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# Genetic diversity of atypical *Aeromonas salmonicida* studied by pulsed-field gel electrophoresis

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## SUMMARY

Pulsed-field gel electrophoresis (PFGE) pattern analysis with *Xba*I restriction enzyme was used to study the genetic heterogeneity of 88 atypical *Aeromonas salmonicida* strains which were earlier or during this study characterized phenotypically, by ribotyping (*Cla*I/*Pst*I) and by plasmid profile analysis. The strains of certain ribotypes were also analysed by digestion with *Spe*I. The strains represented different geographic locations: Finland (72 strains), Iceland (5 strains), Norway (5 strains), Sweden (4 strains) and Denmark (2 strains), and they were from 17 fish species during 1981–97. Thirty-one PFGE genotypes found among these strains correlated well with the ribotypes, and in most cases PFGE pattern analysis subdivided ribotypes into several PFGE genotypes, and further within a PFGE genotype into subtypes. *Xba*I and *Spe*I digests produced concordant results. In most cases, PFGE patterns of strains with the same ribotype shared many fragments, suggesting genetic relatedness. PFGE patterns of most Norwegian and Icelandic strains isolated during an approximately 10-year period had the same ribotype and their PFGE patterns shared most fragments, suggesting close genetic relatedness. Moreover, atypical strains of ribotypes B/B and H/H isolated from the same Finnish fish farms had closely related patterns suggesting genetic stability and persistence of these genotypes. Genotype 29 of Achromogenic strains was strongly associated with disease of Finnish arctic char and grayling. PFGE was shown to be a distinguishing method to study the genetic heterogeneity of atypical *A. salmonicida*. This method is applicable to studies of the epidemiology of these infections.

## INTRODUCTION

According to the current taxonomy, psychrophilic *A. salmonicida* includes three subspecies, subsp. *salmonicida*, subsp. *achromogenes*, and subsp. *masoucida* [1]. A fourth subspecies *A. salmonicida* subsp. *smithia*, has been proposed by Austin and colleagues [2]. *A. salmonicida* subsp. *salmonicida* is genetically homogeneous and well characterized [2–5]. In contrast, the taxonomy of the so-called atypical *A. salmonicida*, which includes strains which do not fulfil the criteria for typical *A. salmonicida*, is unclear, because phenotypic and genetic characteristics of this group are heterogeneous, and many isolates have characteristics that differ from those described for subsp. *achromogenes*, *masoucida* or *smithia* [3, 6–11].

The economic importance of infections caused by atypical *A. salmonicida* is increasing, and in some regions infections caused by atypical *A. salmonicida* may predominate over those caused by typical *A. salmonicida* [11–13]. In future, the relative significance of infections caused by atypical isolates will increase, due to the use of effective vaccines against infections

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Table 1. *Ribotypes and PFGE types of atypical* A. salmonicida *strains isolated from certain Finnish fish farms from 1987–97*

