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Vaccine efficacy in spotted wolffish *Anarhichas minor*: relationship to molecular variation in A-layer protein of atypical *Aeromonas salmonicida*

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ABSTRACT: Atypical *Aeromonas salmonicida* strains comprise a heterogeneous group in terms of molecular and phenotypic characteristics. They cause various conditions of ulcer diseases or atypical furunculosis and are being isolated in increasing number from various fish species and geographical areas. Several marine fish species susceptible to atypical *A. salmonicida*, including spotted wolffish *Anarhichas minor* O., are now being farmed and new vaccines may be needed. A commercial furunculosis vaccine for salmon is reported to protect wolffish poorly against experimental challenge with atypical *A. salmonicida*. The protective antigen(s) in furunculosis vaccines is still unclear, but in oil-adjuvanted vaccine for Atlantic salmon *Salmo salar* L., the surface A-layer was shown to be important for protection. In spotted wolffish, the efficacy of atypical furunculosis vaccines seems to vary with the atypical *A. salmonicida* strains used as bacterin in the vaccine. In the present study we investigated whether differences in the A-layer protein among atypical strains might be responsible for the observed variation in vaccine efficacy. Atypical *A. salmonicida* strains from 16 fish species in 11 countries were compared by genome polymorphism analysis using amplified fragment length polymorphism (AFLP) fingerprinting and by comparative sequencing of the vapA genes encoding the A-protein. The A-protein sequences appeared to be highly conserved except for a variable region between Residues 90 to 170. Surprisingly, the grouping of strains based on AFLP- or A-protein sequence similarities was consistent. In addition, serological differences in the A-protein among the strains were demonstrated by an A-protein-specific monoclonal antibody. Vaccines based on atypical *A. salmonicida* strains possessing genetically and serologically different A-layer proteins were shown to result in significantly different protection in spotted wolffish.

KEY WORDS: Atypical *Aeromonas salmonicida* · Amplified fragment length polymorphism · AFLP · A-layer protein · Vaccine · Spotted wolffish

INTRODUCTION

Aeromonas salmonicida ssp. *salmonicida* comprises a homogeneous group, referred to as typical *A. salmonicida*, and causes furunculosis mostly in salmonid fishes. Atypical *A. salmonicida* strains that cause various conditions of ulcer diseases or atypical furunculosis in both salmonid and marine fish species are heterogeneous in terms of molecular and phenotypic characteristics (review by Wiklund & Dalsgaard 1998). Several subspecies of *A. salmonicida*, i.e. ssp. *achromogenes*, *masoucida*, *smithia* and *pectinolytica* (Smith 1963, Kimura 1969, Austin et al. 1989, Pavan et al. 2000) have been described. In addition, an increasing number of atypical isolates that do not fit into any of these subspecies are reported (reviewed in Wiklund & Dalsgaard 1998). Molecular techniques are now being used to complement the traditional phenotypic methods for classification of atypical *A. salmonicida* strains. Random amplified polymorphic DNA analysis (RAPD) (Kwon al. 1997, O'hIci et al. 2000), ribotyping (Hänninen & Hirvelä-Koski 1997), PCR-typing (Høie et al. 1999), plasmid profiling (Sørum et al. 2000), restriction fragment analysis by pulse-field gel electrophoresis

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(PFGE) (Umelo & Trust 1998, O'hIci et al. 2000) and amplified fragment length polymorphism analysis (AFLP) (Lund et al. 2002a) have been used to demonstrate genetic diversity among the atypical strains. However, these methods are not comparable (Austin et al. 1998), and a reliable method for classification of atypical strains is still lacking.

Several marine fish species susceptible to atypical *Aeromonas salmonicida* infection are now being farmed, and vaccine development will be necessary. It is not known if species-specific vaccines are needed, or if vaccines that protect against all genotypes of atypical *A. salmonicida* in various fish species can be developed. For the past 10 yr furunculosis in Atlantic salmon *Salmo salar* L. has been successfully controlled by the use of highly efficient oil-adjuvanted vaccines. Commercial salmon vaccines have been shown to protect against infection with atypical *A. salmonicida* in salmon (Gudmundsdottir & Gudmundsdottir 1997), but not in spotted wolffish *Anarhichas minor* O. (Lund et al. 2002b). However, in Atlantic salmon a commercial furunculosis vaccine was not as efficient as an autogenous vaccine (Gudmundsdottir & Gudmundsdottir 1997). The reason for the observed variation in efficacy of the furunculosis vaccine when challenged with various atypical strains is not known, but may be due to variation in protective antigens. Several components of *A. salmonicida* have been suggested as protective antigens. Among these is the surface A-layer composed of a single protein subunit, the A-protein, encoded by the virulence array protein gene A (vapA) (Chu et al. 1991). A correlation between the presence of the A-layer and virulence has been suggested (Kay et al. 1981, Austin & Austin 1993), but nevertheless non-virulent strains possessing an A-layer and virulent strains with no detectable 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 lethal effects of phagocytic cells (review by Secombes & Olivier 1997).

The A-layer protein is highly immunogenic, but antibody responses to this antigen could not be correlated with protection (Olivier et al. 1985, Hastings & Ellis 1990, Lund et al. 1991, Bjørnsdottir et al. 1992). However, goldfish with high levels of natural antibody activity against the A-layer protein were largely protected against experimental infection with an atypical strain of *Aeromonas salmonicida* (Sinyakov et al. 2002). Furthermore, a significant positive correlation between survival rates and corresponding antibody levels to the A-layer protein was found in Atlantic salmon vaccinated with commercial oil-adjuvanted furunculosis vaccines (Midtlyng et al. 1996). Also, in these vaccines, the A-layer protein appeared to be crucial for protection since no protection was obtained with an A-layer-negative vaccine strain (Lund et al. 2003)

In farmed spotted wolffish, experimental vaccines for atypical furunculosis based on genetically different strains determined by AFLP-fingerprinting were shown to result in significantly different protection (Lund et al. 2002a). However, protection could not be correlated to specific genes or protective antigens. Thus, the aim of this study was to investigate if variation in the A-protein could be related to differences in vaccine efficacy. Atypical *Aeromonas salmonicida* strains from 16 fish species and 11 countries were subjected to both serological and genetic characterisation using A-protein-specific monoclonal and polyclonal antibodies, AFLP-fingerprinting and comparative sequencing of vapA genes encoding the A-protein. Finally, vaccine efficacy in spotted wolffish was correlated to molecular differences in the A-proteins.

MATERIALS AND METHODS

Bacterial strains and growth conditions. This study comprised a collection of 36 strains of *Aeromonas salmonicida* (see Table 2) from 16 fish species and 11 countries, including the subspecies reference strains *salmonicida* 4010, 4014 and 4112, *smithia* 4109, *masoucida* 4110, *achromogenes* 4111 and *pectinolytica* 4113. The atypical strains from salmonids, cod *Gadus morhua*, halibut *Hippoglossus hippoglossus*, turbot *Scophthalmus maximus* and spotted wolffish were mostly from Norway and Iceland, but additional strains from several other marine fish species from geographically more distant countries were also included; 18 of the atypical strains, including 6 strains previously characterised as the subspecies *achromogenes*, were a gift from Dr. Bjarnheidur Gudmundsdottir (Institute of Experimental Pathology, University of Iceland), while the rest were from the strain collection at Norwegian Institute of Fisheries and Aquaculture Research.

The bacterial strains were stored at –80°C in 15% glycerol until used. They were grown with gentle shaking at 12°C in brain–heart infusion broth (BHI, Difco) supplemented with 2% NaCl. The strains used for challenge were grown for 24 h, harvested by centrifugation, and resuspended in 2% NaCl to OD_{600nm} = 1 (optical density at 600 nm). For vaccine purposes, the bacterial cultures were inactivated with 0.5% vol/vol formaldehyde solution (37%). BHI agar (Oxoid) supplemented with 2% NaCl and 0.005% Coomassie brilliant blue R (Sigma), and blood agar base No. 2 (Oxoid) supplemented with 2% human red blood cells and 1.5% NaCl were used for isolation of the bacteria from the head kidney of dead fish.

