Atypical furunculosis vaccines for Atlantic cod *Gadhus morhua*: impact of reattached Aeromonas salmonicida A-layer protein on vaccine efficacy

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ABSTRACT: Atypical furunculosis caused by atypical Aeromonas salmonicida bacteria is reported as an increasing problem in farmed Atlantic cod *Gadus morhua* in Norway. At present, furunculosis vaccines adapted for cod or other marine fish species are not available. To identify bacterial components important for inducing protection in cod, we compared oil-adjuvanted vaccines based on *A. salmonicida* isolates phenotypically differing in their major cell surface constituents, such as the A-layer protein and lipopolysaccharide O-chains. Also included was an A-layer-deficient isolate with physically reattached A-layer protein. Vaccines containing *A. salmonicida* A-layer-producing cells elicited significantly better protection than vaccines with A-layer-deficient cells or with a supernatant with secreted A-layer protein. The *A. salmonicida* cells with reattached A-layer-protein resulted in significant and equal protection to the A-layer-producing cells and protected significantly better than the A-layer-deficient isolate. These results indicate that the A-layer protein when attached to the cell surface plays a role in inducing protective immunity in cod.

KEY WORDS: Atlantic cod · Aeromonas salmonicida · Furunculosis · Vaccine · A-layer protein

INTRODUCTION

Farming of Atlantic cod *Gadus morhua* is still in its infancy, but even so, bacterial diseases are registered as an increasing problem (Samuelsen et al. 2006). Vibrios has been the major disease since cod farming started in Norway, but is now partly controlled by vaccines adapted to cod (Mikkelsen et al. 2007). Atypical furunculosis caused by atypical *Aeromonas salmonicida* has been the main problem in cod farming in Iceland (Magnadóttir et al. 2002) and is now being reported in increasing numbers of farms along the coast of Norway. Vaccines against atypical furunculosis in marine fish species are not available (Gudmundsdóttir & Björnsdóttir 2007); however, a few reports exist on vaccination of cod. Vaccination, either with an oil-adjuvanted commercial furunculosis vaccine or an experimental bacterin based on the challenge strain, did not elicit significant protection in cod against injection or bath challenge (Gudmundsdóttir et al. 2005, Gudmundsdóttir & Björnsdóttir 2007). An experimental oil-adjuvanted furunculosis vaccine based on an atypical *A. salmonicida* isolate from cod elicited good protection against homologous injection challenge (Mikkelsen et al. 2004). Furthermore, this vaccine protected better than a commercial salmon furunculosis vaccine (Lund et al. 2008a), indicating that vaccines adapted for cod are needed.

Although the protective antigen(s) in typical or atypical furunculosis vaccines are still unclear, several candidates have been suggested. An extra-cellular metallo-protease (Gudmundsdóttir & Magnadóttir 1997), a purified porin (Lutwyche et al. 1995) and both iron-regulated outer-membrane proteins and extra-cellular polysaccharides (Hirst & Ellis 1994, Bricknell et al. 1997, Bricknell et al. 1999) have all been correlated to...
protection in salmonids. Furthermore, a correlation between survival rates and corresponding antibody levels to the A-layer protein has been found in Atlantic salmon *Salmo salar* and goldfish *Carassius auratus* (Midtlyng et al. 1996, Sinyakov et al. 2002). Earlier studies have shown that A-layer-possessing *Aeromonas salmonicida* used in oil-adjuvanted vaccines elicit acceptable protection against homologous challenge in salmon, as well as in marine fish species. However, vaccines based on A-layer-deficient isolates failed to protect in salmon and protected poorly or not at all in cod and Atlantic halibut *Hippoglossus hippoglossus* (Lund et al. 2003a, b, Mikkelsen et al. 2004, Lund et al. 2008a, b).