| Fish farm | Year of isolation | No. of strains | Ribotype | PFGE type* | Genotype | Fish species (number of strains) |
|---|---|---|---|---|---|---|
| 1. The river Paatsjoki basin | | | | | | |
| Farm T | 1987–93 | 11 | G/G | X14a/S14a | 14a | *Salmo trutta* m. *lacustris* (L.) (6), *Salvelinus alpinus* (L.) (4), *Salmo trutta* m. *trutta* (L.) (1) |
| Farm T | 1989 | 1 | B/B | X7a/S7a | 7a | *Salmo trutta* m. *lacustris* (L.) |
| Farm T | 1990 | 1 | ND | X32 | 32 | *Salvelinus alpinus* (L.) |
| Farm R | 1987–90 | 2 | G/G | X14b/S14b | 14b | *Salmo trutta* m. *lacustris* (L.) (2) |
| 2. The river Kemijoki basin | | | | | | |
| Farm M | 1988 | 2 | F/F | X15, X16 | 15, 16 | *Salmo trutta* m. *trutta* (L.) (2) |
| Farm N | 1990–6 | 6 | F/F | X17, X18 X21 (a, b, c) | 17, 18 21 (a, b, c) | *Salmo trutta* m. *lacustris* (L.) (4), *Salvelinus alpinus* (L.) (2) |
| 3. The river Tornio basin | | | | | | |
| Farm O | 1989 | 1 | B/B | X7b/S7b | 7b | *S. trutta* m. *trutta* (L.) |
| Farm P | 1989–94 | 4 | F/F | X19 (a, b), X22 | 19 (a, b) 22 | *Salvelinus alpinus* (L.) (2), *Salmo trutta* m. *lacustris* (L.) (2) |
| 4. The river Oulu basin | | | | | | |
| Farm A | 1992 | 1 | F/F | 20 | 20 | *Salmo trutta* m. *lacustris* (L.) |
| Farm B | 1993–7 | 9 | H/H | X29a/S29a | 29a | *Salvelinus alpinus* (L.) (5), |
| | | 1 | H/H | X29d/S29d | | *Thymallus thymallus* (L.) (4); *T. thymallus* (1) |
| 5. The river Ii basin | | | | | | |
| Farm C | 1988–91 | 10 | B/B | X7a/S7a | 7a | *Salmo trutta* m. *trutta* (L.) (3), *Coregonus* spp. (4), *Salvelinus alpinus* (L.) (1) *Salmo salar* (1), *Salmo trutta* m. *lacustris* (L.) (1) |
| Farm C | 1995 | 1 | ND | X30 | 30 | *Lota lota* (L.) |
| Farm C | 1995–6 | 2 | U/U | X25/S25 | 25 | *Thymallus thymallus* (L.) (2) |
| Farm D | 1991 | 1 | B/B | X7c/S7c | 7c | *Salmo trutta* f. *trutta* (L.) |
| Farm E | 1994 | 1 | U/U | X24/S24 | 24 | *Thymallus thymallus* (L.) |
| 6. The river Kymi basin | | | | | | |
| Farm K | 1988 | 1 | B/B | X7a/S7a | 7a | *Salmo trutta* m. *lacustria* (L.) |
| Farm L | 1990 | 1 | B/B | X7b/S7a | 7b | *Salmo trutta* m. *lacustris* (L.) |
| Farm U | 1990 | 1 | U/U | X26a/S26a | 26a | *Salvelinus alpinus* (L.) |
| 7. The river Ähtävä basin | | | | | | |
| Farm V | 1990 | 1 | U/U | X26b/S26b | 26b | *Salmo trutta* m. *trutta* (L.) |
| 8. The river Vuoksi basin | | | | | | |
| Farm Z | 1988–9 | 2 | H/H | X29b/S29b | 29b | *Salvelinus alpinus* (L.), *Thymallus thymallus* (L.) |
| Farm Z | 1988 | 1 | H/H | X29c/S29c | 29c | *Leuciscus leuciscus* L. |
| 9. Miscellaneous strains | | | | | | |
| 761 | 1988 | 1 | B/B | X7a/S7a | 7a | *Salmo trutta* m. *lacustris* |
| 3419 | 1988 | 1 | U/U | X28/S28 | 28 | *Oncorhynchus mykiss* (L.) |
| 3505 | 1988 | 1 | U/U | X26c/S26c | 26c | *Oncorhynchus mykiss* (L.) |
| 3409 | 1988 | 1 | U/U | X27/S27 | 27 | *Coregonus* spp. |
| Wild | 1996 | 1 | ND | X30 | 30 | *Lota lota* (L.) |
| Wild | 1996 | 1 | ND | X31 | 31 | *Perca fluvialis* |

* All ribotype B/B, G/T, H/H, and U/U strains were digested with *Xba*I and *Spe*I.

caused by typical *A. salmonicida* subsp. *salmonicida*. Atypical *A. salmonicida* causes infections and infective ulcerations in a wide variety of salmonid and non-salmonid fish [12], but because the taxonomy of the causative organisms as well as the description of disease symptoms associated with them has not been systematic, interpretation of the results from different studies is difficult. For a valid diagnosis of atypical *A.*

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Table 2. *Ribotype and PFGE genotype of atypical* A. salmonicida *strains from the Nordic collection*