Characterisation of A-layer proteins. A-layer-producing strains were verified with an A-protein-specific polyclonal rabbit antiserum (R-anti-A) produced in our laboratory against a subspecies *salmonicida* strain. The A-protein-specific monoclonal antibody Mab1B2 (Bjørnsdottir et al. 1992), which is reactive to whole cells as well as epitopes in Western blot, was used to detect amino acid sequence variation in the A-layer protein among the strains. Whole-cell lysates of the atypical *Aeromonas salmonicida* strains were obtained from cells in 1 ml overnight culture. After centrifugation, the cells were suspended in 1 ml 20 mM Tris HCl pH 8.0 and mixed with sample buffer (10 mM Tris HCl pH 8.0, 5% sodium dodecyl sulphate [BIORAD], 10% mercaptoethanol [ROTH], 4% glycerol [Sigma]) in a proportion of 1:1 before being boiled for 5 min. Separation of cell lysates and transfer of proteins (Western blot) onto 0.45 µm nitro-cellulose membranes (Trans-Blot®, BIORAD) were performed in 12% Tris HCl Criterion gels in a Criterion™ cell and Criterion™ blotter (BIORAD) according to the BIORAD instruction manual. The membranes were blocked with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-Tween) and 5% non-fat dry milk. Primary antisera were either R-anti-A or Mab1B2, and secondary antibodies were goat anti-rabbit (DAKO) or goat anti-mouse immunoglobulins (Sigma), both conjugated with alkaline phosphatase. The membranes were incubated in appropriate dilutions of the various antibodies in PBS-Tween with 0.5% non-fat dry milk for 60 min at room temperature with gentle shaking, and washed 3 ¥ 10 min with PBS-Tween between each incubation step. Finally, the membranes were stained by adding the substrate nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate (NBT and BCIP; Gibco BRL) in substrate buffer (0.1 M Tris HCl pH 9.5, 0.1 M NaCl and 50 mM $MgCl_2$) as recommended by the producer.

Genomic DNA extraction. Cells were harvested from overnight cultures by centrifugation of 1 ml at 14 000 ¥ *g* for 3 min followed by extraction of genomic DNA by the use of GenomicPrep™ (Amersham Pharmacia Biotech). High-quality unsheared genomic DNA was confirmed on a 1% agarose gel. The DNA concentration was determined by measuring absorbance at 260 nm in a spectrophotometer (1 A_{260} unit = 50 µg DNA ml^{-1}). The DNA preparations were diluted in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) to 10 µg ml^{-1} and used for both AFLP-fingerprinting and DNA-sequencing.

AFLP-analysis. Amplified fragment length polymorphism analysis was performed using an AFLP™ Microbial fingerprinting kit (PE Applied Biosystems) as previously described (Lund et al. 2002a). Oligonucleotide adaptors, primers and amplification mixture were provided by the producer. Briefly, genomic DNA was digested with restriction enzymes *Eco*RI and *Mse*I (New England BioLabs) followed by ligation of double-stranded restriction-half-site-specific adaptors to the restriction fragments using T4 DNA ligase (Promega). A preselective amplification of the restriction fragments was performed with a primer pair complementary to the adaptors, followed by selective amplification using the *Eco*RI-A/*Mse*I-T primer pair. During the selective amplification, the PCR products were labelled with the 5′ fluorescent dye-label on the *Eco*RI-A primer for use with ABI Prism™ detection technology. Prior to gel-loading, GeneScan-500 was added to each sample as an internal lane-size standard to size all amplification fragments accurately. The PCR products were separated on an ABI PRISM 3100-*Avant* genetic analyser (PE Applied Biosystems), and the results were analysed with ABI Prism™ GeneScan® Analysis and Genotyper® 2.0 software (PE Applied Biosystems). The strains produced a total of 75 amplification fragments evenly dispersed from 50 to 495 bases. Each strain was visually coded for the presence (Code 1) and absence (Code 0) of each DNA fragment, and an AFLP-based similarity dendrogram was derived from unweighted average-pair group cluster analysis using the Pearson similarity coefficient (Wilkinson 1999)

Comparative DNA-sequencing of vapA genes. The vapA gene of *Aeromonas salmonicida* ssp. *salmonicida* Strain A450, encoding an A-protein of 502 residues including a 21 amino acid-signal peptide (Chu et al. 1991, GenBank Accession No. M64655), was used to synthesise 6 forward primers and 1 reverse primer (Eurogentec) (Table 1). The primer pair F1-R1 was used to produce 1565 bp PCR fragments from all strains encoding the mature A-protein. The amplifica-

Table 1. Primers used in PCR for sequencing vapA genes of atypical *Aeromonas salmonicida*. Numbering system according to vapA gene; GenBank Accession No. M64655

Primer	Position	Sequence (5'–3')
Forward		
F1	61–80	TCA ACG GAT AGG TTC AAC CC
F2	256–275	CTG GAC TTC TCC ACT GCT CA
F3	418–437	ACC GGT CTG CTG GTT AAC CC
F4	550–569	GGC AAG CGT CTG CGT CTT GC
F5	838–857	CGT GAT GCG GAT ATT TCT GC
F6	1206–1225	GGC TGA TCT CTT CAT CCT CAC CC
Reverse		
R1	1627–1602	CAG AGT GAA ATC TAC CAG CGG TGC

tion was performed using GeneAmp PCR System 2400 (Perkin Elmer). The 50 µl reaction mixture contained 25 ng genomic DNA, 250 µM dNTPs (Sigma), 1.25 Units *Taq* DNA polymerase, and 5 µl 10 ¥ *Taq* DNA polymerase buffer (Promega), 2 mM $MgCl_2$, 100 ng of each primer and 50 µM tetramethylammoniumchloride. Amplification conditions were 29 cycles of 10 s at 95°C, 10 s at 50°C and 60 s at 72°C, and a final extension of 3 min at 72°C.

The PCR products were purified by separation on 0.8% agarose gel and purified from the gel using QIAEX® II gel extraction kit (QIAGEN). The forward primers were used for DNA-sequencing 1565 bp PCR fragments from 4 of the atypical *Aeromonas salmonicida* strains. Sequencing was performed with an ABI PRISM 3100-*Avant* genetic analyser using the dye-terminator cycle-sequencing method as described in the producer's protocol (ABI PRISM BigDye DNA sequencing kit). The region of the vapA gene encoding the mature A-protein was obtained by primer walking, and a revealed variable region was sequenced from the entire strain collection using the F2 primer (Table 2). Multiple sequence alignment 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).

Fish. Spotted wolffish with an approximate weight of 25 g were from Troms Steinbit AS, Rubbestad, Norway. The vaccination and challenge experiments were performed at the Aquaculture Research Station in Tromsø, Norway. Throughout the whole experimental period, the fish were kept at 12°C with 24 h continuous light. Prior to marking, vaccination or challenge, the fish were anaesthetized with benzocain (50 mg l^{-1} water). Different groups were marked with Visible Implant Fluorescent Elastomer (Northwest Marine Technology) at the basis of the dorsal fin.

Vaccines and vaccination. Four atypical *Aeromonas salmonicida* strains isolated from diseased spotted wolffish were used in the vaccines. Strains 4059, 4065 and 4067 were found to be serologically homogenous, but Strain 4128 was different (see Table 2). Furthermore, according to the A-protein similarity dendrogram based on multiple alignment of partial sequences (Residues 63 to 208) of the gene, Strains 4059, 4065 and 4067 were in the same cluster, whereas Strain 4128 belonged to a closely related cluster (see Fig. 4). Only the cell fractions of formalin-inactivated bacterial cultures were used as vaccine antigens. After centrifugation, the pellet was suspended in 0.9% NaCl to an appropriate bacterial concentration before being emulsified with the mineral oil-adjuvant used in the ALPHA JECT™ 1200 furunculosis vaccine for salmon (Alpharma AS). The amount of bacterial cells used in the wolffish vaccines corresponded to that in ALPHA JECT™ 1200, which was included in the experiment.

