Vol. 61: 257–262, 2004 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published November 4

NOTE

Genetic diversity among A-proteins of atypical strains of *Aeromonas salmonicida*

Vera Lund*, Helene Mikkelsen

Fiskeriforskning, Norwegian Institute of Fisheries and Aquaculture Research, 9291 Tromsø, Norway

ABSTRACT: The virulence array protein gene A (*vapA*) encoding the A-protein subunit of the surface layer of 23 typical and atypical strains of *Aeromonas salmonicida* from salmonids and marine fish species were sequenced, and the deduced A-protein sequences compared. The A-proteins of the typical *A. salmonicida* ssp. *salmonicida* strains were shown to be identical, while amino acid variability was revealed among A-proteins of atypical strains. The highest amino acid variability appears to be in a predicted surface exposed region and is believed to result in antigenic differences among the atypical strains of *A. salmonicida*.

KEY WORDS: Atypical *Aeromonas salmonicida* · A-protein sequence · Surface A-layer

Resale or republication not permitted without written consent of the publisher

Aeromonas salmonicida ssp. *salmonicida* is referred to as typical *A. salmonicida* and causes furunculosis mostly in salmonid fish, while atypical *A. salmonicida* strains including ssp. *achromogenes*, *masoucida*, *smithia* and *pectinolytica* (Smith 1963, Kimura 1969, Austin et al. 1989, Pavan et al. 2000) cause ulcerative diseases or atypical furunculosis in salmonid, non-salmonid and marine fish. In contrast to typical *A. salmonicida* the atypical strains comprise a heterogeneous group in terms of molecular and phenotypic characteristics (reviewed in Wiklund & Dalsgaard 1998). However, the genetic diversity demonstrated among atypical strains of *A. salmonicida* using various molecular methods (Hänninen & Hirvelä-Koski 1997, Umelo & Trust 1998, Høie et al. 1999, O'hIci et al. 2000, Lund et al. 2002) cannot be related to specific genes. Genes of interest for both virulence and epidemiological studies include the hydrophobic A-protein subunit of the surface A-layer. A correlation between the surface A-layer and virulence has been suggested (Kay et al. 1981, Austin & Austin 1993). However, virulent strains with no detectable A-layer and non-virulent strains possessing an A-layer have been reported (Ellis et al. 1988, Olivier 1990, Austin & Austin 1993). The A-layer is also reported to protect the bacteria from the bactericidal activity of both immune and non-immune serum and probably also from the killing activity of phagocytic cells (reviewed in Secombes & Olivier 1997).

The A-protein is encoded by the virulence array protein gene A (*vapA*). The *vapA* gene of an *Aeromonas salmonicida* ssp. *salmonicida* strain has been sequenced and the deduced A-protein sequence consists of 502 amino acids including a 21 amino acid signal peptide (Chu et al. 1991; GenBank accession number M64655). The A-proteins of 4 atypical strains of *A. salmonicida* were shown to have a sequence similarity of 90 to 94% compared to the typical reference sequence M64655 (Lund et al. 2003b). Most of the A-protein sequence is highly conserved, apart from a region between residues 90 and 180. To assess the genetic diversity of A-proteins, the *vapA* genes of an additional 19 typical and atypical strains of *A. salmonicida* were sequenced.

The *Aeromonas salmonicida* strain collection used in this study comprises type strains of the subspecies *salmonicida*, *masoucida*, *achromogenes* and *smithia*, in addition to atypical strains from spotted wolffish *Anarhichas minor*, cod *Gadus morhua*, halibut *Hippoglossus hippoglossus*, turbot *Scophthalmus maximus*, char *Salvelinus alpinus*, flounder *Platichthys flesus*, and roach *Rutilus rutilus* (Table 1). The *achromogenes* type strains ATCC 33659 and NCIMB 1110 have identical origin according to information from

*Email: vera.lund@fiskeriforskning.no

© Inter-Research 2004 · www.int-res.com

Table 1. *Aeromonas salmonicida*. Strain designation, host data and isolation location