Various A-layer functions have been identified by using A-layer-deficient mutants. In addition, physical reconstitution of the A-layer proved to be a useful tool in studying the functions of the *Aeromonas salmonicida* A-layer and identifying A-layer structure–function relationships (Garduño et al. 1995). Mutations leading to lipopolysaccharide (LPS) O-chain-deficient strains gave rise to A-layer-deficient strains that released free sheets of assembled A-layer into the culture medium. This led to the assumption that LPS O-chains were involved in tethering the *A. salmonicida* A-layer to the bacterial outer membrane (Belland & Trust 1985, Dooley et al. 1989). *A. salmonicida* strains lacking the A-layer, but possessing the LPS O-chain, rapidly absorbed secreted A-protein at the cell surface to coat the cells with a single confluent layer (Griffiths & Lynch 1990). Physical reconstitution of the A-layer could be achieved by co-culturing *A. salmonicida* A-layer donor and receiver, or suspending A-layer-deficient cells in a supernatant from A-layer-secreting cells (Garduño et al. 1995). These cells recovered such functions as hydrophobicity, autoaggregation, porphyrin binding, adherence to and invasion of fish macrophages and resistance to macrophage cytotoxicity (Olivier et al. 1986, Garduño & Kay 1992, Garduño et al. 1995, 2000, Daly et al. 1996).

The aim of the present study was to investigate the impact of reattached secreted A-layer protein of atypical *Aeromonas salmonicida* on vaccine efficacy in Atlantic cod.

**MATERIALS AND METHODS**

**Fish.** A total of 880 Atlantic cod were randomly selected from a mixture of 3000 fish originating from 10 families provided by the National Cod Breeding program at Norfima Marin, Tromsø, Norway, and used for vaccination (800 fish) and prechallenge (80 fish). The fish had a mean weight of 30 g (range 25 to 35 g), were healthy and unvaccinated. The experiment was approved by the National Animal Research Authority in Norway. Prior to marking, vaccination and challenge the fish were anaesthetized with Metacainum (70 mg l−1, Norsk Medisinaldepot). Different groups were marked at the operculum with Visible Implant Fluorescent Elastomer (Northwest Marine Technology).

**Bacterial isolates.** *Aeromonas salmonicida* and *Vibrio anguillarum* isolates used in this study are listed in Table 1. Atypical *A. salmonicida* aAs 4099 and subsp. *achromogenes* Asa 4075, both isolated from cod, in addition to subsp. *achromogenes* type strain Asa 4036, are referred to as atypical, while subsp. *salmonicida* type strain Ass 4010 is referred to as typical. Both Ass 4010 and Asa 4036 are A-layer-deficient due to a deletion in the *vapA* gene encoding the A-protein (Lund & Mikkelsen 2004). The Asa 4075 is O-chain negative and therefore secretes the A-protein to the culture medium. The *vapA* gene has been shown to possess a variable region that can be used for grouping of atypical isolates (Lund et al. 2003b). The strains aAs 4099 (GenBank accession number AJ749879), Asa 4036 (AJ49888) and Asa 4075 all group together (Lund & Mikkelsen 2004, authors’ unpubl. data).

Table 1. *Aeromonas salmonicida* and *Vibrio anguillarum* isolates used in this study. A: A-layer, O: polysaccharide O-chain

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Subsp./serotype</th>
<th>Phenotype</th>
<th>Host</th>
<th>Original designation</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. salmonicida</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aAs 4099</td>
<td>atypical</td>
<td>A+/O+</td>
<td>Atlantic cod</td>
<td>93/09/914</td>
<td>D. Colquhouna</td>
</tr>
<tr>
<td>Asa 4036</td>
<td><em>achromogenes</em></td>
<td>A–/O+</td>
<td>Brook trout</td>
<td></td>
<td>ATCC 33659</td>
</tr>
<tr>
<td>Asa 4075</td>
<td><em>achromogenes</em></td>
<td>A+/O–</td>
<td>Atlantic cod</td>
<td>T-233/91</td>
<td>B. Gudmundsdóttirb</td>
</tr>
<tr>
<td>Ass 4010</td>
<td><em>salmonicida</em></td>
<td>A–/O+</td>
<td>Atlantic salmon</td>
<td></td>
<td>NCIMB 1102</td>
</tr>
<tr>
<td><strong>V. anguillarum</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Va 2129</td>
<td>O1</td>
<td>O+</td>
<td>Rainbow trout</td>
<td></td>
<td>NCIMB 2129</td>
</tr>
<tr>
<td>Va 1282</td>
<td>O2</td>
<td>O+</td>
<td>Atlantic cod</td>
<td></td>
<td>Lund et al. 2007</td>
</tr>
</tbody>
</table>

aNational Veterinary Institute, Norway; bInstitute of Experimental Pathology, University of Iceland
The *Aeromonas salmonicida* LPS O-chain has been reported to differ between typical and atypical isolates. All typical isolates so far investigated produce a complete O-chain polysaccharide structure composed of a trisaccharide repeating unit. Atypical isolates, including aAs 4099 and Asa 4036, produce a disaccharide repeating unit (Wang et al. 2005, 2007).

