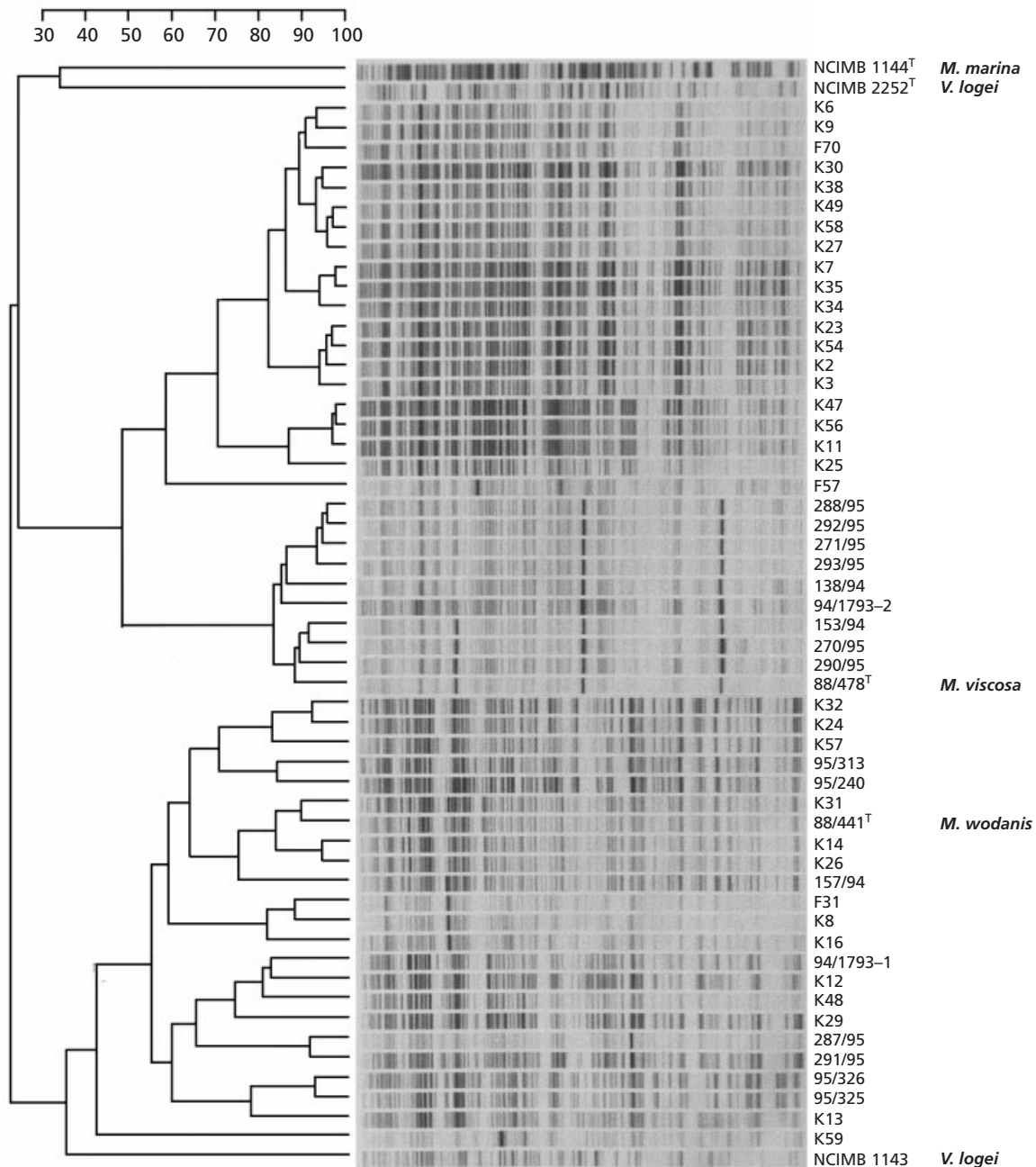


Fig. 2. Dendrogram and digitized AFLP patterns of *M. viscosa* (*V. viscosus*) and *V. wodanis* strains and related reference strains.