A. salmonicida subspecies	Strain no.	Designation	Host	Country of origin
salmonicida	M64655	A450		
salmonicida	4004-Ass	429-R CAE-144	Atlantic salmon *Salmo salar*	Canada
salmonicida	4010-Ass	NCIMB 1102	Atlantic salmon *Salmo salar*	UK
salmonicida	4012-Ass	MT028	Atlantic salmon *Salmo salar*	Scotland
salmonicida	4014-Ass	88/09/1920	Atlantic salmon *Salmo salar*	Norway
salmonicida	4017-Ass	3329/89	Atlantic salmon *Salmo salar*	Norway
masoucida	4035-Asm	ATCC 27013	Masou salmon *Oncorhynchus masou*	Japan
masoucida	4110-Asm	ATCC 27013	Masou salmon *Oncorhynchus masou*	Japan
achromogenes	4036-Asa	ATCC 33659	Brook trout *Salmo trutta*	UK
achromogenes	4111-Asa	NCIMB 1110	Brook trout *Salmo trutta*	UK
smithia	4109-Assm	NCIMB 13210	Roach *Rutilus rutilus*	UK
atypical	4048	99/92	Spotted wolffish *Anarhichas minor*	Norway
atypical	4065	K-0698	Spotted wolffish *Anarhichas minor*	Norway
atypical	4067	K-9/98	Spotted wolffish *Anarhichas minor*	Norway
atypical	4088	92/09/1777	Spotted wolffish *Anarhichas minor*	Norway
atypical	4128	F 98/01	Spotted wolffish *Anarhichas minor*	Norway
atypical	4129	V-01/1001	Spotted wolffish *Anarhichas minor*	Norway
atypical	4050	104/95	Halibut *Hippoglossus hippoglossus*	Norway
atypical	4097	T-01/0900	Halibut *Hippoglossus hippoglossus*	Norway
atypical	4092	88/09/02778	Turbot *Scophthalmus maximus*	Norway
atypical	4099	93/09/914	Cod *Gadus morhua*	Norway
achromogenes	4102	Olivier;81377	Cod *Gadus morhua*	Canada
achromogenes	4043	117-92	Char *Salvelinus alpinus*	Finland
atypical	4122	26-F-16-4	Flounder *Platichthys flesus*	Finland

the culture collections, but in our strain collection are designated 4036-Asa and 4111-Asa, respectively. Similarly, the *masoucida* subspecies 4035-Asm used in our laboratory and the strain 4110-Asm derived from Institute for Experimental Pathology, Iceland, have a common origin in type strain ATCC 27013 (Table 1).

The A-protein sequences were deduced from the *vapA* gene sequences obtained by primer walking of 1565 bp PCR-products as described by Lund et al. (2003b). The nucleotide sequences of 15 *vapA* genes representing A-protein diversity have accession numbers from AJ749879 to AJ749893. Multiple alignment of the predicted protein sequences was produced using Clustal X (1.8) (Thompson et al. 1997), and an unrooted similarity dendrogram based on sequence comparison was derived by the bootstrap neighbour joining method (Felsenstein 1985).

The similarity of the A-protein sequences is at least 92% at the nucleotide level and 85% at the amino acid level, and the number of amino acids varies from 501 to 507. Multiple alignment of the A-protein sequences reveals highest variability in amino acids in the region between residues 90 to 180 (Fig. 1), where amino acids are substituted, inserted or deleted compared to the reference sequence M64655. However, the C-terminal region of approximately 140 residues is highly conserved. For unknown reasons, all sequenced strains have phenylalanine (F) and leucine (L) in position 471 and 472 compared to leucine (L) and valine (V), respectively, in the reference sequence.

The A-protein sequences of the 5 *Aeromonas salmonicida* ssp. *salmonicida* strains sequenced in this study are identical, except for strain 4012 (AJ749882) that has the glutamate (E) in position 124 substituted with lysine (K).

The *vapA* nucleotide sequences of the *masoucida* strains 4035-Asm and 4110-Asm, both originating from ATCC 27013, are identical, including a deletion of a 56 nucleotide sequence (AJ749883). Whether the culture

