Vol. 39: 109–119, 2000 | DISEASES OF AQUATIC ORGANISMS Dis Aquat Org | Published January 14

Genetic diversity of the fish pathogen *Aeromonas salmonicida* demonstrated by random amplified polymorphic DNA and pulsed-field gel electrophoresis analyses

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ABSTRACT: The current taxonomy of *Aeromonas salmonicida* includes 4 subspecies. *A. salmonicida* subsp. *salmonicida* is associated with salmonid furunculosis, and *A. salmonicida* subsp. *achromogenes*, *A. salmonicida* subsp. *masoucida*, and *A. salmonicida* subsp. *smithia* are strains that show variation in some biochemical properties. This classification does not readily encompass isolates from a wide range of fish hosts currently described as atypical *A. salmonicida*. This study examined 17 typical strains, 39 atypical strains and 3 type *A. salmonicida* subspecies strains for genetic similarity using the random amplified polymorphic DNA (RAPD) and pulsed-field gel electrophoresis (PFGE) techniques. On the basis of RAPD- and PFGE-derived profiles, similarity matrices and dendrograms were constructed. The results showed that species *A. salmonicida* constituted a genetically heterogeneous group of strains, encompassing within an homogeneous or clonal lineage comprised solely of typical strains and the *A. salmonicida* subsp. *salmonicida* type strain.

KEY WORDS: *Aeromonas salmonicida* · Fish pathogens · Taxonomy · RAPD · PFGE

INTRODUCTION

Aeromonas salmonicida is the bacterial agent associated with furunculosis, a disease generally confined to salmonid fish species (Popoff 1984). The organism is readily recovered from the kidney or surface lesions of diseased fish and is conventionally identified by the occurrence of dark brown pigmented colonies on tryptone soya agar after incubation at 20 to 25°C for 2 to 4 d. The traditional description of members of this species is: non-motile, fermentative, Gram-negative rods which do not grow at 37°C and which produce catalase and oxidase (Popoff 1984). The current taxonomy of *A. salmonicida* includes 4 subspecies; *A. salmonicida* subsp. *salmonicida* is associated with salmonid furunculosis and has been arbitrarily termed typical to distinguish this taxon from all other forms of *A. salmonicida* (Austin & Austin 1993). *A. salmonicida* subsp. *achromogenes* (Smith 1963), *A. salmonicida* subsp. *masoucida* (Kimura 1969) and *A. salmonicida* subsp. *smithia* (Austin et al. 1989) represent aberrant strains that show variation in some biochemical properties.

This classification scheme is complicated by 2 observations. First, repeated isolations of *Aeromonas salmonicida* subsp. *masoucida* and subsp. *smithia* have not been reported since their initial description (Wiklund & Dalsgaard 1998). Second, there have been many reports describing isolations of *A. salmonicida* from chronic ulcerative diseases found in both salmonid (Evelyn 1971, Paterson et al. 1980) and non-salmonid species including carp (Bootsma et al. 1977), minnow (Hastein et al. 1978), goldfish (Elliot & Shotts 1980, Whittington et al. 1987), Atlantic cod (Cornick et al. 1994), eel (Kitao et al. 1985), sand-eels (Dalsgaard & Paulsen 1986), pike (Wiklund 1990), flounder (Wiklund & Bylund 1991, 1993) and turbot (Pedersen et al. 1994). These isolates are termed atypical *A. salmonicida* and differ in various phenetic traits including colony morphology, growth intensity and temperature range, a

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requirement for salt (1% w/v sodium chloride), fermentation of carbohydrates, amino acid decarboxylation, and oxidase reaction (Austin & Austin 1993, Wiklund & Dalsgaard 1998). Clinical disease signs are often characterized by cutaneous ulceration in comparison to the general septicaemia of typical salmonid furunculosis. The significance of this atypical group of *A. salmonicida* isolates and their expanded host-range with respect to the health of both feral and cultured fish stocks is unclear. However, infections by atypical strains are a cause of increasing economic concern in aquaculture (Austin & Austin 1993), and epizootic infections of wild fish stocks have also been reported (Wiklund et al. 1994). Finally, their position in terms of the current taxonomy of *A. salmonicida* is unresolved.

Characterization of genomes is a useful method for both taxonomic and epizootiological studies of bacterial pathogens, and has been applied to *Aeromonas salmonicida*. DNA:DNA reassociation analysis (Belland & Trust 1988), plasmid profiling (Nielsen et al. 1993), ribotyping (Nielsen et al. 1994), DNA probes (Gustafson et al. 1992, Hiney et al. 1992), restriction endonuclease analysis (McCormick et al. 1990), random amplified polymorphic DNA analysis (Hanninen et al. 1995, Miyata et al. 1995), and pulsed-field gel electrophoresis (PFGE) (Umelo & Trust 1998) have concentrated on *A. salmonicida* subsp. *salmonicida*, revealing this group to be an extremely homogeneous taxon. Only the DNA:DNA reassociation (Belland & Trust 1988) and PFGE (Umelo & Trust 1998) analyses included atypical strains (15 and 16 strains respectively), and these reports concluded that the atypical strains are genetically diverse when compared to typical strains.

Here we report a genotypic analysis of 39 strains of atypical *Aeromonas salmonicida* in comparison with 17 typical strains and the type strains for 3 *A. salmonicida* subspecies (subsp. *smithia* was unavailable). Initially, all strains were examined by screening with a putative *A. salmonicida* species-specific oligodeoxynucleotide DNA probe. Subsequently, the strain collection along with the type *A. salmonicida* strains were analyzed using the random amplified polymorphic DNA (Berg et al. 1994) and PFGE (Schwartz & Cantor 1984) techniques. Finally, the genetic similarities between both the typical and atypical strains and the diversity encompassed by species *A. salmonicida* were evaluated.

MATERIALS AND METHODS

***Aeromonas salmonicida* strains.** *Aeromonas salmonicida* type strains were obtained from the National Collection of Industrial and Marine Bacteria (NCIMB), Aberdeen, Scotland, and the strain designations are listed in Table 1. Typical and atypical *A. salmonicida* strains were collected and the strain designations along with host data and location of isolation (where known) are also listed in Table 1. All typical strains were cultivated in tryptone soya medium (Oxoid, Hampshire, UK) at 22°C for 2 d. All atypical strains were cultivated on either nutrient agar (Oxoid), or columbia base (Oxoid) supplemented with 7% human blood, and incubated at 22°C for between 2 and 5 d.

Genomic DNA extraction. Cells were harvested from 10 ml cultures by centrifugation, 8500 × *g* for 10 min (Centrifuge 5416, Eppendorf, Hamburg, Germany), and genomic DNA extraction was performed using a modification of a previously described method (Ausubel et al. 1992). Briefly, the bacterial pellets were resuspended and washed in 1 ml of sterile H_2O, followed by centrifugation for 5 min at 9650 × *g* (MSE Micro Centaur, Sanyo, UK) and resuspension in 400 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The cells were lysed by the addition of 200 µl of lysozyme (10 mg ml^{-1}) (Boehringer Mannheim GmbH, Germany) and incubation at 37°C for 60 min. The preparation was then incubated for 10 min with 40 µl of Proteinase K (10 mg ml^{-1}) (Boehringer Mannheim) at room temperature, followed by the addition of sodium dodecyl sulphate (SDS) to a final concentration of 1.0% and incubation at room temperature until the preparation was clear. For the atypical strains, the preparation was further incubated at 60°C for 5 min to ensure complete lysis. Eighty µl of 0.5 M EDTA were added, mixed by gentle agitation and the solution was deproteinised by sequential phenol and chloroform-isoamyl alcohol (24:1 v/v) extraction. The genomic DNA was precipitated in ethanol and resuspended in TE buffer. The DNA concentration was estimated by visual comparison with standard DNA size markers after electrophoresis through a 1% agarose (BioGene Ltd, Cambridge, UK) TAE gel stained with 0.5 µg ml^{-1} ethidium bromide (Sigma Chemical Co., St Louis, MO, USA).