The fish were vaccinated by intraperitoneal (i.p.) injection of 0.1 ml vaccine; 5 groups of 80 fish were each vaccinated with 1 of the vaccines, while a control group of 80 fish received 0.9% NaCl emulsified in oil-adjuvant. During the immunisation period of 9 wk, the groups were kept in separate raceways (40 ¥ 20 ¥ 220 cm).

Prechallenge and vaccine challenge. A prechallenge experiment was performed with the atypical *Aeromonas salmonicida* Strain 4067. We injected 3 groups of 15 fish i.p. with approximately 10^4, 10^3 and 10^2 cells $fish^{-1}$. Colony-forming units (cfu) in each dose were determined by counting on agar plates 3 d later. A mortality of 100% occurred in the 2 groups receiving the highest doses. The lowest dose was determined as 26 cfu $fish^{-1}$, and achieved 94% mortality; this was the preferred dose for challenge of the vaccinated fish.

We transferred and redistributed the fish 1 wk prior to challenge, with 40 fish per group into 2 tanks A and B (500 l each). The fish were i.p.-challenged with 0.1 ml of atypical *Aeromonas salmonicida* 4067 containing 32 cfu, as determined by plate-counts. Mortality was recorded daily, and the cause of death verified by isolation of *A. salmonicida* bacteria from the head kidney. Relative percent survival (RPS) was calculated for each group on the last day of the experiment according to the formula RPS = (1 – %mortality in vaccinated group/%mortality in control group) ¥ 100%.

Logistic regression (Type III sum-of-squares) in the GENMOD procedure of SAS 8.1 (SAS Institute 1993) was used to determine statistical differences in mortality between the 2 tanks and between the vaccinated and unvaccinated groups. Comparison of the vaccines was based on the results from duplicate tanks. Each vaccine was individually compared wiht the others, and the results were considered significant if $p < 0.05$.

RESULTS

The polyclonal antibody R-anti-A was used to determine A-layer-producing strains with Western blot. In addition to the atypical strains from cod (4102) and Atlantic salmon (4120) the reference strains of subspecies *salmonicida* 4010 and 4112, *masoucida* 4110, *achromogenes* 4111 and *pectinolytica* 4113 were shown to be A-layer-deficient (Table 2). Thus, the A-protein-specific Mab1B2 did not react with these strains, nor with the subspecies reference strain *smithia* 4109, 4 of the 6 wolffish strains, or the flounder strain 4122 (Table 2), thereby demonstrating antigenic differences in the A-layer protein among the strains.

Genome polymorphism among the strains was demonstrated by AFLP fingerprinting and is presented as a similarity dendrogram in Fig. 1. Comparison using a cluster delineation of 70% similarity resulted in a grouping of the strains into 5 clusters (A to E). A large cluster (A) contained 19 strains, while 4 smaller clusters contained from 2 to 4 strains. In addition, 5 strains were unclustered, including the subspecies reference strains *pectinolytica* 4113 and *smithia* 4109 and 3 strains from goldfish, dab and carp, respectively. The *achromogenes* reference Strain 4111 appeared in a cluster (A) with 5 of the 6 strains previously classified as subspecies *achromogenes* (Table 2) in addition to all 6 wolffish strains, 3 halibut strains and 3 strains from turbot, char and salmon, respectively. The typical *Aeromonas salmonicida* reference strains 4010, 4014 and 4112 all appeared in a cluster (D) with an atypical flounder strain 4122, while the *masoucida* reference strain clustered with 2 atypical strains from halibut and ling cod. For turbot, 3 strains appeared in 1 cluster (B), and the strain from koi carp and char previously classified as subspecies *achromogenes* (Table 2) appeared in another cluster (C).

In order to assess variation in the A-protein, the entire vapA gene of 4 atypical strains from char (4043), halibut (4050), spotted wolffish (4065) and turbot (4092) was sequenced. The deduced protein sequences were compared by sequence alignment with the reference sequence of the typical strain A450 (GenBank M64655) (Fig. 2). The A-protein

Table 2. *Aeromonas salmonicida*. Strain designation, host data, isolation locations and reactivity of A-protein-specific polyclonal (rabbit antiserum, R-anti-A) and monoclonal (MAB1B2) antibodies to the various strains

Strain no.	Designation	*A. salmonicida* subspecies	Host	Country of origin	Antibody reactivity R-anti-A	Mab1B2
4010	NCMB 1102	*salmonicida*	Atlantic salmon	UK	–	–
4014	88/09/1920	*salmonicida*	Atlantic salmon	Norway	+	+
4109	NCIMB13210	*smithia*	Roach	UK	+	–
4110	ATCC 27013	*masoucida*	Masou salmon	Japan	–	–
4111	NCIMB 1110	*achromogenes*	Brook trout	UK	–	–
4112	ATCC 14174	*salmonicida*	Brook trout	USA	–	–
4113	DSM12604	*pectinolytica*	Matanza river water	Argentina	–	–
4078	F63/94	achromogenes	Cod	Iceland	+	+
4099	93/09/914	atypical	Cod	Norway	+	+
4101	F19/99	*achromogenes*	Cod	Iceland	+	+
4102	Olivier; 81377	*achromogenes*	Cod	Canada	–	–
4050	104/95	atypical	Halibut	Norway	+	+
4096	94/09/0273	atypical	Halibut	Norway	+	+
4114	920902656	atypical	Halibut	Norway	+	+
4115	2656/92	atypical	Halibut	Norway	+	+
4091	90/09/2717	atypical	Turbot	Norway	+	+
4092	88/09/02778	atypical	Turbot	Norway	+	+
4116	920720-2/4	atypical	Turbot	Norway	+	+
4117	790901454	atypical	Turbot	Norway	+	+
4059	K-20897	atypical	Spotted wolffish	Norway	+	–
4065	K-0698	atypical	Spotted wolffish	Norway	+	–
4067	K-9/98	atypical	Spotted wolffish	Norway	+	–
4118	920901577	atypical	Wolffish	Norway	+	+
4119	1777/92	atypical	Spotted wolffish	Norway	+	–
4128	F98/01	atypical	Spotted wolffish	Iceland	+	+
4001	265/87	*achromogenes*	Atlantic salmon	Iceland	+	+
4002	D1-03/89	atypical	Char	Norway	+	+
4043	117-92	*achromogenes*	Char	Finland	+	+
4120	143/70	atypical	Atlantic salmon	UK	–	–
4121	87:1147	atypical	Koi carp	Australia	+	+
4122	26-F-16-4	atypical	Flounder	Finland	+	–
4123	V-75/93	atypical	Carp	Yugoslavia	+	+
4124	T5/92	*achromogenes*	Whiting	Iceland	+	+
4125	3 111	atypical	Goldfish	USA	+	+
4126	86/316	atypical	Ling cod	Canada	+	+
4127	920501-3/1	atypical	Dab	Denmark	+	+