Icelandic strains of *V. viscosus* were able to degrade chitin.

No *V. viscosus* strain was able to grow at 25 °C, but all grew at 21 and 4 °C. They showed growth on nutrient agar supplemented with 2 and 3% NaCl, but not on nutrient agar supplemented with 1 or 4% NaCl. *M. marina* did not grow at 21 °C nor on nutrient agar supplemented with 2% NaCl. Almost all *V. viscosus* strains and *M. marina* utilized glucose and ribose as sole carbon source, but none could utilize trehalose,

except when the trehalose medium was supplemented with 0.001% Casamino acids. All of these were able to grow on media containing 1% peptone in synthetic seawater, but not when the amount of peptone was reduced to 0.5%. When 1% L-proline was used as sole source of carbon and nitrogen, two *V. viscosus* strains could grow but not *M. marina*. No *V. viscosus* strain was able to grow on TCBS medium (Oxoid) and neither did *M. marina*.

The *V. viscosus* strains and *M. marina* showed a zone

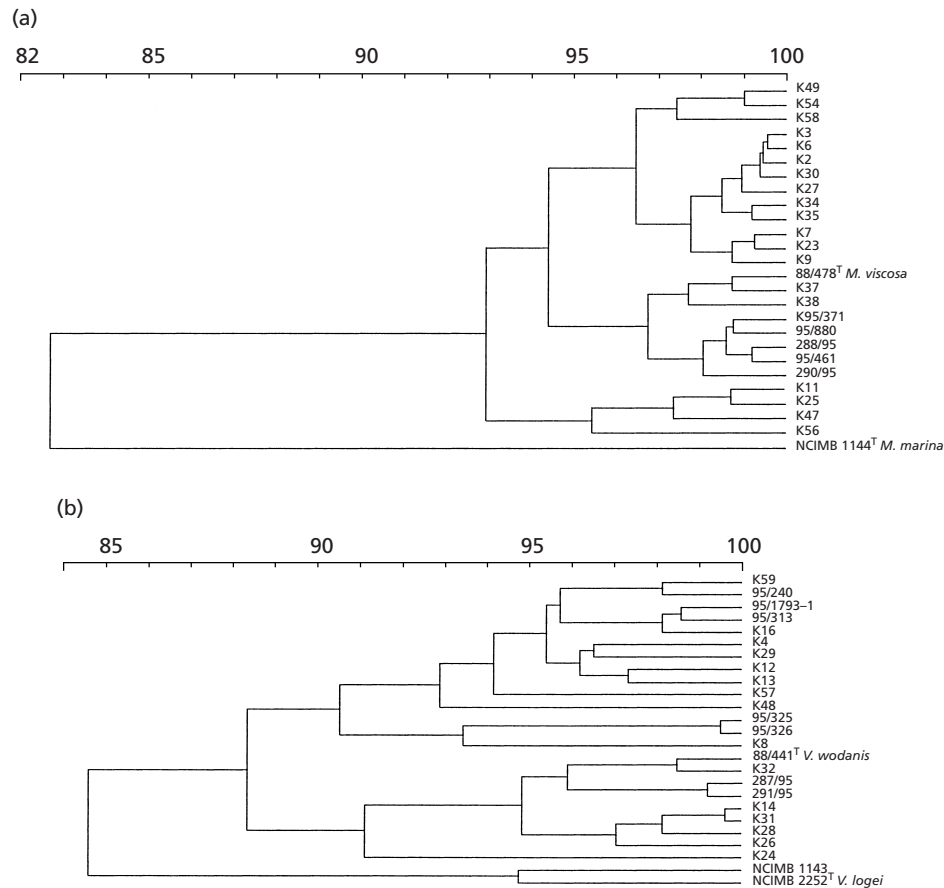


Fig. 3. Dendrogram based on SDS-PAGE protein profiles of whole cells of (a) *M. viscosa* (*V. viscosus*) and (b) *V. wodanis* strains and related reference strains.

of inhibition with 150 µg of the vibriostat 0129 but no zone or a very small zone with 10 µg.