| Strain no./year of isolation | Host country | | Ribotype (*Cla*I/*Pst*I) | PFGE pattern type (*Xba*I/*Spe*I)* | Genotype |
|---|---|---|---|---|---|
| 1977/1988 | N† | *Salmo salar* L. | S/S | X6 | 6 |
| 1777/1992 | N | *Anarhichas lupus* L. | R/I | X5 | 5 |
| 909/1981 | N | *Salmo salar* L. | G/T | X1a/S1a | 1a |
| 2013/1981‡ | N | *Salmo salar* L. | G/T | X4/S4 | 4 |
| 2656/1992 | N | *Hippoglossus hippoglossus* (L.) | G/T | X2/S2 | 2 |
| M45/1989‡ | IS[a] | *Salvelinus alpinus* (L.) | G/T | X1b/S1b | 1b |
| S226/1990 | IS | *Salmo trutta* m. *fario* | G/T | X1c/S1c | 1c |
| M283/1989 | IS | *Salmo salar* L. | G/T | X1b/S1b | 1b |
| T233/1991 | IS | *Gadus morhua* L. | G/T | X3/S3 | 3 |
| T3-A1 | IS | *Melanogrammus aeglefinus* (L.) | G/T | X1d/S1d | 1d |
| No. 1 | FIN[a] | *Salmo trutta* m. *trutta* (L.) | B/B | X7d/S7d | 7d |
| No. 2‡ | FIN | *Salmo trutta* m. *lacustris* (L.) | U/U | X23/S23 | 23 |
| No. 3 | FIN | *Thymallus thymallus* (L.) | U/U | X24/S24 | 24 |
| 3–15 | FIN | *Esox lucius* L. | B/B | X7c/S7c | 7c |
| 921203-2/3‡ | FIN | *Platichthys flesus* (L.) | Q | X9 | 9 |
| 6850319 | DK[a] | *Anguilla anguilla* (L.) | TH | X10 | 10 |
| 860613-1/1‡ | DK | *Salmo salar* L. | G/G | X11 | 11 |
| 420/1988‡ | S[a] | *Salmo trutta* m. *fario* (L.) | B/B | X8a/S8a | 8a |
| 261/1989 | S | *Salmo trutta* m. *fario* (L.) | B/B | X8b/S8b | 8b |
| 329/1989‡ | S | *Salmo salar* L. | U/2S | X12 | 12 |
| 298/1989 | S | *Salvelinus alpinus* (L.) | H/T | X13 | 13 |
| *A. salmonicida* subsp. *achromogenes* | | NCMB 1110 | G/T | X1e/S1e | 1e |
| *A. salmonicida* subsp. *masoucida* | | LMG 3782‖ | ND | X33 | 33 |
| *A. salmonicida* subsp. *salmonicida* | | NCMB§ 1102 | D/D | X34 | 34 |

* All ribotype G/T, B/B, H/H and U/U strains were digested with *Xba*I and *Spe*I.
† N, Norway; IS, Iceland; FIN, Finland; DK, Denmark; S, Sweden.
‡ Included in our earlier study [14].
§ National Collection of Marine Bacteria, Aberdeen, Scotland.
‖ Laboratory of Microbiology, University of Gent, Belgium.

*salmonicida* infections in diseased fish and for possible vaccine production, its taxonomy and other characteristics are important to know. Studies on the epidemiology and transmission of the infection require discriminating typing methods.

In our recent study we continued genetic characterization of atypical *A. salmonicida* isolated from various fish species and from different geographic areas collected during several years. The strains were either from a collection of atypical *A. salmonicida* strains that originated from various fish species and represented different phenotypes. These included representatives from Denmark, Norway, Sweden or Finland (the Nordic collection) [8, 6, 14] or they were Finnish strains isolated during 1988–97 from different fish farms located in eight rivers in southern, central and northern Finland. Pulsed-field gel electrophoresis (PFGE) was used for genetic characterization of the strains, and evaluated as a tool for epidemiologic studies.

## MATERIALS AND METHODS

### Bacterial strains

A total of 88 atypical *A. salmonicida* strains were used. Of these, 21 were from the Nordic collection of atypical strains [6, 8, 10, 15], and the others were isolated at the National Veterinary and Food Research Institute, from diseased fish. The latter strains were chosen from a larger collection, and they represented strains isolated during several years (1988–97) from different geographical areas and from various fish species including wild and farmed fish (Table 1). Included were also the reference strains *A. salmonicida* subsp. *salmonicida* NCMB 1102, *A. sal-*

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*monicida* subsp. *achromogenes* NCMB 1110 and *A. salmonicida* subsp. *masoucida* LGM 3782. Phenotypic and genetic characterization of these strains by ribotyping and plasmid profile analysis has been performed earlier [8] except for 39 new strains, which were characterized during the present study by the methods described earlier [8]. Sixteen of the strains were included in our earlier study on the application of PFGE technique in the studies on *Aeromonas* spp. [16]. The strains from the Nordic collection included in our previous study are marked in Table 2. The strains were stored at $-70$ °C before use in the experiments and were grown at 22 °C on blood agar plates for 2 days to confirm their purity.

## PFGE

The bacterial cells were grown in 10 ml of BHI broth (Difco Laboratories, Detroit, MI, USA). Chromosomal DNA for PFGE was prepared as described previously [16], and the methods of Maslow and colleagues [17] were followed. After digestion of DNA in the agar plugs, the DNA fragments were separated with Gene Navigator (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) in 1% agarose gel in 0·5 × TBE (45 mM Tris; 45 mM boric acid; 1 mM EDTA) buffer. Several restriction enzymes were tested in our earlier study [8]. Restriction enzymes *Xba*I and *Spe*I were found to produce 20–40 fragments, and *Xba*I was chosen for further studies. The strains of ribotypes B/B, G/T, U/U and H/H which were shown to have related patterns with *Xba*I, were further analysed with *Spe*I. The DNA fragments produced with *Xba*I and *Spe*I were separated at 200 V for 20 h with ramped pulse times from 0·5 to 18 s. Bacteriophage $\lambda$ concatamers and PFG Mid Range Marker (New England Biolabs) were run in parallel as standard size-markers. At completion of the electrophoresis, the gels were stained with ethidium bromide and photographed.