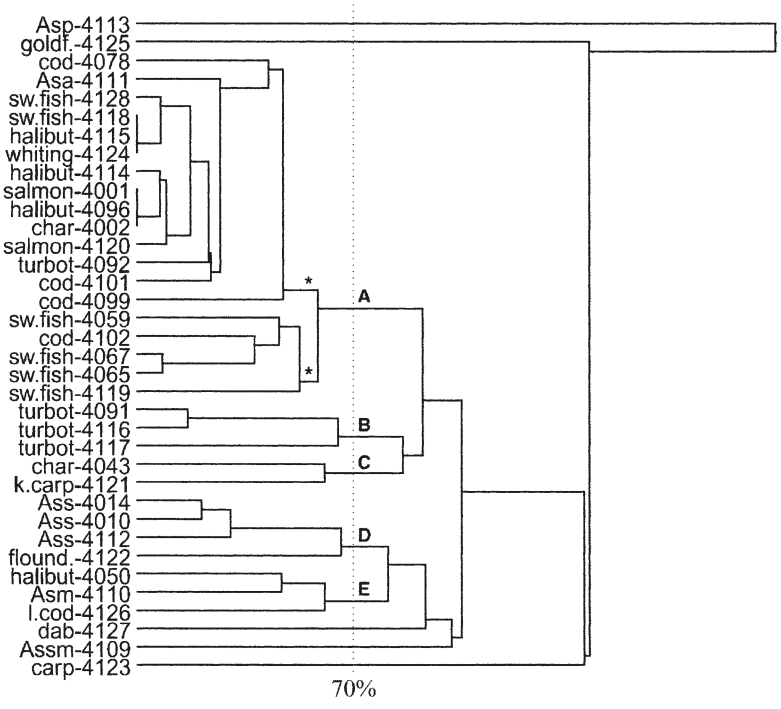

Fig. 1. *Aeromonas salmonicida.* Amplified fragment length polymorphism (AFLP) similarity dendrogram derived from cluster analysis of AFLP fingerprints of atypical strains isolated from various fish species, including subspecies reference strains *salmonicida* (Ass-4010 and Ass-4112), *achromogenes* (Asa-4111), *masoucida* (Asm-4110), *smithia* (Assm-4109) and *pectionolytica* (Asp-4113). Vertical dashed line denoting hypothetical node of 70% added for comparison purpose. Clusters labelled A to E, subclusters indicated by asterixes. goldf: goldfish, sw.fish: spotted wolffish,k.carp: koi carp, l.cod: ling cod

sequences appeared very conserved, displaying from 90 to 94% protein sequence identity compared to the typical reference sequence. However, the variation in the protein sequence was restricted to the region between Residues 90 to 170. Thus, for the rest of the strains only the protein sequence corresponding to this region was compared (Fig. 3). The multiple alignment of the partial A-protein sequences showed a variable region of approximately 80 amino acids flanked by more conserved regions. In addition to variation in amino acids between the sequences, insertions and deletions of amino acids resulted in varying sequence lengths, from 140 to 151 residues. Unfortunately, it was impossible to obtain sequences from the 2 subspecies reference strains *salmonicida* 4112 and *pectinolytica* 4113 with the primer-set used for the other strains.

A similarity dendrogram describing the approximate grouping of the *Aeromonas salmonicida* strains based on comparison of the partial A-protein sequences is shown in Fig. 4. The largest group contained 14 nearly identical sequences from atypical strains isolated from various fish species such as cod, halibut, turbot, spotted wolffish, char, salmon and whiting. Included was also the *achromogenes* reference strain 4111 and 4 of the 6 strains previously classified as *achromogenes* (Table 2). The closest neighbouring group contained 4 strains from spotted wolffish and 1 from cod, while the atypical flounder strain 4122 was the most distantly related strain. The 2 *salmonicida* reference strains 4010 and 4014 were identical to the typical reference sequence, and grouped with the atypical strain 4123 from carp. The *masoucida* reference strain grouped with 2 strains from halibut and ling cod, and the *smithia* reference strain with 2 strains from goldfish and koi carp. There seemed to be no clear correlation between grouping of strains and fish species or geographical location. Several strains with identical partial A-protein sequences originated from different fish species and locations, such as the cod strain 4102 from Canada and the spotted wolffish strain 4119 from Norway, or the goldfish strain 4125 from USA and the koi carp Strain 4121 from Australia.

In order to compare vaccine efficacy, groups of spotted wolffish were vaccinated with atypical strains possessing serologically and genetically different A-layer proteins. The atypical *Aeromonas salmonicida* strains from spotted wolffish used as vaccine antigens were either Mab1B2-negative (4059, 4065 and 4067) or Mab1B2-positive (4128) (Table 2). The vaccine ALPHA JECT™ 1200 containing a Mab1B2-positive strain was included for comparison. The vaccinated fish were challenged in parallel tanks with Mab1B2-negative Strain 4067, which was also used in 1 of the vaccines (Fig. 5). The total mortality in the parallel tanks did not differ significantly ($p = 0.7896$), and the mortality in the control groups was 46 and 41%, respectively. In both tanks, the vaccines containing Mab1B2-negative strains gave the best protection, while the vaccines with Mab1B2-positive wolffish Strain 4128 or the ALPHA JECT strain appeared to be less efficient (Fig. 5). A statistical comparison based on the results from both tanks demonstrated that all vaccines yielded significant protection (Table 3). Vaccines containing Mab1B2-negative strains (4059, 4065 or 4067) did not differ from each other, but gave significantly better protection compared to vaccines with Mab1B2-positive strains (4128 or the ALPHA JECT strain), which did not differ from each other.

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M64655    MFKKTLIAAAIVVGSAAPAFADVVISPNDNTFVTTSLASVTKQPVLDFSTAQQNLTLNFS     60
4043      MFKKTLIAAAIVVGSAAPAFAHVVISPNDNTFVTTSLASVTKQPVLDFSTAQQNLTLHFS     60
4050      MFKKTLIAAAIVVGSAAPAFADVVISPNDNNFVTTSLASVTKQPVLDFSTAQQNLTLNFS     60
4065      MFKKTLIAAAIVVGSAAPAFADVVISPNDNTFVTTSLASVTKQTVLDFSTAQQTLTLNFS     60
4092      MFKKTLIAAAIVVGSAAPAFADVVISPNDNTFVTTSLASVTKQQVLDFSTAQQTLTLNFS     60
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M64655    EVGDLKNNGFIVLEIQGEGQFNDAEIRQWLSNGFWRRPFTGLLVNPND---HGNF--ANS     115
4043      D-GDLKNNGFIVLEIQGEGQFNDAEIRKWLSNSVAGRSFTGLLVSATRNRIFANGVFVHS     119
4050      EVGDLKNNGFIVLEIQGEGQFNDAEIRQWLSNRFWRDPFTGLLVSPND---YGSR--GNS     115
4065      EVGDLKNNGFIVLEIQGEGQFNDAEIRQWLSNRYWNSSFTGLQVGPRT---FRNGSISNS     117
4092      EVGDLKNNGFIVLEIQGEGQFNDAEIRQWLSNRYWNSPFTGLLVGPRN---FRNG--ANS     115
          : ***************************:****     .**** *..    . .    :*

M64655    GEVNDVRKFFKIISDGTQLTIVHTIDSNGKRLRLALASDVEETINFADAEVELKLNLANQ     175
4043      GRVENVQRFFKTTSDGAQLTIDHTIDNNGKRLRLALASDVETTS---DAEVELKLNLANQ     176
4050      GEVAHVRQFFKIISDGTQQTIDHTIDNNGKRLRLALASDVETTA-IADAKVELKLNLANQ     174
4065      GEFGYVRQFFKIISDGTQQTIDHTIDKSGKRLRLALASDVESNA-IADLRVVLKLNLANQ     176
4092      GELNYVRQFFKIISDGTQQTIDHTIDKSGKRLRLALASDVETAA-VADQRVVLKLNLANQ     174
          *..   *:***  ***:* ** **** ************   . : * .* ********

M64655    AFKLTSGSQGTVALTAGALWNASYTADPVATKPLFKLGKLFQLSLTNAGKATALVSEGFL     235
4043      AFKLTSGSQGTVSLTVGALWNASYTADPVATKPLFKLGKLFQLSLTNAGKATALVSEGFL     236
4050      AFKLTSGSQGTVALTAGALWNASYTADPVATKPLFKLGKLFQLSLTNGGKATALVSEGFL     234
4065      AFKLTSGSQGTVALTAGALWNASYTADPVATKPLFKLGKLFQLSLTNAGNAIALVSEGFL     236
4092      AFKLTSGSQGTVALTAGALWNASYTADPVATKPLFKLGKLFQLSLTNAGNAIALVSEGFL     234
          *************.**.*********************************.:* ********