Vibrio anguillarum isolates used were of serotype O1 (Va 2129), possessing high molecular weight O-chains of homogenous length (Knappskog et al. 1993) and serotype O2 (Va 1282) with O-chains of variable length (Lund et al. 2007).

**Media and growth conditions.** Bacterial isolates stored in glycerol at –80°C were inoculated on Tryptic Soya Agar (Oxoid) supplemented with 5% human blood and 1.5% NaCl (BA plate) and incubated for 3 d at 12°C. *Aeromonas salmonicida* was grown in brain heart infusion broth (BHI, Difco) and *Vibrio anguillarum* in marine broth (MB-2216, Difco) at 12°C for 24 to 30 h until an optical density at 600 nm (OD_{600nm}) of approximately 1.0. The bacterial cultures were either used for challenge and attachment of A-layer protein or inactivated by adding formaldehyde solution (37%) to a final concentration of 0.5% (v/v), before being used as vaccines or as antigens on Western blot. BA plates were used to determine cfu of challenge doses. BHI agar supplemented with 2% NaCl and 0.005% Coomassie Brilliant Blue R (Sigma) (BHI-CBB plates) was used for re-isolation of *A. salmonicida* from moribund fish. Growth of *A. salmonicida* was confirmed by Mono-As agglutination test (Bio-Nor).

**Electrophoresis and Western blotting.** *Aeromonas salmonicida* isolates were characterised by sodium dodecyl sulphate-polyacrylamid gel electrophoresis (SDS-PAGE) and Western blotting using the Bio-Rad Criterion system including premixed running buffer and XT MOPS sample buffer with reducing agents (Lund et al. 2007). Briefly, lysates of bacterial cells or supernatants were separated in 12% Bis-Tris gels (Lund et al. 2006). Bacterial cells (C) washed once in saline after centrifugation or supernatant (S) containing secreted A-layer protein (Table 2). B and C vaccines contained 10^{10} cells ml^{-1}. The bacterial pellet was re-suspended in saline or supernatant to the appropriate concentration, whereby OD_{600nm} = 1 corresponds to approximately 10^{8} cells ml^{-1}. Vaccine 4036(A–)+C was prepared by suspending the washed Asa 4036(A–) cell pellet in Asa 4075 supernatant (OD_{600nm} = 1). The mixture was incubated for 30 min with gentle shaking before being inactivated as described above and emulsified with the

**Table 2. Gadus morhua** vaccinated with oil-adjuvanted vaccines and intraperitoneal (ip) challenged with *Aeromonas salmonicida* aAs 4099. Vaccines are named according to the isolate number. The control group received saline without oil. Relative percent survival (RPS) calculated for each group (Gr.) as average in 2 tanks. Average mortality in control groups was 82% (see Fig. 4).

<table>
<thead>
<tr>
<th>Gr.</th>
<th>Vaccine</th>
<th>Vaccine antigen</th>
<th>RPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4099-C</td>
<td>aAs 4099 cells</td>
<td>78</td>
</tr>
<tr>
<td>2</td>
<td>4099-B</td>
<td>aAs 4099 bacterin</td>
<td>73</td>
</tr>
<tr>
<td>3</td>
<td>4036(A–)+C</td>
<td>Asa 4036 cells</td>
<td>35</td>
</tr>
<tr>
<td>4</td>
<td>4036(A–)+C</td>
<td>Asa 4036 cells with reattached Asa 4075 A-protein</td>
<td>76</td>
</tr>
<tr>
<td>5</td>
<td>4075-C</td>
<td>Asa 4075 cells</td>
<td>36</td>
</tr>
<tr>
<td>6</td>
<td>4075-B</td>
<td>Asa 4075 bacterin</td>
<td>39</td>
</tr>
<tr>
<td>7</td>
<td>4075-S</td>
<td>Asa 4075 supernatant with secreted A-protein</td>
<td>35</td>
</tr>
<tr>
<td>8</td>
<td>Saline</td>
<td>Saline</td>
<td></td>
</tr>
</tbody>
</table>
Vaccine 4075-S contained supernatant (total protein 3.2 mg ml−1) emulsified in oil adjuvant.