Fig. 1. Multiple alignment of A-protein sequences deduced from nucleotide sequences of virulence array protein gene A (*vapA*) of *Aeromonas salmonicida* strains from various fish species, including the subspecies strains *salmonicida* (4004-Ass, 4010-Ass, 4012-Ass, 4014-Ass and 4017-Ass), *masoucida* (4110-Asm), *achromogenes* (4036-Asa and 4111-Asa) and *smithia* (4109-Assm). The A-protein sequence of a typical *A. salmonicida* strain (GenBank M64655) is used as reference, and residues identical to the reference sequence have been replaced with dots (.). In the consensus line positions with a fully conserved residue, or strong or weaker groups fully conserved are indicated with (*), (:) or (.), respectively. Due to amino acid insertions and deletions in the atypical strains, the residue positions are different from those used in GenBank. The C-terminal end of the A-protein of strains 4043, 4048, 4050 and 4065 was not sequenced. sw.fish: spotted wolffish

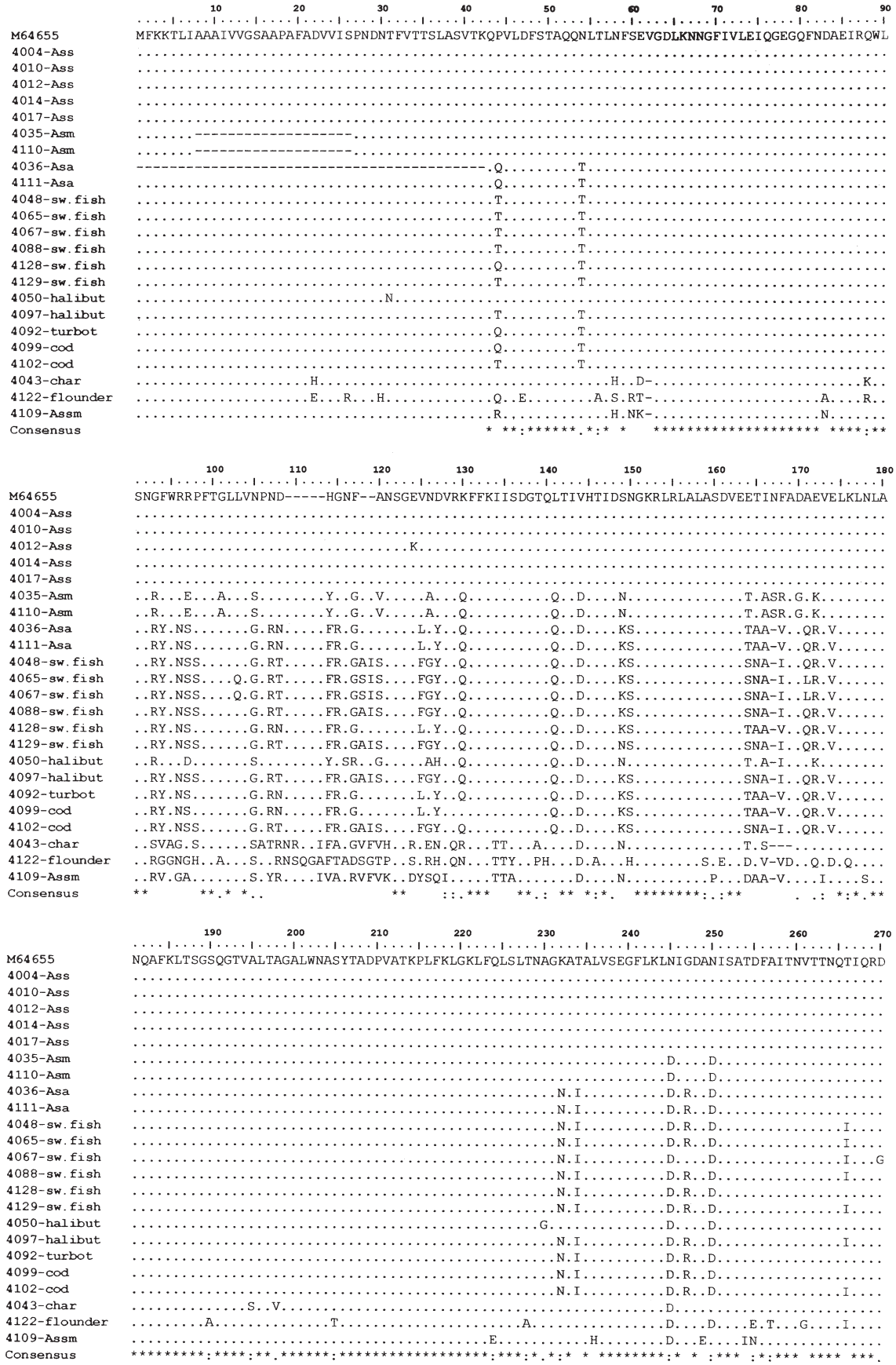

Fig. 1 (continued on next page)