16S rRNA gene analysis. The PCR primer designations, sequences, and reference positions on alignments of 16S rRNA sequences are EB, 5'-GAGTTTGATCCTGGCTCAG-3', (bases 3–25) and UN, 5'-ACGGNWACCTTGTTACGAGTT-3' (bases 1423–1402) (standard IUPAC nomenclature: N is G, A, T, or C; W is A or T). Both PCR primers have been described as specific for most bacterial species (Lane 1991). The 50 µl reaction contained 10× KCl buffer (Biotaq, Bioline Ltd, UK) including 1.5 mM $MgCl_2$, 200 µM dNTPs (Pharmacia, Uppsala, Sweden), 1 U *Taq* DNA polymerase (Biotaq, Bioline Ltd), 170 pmol of primer EB, 157 pmol of primer UN and 20 ng of genomic DNA, overlayed with 30 µl of mineral oil. Negative controls containing no target DNA were included. The reactions were then amplified using a

TRIO-thermoblock thermocycler (Biometra GmbH, Göttingen, Germany) through 30 cycles of 1 min at 94°C, 1 min at 52°C and 1 min at 72°C followed by a final extension of 5 min at 72°C, and then held at 15°C. Five µl aliquots of the amplification products were analyzed by electrophoresis through 1% agarose (BioGene) TAE gels stained with 0.5 µg ml^{-1} ethidium bromide (Sigma). Slot blot hybridization analysis using the *Aeromonas salmonicida* specific oligodeoxynucleotide (bases 469–486 of *A. salmonicida* subsp. *salmonicida* 16S rRNA gene) was performed by end labelling 100 ng of the DNA probe, 5'-GGCGCCTAATACGTGTCA-3', with 50 µCi (γ-32P) ATP (DuPont NEN, Hitchin, UK) and 1 U T4 polynucleotide kinase (Promega, Madison, WI, USA). 16S rRNA gene PCR products were denatured by addition of a half volume of 1 M NaOH and incubation at 55°C for 5 min followed by neutralization by addition of an equal volume of 1 M HCl prior to application onto a nylon membrane (Nytran, Schleicher & Schuell, Dassel, Germany) with a 96-well slot blot apparatus (Schleicher & Schull). Standard hybridization (6× SSC, 1× Denhardt's solution, 0.1% SDS) was performed for 8 h at 51°C. The filter was then washed twice in 2× SSC for 15 min at room temperature, 1× SSC for 15 min at room temperature, and finally 0.1× SSC for 15 min at 40°C before autoradiography.

Random amplified polymorphic DNA (RAPD). The 3 previously described RAPD primers (Caetano-Anolles et al. 1991) used in the study were H1, 5'-TGCCGAGCTG-3'; H2, 5'-AGTCAGCCAC-3'; and H3, 5'-CGCGCCGG-3'. Amplification was performed using a hot bonnet thermocycler (Omnigene TR3 CM220, Hybaid Ltd, Middlesex, UK) with no overlay of mineral oil. The 50 µl reaction contained 10× NH_4 buffer (Bioline Ltd), 4.0 mM $MgCl_2$, 200 µM of dNTPs (Pharmacia), 2 U *Taq* DNA Polymerase (Bioline Ltd), 60 to 80 pmol of primer and 20 ng of genomic DNA. Negative control reaction mixtures lacking DNA template were also included. Amplification conditions were

Table 1. Strains of *Aeromonas salmonicida* examined in this study. In parentheses: c, isolated from cultivated host; w, isolated from wild host; a, isolated from host in aquarium; u, host status unknown; A, growth on non-supplemented media; B, growth on supplemented media only

Type strains	
NCIMB 1102	*A. salmonicida* subsp. *salmonicida*
NCIMB 1110	*A. salmonicida* subsp. *achromogenes*
NCIMB 2020	*A. salmonicida* subsp. *masoucida*
Typical strains	
Isolate	Source
SRT480	Atlantic salmon *Salmo salar*, Ireland (c)
0299/M	Atlantic salmon *Salmo salar*, Ireland (w)
BMB2	Atlantic salmon *Salmo salar*, Scotland (c)
BMA1	Atlantic salmon *Salmo salar*, Scotland (c)
MT1014	Brown trout *Salmo trutta*, Norway (u)
129/91	Salmonid (species unknown), Norway (u)
028	Brown trout *Salmo trutta*, USA (u)
80204	Atlantic salmon *Salmo salar*, New Brunswick, Canada (c)
SS70.1	Coho salmon *Oncorhynchus kitsutch*, USA (u)
95063	Atlantic salmon *Salmo salar*, Nova Scotia, Canada (c)
BC-7	Atlantic salmon *Salmo salar*, British Columbia, Canada (c)
1018	Atlantic salmon *Salmo salar*, Quebec, Canada (c)
65-R	Salmonid (species unknown), France (u)
810	Atlantic salmon *Salmo salar*, Quebec, Canada (c)
BJ	Atlantic salmon *Salmo salar*, New Brunswick, Canada (c)
NS4	Atlantic salmon *Salmo salar*, Nova Scotia, Canada (c)
A450	Brown trout *Salmo trutta*, France (u)
Atypical strains	
Isolate	Source
M-1	Masou salmon *Oncorhynchus masou*, Japan (u, A)
86-316	Ling cod *Ophiodon elongatus*, British Columbia, Canada (w, A)
F661-2/89	Atlantic salmon, *Salmo salar*, Norway (c, A)
81377	Atlantic cod *Gadus morhua*, Canada (w, A)
87048	Atlantic salmon, *Salmo salar*, New Brunswick,Canada (w, A)
F1542-2	Atlantic salmon *Salmo salar*, Norway (u, A)
K3	Brook trout *Salvelinus fontinalis*, Newfoundland, Canada (w, A)
88301	Tom cod *Gadus microgadus*, New Brunswick, Canada (w, A)
94504	Tom cod *Gadus microgadus*, New Brunswick, Canada (w, A)
94326-2	Tom cod *Gadus microgadus*, New Brunswick, Canada (w, A)
94326-4	Tom cod *Gadus microgadus*, New Brunswick, Canada (w, A)
94326-5	Tom cod *Gadus microgadus*, New Brunswick, Canada (w, A)
Fin3	Brown trout *Salmo trutta*, Finland (u, A)
143/70	Atlantic salmon *Salmo salar*, Scotland (u, A)
MT373	Atlantic salmon *Salmo salar*, Scotland (u, A)
MT194	Atlantic salmon *Salmo salar*, Scotland (u, A)
MT675	Atlantic salmon *Salmo salar*, Scotland (u, A)
88165	Atlantic salmon *Salmo salar*, Newfoundland, Canada (c, B)
NFD'95	Arctic charr *Salvelinus alpinus*, Newfoundland, Canada (c, B)
87089	Atlantic salmon *Salmo salar*, New Brunswick, Canada (c, B)
86663	Atlantic salmon *Salmo salar*, New Brunswick, Canada (c, B)
87480-an	Atlantic salmon *Salmo salar*, Nova Scotia, Canada (w, B)
87445-1	Atlantic salmon *Salmo salar*, Canada (c, B)
87445-2	Atlantic salmon *Salmo salar*, Canada (c, B)
87445	Atlantic salmon *Salmo salar*, Nova Scotia, Canada
94450	Atlantic salmon *Salmo salar*, Nova Scotia, Canada
91518	Eel *Anguilla anguilla*, Nova Scotia, Canada (w, B)
91549	Eel *Anguilla anguilla*, Nova Scotia, Canada (w, B)
1618.92	Eel *Anguilla anguilla*, New Brunswick, Canada (w, B)
Fin5	Brown trout *Salmo trutta*, Finland (u, B)
FP9	Unknown host, England (u, B)
Fin6	Brown trout *Salmo trutta*, Finland (u, B)
Fin7	Brown trout *Salmo trutta*, Finland (u, B)
Fin8	Brown trout *Salmo trutta*, Finland (u, B)
Fin9	Brook trout *Salvelinus fontinalis*, Finland (u, B)
3.111	Goldfish *Carassisus auratus*, USA (a, B)
82-83	Goldfish *Carassisus auratus*, Germany (a, B)
V234/81	Carp *Cyprinus carpio*, Netherlands (u, B)
T5	Flounder *Platichthys flesus*, Finland (w, B)