**Vaccination and challenge.** Seven groups of 100 fish were vaccinated by intraperitoneal (ip) injection of 0.1 ml vaccine fish−1 (Table 2). The control group received 0.1 ml saline fish−1. The groups were kept in separate tanks (500 l) at 10°C for 7 wk. Five days prior to challenge the 8 groups were distributed as follows; 50 fish from each group were placed into duplicate tanks resulting in a total of 400 fish in each tank (900 l).

Prechallenge and challenge were performed at 12°C. Prechallenge was performed 3 wk prior to challenge of vaccinated fish in order to determine the dose of aAs 4099 (cfu fish−1) needed to obtain 60 to 90% mortality in an unvaccinated control group. Three groups of 20 fish were marked before receiving 0.1 ml doses of 10-fold dilutions of an aAs 4099 culture and transferred to a 500 l tank. In addition a control group of 20 fish injected with 0.1 ml saline was included. The exact dose was determined by plating on BA-plates. Doses of 7.3 × 10^5, 7.3 × 10^4 and 7.3 × 10^3 cfu fish−1 resulted in 100, 45 and 31% cumulative mortality, respectively.

The vaccinated groups were challenged in parallel tanks by ip injection of 0.1 ml fish−1 of a bacterial dilution containing 1.6 × 10^5 cfu of aAs 4099. Dead and moribund fish were recorded and removed daily. Cause of death was verified by re-isolation of *Aeromonas salmonicida* from head kidney seen as blue colonies on BHI-CBB plates. Relative percent survival (RPS) was calculated according to the method of Amend (1981).

**Statistical analysis.** Logistic regression (Type III sum of squares) in the GENMOD procedure of SAS 9.1.3 (SAS 1993) was used to determine statistical differences in mortality between duplicate tanks and between the vaccinated and unvaccinated groups. Comparison of the vaccines was based on the results from duplicate tanks and each vaccine was compared to the others one by one. The results were considered significant for p < 0.05.

**RESULTS**

*Aeromonas salmonicida* isolates (Table 1) used as vaccines and the additional isolate Ass 4010 were characterised by SDS-PAGE and Western blotting (Fig. 1). Protein staining of the gel indicated that only the aAs 4099 cells and the Asa 4075 supernatant contained a major protein of 50 kDa (Fig. 1A), confirmed to be the A-layer protein by the R-α-A serum by Western blot (Fig. 1B,C). All *A. salmonicida* isolates except Asa 4075 contained high molecular weight O-chains as identified by the LPS O-chain specific mAb 2E6 (Fig. 1D). Only the Asa 4036 supernatant contained considerable amounts of O-chains (Fig. 1E), and only the LPS O-chain-negative Asa 4075 secreted A-protein into the culture supernatant (Fig. 1C). Hereafter, the *A. salmonicida* isolates are referred to as aAs 4099 A+/O+, Asa 4036 A−/O+, Asa 4075 A+/O− and Ass 4010 A−/O+.

Fig. 1. *Aeromonas salmonicida* isolates characterised by SDS-PAGE and Western blot. (A) Coomassie staining of gel with cell lysates and supernatants. Western blot with (B) cell lysates, (C) supernatants stained with R-α-A serum, (D) cell lysates and (E) supernatants stained with lipopolysaccharide (LPS) O-chain-specific mAb 2E6. Lanes: 1, Asa 4099 cells; 2, Asa 4099 supernatant; 3, Asa 4036 cells; 4, Asa 4036 supernatant; 5, Asa 4075 cells; 6, Asa 4075 supernatant; 7, Ass 4010 cells; 8, Ass 4010 supernatant. Molecular weight marker (M) indicated in kDa.
4036 A−/O+ isolates, but not to O-polysaccharide possessing V. anguillarum serotype O1 or O2 (Fig. 2). Repeated washing, up to 5 times, of the Asa 4036 cells did not remove the A-layer protein that was firmly attached to the cell surface (Fig. 3).

Protection elicited in Atlantic cod by vaccines based on Aeromonas salmonicida isolates possessing or lacking the A-layer protein and/or the O-chains, or an isolate with reattached A-layer protein, was compared in duplicate tanks when ip challenged with the aAs 4099 A+/O+ isolate (Table 2). The results are presented as average RPS (Table 2) and as cumulative mortality (%) in each group in duplicate tanks (Fig. 4).