All *V. viscosus* strains isolated from salmonids in both countries agglutinated in sera prepared against *V. viscosus* K2, but the single strain F57 did not react with the antisera and neither did *M. marina*.

The *V. wodanis* strains produced acid from glucose, *N*-acetylglucosamine, ribose, glycerol, maltose and trehalose, and 43% of the strains produced acid from mannitol, 96% from mannose and 78% from sucrose. All strains were negative in acid production from arabinose, cellobiose, lactose and sorbitol.

All *V. wodanis* strains utilized glucose, but an overall heterogeneity was observed in the utilization of ribose, trehalose and L-proline. One *V. wodanis* isolate grew at 1% NaCl, all grew at 2, 3 and 4%, and 16 of the 23 *V. wodanis* isolates grew at 5% NaCl. All *V. wodanis* strains were able to grow on medium containing 1% peptone in synthetic seawater, but 21 of 25 *V. wodanis* strains tested were able to grow on 0.5% peptone. Only 30% of the *V. wodanis* strains showed growth on TCBS agar. In none of the tests was grouping of *V. wodanis* according to geographical origin noted, except

growth at 25 °C: 13 of 14 *V. wodanis* strains isolated in Iceland did not grow at 25 °C, while seven of nine *V. wodanis* strains isolated in Norway did.

Challenge tests

All fish challenged with 1.1×10^3 c.f.u. or more of K56 died, as did four of ten fish challenged with 1.1×10^2 c.f.u. All fish challenged with 5.5×10^7 c.f.u. of F57 died, and so did nine, one and no fish challenged with 5.5×10^6 , 5.5×10^5 and 5.5×10^4 , respectively, with the LD₅₀ calculated to be 1.7×10^6 c.f.u.

Pathology

The skin colour of affected fish receiving all three strains became darker around the injection site and subsequently turned greyish with necrotic skin and occasionally slight petechial haemorrhaging. Generally there was liquefactive necrosis with a varying degree of haemorrhaging in the underlying muscle. Internal organs, especially the liver of fish receiving strain F57, were frequently pale. Internal lesions of fish receiving strains 88/478^T and K56 were characterized