35 cycles of 10 s at 94°C, 30 s at 37°C, 60 s at 72°C and a final extension of 5 min at 72°C. Five µl aliquots of the amplification products were analyzed by electrophoresis through 1% agarose (BioGene), 1% NuSieve (FMC Bioproducts, Rockland, ME, USA) TAE gels stained with 0.5 µg ml^{-1} ethidium bromide and visualized over UV using the Bio-Profil imaging system (Vilber Lourmat, Marne La Vallee, France). The 100 bp DNA ladder (Life Technologies, Gibco BRL, Renfrewshire, UK) standard length DNA markers were included in each electrophoresis.

Pulsed field gel electrophoreses (PFGE). Colonies of each strain were diluted in sterile H_2O until an optical density (OD) at 600 nm of approximately 0.1 was reached. Genomic DNA was prepared in chromosomal grade ultra-pure DNA agarose (Bio-Rad Laboratories, Richmond, USA) as previously described (Cameron et al. 1994). Digestion with restriction enzymes was performed by cutting a slice from 1 DNA-containing agarose plug and incubating for 1 h in the relevant restriction enzyme reaction buffer followed by 4 h with 20 U of restriction enzyme. *Spe*I enzyme and reaction buffer were obtained from New England Biolabs (Hertfordshire, UK) and *Xba*I enzyme and reaction buffer were obtained from Promega. The DNA fragments were then subjected to electrophoresis in a 1% SeaKem agarose (FMC BioProducts) gel submerged in 0.5× Tris-borate-EDTA buffer using a modified contour clamped homogenous electric field (Pulsaphor Plus, Pharmacia). The running conditions were 12 V cm^{-1} at 14°C for 22 h. The pulse times were increased by stepping as follows: 2 s for 5 h, 5 s for 6 h, 9 s for 6 h, and 12 s for 5 h for *Xba*I digestions; and 5 s for 3 h, 9 s for 5 h, 12 s for 5 h, 20 s for 4 h, 25 s for 3 h and 30 s for 2 h for *Spe*I digestions. The concatenated lambda DNA ladder (Pharmacia) was included in each electrophoresis. After electrophoresis, gels were stained in 2 µg ml^{-1} ethidum bromide for 15 min, destained in distilled H_2O for 15 min, and visualized over UV using the Bio-Profil imaging system (Vilber Lourmat).

RAPD and PFGE cluster analysis. The molecular lengths of the RAPD- and PFGE-generated DNA fragments were determined using the Bio-Gene software package (Vilber Lourmat) on the Bio-Profil imaging system. Each strain was then visually coded for the presence (coded 1) or absence (coded 0) of each DNA fragment. The data obtained for all 3 RAPD primers were combined into 1 data matrix which was then analyzed using the inverse of Jaccard's similarity coefficient as previously described (Morgan et al. 1993). A similar analysis was performed on the PFGE profiles generated by both *Spe*I and *Xba*I restriction enzymes. The similarity dendrograms were generated using the UPGMA clustering method of the PHYLIP phylogeny inference package (Felsenstein 1993).

RESULTS

16S rRNA gene analysis

Initially, to confirm that all the atypical strains were members of the species *Aeromonas salmonicida*, their 16S rRNA encoding genes were amplified by PCR and the amplification products were screened with an oligodeoxynucleotide DNA probe designed to specifically target the species *A. salmonicida*. Nucleotide sequences have been described for the 16S rRNA genes of *A. salmonicida* subsp. *salmonicida* (GenBank accession number X71836), subsp. *achromogenes* (X60407), subsp. *masoucida* (X74680), subsp. *smithia* (AJ009859) along with the related aeromonad species, *A. hydrophila* (X74677), *A. sobria* (X74683), *A. media* (X74679), and *Haemophilus piscium* (AJ009860) (Martinez-Murcia et al. 1992, Thornton et al. 1999). A DNA sequence alignment was performed using these sequences and an 18 base oligodeoxynucleotide DNA probe was designed complementary to bases 469 to 486 of the *A. salmonicida* subsp. *salmonicida* 16S rRNA sequence. Under the appropriate hybridization conditions, this DNA probe will only recognise the 16S rDNA sequences of the 4 accredited *A. salmonicida*

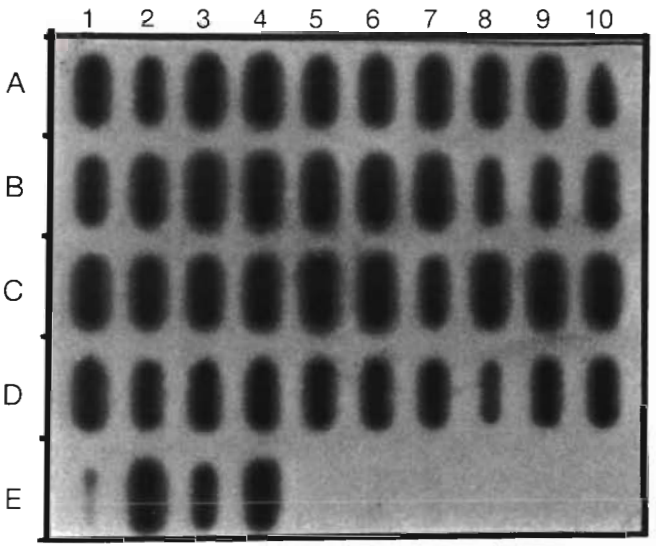

Fig. 1. *Aeromonas salmonicida* specific 16S rDNA slot blot hybridization. Row A: 1, M-1; 2, 86-316; 3, F661-2/89; 4, 81377; 5, 87048; 6, F1542-2; 7, K3; 8, 88301; 9, 94504; 10, 94326-2. Row B: 1, 94326-4; 2, 94326-5; 3, Fin3; 4, 143/70; 5, MT373; 6, MT194; 7, MT675; 8, 88165; 9, NFD'95; 10, 87089. Row C: 1, 86663; 2, 87480-an; 3, 87445-1; 4, 87445-2; 5, 87445; 6, 94450; 7, 91518; 8, 91549; 9, 1618.92; 10, Fin5. Row D: 1, FP9; 2, Fin6; 3, Fin7; 4, Fin8; 5, Fin9; 7, 3.111; 8, 82-83; 9, V234/81; 10, T5. Row E: 1, *A. sobria*; 2, NCIMB 1102; 3, NCIMB 1110; 4, NCIMB 2020; 5, *A. media*; 6, *A. hydrophila*; 7, *E. coli*. Note: Row D, 6 represents 1 atypical strain that was later removed from this study due to subsequent bacterial contamination of stocks