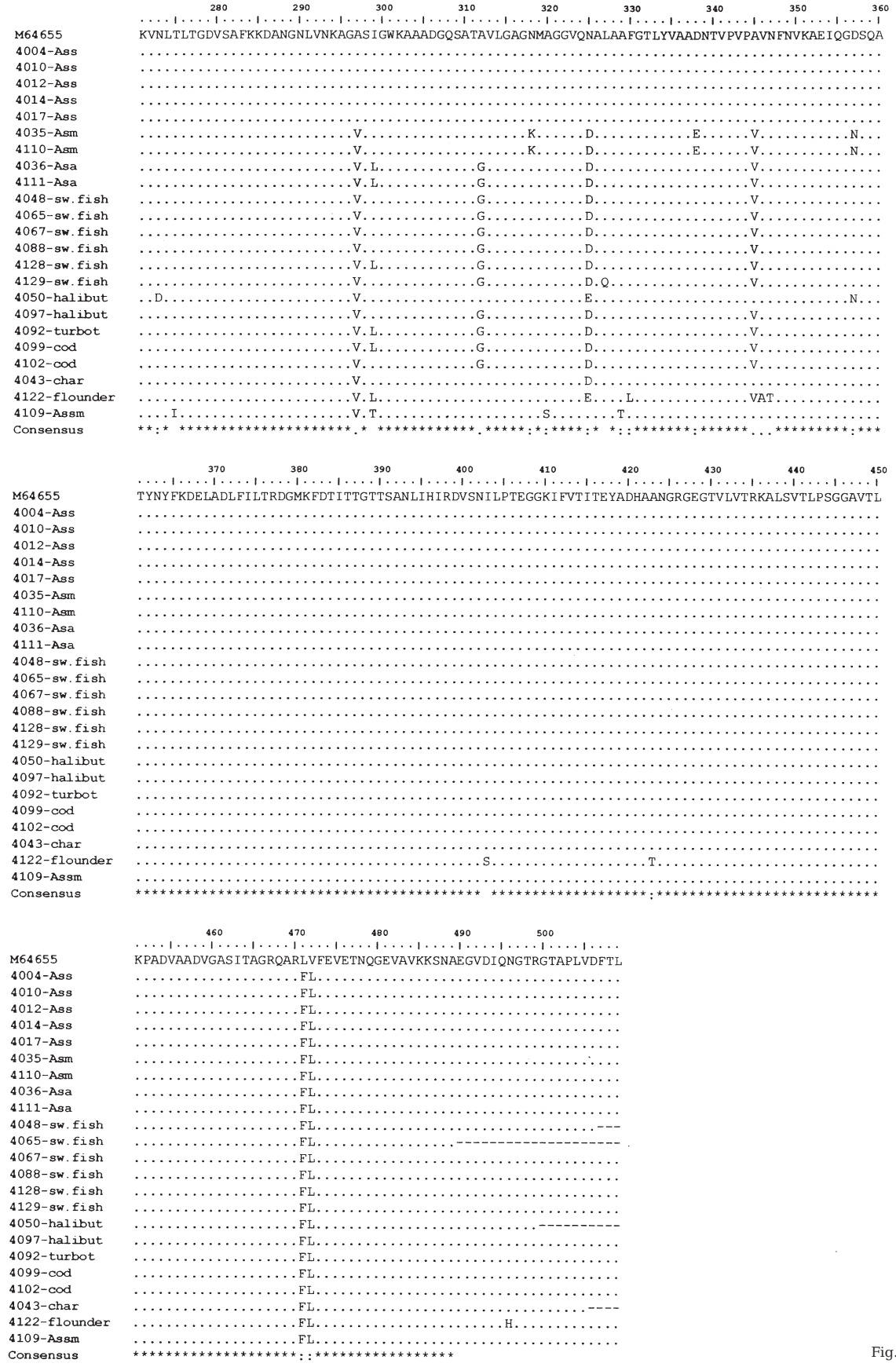

Fig. 1 (continued)

collection contains a *masoucida* type strain with a deletion in the *vapA* gene or identical deletions have occurred in 2 different laboratories, is unknown. These strains do not express the surface A-layer as demonstrated by lack of binding of A-protein-specific antibodies to cell lysate on Western blot (not shown). The lack of A-layer is probably a result of the translation being out of the reading frame downstream of the deletion. For comparison, the deduced A-protein sequence is adjusted to correct the reading frame, resulting in 19 residues missing in the N-terminal region of the protein (Fig. 1). In addition to the deletion, the *masoucida* strains differ from the reference sequence M64655 by having 28 amino acids substituted.