### Analysis of PFGE patterns

*Xba*I produced 20–30 fragments of molecular sizes from approx. 15–340 kb. *Spe*I digests had molecular sizes from < 15–400 kb. The patterns were analysed by an extensive visual fragment-for-fragment comparison. A pattern was designated a type 1, 2, 3... if it differed by more than seven fragments from other patterns; if patterns were related and differed by 1–7 fragments they were designated subtypes and marked by the letters a, b, c... [18].

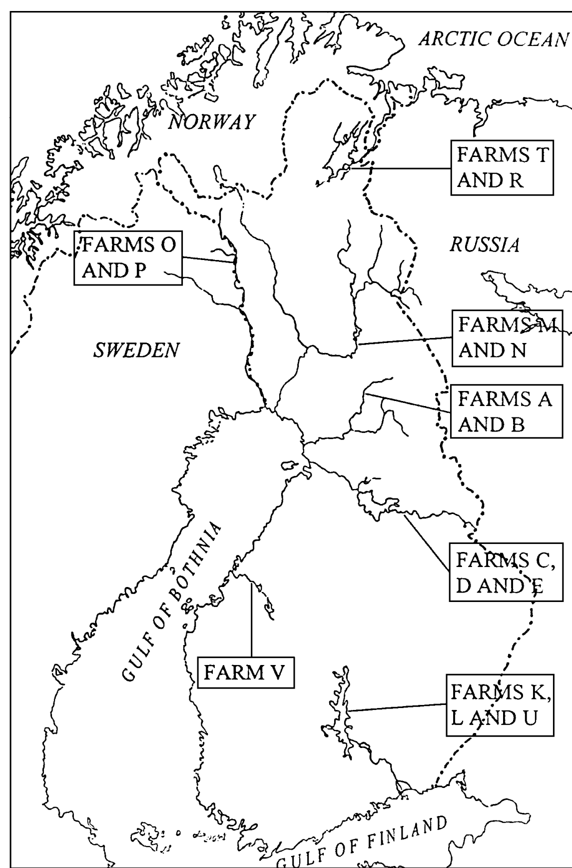


**Fig. 1.** A schematic map of Finland showing the approximate location of the fish farms.

## RESULTS

A total of 33 PFGE genotypes were found among 90 atypical *A. salmonicida* strains including the type strains *A. salmonicida* subsp. *achromogenes* and subsp. *masoucida*. The pattern analysis with *Xba*I (X1, X2, ...) and *Spe*I (S1, S2...) produced concordant results. Combined *Xba*I/*Spe*I patterns were designated as genotypes (Tables 1, 2). The geographic origin, year of isolation, PFGE types and the respective ribotypes of the strains from the Nordic collection are shown in Table 2 and those of additional strains from Finnish fish farms in Table 1. A schematic map shows the locations of most of the studied Finnish fish farms (Fig. 1). Included were also patterns of the strains isolated from diseased fish caught from the surroundings of the fish farms (burbot, dace, perch and whitefish). PFGE typing subdivided all ribotypes that included more than one strain. In most cases, PFGE

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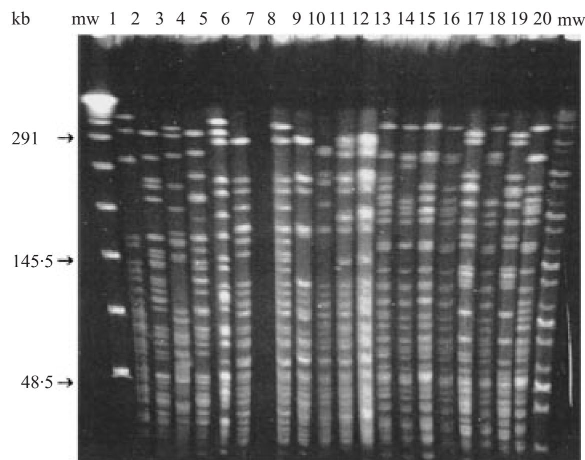