subspecies and *H. piscium* and should not react with other aeromonads. Fig. 1 shows that the 16S rDNA amplification products from the atypical *A. salmonicida* strains (Fig. 1, Rows A, B, C & D) along with the type strains *A. salmonicida* subsp. *salmonicida*, *A. salmonicida* subsp. *achromogenes*, and *A. salmonicida* subsp. *masoucida* (Fig. 1, Row E, Columns 2, 3 & 4 respectively), were recognised by this DNA probe. Other species such as *A. media*, *A. hydrophila* and *E. coli* showed no hybridization (Fig. 1., Row E, Columns 5, 6 & 7 respectively), and *A. sobria* revealed only a low-intensity hybridization signal (Fig. 1, Row E, Column 1). Bases 469–486 of the 16S rDNA of *A. sobria* are the most similar in sequence homology to the respective region of the *A. salmonicida* 16S rDNA with only 2 variant bases. All the atypical *A. salmonicida* strains analyzed in this study reacted with this *A. salmonicida* species-specific DNA probe providing evidence for their membership of this species.

RAPD analysis

Using the RAPD conditions described, the 3 RAPD primers reproducibly amplified 100 DNA fragments that could be scored for similarity between the *Aeromonas salmonicida* strains. Fig. 2 shows the RAPD DNA profiles produced for the typical *A. salmonicida* strains using RAPD primer H1 (Fig 2A), RAPD primer H2 (Fig. 2B) and RAPD primer H3 (Fig. 2C). None of the 3 type *A. salmonicida* subspecies strains showed a pattern identical to one of the other strains with any of the 3 RAPD primers (Fig. 2, lanes 2, 3 & 4 in gels A, B, & C) although almost all the RAPD fragments amplified from *A. salmonicida* subsp. *masoucida* NCIMB 2020 (Fig. 2, lane 4) were contained within the RAPD profile of *A. salmonicida* subsp. *salmonicida* NCIMB 1102 (Fig. 2, lane 2). The RAPD profiles amplified from the typical *A. salmonicida* strains were clearly similar to one another with both the H1 and H2 primers, while differences between some typical strains could be noted with the H3 primer (Fig. 2, lanes 5 to 21 in gels A, B, & C). The RAPD profiles of these typical strains produced by primers H1 and H2 were very similar to those amplified from the type *A. salmonicida* subsp. *salmonicida* strain (Fig. 2A, lane 2; and Fig. 2B, lane 2).

Fig. 3 shows the RAPD DNA profiles produced for many of the atypical *Aeromonas salmonicida* strains using RAPD primer H1 (Fig. 3A), RAPD primer H2 (Fig. 3B) and RAPD primer H3 (Fig. 3C). It was readily apparent that, regardless of the RAPD primer used, none of the atypical strains produced a RAPD DNA profile similar to any of the type *A. salmonicida* subspecies strains (Fig. 3, lanes 2, 3 & 4 in gels A, B, & C). Also, when the results of all 3 RAPD primers were combined, no 2 atypical strains produced an identical RAPD DNA profile to each other, although similar RAPD DNA profiles among some atypical strains could be seen with individual RAPD primers (Fig. 3A, lanes 9 & 10 or lanes 13 & 14; 3B, lanes 7 & 8, lanes 15 & 16, or lanes 17 to 21; 3C, lanes 19 & 20). While primer H2 resulted in the greatest number of common DNA fragments between various atypical strains, primer H3 produced individual DNA patterns with each strain with few common DNA fragments.

The lengths of the DNA fragments generated by the RAPD primers were determined and the combined RAPD profiles of the typical, atypical and type strains were resolved into numerical matrices and subsequent dendrograms describing genetic similarity. Fig. 4 shows the RAPD-based similarity dendrogram prepared for the typical *Aeromonas salmonicida* strains.

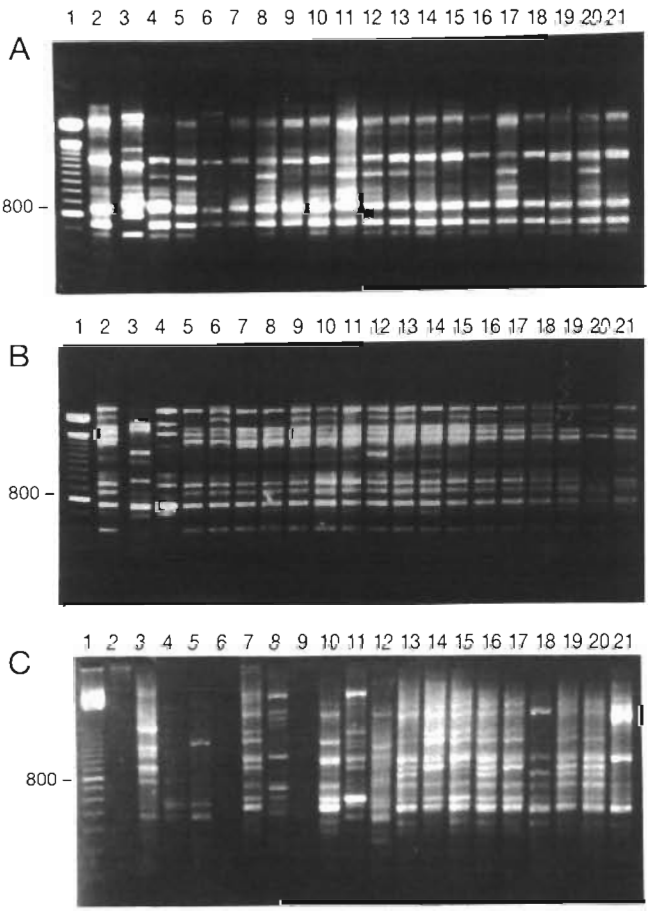

Fig. 2. RAPD profiles of typical *Aeromonas salmonicida* strains. (A) RAPD primer H1; (B) RAPD primer H2; (C) RAPD Primer H3. Lanes: 1, 100 bp DNA molecular size markers (800 bp marker noted on the left); 2, NCIMB 1102; 3, NCIMB 1110; 4, NCIMB 2020; 5, 0229/M; 6, SRT480; 7, MT1014; 8, 129/91; 9, BMB2; 10, BMA1; 11, 028; 12, BC-7; 13, 1018; 14, BJ; 15, SS70.1; 16, NS4; 17, 80204; 18, 95063; 19, 65-R; 20, 810; 21, A450