A deletion probably starting upstream of the start codon was detected in the *vapA* gene of the 4036-Asa of the *achromogenes* type strain (ATCC 33659, AJ749888), and as in the *masoucida* strains, this strain does not express the surface A-layer. In spite of the identical origin, the deletion is not present in the *achromogenes* strain 4111-Asa (NCIMB 1110, AJ749889), and it is unknown when the deletion has occurred. Apart from the deletion, the *achromogenes* strains have identical nucleotide and amino acid sequences. However, they differ from the reference sequence M64655 in that they have 33 amino acids substituted and, as in most of the atypical strains, the asparagine (N) in position 167 is deleted. The A-proteins of the *smithia* type strain 4109-Assm and the atypical strains from char (4043) and flounder (4122) have in addition the valine (V) in position 62 deleted. Furthermore, several of the atypical strains have from 2 to 7 amino acids inserted between positions 108 to 120.

Although the highest amino acid variability is detected between residues 90 and 180, most of the atypical strains also have an additional 10 to 15 amino acids substituted outside this region. Compared to the reference sequence M64655, half of the substitutions involve changes between hydrophilic and hydrophobic amino acids that may alter the hydrophobicity and the surface A-protein.

A similarity dendrogram based on multiple alignment of the A-protein sequences available for all strains, i.e. between position 44 and 489, is shown in Fig. 2. The strains appear in 3 different clusters, with all the subspecies *salmonicida* strains in 1 cluster, and the *masoucida* type strains together with a strain from halibut (4050) in another. The third cluster contains the *achromogenes* type strains together with 10 atypical strains from the fish species cod, turbot, halibut and spotted wolffish, while the *smithia* type strain (4109) and the char and flounder strains (4043 and 4122) are unclustered.

The impact of the molecular variation in the A-proteins on the properties of the surface A-layer of the various atypical *Aeromonas salmonicida* strains is not known. The conserved regions are probably important for folding of the A-protein, assembling and anchoring of the surface A-layer to the membrane, while the variable region may affect the A-layer as a virulence factor. A study of the surface topography of the A-layer revealed that the conserved C-terminal region contained most of the surface-accessible sequences, while the N-terminal region contained the majority of the inaccessible residues (Doig et al. 1993). However, 65 surface-accessible residues in 5 linear epitopic clusters, of which 3 appear to be within the revealed variable region between residues 90 to 180, were