**Fig. 2.** Examples of PFGE patterns of *Xba*I-digested DNA of atypical *A. salmonicida* strains of ribotypes F/F (lanes 2–5), B/B (lanes 6–12), G/T (lanes 13–16, 18), and G/G (lanes 17, 19, 20) showing subdivision of certain ribotypes. Lane 2, strain 3735/1988 (strain number/year of isolation), farm M, pattern X15; lane 3, strain 3723/1/1988, farm M, pattern X16; lane 4, strain 2490/1990, farm N, pattern X17; lane 5, strain 519/1992, farm A, pattern X20; lane 6, Swedish strain 420/1988, pattern X8a; lanes 7–8, Swedish strain 201/1989, pattern X8b; lane 9, strain 2968/1989, pattern X7b; lane 10, strain 3–15, pattern X7c; lane 11, strain 3114/1988, pattern X7a; lane 11, strain 3042/1988, pattern X7a; lane 12, strain 761/1988, pattern X7b; lane 13, Norwegian strain 909/1981, pattern X1a; lane 14, Norwegian strain 2656/1992, pattern X2; lane 15, Icelandic strain M45/1989, pattern X2b; lane 16, Icelandic strain S266/1990, pattern X1c; lane 17, strain 3493/1987, farm T, pattern X14a; lane 18, pattern X1e, NCMB 1102; lane 19, pattern X13a, strain 4424/1994, farm T; lane 20, pattern 11, strain 860613-1/1 from the Faroe Islands; lane 1, type 4, Norwegian strain 1977/1988. Molecular size marker lambda concatamer (48·5 kb) on the left side and selected molecular sizes (kb) are marked on the left.

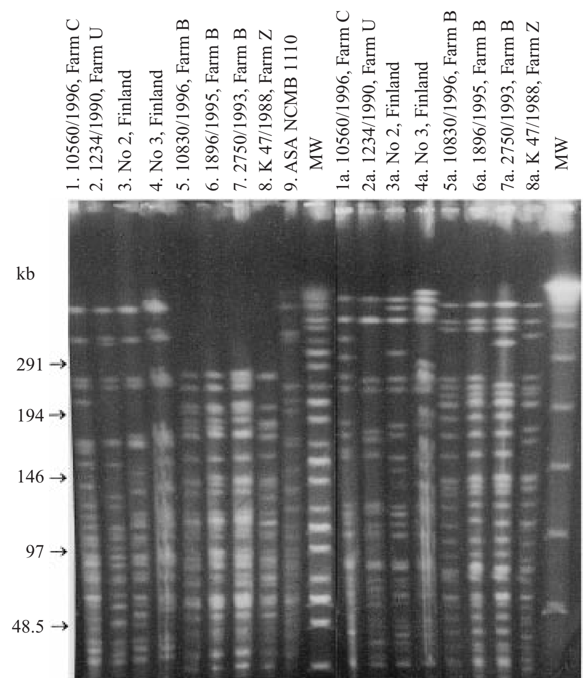


**Fig. 3.** Examples of *Xba*I and *Spe*I patterns of achromogenic strains of ribotype U/U (lanes 1–4, 1a–4a, respectively) and H/H (lanes 5–8, 5a–8a, respectively) showing the close relatedness of the patterns within the ribotypes. Lane 1, pattern 25; lane 2, pattern 26a; lane 3, pattern 23, lane 4, pattern 24; lanes 5, 6, pattern 29a; lane 7, patterns 29d, 29b; lane 9, *A. salmonicida* subsp. *achromogenes* NCMB 1110. Molecular size markers PFG mid range (15 kb) and lambda concatamer (48·5 kb) in the middle and on the left, respectively.

patterns within a ribotype were more closely related to each other than to the patterns within any other ribotype, although all atypical strains shared small fragments. Examples of PFGE patterns are shown in Figures 2–4. PFGE patterns within ribotypes G/T, G/G, B/B, and H/H formed lineages with several subtypes with most shared fragments. The patterns of the ribotypes F/F and U/U strains were more heterogeneous (Table 1).

### Subdivision of ribotypes

PFGE subdivided eight strains of ribotype G/T into four related genotypes 1–4 (Table 2). Genotype 1 included five strains from Norway and Iceland (Fig. 2, lanes 13–16 and Fig. 4, lanes 1–6, 8), and the type strain NCMB 1110 (Fig. 2, lane 18, and Fig. 4, lane 7) and was divided into five closely related subtypes: 1a, 1b, 1c, 1d and 1e which differed from each other by 1–5 fragments (Table 2). The *Xba*I pattern 1e of type strain NCMB 1110 differed by 3–4 fragments from the pattern 1b of Icelandic strains (Fig. 2, lanes 15, 16, 18) and 7 fragments from the Norwegian strain 909/1981. Most strains of ribotype G/T shared most of the fragments and formed a lineage of related patterns. The exception was strain 2013/1981 which differed by most fragments from other ribotype G/T strains (Fig. 4, lane 8). The Norwegian strain 1977/88 from salmon with PFGE genotype 6 shared several fragments with type 1 strains (Fig. 2, lane 1) and similarly, Icelandic strain T233/1991 with genotype 3 shared most fragments with type 1 strains (Fig. 4, lane 6).