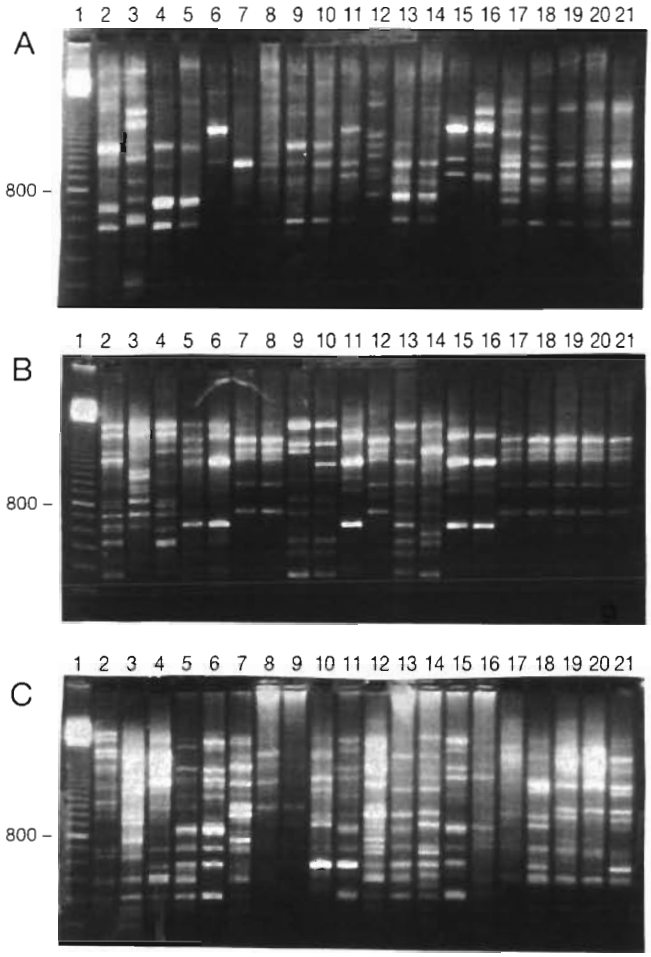

Fig. 3. RAPD profiles of atypical *Aeromonas salmonicida* strains. (A) RAPD primer H1; (B) RAPD primer H2; (C) RAPD Primer H3. Lanes: 1, 100 bp DNA molecular size markers (800 bp marker noted on the left); 2, NCIMB 1102; 3, NCIMB 1110; 4, NCIMB 2020; 5, M-1; 6, 86-316; 7, F661-2/89; 8, 81377; 9, 87048; 10, F1542-2; 11, K3; 12, 88301; 13, 94504; 14, 94326-2; 15, 94326-4; 16, 94326-5; 17, Fin3; 18, 143/70; 19, MT373; 20, MT194; 21, MT675

The most distantly related strain was the type *A. salmonicida* subsp. *achromogenes* strain (NCIMB 1110), which showed less than 70% RAPD-based similarity to all other typical and type strains. The remaining strains were separated into 2 clusters which bifurcated at a point just greater than 70% RAPD-based similarity. The type *A. salmonicida* subsp. *salmonicida* strain (NCIMB 1102) formed a cluster which, with the exception of the French isolates, strains 65-R and A450, contained 8 typical strains isolated in North America. The type *A. salmonicida* subsp. *masoucida* strain (NCIMB 2020) formed a cluster which, with the exception of strain 028 (isolated from brown trout in the USA) contained 6 typical strains isolated in northern Europe.

Fig. 5 shows the RAPD-based similarity dendrograms prepared for the atypical *Aeromonas salmonicida* strains. By comparison with the typical strains, genetic heterogeneity among the atypical strains was readily apparent with 23 of 39 atypical strains showing less than 70% RAPD-based similarity to their closest neighbour. No atypical strain showed a 70% or greater RAPD-based similarity to any of the 3 type *A. salmonicida* subspecies strains. The only well-defined cluster contained 4 atypical strains (143/70, MT675, MT373 and MT194) isolated from Atlantic salmon in Scotland.

PFGE analysis

Initially, 10 atypical *Aeromonas salmonicida* isolates were screened by PFGE using the enzymes *Not*I, *Xho*I, *Xba*I and *Spe*I to determine which restriction endonu-

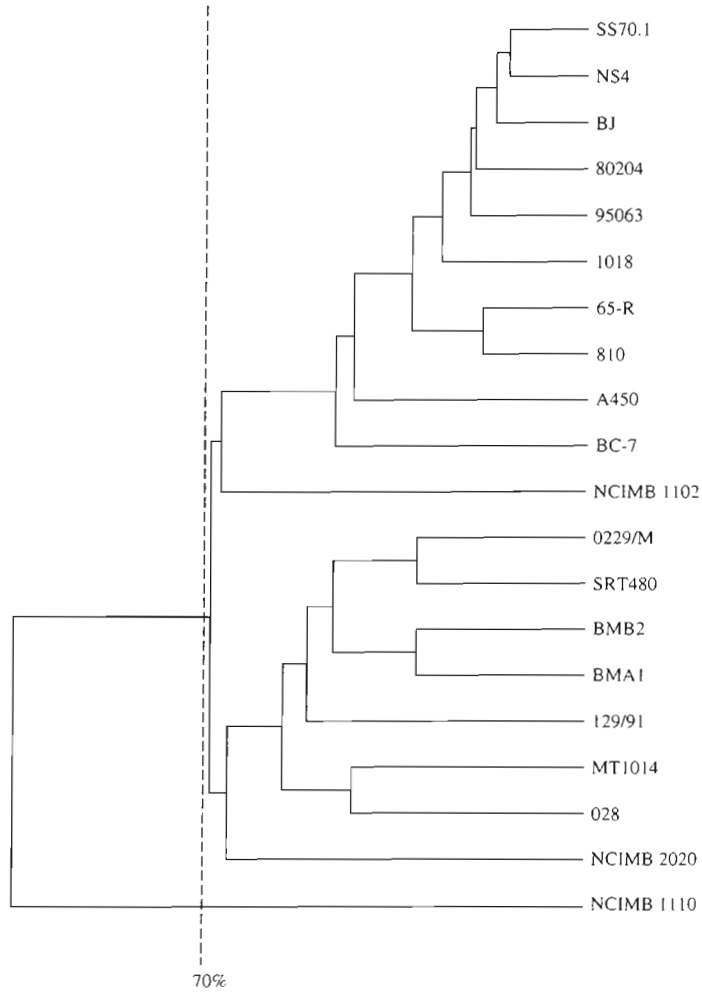

Fig. 4. UPGMA dendrogram describing RAPD-based genetic similarity of typical *Aeromonas salmonicida* strains. A vertical line denoting a hypothetical node of 70% RAPD-based similarity is added for comparative analysis