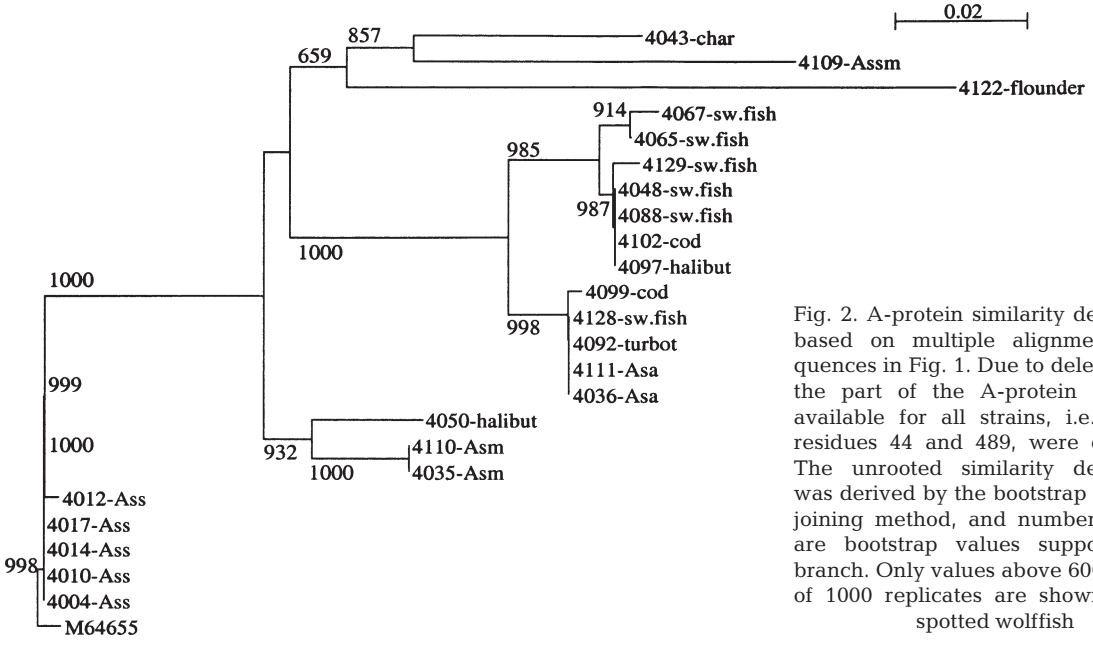

Fig. 2. A-protein similarity dendrogram based on multiple alignment of sequences in Fig. 1. Due to deletions, only the part of the A-protein sequences available for all strains, i.e. between residues 44 and 489, were compared. The unrooted similarity dendrogram was derived by the bootstrap neighbour joining method, and numbers at forks are bootstrap values supporting the branch. Only values above 600 of a total of 1000 replicates are shown. sw.fish: spotted wolffish

dispersed approximately between residues 30 and 240. Thus, most of the A-protein sequence variability appears to be restricted to a predicted surface exposed and immunogenic region, which we believe may result in antigenic differences among the atypical strains. This is supported by the fact that although a polyclonal antiserum raised against a typical *A. salmonicida* strain and adsorbed with atypical strains resulted in a great reduction of the titre, significant reactivity remained, indicating antigenic differences between the atypical and typical strains (Doig et al. 1993). Further, an A-protein-specific monoclonal antibody was shown to react with a restricted number of atypical strains of *A. salmonicida* (Lund et al. 2003b). However, as demonstrated in our A-protein sequences, the C-terminal region appears to be very conserved as 8 different monoclonal antibodies specific to this region of the A-protein reacted with all 25 typical and atypical strains of *A. salmonicida* studied (Doig et al. 1993).

Our interest in the surface A-layer is related to its possible role as a protective antigen in furunculosis vaccines. In oil-adjuvanted furunculosis vaccines for salmon, the A-layer appeared to be important, since vaccines containing strains without A-layer did not provide any protection (Lund et al. 2003a). Also, vaccines based on atypical *Aeromonas salmonicida* strains from the 3 different clusters shown in Fig. 2 showed significant differences in protection of spotted wolffish when challenged with strain 4067 (Lund et al. 2003b). Today, atypical furunculosis in farmed marine fish species is an increasing problem. Vaccination with the very efficient furunculosis vaccines for salmon appears to give poor protection against infection with atypical *A. salmonicida* in halibut farms in Norway (K. Gravningen pers. comm.). Thus, if the A-protein is a protective antigen, the vaccine efficacy may depend on the antigenic similarity between the vaccine and the infecting strain.

Acknowledgements. This study was supported by The Norwegian Industrial and Regional Development Fund (SND) in addition to funding from Fiskeriforskning. The control sequencing of some of the strains by Dr. Duncan Colquhoun, National Veterinary Institute of Norway, is gratefully appreciated.

LITERATURE CITED

Austin B, Austin DA (1993) Aeromonadaceae representatives (*Aeromonas salmonicida*). In: Austin B, Austin DA (eds) Bacterial fish pathogens disease in farmed and wild fish, 2nd edn. Ellis Horwood, Chichester, p 86–170

Austin DA, McIntosh D, Austin B (1989) Taxonomy of fish associated *Aeromonas* spp., with the description of *Aeromonas salmonicida* ssp. *smithia* ssp. nov. System Appl Microbiol 11:277–290

Chu S, Cavaignac S, Feutrier J, Phipps BM, Kostrzynska M, Kay WW, Trust TJ (1991) Structure of the tetragonal surface virulence array protein and gene of *Aeromonas salmonicida*. J Biol Chem 266:15258–15265