Fourteen strains within ribotype G/G had two PFGE patterns, 11 and 14. Two subtypes, 14a and 14b, were from two fish farms T and R from the river Paatsjoki (Table 1, Fig. 1, Fig. 2, lanes 17 and 19, subtype 14a) and a strain with pattern 11 was from *S.*

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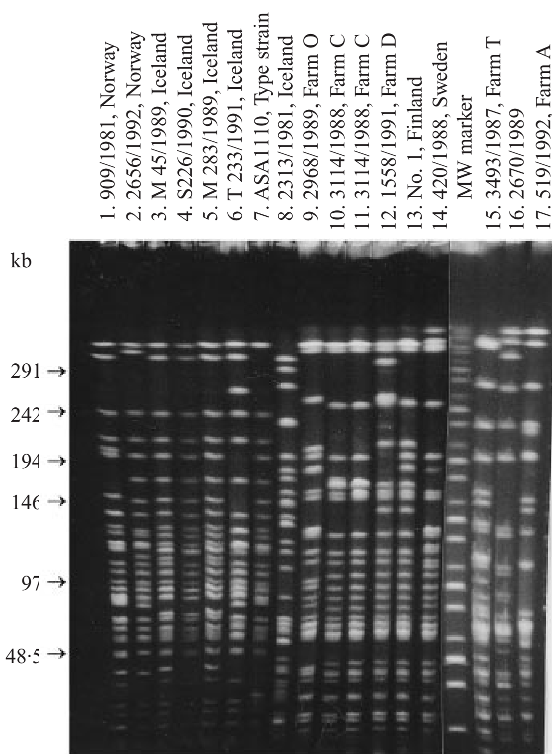


**Fig. 4.** Examples of *Spe*I digests showing relatedness of ribotype G/T strains (lanes 1–8) and ribotype B/B strains (lanes 9–14). Lane 1, pattern S1a; lane 2, pattern S1b; lane 3, pattern S1c; lane 4, pattern S1d; lane 5, pattern S1c; lane 6, pattern S3; lane 7, pattern S1e; lane 8, pattern S2; lane 9, pattern S7b; lane 10, pattern S7a; lane 11, pattern S7a; lane 12, pattern S7c; lane 13, pattern S7d; lane 14, pattern S7a; lane 15, pattern S14a (ribotype G/G); lane 16, pattern S18a (ribotype F/F); lane 17, pattern S20 (ribotype F/F). Molecular size marker is PFG mid range marker (MW).

*salar* (L.) from the Faroe Islands (Table 2, Fig. 2, lane 20).

All 16 Finnish strains within ribotype B/B had closely related PFGE banding patterns differing by 1–4 fragments, and 4 subtypes were found: 7a, 7b, 7c and 7d (Table 1; Fig. 1; Fig. 2, lanes 9–12; Fig. 3, lanes 9–14). Ten Finnish strains isolated during 1988–9, either from whitefish, arctic char or sea trout from fish farm C in the Ii river, were of same genotype 7a and differed by two fragments from the pattern 7c from a strain isolated from sea trout from fish farm D in the same river (Fig. 1, Table 1). Two strains isolated from brown trout in the river Kymi basin (farms K and L) in 1988 and 1990 had patterns 7a and 7b, identical to the strains isolated from the farms C, T and O (Table 1; Fig. 1; Fig. 3, lanes 9–11). PFGE pattern analysis distinguished two Swedish strains of ribotype B/B from the respective Finnish strains (Table 2; Fig. 2, lanes 6, 8; Fig. 3, lane 14). Small fragments (MW < 150 kb) of *Xba*I digests of genotypes 7 and 8 were similar, and many of these shared fragments with ribotype G/T strains (Fig. 2, lanes 6–16).

The ribotype F/F included 13 strains, and these had 8 different PFGE patterns (Table 1; Fig. 1, lanes 2–5; Fig. 4, lanes 16, 17). Eight of the strains were from the Kemijoki river from farms M and N, from sea trout, lake trout or arctic char.

PFGE genotype 29/ribotype H/H included 13 Finnish strains isolated from two farms (B and Z; Fig. 1) from grayling or arctic char (Table 1). These strains had homogeneous PFGE patterns, and this group was divided into four PFGE subtypes; 29a, 29b, 29c and 29d (Fig. 3, lanes 5–8, *Xba*I digests and lanes 5a–8a, *Spe*I digests). Nine of the strains with identical PFGE patterns were isolated from one fish farm in the river Oulu basin during 1993–7 (Table 1). The strain from wild dace showed a highly similar PFGE pattern X29c/S29c (results not shown).