M64655    KLNIGDANISATDFAITNVTTNQTIQRDKVNLTLTGDVSAFKKDANGNLVNKAGASIGWK     295
4043      KLDIGDANISATDFAITNVTTNQTIQRDKVNLTLTGDVSAFKKDANGNLVNKAGVSIGWK     296
4050      KLDIGDADISATDFAITNVTTNQTIQRDKVDLTLTGDVSAFKKDANGNLVNKAGVSIGWK     294
4065      KLDIRDADISATDFAITNVTTNQIIQRDKVNLTLTGDVSAFKKDANGNLVNKAGVSIGWK     296
4092      KLDIRDADISATDFAITNVTTNQTIQRDKVNLTLTGDVSAFKKDANGNLVNKAGVSLGWK     294
          **:* **:***************** ******:**********************.*:***

M64655    AAADGQSATAVLGAGNMAGGVQNALAAFGTLYVAADNTVPVPAVNFNVKAEIQGDSQATY     355
4043      AAADGQSATAVLGAGNMAGGVQDALAAFGTLYVAADNTVPVPAVNFNVKAEIQGDSQATY     356
4050      AAADGQSATAVLGAGNMAGGVQEALAAFGTLYVAADNTVPVPAVNFNVKAEIQGNSQATY     354
4065      AAADGQSATGVLGAGNMAGGVQDALAAFGTLYVAADNTVPVPVVNFNVKAEIQGDSQATY     356
4092      AAADGQSATGVLGAGNMAGGVQDALAAFGTLYVAADNTVPVPVVNFNVKAEIQGDSQATY     354
          *********.***********:******************.***********:*****

M64655    NYFKDELADLFILTRDGMKFDTITTGTTSANLIHIRDVSNILPTEGGKIFVTITEYADHA     415
4043      NYFKDELADLFILTRDGMKFDTITTGTTSANLIHIRDVSNILPTEGGKIFVTITEYADHA     416
4050      NYFKDELADLFILTRDGMKFDTITTGTTSANLIHIRDVSNILPTEGGKIFVTITEYADHA     414
4065      NYFKDELADLFILTRDGMKFDTITTGTTSANLIHIRDVSNILPTEGGKIFVTITEYADHA     416
4092      NYFKDELADLFILTRDGMKFDTITTGTTSANLIHIRDVSNILPTEGGKIFVTITEYADHA     414
          ************************************************************

M64655    ANGRGEGTVLVTRKALSVTLPSGGAVTLKPADVAADVGASITAGRQARLVFEVETNQGEV     475
4043      ANGRGEGTVLVTRKALSVTLPSGGAVTLKPADVAADVGASITAGRQARFLFEVETNQGEV     476
4050      ANGRGEGTVLVTRKALSVTLPSGGAVTLKPADVAADVGASITAGRQARFLFEVETNQGEV     474
4065      ANGRGEGTVLVTRKALSVTLPSGGAVTLKPADVAADVGASITAGRQARFLFEVETNQGEV     476
4092      ANGRGEGTVLVTRKALSVTLPSGGAVTLKPADVAADVGASITAGRQARFLFEVETNQGEV     474
          ************************************************::***********

M64655    AVKKSNAEGVDIQNGTRGTAPLVDFTL   502
4043      AVKKSNAEGVDIQNG-----------   491
4050      AVKKSNAEGVDIQNGT----------   490
4065      AVKKSNA-------------------   483
4092      AVKKSNAEGVDIQNGTRGTAPLVDFTL   501
          *******
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Fig. 2. *Aeromonas salmonicida.* A-layer protein sequences deduced from nucleotide sequences of the vapA genes of atypical strains from char (Strain 4043), halibut (Strain 4050), spotted wolffish (Strain 4065) and turbot (Strain 4092) compared to reference sequence of typical strain, Strain A450 (GenBank Accession No. M64655)