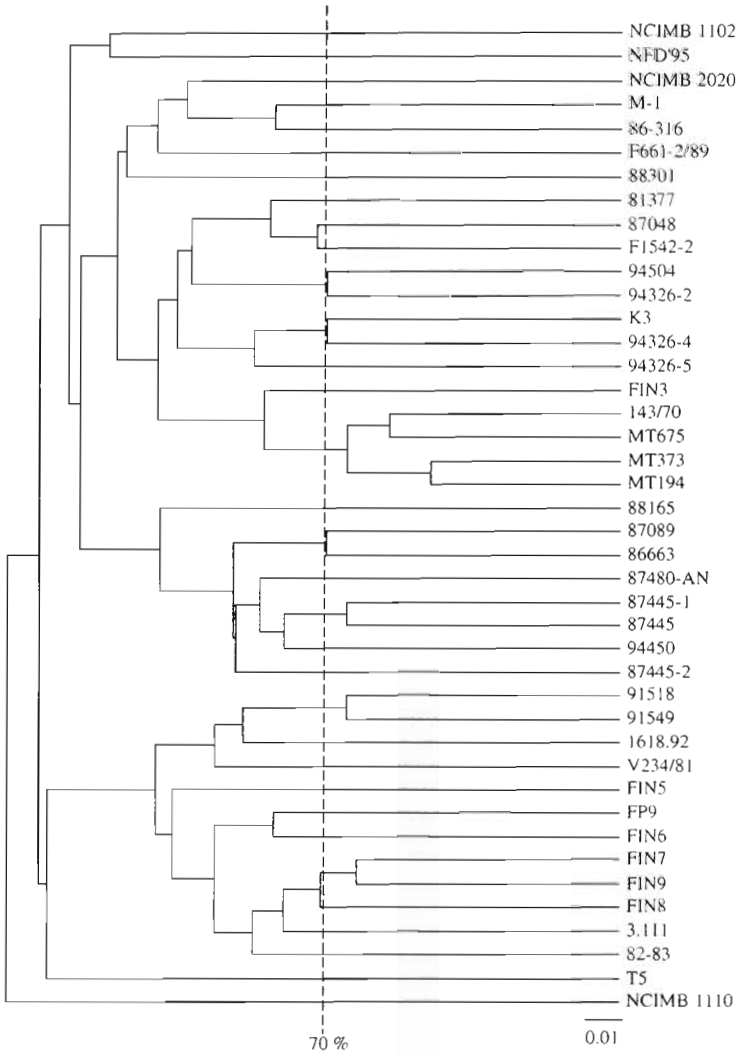

Fig. 5. UPGMA dendrogram describing RAPD-based genetic similarity of atypical *Aeromonas salmonicida* strains. A vertical line denoting a hypothetical node of 70% RAPD-based similarity is added for comparative analysis

cleases produced the most informative DNA profiles for strain comparison. *Not*I and *Xho*I digestion resulted in greater than 30 DNA fragments of less than 150 kb in length, *Xba*I digestion resulted in approximately 40 DNA fragments of between 20 and 250 kb in length, and *Spe*I digestion resulted in approximately 30 DNA fragments of between 20 and 500 kb in length. Based on these results, the endonucleases *Xba*I and *Spe*I were selected for analysis of genome similarity among the *A. salmonicida* strains. Subsequently, however, restriction endonuclease and PFGE analysis could not be performed on 6 of the atypical strains (strains 94504, K3, 94326-4, T5, 86663 and 94450) as the genomic DNA was severly degraded. Despite repeated attempts, this problem could not be solved and it may be that these particular atypical *A. salmonicida* strains contained sufficient endogenous nucleases to degrade the genomic DNA within the agarose plugs prepared for PFGE.

Fig. 6 shows the PFGE DNA profiles produced for the type and typical *Aeromonas salmonicida* strains using the restriction endonucleases *Xba*I (Fig. 6A) and *Spe*I (Fig. 6B). In a similar manner to the RAPD analysis, none of the 3 type *A. salmonicida* subspecies strains showed an identical PFGE pattern to one another (Fig. 6A, lanes 2, 3 & 4) although *A. salmonicida* subsp. *masoucida* (NCIMB 2020) shared many similar-length PFGE DNA fragments with *A. salmonicida* subsp. *salmonicida* (NCIMB 1102). The PFGE profiles found among the typical strains were very similar to one another and identical among the majority of strains (Fig. 6A, lanes 5 to 20; and Fig. 6B, lanes 2 to 18). This PFGE profile was also clearly similar to that of the type *A. salmonicida* subsp. *salmonicida* strain (Fig. 6A, lane 2). Fig. 7 shows the PFGE DNA profiles produced for many of the atypical *A. salmonicida* strains using the restriction endonucleases *Xba*I (Fig. 7A) and *Spe*I (Fig. 7B). Only 2 atypical strains, 87445 and 87445-2, produced an identical PFGE DNA profile with both *Xba*I and *Spe*I. Very similar, but not identical, PFGE DNA profiles could be seen among some of the atypical strains (Fig. 7A, lanes 7, 8, 9 & 10 or lanes 11, 12 & 13; Fig. 7B, lanes 7, 8, 9, 10 & 11 or lanes 12, 13 & 14).

Cluster analysis was performed using the PFGE data in a similar manner to the analysis of the RAPD data. However, no analysis was performed on the typical *Aeromonas salmonicida* strains as there was insufficient variation among the strain profiles, i.e. all the typical strains showed a 90% or greater similarity to each other. With respect to the atypical strains, only DNA fragments greater than 100 kb in length were scored in this analysis. Using both *Xba*I and *Spe*I restriction endonucleases, 50 DNA fragments were scored for similarity between the atypical *A. salmonicida* strains. Fig. 8 shows the PFGE-based similarity dendrogram prepared for the atypical *A. salmonicida* strains. As with the RAPD analysis, genetic heterogeneity among the atypical strains was readily apparent with 21 of 32 atypical strains showing less than 70% PFGE-based similarity to their closest neighbour. No atypical strain showed a 70% or greater PFGE-based similarity to the type *A. salmonicida* subsp. *salmonicida* (NCIMB 1102) or subsp. *achromogenes* (NCIMB 1110) strains, while 3 atypical strains from Finland showed a 75% PFGE-based similarity to the type subsp. *masoucida* (NCIMB 2020) strain.