The ribotype U/U included 10 Finnish strains, which had 6 PFGE genotypes; 23–28. Genotype 26 was divided into three subtypes (Tables 1, 2; Fig. 4, lanes 1–4, *Xba*I digests and lanes 1a–4a, *Spe*I digests). The strain 3505 isolated from wild whitefish had genotype X26c/S26c (Table 1).

## DISCUSSION

We used PFGE for genetic characterization of atypical *A. salmonicida* strains with known phenotypes, ribotypes and plasmid profiles determined either during our earlier study [8, 16] or during this study. PFGE pattern-analysis further confirmed the earlier results on the genetic heterogeneity of the atypical *A. salmonicida* group [3, 6, 8, 10, 14, 16], because 32 genotypes were identified among 88 strains which represented various geographic locations and different fish species and were isolated during an extended time-span of several years. Reference strains *A. salmonicida* subsp. *achromogenes* NCMB 1110 and subsp. *masoucida* LGM 3782 had differing genotypes 1e and 33, respectively. Our results indicated that this method is useful in the studies on the molecular epidemiology of atypical *A. salmonicida* infections, because strains without any association mostly generated different patterns, but strains which came from the same fish farm or same location had in most cases identical or closely related patterns. PFGE pattern analysis has been extensively applied in studies on the epidemiology of human and animal infections to trace

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the source of infection and find the route(s) of transmission (17, 18]. PFGE pattern analysis of atypical *A. salmonicida* supported our earlier ribotyping results with *Cla*I and *Pst*I restriction enzymes, because in most cases strains within a ribotype had closely related PFGE patterns, and one PFGE pattern was not distributed among several ribotypes.

All Finnish strains were isolated from diseased fish either from skin lesions or from the kidney. Finnish genotypes 7 and 14, which were identified several times, were isolated from several fish species, suggesting that these genotypes do not have species-specific virulence factors. Several genotypes were identified from sea trout, brown trout, salmon, and arctic char, further indicating that virulence is widely distributed among atypical *A. salmonicida*. On the other hand, PFGE genotype 29 and genotypes 24–28 (ribotype U/U) of achromogenic strains were strongly associated with arctic char and grayling, suggesting a host–pathogen association to be important in the virulence of these particular Finnish genotypes. For example, at the farm C, where several genotypes existed during the study period of 1988–96, but genotype 25 was always associated with grayling. Moreover, the strains isolated from two wild burbots from different locations had similar phenotypic characteristics and identical PFGE patterns, suggesting that genotype 30 is burbot-associated. Because pathological features of the disease caused by different atypical *A. salmonicida* strains in various fish species are not well defined, the interpretation of our results suggests that further pathogenicity studies with pheno- and genotypically characterized strains are needed. Pathogenicity studies in several fish species performed on atypical strains by Austin and colleagues [14] showed that most strains caused disease in two or more fish species, although some of the strains showed host specificity, thus supporting the results of our present study. Gudmundsdottir [15] studied atypical strains from the same Nordic collection as our strains, and found at least three distinct groups based on different characteristics of their extracellular proteolytic enzymes. Her results are at least partly in accordance with our genetic studies, because all our PFGE genotype 1 strains including NCMB 1110 fell into one of her pathogenicity groups.

As expected from our preliminary study of 18 atypical *A. salmonicida* strains [16], PFGE was a better method for distinguishing strains than was ribotyping. PFGE has been revealed to be one of most discriminating methods in the studies of bacterial pathogens [18]. Ribotyping is based on RFLP (restriction fragment length polymorphism) of conserved ribosomal genes [19], and PFGE is based on RFLP of the whole genome with rare-cutting enzymes [17]. Thus, various genetic events, such as recombinations, insertions, deletions, and mutations may be detected by fragment patterns that change. We tested an extensive collection of rare-cutting enzymes and used *Xba*I and *Spe*I. Both enzymes produced a rather high number of fragments which increased the possibility of recognizing genetic heterogeneity. *Xba*I and *Spe*I digests produced concordant results, further suggesting that strains with identical *Cla*I/*Pst*I ribopatterns and identical or closely related PFGE patterns represented genetically related entities. Computerized methods have been extensively used for numerical analysis of PFGE patterns [18]. The high number of relatively small, insufficiently separated fragments (< 48 kb) hindered the use of computerized algorithmic analysis to delineate the relationships of different types. Thus visual comparison of restriction patterns was shown to be the best way in the evaluation of the relatedness.