DISCUSSION

A high degree of conservation of the antigenic structure of the A-protein of 25 typical and atypical strains of *Aeromonas salmonicida* has been demonstrated by the use of A-protein-specific polyclonal and monoclonal antibodies (Doig et al. 1993): 8 different monoclonal antibodies were shown to bind epitopes in the C-terminal region of the A-protein and to react with all the strains, indicating this region of the protein to be

```
M64655          DLKNNGFIVLEIQGEGQFNDAEIRQWLSNGFWRRPFTGLLVNPND-----HGN--FANSGEVNDVRKFFKIISDGT  69
4010-Ass        DLKNNGFIVLEIQGEGQFNDAEIRQWLSNGFWRRPFTGLLVNPND-----HGN--FANSGEVNDVRKFFKIISDGT  69
4014-Ass        DLKNNGFIVLEIQGEGQFNDAEIRQWLSNGFWRRPFTGLLVNPND-----HGN--FANSGEVNDVRKFFKIISDGT  69
4109-Assm       DLKNNGFIVLEIQGEGQFNNAEIRQWLSNRVWGAPFTGLLVSPYR----IVANRVFVKSGDYSQIRKFFKTTADGT  72
4110-Asm        DLKNNGFIVLEIQGEGQFNDAEIRQWLSNRFWREPFTALLVSPND-----YGNG--VNSGEVADVRQFFKIISDGT  69
4111-Asa        DLKNNGFIVLEIQGEGQFNDAEIRQWLSNRYWNSPFTGLLVGPRN-----FRNG--ANSGELNYVRQFFKIISDGT  69
4078-cod        DLKNNGFIVLEIQGEGQFNDAEIRQWLSNRYWNSPFTGLLVGQRN-----FRNG--ANSGELNYVRQLFKIISDGT  69
4099-cod        DLKNNGFIVLEIQGEGQFNDAEIRQWLSNRYWNSPFTGLLVGPRN-----HRNG--ANSGELNYVRKFFKIISDGT  69
4101-cod        DLKNNGFIVLEIQGEGQFNDAEIRQWLSNRYWNSPFTGLLVGPRN-----FRNG--ANSGELNYVRQFFKIISDGT  69
4102-cod        DLKNNGFIVLEIQGEGQFNDAEIRQWLSNRYWNSSFTGLLVGPRT-----FRNGAISNSGEFGYVRQFFKIISDGT  72
4050-halibut    DLKNNGFIVLEIQGEGQFNDAEIRQWLSNRFWRDPFTGLLVSPND-----YGSR--GNSGEVAHVRQFFKIISDGT  69
4096-halibut    DLKNNGFIVLEIQGEGQFNDAEIRQWLSNRYWNSPFTGLLVGPRN-----FRNG--ANSGELNYVRQFFKIISDGT  69
4114-halibut    DLKNNGFIVLEIQGEGQFNDAEIRQWLSNRYWNSPFTGLLVGPRN-----FRNG--ANSGELNYVRQFFKIISDGT  69
4115-halibut    DLKNNGFIVLEIQGEGQFNDAEIRQWLSNRYWNSPFTGLLVGPRN-----FRNG--ANSGELNYVRQFFKIISDGT  69
4091-turbot     DLKNNGFIVLEIQGEGQFNATEIRQWLSNTFWNAPFTGLLVSPQRNRQ-GVAEGAVAKSGTVTNVRKFFKLVSDGH  75
4092-turbot     DLKNNGFIVLEIQGEGQFNDAEIRQWLSNRYWNSPFTGLLVGPRN-----FRNG--ANSGELNYVRQFFKIISDGT  69
4116-turbot     DLKNNGFIVLEIQGEGQFNATEIRQWLSNTFWNAPFTGLLVSPQRNRQ-GVAEGAVAKSGTVTNVRKFFKLVSDGH  75
4117-turbot     DLKNNGFIVLEIQGEGQFNDAEIRKWLSNSVAGRSFTGLLVSATRNR--IFANGVFVHSGRVENVQRFFKTTSDGA  74
4059-sw.fish    DLKNNGFIVLEIQGEGQFNDAEIRQWLSNRYWR-SFTGLQVGPRT-----FRNGSITNSGEFGYVRKFFKIISDGT  70
4065-sw.fish    DLKNNGFIVLEIQGEGQFNDAEIRQWLSNRYWNSSFTGLQVGPRT-----FRNGSISNSGEFGYVRQFFKIISDGT  71
4067-sw.fish    DLKNNGFIVLEIQGEGQFNDAEIRQWLSNRYWNSSFTGLQVGPRT-----FRNGSISNSGEFGYVRQFFKIISDGT  71
4118-sw.fish    DLKNNGFIVLEIQGEGQFNDAEIRQWLSNRYWNSPFTGLLVGPRN-----FRNG--ANSGELNYVRQFFKIISDGT  69
4119-sw.fish    DLKNNGFIVLEIQGEGQFNDAEIRQWLSNRYWNSSFTGLLVGPRT-----FRNGAISNSGEFGYVRQFFKIISDGT  71
4128-sw.fish    DLKNNGFIVLEIQGEGQFNDAEIRQWLSNRYWNSPFTGLLVGPRN-----FRNG--ANSGELNYVRQFFKIISDGT  69
4001-salmon     DLKNNGFIVLEIQGEGQFNDAEIRQWLSNRYWNSPFTGLLVGPRN-----FRNG--ANSGELNYVRQFFKIISDGT  69
4002-char       DLKNNGFIVLEIQGEGQFNDAEIRQWLSNRYWNSPFTGLLVGPRN-----FRNG--ANSGELNYVRQFFKIISDGT  69
4043-char       DLKNNGFIVLEIQGEGQFNDAEIRKWLSNSVAGRSFTGLLVSATRNR--IFANGVFVHSGRVENVQRFFKTTSDGA  74
4120-salmon     DLKNNGFIVLEIQGEGQFNDAEIRQWLSNRYWNSPFTGLLVGPRN-----FRNG--ANSGELNYVRQFFKIISDGT  69
4121-koi_carp   DLKNNGFIVLEIQGEGQFNDAEIRQWLSNSFWNHPFTGVLVSP---------NRNRVKSGQIVDVRKFFKTTSDGT  67
4122-flounder   DLKNNGFIVLEIQGEGQFNAAEIRRWLSNRGGNGHFTALLVSPNRNSHGAFTADSGTPSGSVRHVQNFFKTTYDGP  76
4123-carp       DLKNNGFIVLEIQGEGQFNDAEIRQWLSNRFWNAPFTGLLVSPSR-----NGNAIVANSGKIVDVRRFFKIISDGT  71
4124-whiting    DLKNNGFIVLEIQGEGQFNDAEIRQWLSNRYWNSPFTGLLVGPRN-----FRNG--ANSGELNYVRQFFKIISDGT  69
4125-goldfish   DLKNNGFIVLEIQGEGQFNDAEIRQWLSNSFWNHPFTGVLVSP---------NRNRVKSGQIVDVRKFFKTTSDGT  67
4126-ling_cod   DLKNNGFIVLEIQGEGQFNDAEIRQWLSNRFWREPFTALLVSPND-----YGID--ANSGEVADVRQFFKIISDGT  69
4127-dab        DLKNNGFIVLEIQGEGQFNGAEIRQWLSNQIGHQSFTGLLVGPNRPE--VFVHGVVVNSGDVRDVRRFFKTTSDGT  74
                ****************** :***:****      **.: *.                      ::.:**   **

M64655          QLTIVHTIDSNGKRLRLALASDVEETINFADAEVELKLNLANQAFKLTSGSQGTVALTAGALWNASYTADPVATKP 145
4010-Ass        QLTIVHTIDSNGKRLRLALASDVEETINFADAEVELKLNLANQAFKLTSGSQGTVALTAGALWNASYTADPVATKP 145
4014-Ass        QLTIVHTIDSNGKRLRLALASDVEETINFADAEVELKLNLANQAFKLTSGSQGTVALTAGALWNASYTADPVATKP 145
4109-Assm       QLTIDHTIDNNGKRLRLALAPDVEDAA-VADAEIELKLSLANQAFKLTSGSQGTVALTAGALWNASYTADPVATKP 147
4110-Asm        QQTIDHTIDNNGKRLRLALASDVETTASRAGAKVELKLNLANQAFKLTSGSQGTVALTAGALWNASYTADPVATKP 145
4111-Asa        QQTIDHTIDKSGKRLRLALASDVETAA-VADQRVVLKLNLANQAFKLTSGSQGTVPLTAGALWNASYTADPVATKP 144
4078-cod        QQTIDHTIDKSGKRLRLALASDVETAA-VADQRVELKLNLANQAFKLTSGSQGTVALTAGALWNASYTADPVATKP 144
4099-cod        QQTIDHTIDKSGKRLRLALASDVETAA-VADQRVVLKLNLANQAFKLTSGSQGTVALTAGALWNASYTADPVATKP 144
4101-cod        QQTIDHTIDKSGKRLRLALASDVETAA-VADQRVVLKLNLANQAFKLTSGSQGTVALTAGALWNASYTADPVATKP 144
4102-cod        QQTIDHTIDKSGKRLRLALASDVESNA-IADQRVVLKLNLANQAFKLTSGSQGTVALTAGALWNASYTADPVATKP 146
4050-halibut    QQTIDHTIDNNGKRLRLALASDVETTA-IADAKVELKLNLANQAFKLTSGSQGTVALTAGALWNASYTADPVATKP 144
4096-halibut    QQTIDHTIDKSGKRLRLALASDVETAA-VADQRVVLKLNLANQAFKLTSGSQGTVALTAGALWNASYTADPVATKP 144
4114-halibut    QQTIDHTIDKSGKRLRLALASDVETAA-VADQRVVLKLNLANQAFKLTSGSQGTVALTAGALWNASYTADPVATKP 144
4115-halibut    QQTIDHTIDKSGKRLRLALASDVETAA-VADQRVVLKLNLANQAFKLTSGSQGTVALTAGALWNASYTADPVATKP 144
4091-turbot     QLIIDHAIDSNGKRLRLALSAGVENTV-VDDAEVELKLNVANQAFKLTSGAQGTVALTAGALWNASYTADPVATKP 150
4092-turbot     QQTIDHTIDKSGKRLRLALASDVETAA-VADQRVVLKLNLANQAFKLTSGSQGTVALTAGALWNASYTADPVATKP 144
4116-turbot     QLIIDHAIDSNGKRLRLALSAGVENTV-VDDAEVELKLNVANQAFKLTSGAQGTVALTAGALWNASYTADPVATKP 150
4117-turbot     QLTIDHTIDNNGKRLRLALASDVETTS---DAEVELKLNLANQAFKLTSGSQGTVSLTVGALWNASYTADPVATKP 147
4059-sw.fish    HQTIDHTIDKSGKRLRLALASDVESNA-IADLRAVLKLNLANQAFKLTSGSQGTVALTAGALWNASYTADPVATKP 145
4065-sw.fish    QQTIDHTIDKSGKRLRLALASDVESNA-IADLRVVLKLNLANQAFKLTSGSQGTVALTAGALWNASYTADPVATKP 146
4067-sw.fish    QQTIDHTIDKSGKRLRLALASDVESNA-IADLRVVLKLNLANQAFKLTSGSQGTVALTAGALWNASYTADPVATKP 146
4118-sw.fish    QQTIDHTIDKSGKRLRLALASDVETAA-VADQRVVLKLNLANQAFKLTSGSQGTVALTAGALWNASYTADPVATKP 144
4119-sw.fish    QQTIDHTIDKSGKRLRLALASDVESNA-IADQRVVLKLNLANQAFKLTSGSQGTVALTAGALWNASYTADPVATKP 146
4128-sw.fish    QQTIDHTIDKSGKRLRLALASDVETAA-VADQRVALKLNLANQAFKLTSGSQGTVALTAGALWNASYTADPVATKP 144
4001-salmon     QQTIDHTIDKSGKRLRLALASDVETAA-VADQRVVLKLNLANQAFKLTSGSQGTVALTAGALWNASYTADPVATKP 144