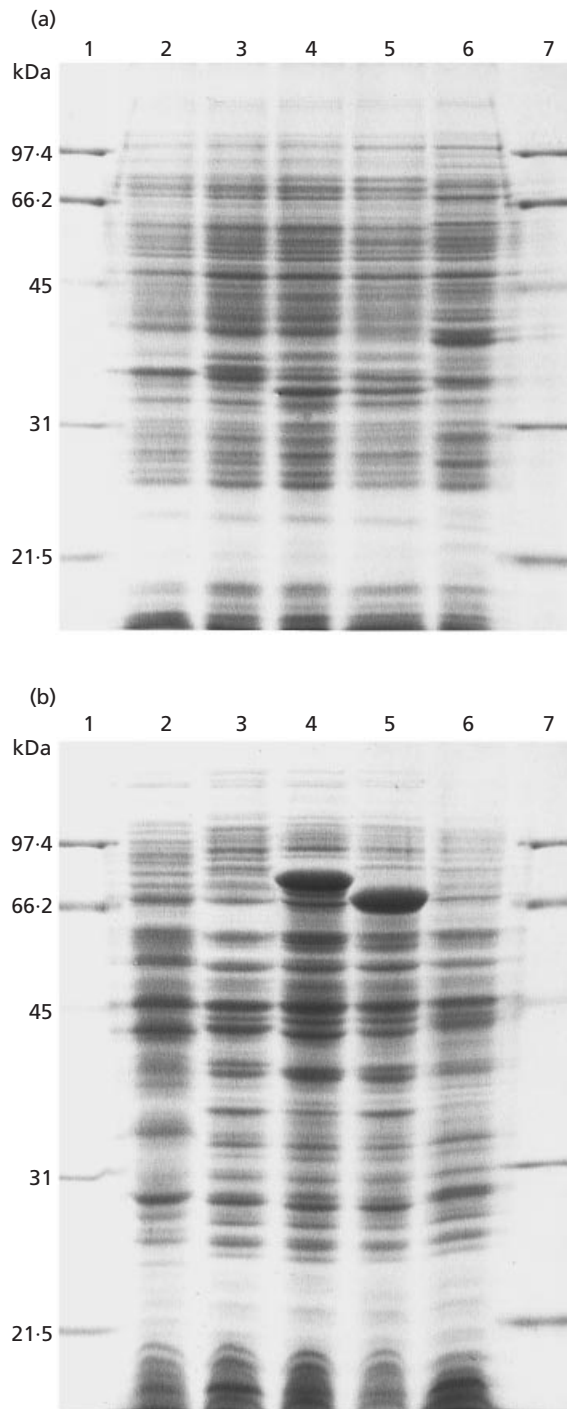


Fig. 4. SDS-PAGE protein profiles of whole cells. (a) Lanes: 1 and 7, molecular mass markers; 2, F57; 3, K56; 4, K58; 5, *V. viscosus* (*M. viscosa*) NVI 88/478^T; 6, *M. marina* NCIMB 1144^T. (b) Lanes: 1 and 7, molecular mass markers; 2, *V. logei* NCIMB 2252^T; 3, *V. wodanis* NVI 88/441^T; 4, 95/325; 5, K59; 6, K32.

by rather heavy disseminated haemorrhages in the liver, sometimes with diffuse and heavy petechial haemorrhages in the perivisceral fat around the pyloric caeca and frequently with mucosal and submucosal

disseminated haemorrhages of the small intestines and the pyloric area. These changes were not seen in fish receiving strain F57.

DISCUSSION

Molecular characterization by AFLP offers a high degree of discrimination and the results presented here demonstrate its use in epidemiological analysis. The present results of the AFLP analysis indicate a clear correlation between the grouping and the origin of the *V. viscosus* strains: almost identical patterns of strains of *V. viscosus* appeared according to their geographical location of isolation. This indicates a common clonal origin of *V. viscosus* within particular geographical areas (Ørskov & Ørskov, 1983) and that clones of *V. viscosus* are spread within fish farms and even between farms within a given geographical area.

Strains originating in Iceland grouped into two clusters and a single strain that were more related to each other than to strains originating in Norway, which only clustered into one group. The isolates from Norway that were included in the AFLP analysis originated from 62 to 65° N, but isolates from other geographical areas or isolates originating from sources other than salmonid fish would probably give another pattern. The differences between the AFLP clusters were reflected by differences in one to three biochemical tests and within each AFLP group strains were biochemically homogeneous. The difference between the two groups isolated in Iceland was detected by SDS-PAGE protein profile analysis, but not the difference between the genetically less related groups of strains of *V. viscosus* originating in Norway and south-west Iceland. The homogeneous results from the SDS-PAGE protein profile analysis make this method well suited for species identification.

Representatives of the two AFLP groups of *V. viscosus* isolated in south-west Iceland and in Norway have been shown to be virulent for salmonid fish (Benediktsdóttir *et al.*, 1998; Lunder *et al.*, 1995). In this study a representative of the AFLP group isolated in north Iceland was shown to be virulent as well and the pathological symptoms were the same. A single strain isolated from the gills of a healthy lump sucker, was low- or non-virulent, indicating the existence of non-virulent clones of *V. viscosus* in the environment.