Doig P, McCubbin WD, Kay CM, Trust TJ (1993) Distribution of surface-exposed and non-accessible amino acid sequences among the two major structural domains of the S-layer protein of *Aeromonas salmonicida*. J Mol Biol 233:753–765

Ellis AE, Burrows AS, Stapleton KJ (1988) Lack of relationship between virulence of *Aeromonas salmonicida* and the putative virulence factors: A-layer, extracellular proteases and extracellular haemolysin. J Fish Dis 11:309–323

Felsenstein J (1985) Confidence limits on phylogenies: an approach using bootstrap. Evolution 39:783–791

Hänninen ML, Hirvelä-Koski V (1997) Molecular and phenotypic methods for the characterization of atypical *Aeromonas salmonicida*. Vet Microbiol 56:147–158

Høie S, Dalsgaard I, Aase IL, Heum M, Thornton JM, Powell R (1999) Polymerase chain reaction (PCR)-based typing analysis of atypical isolates of the fish pathogen *Aeromonas salmonicida*. System Appl Microbiol 22:403–411

Kay W, Buchley JT, Ishiguro EE, Phipps BM, Monette JPL, Trust TJ (1981) Purification and disposition of a surface protein associated with virulence of *Aeromonas salmonicida*. J Bacteriol 147:1077–1084

Kimura T (1969) A new subspecies of *Aeromonas salmonicida* as an etiological agent of furunculosis on 'sakuramasu' (*Oncorhynchus masou*) and pink salmon (*O. gorbuscha*) rearing for maturity. Part 1. On the morphological and physiological properties. Fish Pathol 3:34–44

Lund V, Jenssen LM, Wesmajervi MS (2002) Assessment of genetic variability and relatedness among atypical *Aeromonas salmonicida* from marine fishes, using AFLP-fingerprinting. Dis Aquat Org 50:119–126

Lund V, Arnesen JA, Coucheron D, Modalsli K, Syvertsen C (2003a) The *Aeromonas salmonicida* A-layer protein is an important protective antigen in oil-adjuvanted vaccines. Fish Shellfish Immunol 15:367–372

Lund V, Espelid S, Mikkelsen H (2003b) Vaccine efficacy in spotted wolffish (*Anarhichas minor* O.) related to molecular variation in the A-layer protein of atypical *Aeromonas salmonicida*. Dis Aquat Org 56:31–42

Lund V, Jenssen LM, Wesmajervi MS (2002) Assessment of genetic variability and relatedness among atypical *Aeromonas salmonicida* from marine fishes, using AFLP-fingerprinting. Dis Aquat Org 50:119–126

O'hIci B, Olivier G, Powell R (2000) Genetic diversity of the fish pathogen *Aeromonas salmonicida* demonstrated by random amplified polymorphic DNA and pulsed-field gel electrophoresis analyses. Dis Aquat Org 39:109–119

Olivier G (1990) Virulence of *Aeromonas salmonicida*: lack of relationship with phenotypic characteristics. J Aquat Anim Health 2:119–127

Pavan ME, Abbott SL, Zorzopulos J, Janda JM (2000) *Aeromonas salmonicida* subsp *pectinolytica* subsp nov., a new pectinase-positive subspecies isolated from a heavily polluted river. Int J System Bacteriol 50:1119–1124

Secombes CJ, Olivier G, (1997) Host-pathogen interactions in salmonids. In: Bernoth EM, Ellis AE, Midtlyng PJ, Olivier G, Smith P (eds) Furunculosis — multidiciplinary fish disease research. Hartnolls Ltd, Bodmin, p 269–296

Smith IW (1963) The classification of *Bacterium salmonicida*. J Gen Microbiol 33:263–274

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DJ (1997) The ClustalX windows interface: flexible strategies for multiple sequences alignment aided by quality analysis tools. Nucleic Acids Res 24:4876–4882

Umelo E, Trust TJ (1998) Physical map of the chromosome of *Aeromonas salmonicida* and genomic comparisons between *Aeromonas* strains. Microbiology UK 144:2141–2149

Wiklund T, Dalsgaard I (1998) Occurrence and significance of atypical *Aeromonas salmonicida* in non-salmonid fish species: a review. Dis Aquat Org 32:49–69

Editorial responsibility: Carey Cummingham, Aberdeen, UK

Submitted: December 18, 2003; Accepted: July 23, 2004
Proofs received from author(s): October 15, 2004