Although the typing system proposed by Tenover and colleagues [18] for a definition of genotype was originally developed for studies on molecular epidemiology of strains collected during a rather short time-span, it was found to be applicable for *Xba*I/*Spe*I-digested patterns of atypical *A. salmonicida* strains collected during approximately a decade. In most cases, PFGE patterns within a ribotype formed their own lineage of related banding patterns, suggesting genetic clonality for these strains. For example, Norwegian and Icelandic strains of ribotype G/T shared 70–95% of their fragments and formed a lineage among atypical *A. salmonicida*. Three Icelandic strains from arctic char and salmon had the almost identical patterns 1b and 1c, which differed by 4–5 fragments from the Norwegian genotype 1 strains. Without any information on the epidemiology of the infections in Norway or Iceland the interpretation of the significance of the relatedness of the patterns is difficult. According to Gudmundsdottir [12], infections caused by atypical *A. salmonicida* appeared in 1980 in Iceland. An approximate 10-year difference in the isolation dates of related genotypes 2–4 and four subtypes 1a, 1b, 1c, and 1d with same ribotype from six fish species suggests that these strains may have a clonal origin, and that this lineage is persistent [18]. The reference strain NCMB 1110 of Scotch origin isolated in the 1960s [20] had subtype 1e differing from

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Icelandic subtype 1c strains by four fragments and from Norwegian subtype 1a and 2 strains by two or four fragments. These results suggest that the time-difference in the isolation dates cannot alone explain the differences seen in the banding patterns of Norwegian, Icelandic and NCMB 1110 strains. Genotype 7 and 8 strains isolated in Finland and Sweden, respectively, shared many small fragments with strains of genotype 1–4 strains from Norway and Iceland, suggesting genetic relatedness.

Genotype 7a was identified from sea trout, whitefish, arctic char, salmon and brown trout from one fish farm during a 4-year period. The same genotype 7a was also identified in central, northern, and southern Finland. The reason for this may be that during the 1980s, central Finland was an important area for producing fingerlings of salmonids for stocking and rearing throughout the country [13]. Because two Swedish genotype 8 strains from brown trout with the same ribotype B/B as Finnish genotype 7 strains shared most fragments with genotype 7, these genotypes may represent the same genetic lineage. The Bothnian Bay borders both Finland and Sweden, and atypical *A. salmonicida* strains can be transmitted between Finland and Sweden, explaining related genotypes seen in both countries. Certain Finnish and Swedish PFGE types of *A. salmonicida* subspecies *salmonicida* were also shown to be identical, further suggesting transmission of strains between these two countries [5]. Major patterns identified from strains isolated from the river Paatsjoki basin from two fish farms had closely related patterns (14a, 14b). The strains were isolated from brown trout and arctic char during a 6-year time-span, suggesting genetic stability of strains infecting fish on these farms. The river Paatsjoki basin is connected with the Arctic Ocean. One strain isolated from salmon caught in the Faroe Islands with the same ribotype as the strains from Paatsjoki had a different PFGE genotype, revealing the discriminatory power of PFGE typing to distinguish between epidemiologically unrelated strains.

Stability of genotypes is one of the most important criteria in the evaluation of applicability of a technique for use as a typing method [18]. During a large multistate outbreak of EHEC (enterohaemorrhagic *Escherichia coli*) in the USA, PFGE typing of the epidemic strains revealed one-fragment differences between strains [21], suggesting that the genotype had changed during the epidemic. Similarly, a set of closely related PFGE banding patterns of *Campylobacter jejuni* were identified in a batch of chicken meat samples [22]. Atypical *A. salmonicida* strains of genotypes 1, 7, 8, 14, 26 and 29 had several subtypes differing by 1–7 fragments; these were collected over an extended time-span. These results suggest that random genetic rearrangements occur and are visible as changed PFGE patterns, but stable ribotypes.

Genotypes 24–29 were all achromogenic variants of atypical *A. salmonicida* and were in most cases isolated either from Finnish grayling or arctic char. Achromogenic strains have been associated with serious disease in these fish species in Finland [13], and development of a vaccine against this disease is in progress at the National Veterinary and Food Research Institute. These genotypes have persisted, because the oldest isolates in our study were from 1988 and the latest isolations were made in 1996. Our genotyping studies suggest that these fish species are infected with two homogeneous groups of strains, and later studies will indicate whether one vaccine is effective against infections caused by either of these genotypes.

In conclusion, whole-genome analysis using PFGE combined with ribotyping data was shown to be a useful tool to analyse distribution of different genotypes among atypical *A. salmonicida*. The PFGE method was found to be a method better for distinguishing strains than was ribotyping, and it is applicable to studies on the epidemiology of skin ulceration caused by atypical *A. salmonicida*. The association of specific genotypes with pathogenicity requires further studies. Genetic analysis of strains causing infections within one country or in a fish species may help in the development of a vaccine because of the heterogeneity of the atypical *A. salmonicida* group.

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