Results from AFLP, SDS-PAGE and biochemical tests indicate that *V. wodanis* found in winter ulcers is a defined but heterogeneous species, not showing any sign of clonal spread that is often characteristic of primary pathogens. This result is consistent with challenge tests with these bacteria performed separately in Norway and Iceland that did not indicate any pathogenicity to salmon parr (Lunder *et al.*, 1995; Benediktsdóttir *et al.*, 1998). However, the independent and repeated findings of *V. wodanis* in winter ulcers in Norway, Iceland and Scotland indicate strongly that *V. wodanis* is an important opportunistic

pathogen that may suppress the healing process of skin by colonizing ulcers of salmon infected primarily with *V. viscosus*.

V. viscosus is rather inert biochemically and often needs prolonged incubation times on test media; in addition it does not grow readily on all test media that support growth of vibrios. In this report we show that for most strains 1% peptone is needed for growth. This finding includes the fact that *V. viscosus* does not grow in salt-supplemented Hugh and Leifson's OF Medium and other media often used for fermentation tests for vibrios. Faint growth of two strains of *V. viscosus* on TCBS agar was observed and not all strains of *V. wodanis* could grow on TCBS. The growth of *V. viscosus* and of *V. wodanis* in different salt concentrations observed in this study is inconsistent with the results of Lunder *et al.* (2000) who reported growth of the *V. viscosus* strains in 1–4% NaCl and of the *V. wodanis* strains in 0.5–5% NaCl. They also reported growth of *V. viscosus* strains at 25 °C that was not observed at this laboratory, with repeated testing both on agar media with and without blood, using an incubator controlled with a calibrated thermometer.

Colwell & Morita (1964) reported growth of *M. marina* ATCC 15381^T (NCIMB 1144^T) on proline after the first isolation of this species. The strain seems to have lost this property: no growth of *M. marina* and growth of only two strains of *V. viscosus* was observed using proline as sole source of carbon and nitrogen. However, the invariant results obtained from the utilization tests in this study indicate that these kinds of tests should be used with great caution for *M. marina* and *V. viscosus*. Both species often render themselves non-cultivable after being kept on an agar or broth medium for some time, resulting in possible false negative results.

The sequencing of 16S rRNA of *V. wodanis* revealed that this species clusters within the *V. fischeri* assemblage. *V. fischeri* and *V. logei* are formerly members of the genus *Photobacterium* (Bang *et al.*, 1978), but were transferred to the genus *Vibrio* because evolutionary divergence was indicated by amino acid sequencing of the glutamine synthetase and superoxide dismutase enzymes (Baumann & Baumann, 1980). 16S rRNA sequences indicate that these species, including *V. wodanis*, are more related to each other than to other bacteria of the *Vibrio* genus, but further studies are needed to determine if they should be transferred to a separate genus.

V. viscosus clustered within the *Pseudoalteromonas/Shewanella* group and it shared 99.1% similarity to *M. marina*. We therefore propose that *Vibrio viscosus* should be reclassified and renamed *Moritella viscosa* comb. nov.

M. viscosa has a thymine–thymine insertion between bases 206 and 207 (*Escherichia coli* numbering), supporting the suggestion of Urakawa *et al.* (1998) that this is a feature that distinguishes *Moritella* species from other close taxa.

One barophilic strain included in the study of Urakawa *et al.* (1998) has been described as a new species, *Moritella japonica* (Nogi *et al.*, 1998). This strain differs from the *M. viscosa* strains in acid production from glycerol and its total resistance to 0129. *M. viscosa* is the only species of the genus at present that is associated with fish and is virulent. Other strains were isolated from water or sediment at depths from 500 to 8600 m and at temperatures between 1 and 11 °C (Urakawa *et al.*, 1998). A common property of *Moritella* strains is their psychrophily: 15 strains designated MP and described by Colwell & Morita (1964) as *M. marina* were all psychrophilic and did not grow at temperatures above 24 °C. Urakawa *et al.* (1998) reported that the 11 *Moritella* strains they isolated were psychrophiles.

Description of *Moritella viscosa* comb. nov.

Moritella viscosa (vis.co'sa. L. fem. adj. *viscosa* viscous, because of its thread-forming, adherent colonies).