4002-char       QQTIDHTIDKSGKRLRLALASDVETAA-VADQRVVLKLNLANQAFKLTSGSQGTVALTAGALWNASYTADPVATKP 144
4043-char       QLTIDHTIDNNGKRLRLALASDVETTS---DAEVELKLNLANQAFKLTSGSQGTVSLTVGALWNASYTADPVATKP 147
4120-salmon     QQTIDHTIDKSGKRLRLALASDVETAA-VADQRVVLKLNLANQAFKLTSGSQGTVALTAGALWNASYTADPVATKP 144
4121-koi_carp   QLTIDHTIDNNGKRLRLALASAVEDAA-IA--EVELKLSLANQAFKLTSGSQGTVALTVAALWNASYTADPVATKP 140
4122-flounder   HLTIDHAIDSHGKRLRLALSSEVEDTV-VDDAQVDLQLNLANQAFKLTSGAQGTVALTAGALWNASYTADPVATKP 151
4123-carp       QRTIEHTIDKNGKRLRLALASDVEDTIANADAEVELKLNLANQAFKLTSGSQGTVALTAGALWNASYTADPVATKP 147
4124-whiting    QQTIDHTIDKSGKRLRLALASDVETAA-VADQRVVLKLNLANQAFKLTSGSQGTVALTAGALWNASYTADPVATKP 144
4125-goldfish   QLTIDHTIDNNGKRLRLALASAVEDAA-IA--EVELKLSLANQAFKLTSGSQGTVALTVAALWNASYTADPVATKP 140
4126-ling_cod   RQIIDHTIDNNGKRLRLALASDVETTASKAGAKVELKLNLANQAFKLTSGSQGTVALTAGALWNASYTADPVATKP 145
4127-dab        QLTIDHTIDNNGKRLRLALASAVENRA-LHGADVELKLNLANQAFKLTSGSQGTVALTAGALWNASYTADPVATKP 149
                :  * *:**. ********:. **          *:*.:**********:****.**..**************
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Fig. 3. *Aeromonas salmonicida.* Multiple alignment of partial A-protein sequences deduced from nucleotide sequences of 34 strains from various fish species, including subspecies reference strains *salmonicida* (4010-Ass and 4014-Ass), *achromogenes* (4111-Asa), *masoucida* (4110-Asm) and *smithia* (4109-Assm) and reference sequence of typical *A. salmonicida* Strain A450 (GenBank M64655). Sequences correspond to region between Residues 63 and 208 of the typical reference sequence in Fig. 2

conserved. However, polyclonal antiserum raised against a typical *A. salmonicda* strain and adsorbed with atypical strains resulted in a great reduction of the titre, but significant reactivity remained, demonstrating antigenic differences among atypical and typical strains (Doig et al. 1993). Our results show the deduced A-protein sequences of 5 strains to be very conserved (Fig. 2) apart from a variable region between Amino Acid Residues 90 and 170, which may be responsible for antigenic variation among the strains.

The A-protein-specific polyclonal R-anti-A antiserum revealed 7 A-layer-deficient strains, including subspecies reference strains *salmonicida* (4010 and 4112) and *masoucida* (4110), previously reported to be A-layer-deficient (Austin et al. 1998), the *achromogenes* (4111) and *pectinolytica* (4113) reference strains, and the atypical Strain 4102 from cod and Strain 4120 from salmon (Table 2). The A-protein-specific Mab1B2 reacted with all A-layer-producing strains except the *smithia* reference strain, 4 of 6 atypical strains from spotted wolffish, and flounder Strain 4122. The serological difference demonstrated by Mab1B2 is assumed to be in the variable region, since only minor amino acid differences were found in the remaining A-protein sequences, including both Mab1B2-positive and -negative strains (Fig. 2).

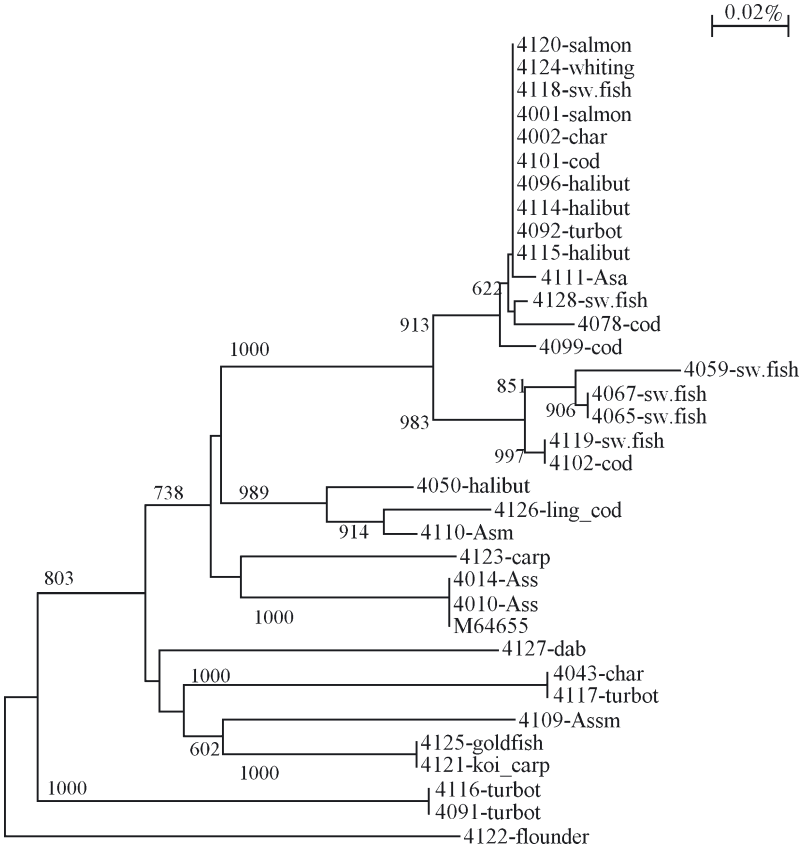

Fig. 4. *Aeromonas salmonicida.* A-protein similarity dendrogram based on multiple alignment of partial A-protein sequences of strains from various fish species, including subspecies reference strains *salmonicida* (4010-Ass and 4014-Ass), *achromogenes* (4111-Asa), *masoucida* (4110-Asm), *smithia* (4109-Assm) and typical reference sequence (GenBank M64655). Only sequences corresponding to Residues 63 to 208 of the typical reference sequence in Fig. 2 are compared. Unrooted similarity dendrogram was derived by bootstrap neighbour-joining method, and numbers at forks are bootstrap values supporting the branch. Only values above 600 of a total of 1000 replicas are shown. sw.fish: spotted wolffish

Genome polymorphism demonstrated by AFLP-fingerprinting has previously been reported among atypical *Aeromonas salmonicida* strains (Lund et al. 2002a), and was also confirmed in the present study. Some of the strains used in the previous study were included here in addition to other subspecies reference strains; 3 *salmonicida* subspecies reference strains appeared in the same cluster as an atypical strain from flounder, while the *smithia* and *pectinolytica* reference strains were unclustered. Of the 6 atypical strains classified as subspecies *achromogenes* (Table 2), 5 clustered with the *achromogenes* reference, while in the previous study they clustered with a *masoucida* reference strain (Lund et al. 2002a). This illustrates that AFLP-based grouping of atypical strains does not support the existing subspecies classification of *A. salmonicida*. There is no clear correlation between grouping of strains and fish species origin or geographical location, as was shown by AFLP fingerprinting of *Vibrio viscosus*, for which strains from Iceland and Norway appeared in different subgroups (Benediktsdottir et al. 2000).

Surprisingly, grouping of *Aeromonas salmonicida* strains based on genome polymorphism was reflected in the similarity dendrogram based on a partial sequence of the single vapA gene (Figs. 1 & 4). The large AFLP-cluster (A) of 19 strains contained 2 sub-clusters, with 14 and 5 strains that were identical to the 2 neighbouring groups in the A-protein similarity dendrogram. The 4 wolffish strains in the AFLP sub-cluster possessed Mab1B2-negative A-layer proteins, while the cod strain 4102 was Mab1B2-negative because of its A-layer deficiency. Also, in both dendrograms, the *smithia* reference strain 4109 grouped with a halibut and ling cod strain. The partial A-protein sequences of the 2 *salmonicida* ref-

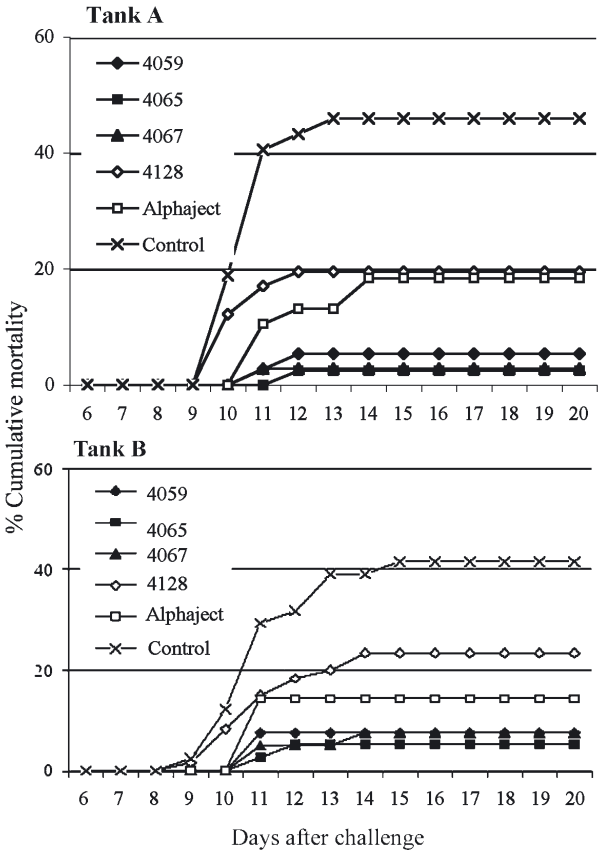

Fig. 5. *Anarhichas minor.* Cumulative mortality in vaccinated spotted wolffish after challenge (i.p.) with atypical *Aeromonas salmonicida* Strain 4067 (32 cfu fish^{-1}). The 4 wolffish vaccines contained serologically and genetically different strains of atypical *A. salmonicida*, either Strains 4059, 4065, 4067 or 4128. Salmon furunculosis vaccine ALPHA JECT™ 1200 was used as reference vaccine and the control group received 0.9% NaCl in oil-adjuvant. The challenge was performed in 2 parallel tanks (A and B)