Total cumulative mortality did not differ between the tanks (p = 0.0852). The vaccine based on aAs 4099 A+/O+ cells (4099-C) or bacterin (4099-B) did not differ significantly. Furthermore, the vaccine based on Asa 4036 A−/O+ cells with reattached A-layer protein, 4036(A+)-C did not differ from the 4099-C vaccine, while vaccines 4036(A−)-C and 4075-C with A-layer-deficient cells, or vaccine 4075-S containing supernatant with secreted A-layer protein, protected significantly less. Compared to the control group, all vaccines elicited significant protection (Table 2), but vaccines based on A. salmonicida A+/O+ cells elicited equal and significantly better protection (RPS 73 to 78) than vaccines based on A−/O+ or A−/O− cells or on A+ supernatant (RPS 35 to 39).
DISCUSSION

Physical reconstitution of the Aeromonas salmonicida A-layer has demonstrated recovered functions absent from A-layer-deficient mutants such as hydrophobicity, autoaggregation, adherence to fish cell lines and survival in head kidney macrophages (Garduño et al. 1995, 2000). In the present study we have used the same approach to investigate the importance of the A. salmonicida A-layer for protection in furunculosis vaccines for Atlantic cod. Ideally, A. salmonicida strains with a genetically reconstituted A-layer should be used, but as such were not available, A-layer protein was physically reattached to an A-layer-deficient isolate.

The Aeromonas salmonicida A-layer forms a complex structure partly embedded in the O-polysaccharide chains of homogenous length (Chart et al. 1984), of which only a portion penetrate the A-layer becoming accessible at the cell surface. Both the A-protein (Lund & Mikkelsen 2004) and O-polysaccharide (Wang et al. 2007) have been shown to vary between typical and atypical A. salmonicida isolates, giving rise to putative variation in surface determinants between the isolates. In the present study A-layer protein secreted by Asa 4075 and genetically identical to that of the challenge isolate aAs 4099 (authors’ unpubl. data) was attached to Asa 4036 A–/O+ cells producing the same O-chain type as aAs 4099 (Wang et al. 2007). Therefore, the 4099-C and 4036(A+)-C vaccines should be comparable with regard to the A-protein and O-chain. These were shown to protect equally well and significantly better than the A-layer-deficient vaccines 4036(A–)-C and 4075-C, against challenge with aAs 4099. The A-layer-deficient Asa 4036 has been shown to be non-virulent (authors’ unpubl. data) and could not be used for challenge.

Interestingly, supernatant with A-layer protein as the major component protected poorly, as did a vaccine based on purified A-protein in a previous study (Lund et al. 2008a). Mixing of Asa 4036 A–/O+ cells with Asa 4075 A+ supernatant resulted in cells with reattached A-layer protein that elicited protection. Together with previous results that Aeromonas salmonicida A+/O+ isolates elicited acceptable protection against unrelated gram-negative bacteria (Griffiths & Lynch 1990, Garduño et al. 1995). Similarly, in the present study the A-layer protein did not associate with the cell surface of unrelated gram-negative bacteria (Griffiths & Lynch 1990, Garduño et al. 1995). Interestingly, although the O-polysaccharides of typical and atypical A. salmonicida represents distinct structural types composed of trisaccharide or disaccharide repeating units, respectively (Wang et al. 2007), the A-layer protein appeared to reattach onto both typical and atypical A–/O+ cells. This may result in differences in surface topography and antigenic determinants and may have impact on vaccine efficacy, which should be further assessed.

The reason the A-layer protein appears protective on the cell surface and not in solution may be that protective epitopes are recovered either as sole A-layer protein epitopes in correct conformation guided by the O-chains or in combination with the O-chains or with
other membrane components. Both a dissociable inter-
action between A-protein subunits and the O-polysac-
ccharide chains (Garduño et al. 1995) and a tight inter-
action with outer membrane proteins, probably a porin, have been suggested (Garduño et al. 1994).

In summary, the present study confirms previous results that vaccines based on *Aeromonas salmonicida* A-layer-possessing isolates elicit significantly better protection in cod than a vaccine based on an A-layer-deficient isolate. Reattachment of the A-layer protein on an A-layer-deficient isolate was performed in order to produce *A. salmonicida* cells with an A-layer protein and an O-polysaccharide chain structure comparable to the challenge isolate. Such cells elicited significant and equal protection to that seen with the homologous vac-
cine. These results indicate that the A-layer protein is important for achieving protection. Physical reattach-
ment of A-layer sheets or purified A-protein should prove useful in elucidating the role of the A-layer in in-
ducing protection in various farmed fish species.

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