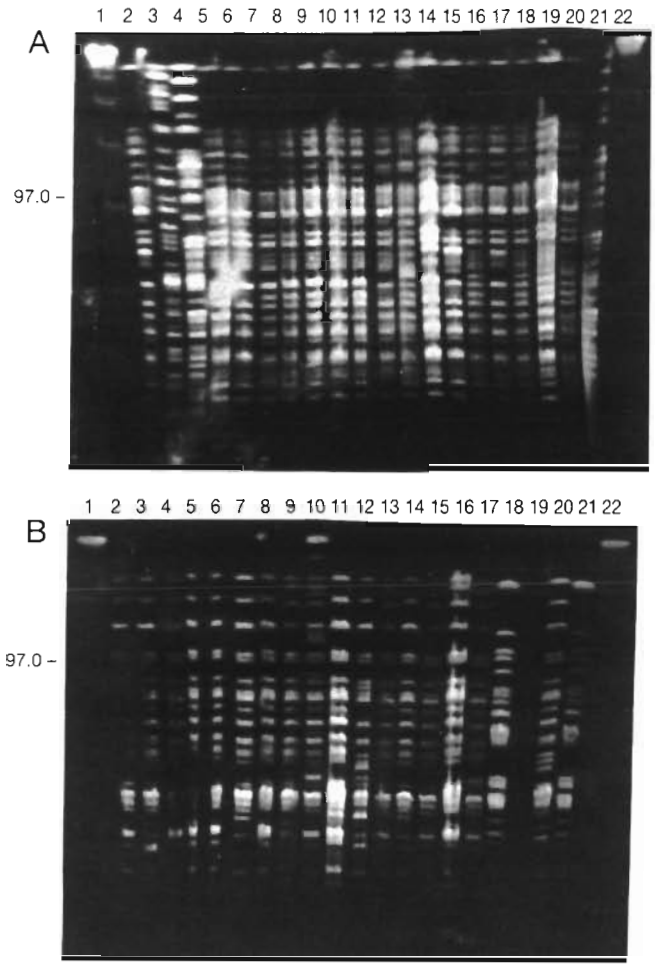

Fig. 6. PFGE profiles of typical *Aeromonas salmonicida* strains. (A) *Xba*I digestions, Lanes: 1 & 22, concatenated phage lambda DNA (48.5 kb) molecular size markers (97 kb marker noted on the left); 2, NCIMB 1102; 3, NCIMB 1110; 4, NCIMB 2020; 5, SRT480; 6, 0299/M; 7, BMB2; 8, BMA1; 9, 129/91; 10, MT1014; 11, 028; 12, BJ; 13, 1018; 14, BC-7; 15, 65-R; 16, 80204; 17, SS70.1; 18, 95063; 19, NS4; 20, 810; 21, A450. (B) *Spe*I digestions, Lanes: 1 & 22, concatenated phage lambda DNA (48.5 kb) molecular size markers (97 kb marker noted on the left); 2, SRT480; 3, 0229/M; 4, BMB2; 5, BMA1; 6, 129/91; 7, MT1014; 8, 028; 9, BJ; 10, 1018; 11, BC-7; 12, 65-R; 13, 80204; 14, SS70.1; 15, 95063; 16, NS4; 17, 810; 18, A450; 19, blank; 20 & 21, non-aeromonad strains

DISCUSSION

This study describes a genotypic analysis of 17 typical and 39 atypical strains of *Aeromonas salmonicida* isolated from a variety of host species and geographic locations and also included type strains representing 3 *A. salmonicida* subspecies. Initially, all the atypical strains were screened with an oligodeoxynucleotide DNA probe designed to specifically recognise members of the species *A. salmonicida*. All the atypical strains reacted positively with this DNA probe reflecting a common genetic relationship between these members of the species *A. salmonicida*. To further resolve this relationship, the strain collection was examined using the RAPD and PFGE techniques.

DNA profiling using the RAPD technique has been shown to be a very useful method for discriminating among strains of a species (Berg et al. 1994). It has been used successfully to demonstrate genetic heterogeneity among field isolates of *Mycoplasma hyopneumoniae*

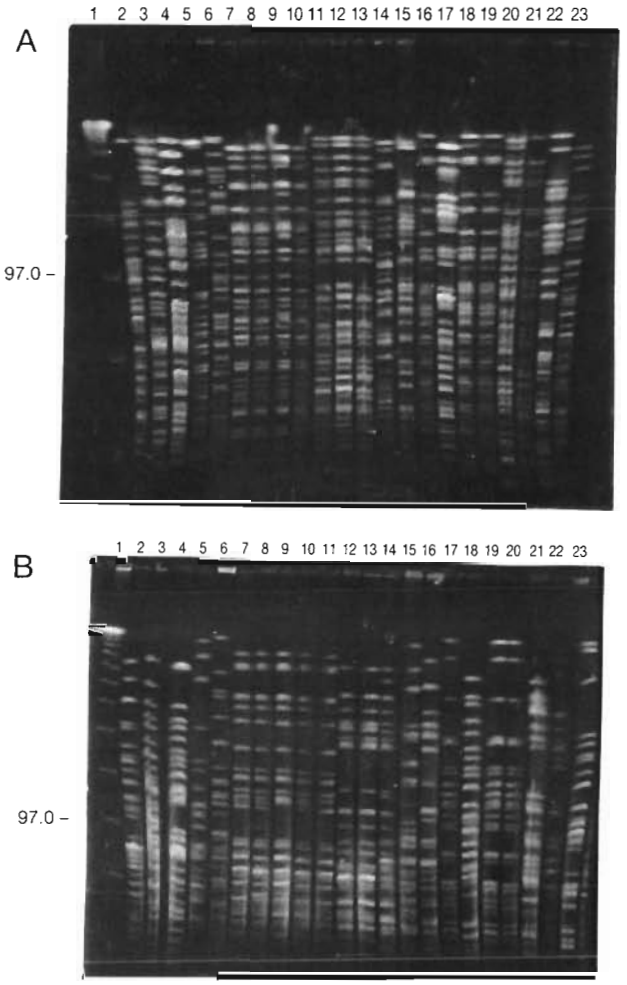

Fig. 7. PFGE profiles of atypical *Aeromonas salmonicida* strains. (A) *Xba*I digestions, Lanes: 1, concatenated phage lambda DNA (48.5 kb) molecular size markers (97 kb marker noted on the left); 2, NCIMB 1102; 3, NCIMB 1110; 4, NCIMB 2020; 5, 88165; 6, NFD'95; 7, 87089; 8, 87480-an; 9, 87445-1; 10, 87445-2; 11, 87445; 12, 91518; 13, 91549; 14, 1618.92; 15, Fin5; 16, FP9; 17, Fin6; 18, Fin7; 19, Fin8; 20, Fin9, 21, 3.111; 22, 82-83; 23, V234/81. (B) *Spe*I digestions, Lanes: 1, concatenated phage lambda DNA (48.5 kb) molecular size markers (97 kb marker noted on the left); 2, NCIMB 1102; 3, NCIMB 1110; 4, NCIMB 2020; 5, 88165; 6, NFD'95; 7, 87089; 8, 87480-an; 9, 87445-1; 10, 87445-2; 11, 91518; 12, 91549; 13, 1618.92; 14, Fin5; 15, FP9; 16, Fin6; 17, Fin7; 18, Fin8; 19, Fin9; 20, 3.111; 21, 82-83; 22, V234/81; 23, T5