erence strains were identical to the typical reference sequence (GenBank M64655). However, they grouped with carp strain 4123 in the A-protein-based dendrogram and with the flounder strain 4122 in the AFLP-based dendrogram. The *pectinolytica* reference Strain 4113 was the most distant strain in the AFLP-based dendrogram, joining the other strains at a similarity of 10 to 15%, which may explain why no PCR-product was obtained from this strain. The PCR-product obtained from the subspecies *salmonicida* Strain 4112 was shorter than expected, and may contain a deletion in the vapA gene, which would explain why this strain is A-layer-deficient (Table 2) and why no DNA-sequence of the variable region could be obtained.

Vaccines containing Mab1B2-negative or -positive strains were compared in spotted wolffish challenged with a Mab1B2-negative strain. Although the challenge dose was comparable to that resulting in 94% mortality in the prechallenge experiment, only 46 and 41% mortality was obtained in the control groups in Tanks A and B, respectively. In contrast to the fish used for prechallenge, the control groups had received an injection of 0.9% NaCl in oil-adjuvant, which may have resulted in unspecific protection. However, an unspecific protection due to the oil-adjuvant alone was not observed in a previous experiment (Lund et al. 2002b), and was the reason for not including 2 control groups in the present study, with one receiving 0.9% NaCl alone and the other receiving 0.9% NaCl in oil-adjuvant. Despite the low mortality in the control groups, statistical analysis based on the results from both tanks demonstrated all vaccines to yield significant protection. However, the vaccines containing Mab1B2-negative strains resulted in a significantly better protection compared to the vaccines with Mab1B2-positive strains, including vaccine ALPHA JECT™ 1200. In a previous experiment, ALPHA JECT™ 1200 resulted in no protection of spotted wolffish challenged with a 100¥ higher dose (3.5 ¥ 10^3 cfu fish^{-1}) of the same atypical Strain 4067 (Lund et al. 2002b).

The protective antigens in the vaccines must be cellular, since only the bacterial cells of the bacterin were used in our experiments. Components of the cell surface are fundamental candidates as protective antigens in vaccines, since they form the initial contact

Table 3. *Anarhichas minor.* Vaccine efficacy as relative percent survival (RPS) in 2 tanks and statistical differences between vaccines when individually compared. Mortality in control group in the 2 tanks was 41 and 46%, respectively (ALPHA JECT™ 1200: furunculosis vaccine for salmon).

Vaccine	RPS		p-values of strains				
	Tank A	Tank B	4059	4065	4067	4128	ALPHA JECT™
4059	88	82	–				
4065	95	87	0.4498	–			
4067	94	82	0.5938	0.8303	–		
4128	58	43	<0.0001	<0.0001	<0.0001	–	
ALPHA JECT™	60	65	0.0005	0.0027	0.0021	0.3010	–
Control			<0.0001	<0.0001	<0.0001	0.0181	0.0013

between host and pathogen. The iron-binding outer-membrane protein and a conserved porin have been reported to be protective (Hirst & Ellis 1994, Lutwyche et al. 1995). In addition, the A-layer has been shown to be an important protective antigen in oil-adjuvanted furunculosis vaccines for salmon (Lund et al. 2003). Although many cellular antigens may vary among the strains used in the vaccines, the A-protein is the only antigen known to differ both serologically and genetically. The authors therefore questioned if serological differences in the A-protein could be responsible for the observed variation in vaccine efficacy in spotted wolffish (Lund et al. 2002b). The deduced A-protein sequence of 4 atypical strains from various fish species revealed a sequence identity of 90 to 94% with the typical reference sequence. However, most of the sequences were highly conserved, apart from the region between Residues 90 and 170. Comparison of this A-protein region in the whole strain collection demonstrated a sequence identity of 70 to 100% with the typical reference sequence (GenBank M64655).

If antibody responses towards the A-layer protein were important for protection, then differences in protection obtained with the various strains should correlate with the variable region of the A-protein. By the use of mimeotope analysis and electron microscopy using polyclonal and monoclonal antibodies, the surface topography of the A-layer was defined (Doig et al. 1993). Inaccessible or non-epitopic residues accounted for 70% of the protein. While the strongly antigenically conserved C-terminal region contained most of the A-layer surface-accessible sequences, the N-terminal region contained the majority of the inaccessible residues. However, dispersed among these were 65 surface-accessible residues in 5 linear epitopic clusters, and 3 of these clusters appeared to be within the revealed variable region between Residues 90 and 170. Since, thus far, A-protein sequence variability appears to be restricted to a possible surface-exposed and immunogenic region, it is logical to consider it of importance. However, the vapA gene of additional typical and atypical strains should be fully sequenced to investigate the possibility of other variable regions of the A-protein. According to Høie et al. (1999), 72 of 205 *Aeromonas salmonicida* strains did not produce PCR products with a primer-set targeting the C-terminal region of the A-protein, indicating sequence variation in this region.

Vaccine efficacy in different fish species may depend on the antigenic similarity between the A-layer protein of the vaccine and challenge strains of atypical *Aeromonas salmonicida*. Commercial salmon furunculosis vaccines have been shown to protect against infections with atypical *A. salmonicida* in salmon and cyprinid fishes (Jones et al. 1996, Gudmundsdottir & Gudmundsdottir 1997) and in spotted wolffish (present study). ALPHA JECT™ 1200 was reported to protect halibut very well against experimental challenge with an atypical strain from cod (B. Gudmundsdottir pers. com.), but it gave poor protection against a natural outbreak of atypical furunculosis in a halibut farm in Norway (K. Gravningen, Alpharma AS, pers. com.). However, there is no data on the similarity between the A-layer proteins of the vaccine and the infecting strains in these cases. Thus, a possible correlation between serological and genetic differences in the A-protein among atypical strains of *A. salmonicida* and protection calls for further investigation.

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