The description of *Moritella viscosa* comb. nov. is identical to the description given for *Vibrio viscosus* by Lunder *et al.* (2000). In addition, the production of lysine decarboxylase and the production of acid from maltose and mannose are different, depending on subgroups. *M. viscosa* is partially sensitive to 0129, showing a very small or no clear zone around a disc containing 10 µg 0129 and negative for growth on TCBS agar. The type strain is NVI 88/478^T.

ACKNOWLEDGEMENTS

We thank the Laboratory of Microbiology, University of Ghent, for hosting E.B. for 2 months. We also thank Dirk Dewettinck, Sylvie Van Eygen and Ágústa Thóra Jónsdóttir for excellent technical assistance. This work was supported by the Research Fund of the University of Iceland and the Research Council of Iceland.

REFERENCES

- Arias, C. R., Verdonck, L., Swings, J., Garay, E. & Aznar, R. (1997). Intraspecific differentiation of *Vibrio vulnificus* biotypes by amplified fragment length polymorphism and ribotyping. *Appl Environ Microbiol* **63**, 2600–2606.
- Bang, S. S., Baumann, P. & Nealson, K. H. (1978). Phenotypic characterization of *Photobacterium logei* (sp. nov.), a species related to *P. fischeri*. *Curr Microbiol* **1**, 285–288.
- Baumann, L. & Baumann, P. (1980). Immunological relationships of glutamine synthetases from marine and terrestrial enterobacteria. *Curr Microbiol* **3**, 191–196.
- 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, p. 125.
- Benediktsdóttir, E., Helgason, S. & Sigurjónsdóttir, H. (1998). *Vibrio* spp. isolated from salmonids with shallow skin lesions and reared at low temperature. *J Fish Dis* **21**, 19–28.
- Bruno, D. W., Griffiths, J., Petrie, J. & Hastings, T. S. (1998). *Vibrio viscosus* in farmed Atlantic salmon *Salmo salar* in Scotland:

- Field and experimental observations. *Dis Aquat Org* **34**, 161–166.
- Colwell, R. R. & Morita, R. Y. (1964).** Reisolation and emendation of description of *Vibrio marinus* (Russell) Ford. *J Bacteriol* **88**, 831–837.
- DeSoete, G. (1983).** A least square algorithm for fitting additive trees to proximity data. *Psychometrika* **48**, 621–626.
- Gauthier, G., Gauthier, M. & Christen, R. (1995).** Phylogenetic analysis of the genera *Alteromonas*, *Shewanella*, and *Moritella* using genes coding for small-subunit rRNA sequences and division of the genus *Alteromonas* into two genera, *Alteromonas* (emended) and *Pseudoalteromonas* gen. nov., and proposal of twelve new species combinations. *Int J Syst Bacteriol* **45**, 755–761.
- Huys, G., Coopman, R., Janssen, P. & Kersters, K. (1996).** High-resolution genotypic analysis of the genus *Aeromonas* by AFLP fingerprinting. *Int J Syst Bacteriol* **46**, 572–580.
- Janssen, P., Coopman, R., Huys, G., Swings, J., Bleeker, M., Vos, P., Zabeau, M. & Kersters, K. (1996).** Evaluation of the DNA fingerprinting method AFLP as a new tool in bacterial taxonomy. *Microbiology* **142**, 1881–1893.
- Jukes, T. H. & Cantor, C. R. (1969).** Evolution of protein molecules. In *Mammalian Protein Metabolism*, vol. 3, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.
- Keim, P., Kalif, A., Schupp, J. & 7 other authors (1997).** Molecular evolution and diversity in *Bacillus anthracis* as detected by amplified fragment length polymorphism markers. *J Bacteriol* **179**, 818–824.
- Kersters, K. (1985).** Numerical methods in the classification of bacteria by protein electrophoresis. In *Computer-Assisted Bacterial Systematics*, pp. 337–365. Edited by M. Goodfellow, D. Jones & F. G. Priest. London: Academic Press.
- Kita-Tsukamoto, K., Oyaizu, H., Nanba, K. & Simidu, U. (1993).** Phylogenetic relationships of marine bacteria, mainly members of the family *Vibrionaceae*, determined on the basis of 16S rRNA sequences. *Int J Syst Bacteriol* **43**, 8–19.
- Laidler, L. A., Grant, A. N. & Wadsworth, S. (1999).** Preliminary investigations into the bacteriology of skin lesions of Atlantic salmon reared in seawater in Scotland. *Fish Vet J* **3**, 27–31.
- Lee, J. V., Shread, P., Furniss, A. L. & Bryant, T. N. (1981).** Taxonomy and description of *Vibrio fluvialis* sp. nov. (Synonym group F Vibrios, Group EF6). *J Appl Bacteriol* **50**, 73–94.
- Lunder, T. (1992).** ‘Winter ulcer’ in Atlantic salmon. A study of pathological changes, transmissibility, and bacterial isolates. DSc thesis, 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. *Dis Aquat Org* **23**, 39–49.
- Lunder, T., Sørum, H., Holstad, G., Steigerwalt, A. G., Mowinckel, P. & Brenner, D. J. (2000).** Phenotypic and genotypic characterization of *Vibrio viscosus* sp. nov. and *Vibrio wodanis* sp. nov. isolated from Atlantic salmon (*Salmo salar*) with ‘winter ulcer’. *Int J Syst Bacteriol* **50**, 427–450.
- MacDonell, M. T. & Colwell, R. R. (1985).** Phylogeny of the *Vibrionaceae*, and recommendation for two new genera, *Listonella* and *Shewanella*. *Syst Appl Microbiol* **6**, 171–182.
- Maidak, B. L., Olsen, G. J., Larsen, N., Overbeek, R., McCaughey, M. J. & Woese, C. R. (1997).** The RDP (Ribosomal Database Project). *Nucleic Acids Res* **25**, 109–110.
- Nogi, Y., Kato, C. & Horikoshi, K. (1998).** *Moritella japonica* sp. nov., a novel barophilic bacterium isolated from a Japan Trench sediment. *J Gen Appl Microbiol* **44**, 289–295.
- Ørskov, F. & Ørskov, I. (1983).** Summary of a workshop on the clone concept in the epidemiology, taxonomy, and evolution of the *Enterobacteriaceae* and other bacteria. *J Infect Dis* **148**, 346–357.
- Pitcher, D. G., Saunders, N. A. & Owen, R. J. (1989).** Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett Appl Microbiol* **8**, 151–156.
- Rainey, F. A., Dorsch, M., Morgan, H. W. & Stackebrandt, E. (1992).** 16S rDNA analyses of *Spirochaeta thermophila*: its phylogenetic position and implications for the systematics of the order *Spirochaetales*. *Syst Appl Microbiol* **15**, 197–202.
- Sneath, P. H. A. & Sokal, R. R. (1973).** *Numerical Taxonomy: the Principles and Practice of Numerical Classification*. San Francisco: Freeman.
- Steven, S. E. (1990).** *Molecular systematics of Vibrio and Photobacterium*. PhD dissertation. University of Maryland, College Park, MD, USA.
- Urakawa, H., Kita-Tsukamoto, K., Steven, S. E., Ohwada, K. & Colwell, R. R. (1998).** A proposal to transfer *Vibrio marinus* (Russell 1891) to a new genus *Moritella* gen. nov. as *Moritella marina* comb. nov. *FEMS Microbiol Lett* **165**, 373–378.
- Vauterin, L. & Vauterin, P. (1992).** Computer-aided objective comparison of electrophoresis patterns for grouping and identification of microorganisms. *Eur Microbiol* **1**, 37–41.
- Vos, P., Hogers, R., Bleeker, M. & 8 other authors (1995).** AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res* **23**, 4407–4414.