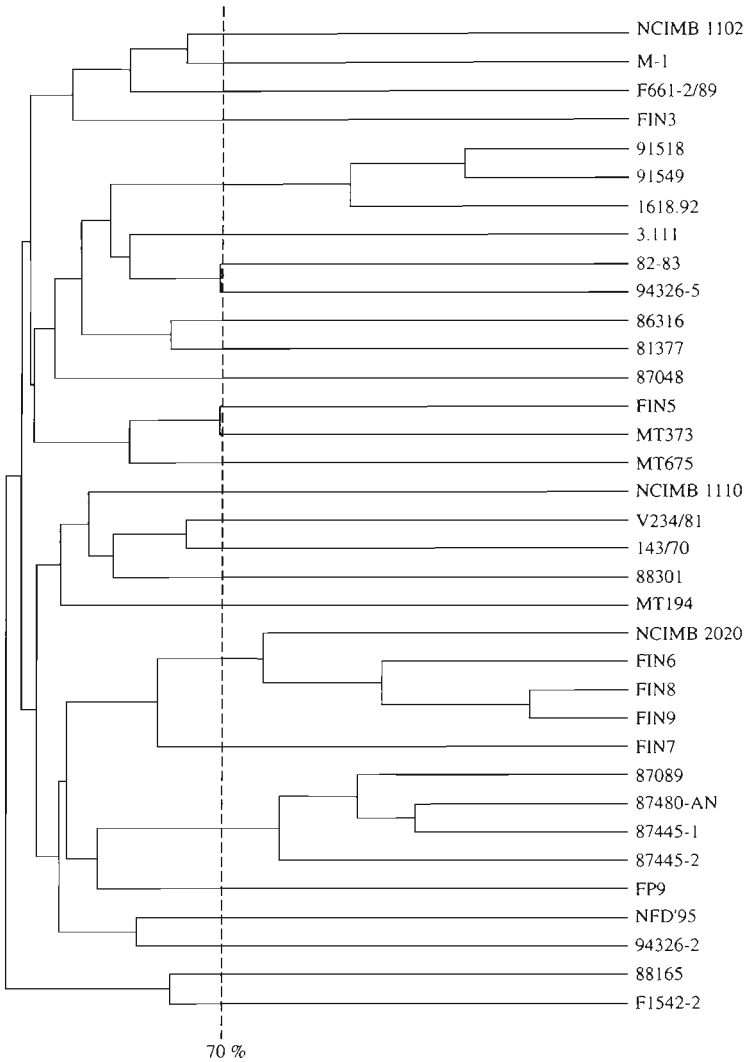

Fig. 8. UPGMA dendrogram describing PFGE-based genetic similarity of the atypical *Aeromonas salmonicida* strains. A vertical line denoting a hypothetical node of 70% PFGE-based similarity is added for comparative analysis

(Artiushin & Minion 1996), to differentiate strains of *Salmonella enteritidis* (Lin et al. 1996) and to examine reports of clonal lineages of *Pseudomonas aeruginosa* associated with human disease (Mahenthiralingam et al. 1996). One previous study describing RAPD analysis of 13 typical *Aeromonas salmonicida* subsp. *salmonicida* strains isolated from the UK, USA and Japan found identical RAPD profiles among all the typical strains (Miyata et al. 1995). A second RAPD study of typical *A. salmonicida* strains also found very homogeneous patterns with few polymorphic loci (Hanninen et al. 1995). In this study, the use of 3 RAPD primers that amplified 100 DNA fragments for use in strain comparison also showed the close genetic relationship between the typical *A. salmonicida* strains. However, unlike the previous RAPD studies of typical *A. salmonicida*, it was possible to resolve the strains into 2 clusters within which the majority of the strains correlated with the geographic area of isolation, i.e. one cluster contained predominantly North American strains (8 of 10 strains) and the second cluster contained predominantly northern European strains (6 of 7 strains). With respect to the atypical *A. salmonicida* strains examined, the RAPD analysis highlighted their genetically heterogeneous nature, with none of the atypical strains showing any close similarity with the type *A. salmonicida* subspecies strains. Within the atypical strains, little clustering of strains correlating with fish host and geographical location were found. The only 2 correlations of note were strains 143/70, MT675, MT373 and MT194, which were all isolated from Atlantic salmon in Scotland, and strains 91518 and 91549, which were isolated from eel in Nova Scotia. The only other eel isolation in the collection, strain 1618-92 from New Brunswick, was placed as their closest neighbour although at less than 70% RAPD-based similarity.

In a similar fashion to RAPD analysis, PFGE of large genomic DNA fragments has been shown to be a valuable typing method for epidemiological investigations (Lin & Johnson 1995), including clonal studies (Skov et al. 1995), and has proved to be highly differentiating when other methods were not suitable (Wong et al. 1996). In this study, the PFGE analysis using 2 restriction endonucleases showed the typical *Aeromonas salmonicida* strains to have greater than 90% similarity to one another and also to the type *A. salmonicida* subsp. *salmonicida* strain (NCIMB 1102). This data supported the results found from I-*Ceu*I digestion and subsequent PFGE analysis of 9 typical *A. salmonicida* strains reported previously (Umelo & Trust 1998). With respect to the atypical strains examined, the PFGE analysis also highlighted their genetic heterogeneity. In this case, the noteworthy correlations with strain provenance included the clustering of the 3 Canadian eel isolates, 91518, 91549 and 1618-92, and a cluster of Canadian Atlantic salmon isolates, 87089, 87480-an, 87445-1 and 87445-2.

While both the RAPD and PFGE analyses showed the close genetic similarity of typical strains and *Aeromonas salmonicida* subsp. *salmonicida*, and the heterogeneity present among atypical strains, there was little correlation between the 2 methods in identifying clusters of similar atypical strains. Primarily, this is likely due to the small number of strains examined and the large genetic variation detected by both methods

in this study. Also, discrepancies between strain typing by RAPD and PFGE have been reported for other bacteria (Struelens et al. 1993, Barbier et al. 1996), and may be explained by the fact that these methods explore different types of DNA polymorphisms. PFGE is based on restriction enzyme polymorphisms, while RAPD analyzes sequence polymorphisms of regions complementary to the amplification primers and also length polymorphisms of the regions amplified. Furthermore, it has been reported that plasmids represent approximately 20% of the *A. salmonicida* genome (Belland & Trust 1988) although less information is available on the plasmid content of atypical *A. salmonicida* strains. These endogenous plasmids may act as targets for RAPD primers, resulting in the amplification of DNA fragments that would interfere with the similarity analysis. In this analysis, we sought to remove interference by plasmids within the PFGE analysis by analyzing only DNA fragments greater than 100 kb in length. However, no such limitation could be made on the RAPD analysis. This may explain some of the differences found between the RAPD and PFGE strain clusters, and it shows that molecular typing methods may have some of the disadvantages, including subjectivity, associated with strain characterisation using phenetic traits. These problems have previously been noted in studies of atypical *A. salmonicida* (Austin et al. 1998). In this study, the RAPD technique produced 100 loci for strain comparison, which was twice the number of scorable loci produced by PFGE. Based on the results, modifications of both the RAPD and PFGE conditions or other genotype methods producing a lower number of comparable loci may be more suitable for the identification of strain clusters of epizootiological significance among the atypical *A. salmonicida* strains. Also, in terms of technique suitability or robustness, problems were experienced with both techniques. Reproducibility of DNA profiles using the RAPD technique proved onerous if different thermocylcers were used, and some of the atypical strains could not be profiled with the PFGE technique.

Finally, in terms of population structure, one previous study using multilocus enzyme electrophoretic analysis differentiated *Aeromonas salmonicida* into 2 clones (Boyd et al. 1994), one containing typical *A. salmonicida* strains and the other containing atypical strains. However, the RAPD and PFGE analyses showed that while the typical *A. salmonicida* strains represented a clonal population, the atypical strains examined showed a far greater genetic heterogeneity and cannot be grouped into a clonal population. The data supported the previous suggestion based on DNA:DNA reassociation analysis that the atypical strains encompass sufficient diversity to possibly warrant further subdivision in the future (Belland & Trust 1988).

Acknowledgements. We are grateful to colleagues for the gifts of strains, and the support of the E.U. AIR Programme (AIR3-CT94-1884) and BioResearch Ireland. B.O'hI. was supported by NUIG and Enterprise Ireland post-graduate fellowships, and is grateful to the European Molecular Biology Organisation (EMBO), and J. L. Larsen, M. N. Skov and K. Pedersen of the Royal Veterinary and Agricultural University, Frederiksberg, Denmark, for their help with PFGE.

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Editorial responsibility: Larry Vaughan, Portsmouth, New Hampshire, USA

Submitted: June 13, 1999; Accepted: November 21, 1999
Proofs received from author(s): January 6, 2000