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A Conserved *Aeromonas salmonicida* Porin Provides Protective Immunity to Rainbow Trout

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A protein with an apparent M_r of 28,000 was isolated from outer membrane preparations of *Aeromonas salmonicida* A440. The protein was tested for the ability to form pores, using a planar lipid bilayer model membrane system. The protein appeared to be a monomer with a single-channel conductance in 1.0 M KCl of 1.96 nS and a cation/anion permeability ratio of 2.91 ± 0.68. These data show that the porin channel is comparable in size to OmpC and OmpF of *Escherichia coli* and is relatively nonselective, having some preference for cations over anions. The porin was purified by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and a polyclonal antibody was raised. Immunoblot analysis showed that an immunologically cross-reactive protein was present in other *Aeromonas* strains but not in strains of *Vibrio* or *Yersinia*. The N-terminal amino acid sequence of the porin was determined and was found to show some homology to an *Aeromonas hydrophila* outer membrane protein. This is the second porin species of *A. salmonicida* to be described, and it differs from the other in subunit molecular weight, aggregation properties, peptidoglycan association, pore size, and antigenicity. Rainbow trout (*Oncorhynchus mykiss*) immunized intraperitoneally with the purified porin protein were significantly protected from experimental *A. salmonicida* challenge. This is the first report of successful vaccination against *A. salmonicida* with a purified outer membrane component.

The gram-negative bacterium *Aeromonas salmonicida* is the causative agent of furunculosis, a systemic disease of salmonid fish that causes significant yearly losses to the aquaculture industry. The precise mechanism of pathogenesis of this organism is not known, although the paracrystalline surface protein array (A-layer) is thought to be essential for virulence (23), shielding the bacteria from the bacteriocidal action of serum components (35) and promoting adhesion to a variety of molecules such as porphyrins (27), immunoglobulins (39), and laminin and fibronectin (9) as well as macrophages (16, 45). Little is known about the anchoring of this layer to the outer membrane, but Tn*5* insertion mutagenesis has identified several genes important in the secretion and organization of an ordered, functional A-layer (2). The presence of a normal lipopolysaccharide (LPS) moiety is essential for anchoring (2, 26), and cross-linking experiments have identified several outer membrane proteins (including the subject of this study) that are closely associated with the A-layer (26). The outer membrane of virulent *A. salmonicida* is masked by the A-layer but is known to contain a porin (7) and receptors for bacteriophage (24) and is permeable to small molecules and LPS O chains.

Commercial vaccines against *A. salmonicida* normally consist of killed cells, extracellular fractions, cell culture supernatants, and avirulent strains (10, 13, 18, 37). Although both *A. salmonicida* A-protein and LPS have been shown to stimulate antibody production in fish (1, 5, 18, 31), the protective effects of these cell surface components have not been clearly established.

Porins are essential for bacterial metabolism and act as the major uptake channels for certain antibiotics. Porins are most often heat modifiable (i.e., their migration on sodium dodecyl sulfate [SDS]-polyacrylamide gels differs depending on the solubilization temperature [8]) and associated with peptidoglycan (PG) (41). This study characterizes an outer membrane porin from *A. salmonicida* with an apparent subunit M_r of 28,000 that creates a relatively nonspecific water-filled pore comparable in dimensions to well-characterized *Escherichia coli* trimeric porins (36). The porin is heat modifiable, with its apparent molecular weight (determined by SDS-polyacrylamide gel electrophoresis [PAGE]) increasing after prolonged heating, but it is not peptidoglycan associated. This porin appears to be a conserved feature of the genus *Aeromonas* and is capable of conferring protective immunity against *A. salmonicida* to salmonid fish. This finding indicates that at some point in the infective cycle, the barrier activity of the A-layer may be compromised, exposing the outer membrane to the surrounding milieu in vivo.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study and their origins are detailed in Table 1. Stock cultures were maintained at −70°C in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) containing 15% glycerol. Cultures were routinely grown on tryptone soy agar (Difco Laboratories, Detroit, Mich.) or tryptic soy broth (Difco) for 36 h at 20°C. Atypical *A. salmonicida* strains were grown at 20°C on Trypticase soy agar supplemented with 5% (vol/vol) horse blood.

Outer membrane preparations. Cell envelopes were prepared by passing suspensions of *A. salmonicida* A440 through a French pressure cell three times in succession. Intact cells were then removed by centrifugation at 4,000 × *g*, and fractured membranes were recovered from the supernatant by centrifugation at 43,000 × *g*. Outer membranes were prepared by differential solubilization of the inner membrane, using the detergent sodium lauryl sarcosinate (Sarkosyl; Sigma) as described by Marchesi and Andrews (32). Total membranes were shaken for 30 min at a protein/detergent ratio of 1:6 (milligrams per milliliter), and the preparation was centrifuged at 43,000 × *g* for 30 min at 4°C. The pellet of fractured outer membranes was washed three times in 20 mM Tris-HCl (pH 7.4).

Electrophoresis. Qualitative SDS-PAGE was performed in a Bio-Rad minigel

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TABLE 1. Strains used

Strain	Source
Aeromonas salmonicida	
Typical	
A202	Japan
A203	Japan
A362	Atlantic salmon, United Kingdom
A438	Coho salmon, British Columbia
A440	Ex-ATCC 14174, brook trout, United States
A449	Brown trout, Eure, France
A500	Atlantic salmon
Atypical	
A206	Masou, Japan
A419	Goldfish, Arkansas
A. hydrophila	
A274	Sloth, Australia
TF7	Trout lesion, Quebec, Canada
A. veronii biotype sobria	
A225	Trout, Quebec, Canada
Vibrio anguillarum	
PG75-834	Sockeye salmon
Yersinia ruckeri	
2TC74-3kd	Steelhead trout, Washington
1ST75-3kd	South Tacoma, Wash.

apparatus by the method of Laemmli (29). Proteins solubilized in sample buffer (0.125 M Tris [pH 6.8], 4% [wt/vol] SDS, 20% [vol/vol] glycerol, 10% [vol/vol] 2-mercaptoethanol, 5 μg of bromphenol blue per ml) were stacked in a 4.5% (wt/vol) acrylamide gel and separated on 7.5, 12.5, or 15% (wt/vol) acrylamide gels for 45 min at 150 V. The gels were stained with Coomassie blue R-250. For visualization of LPS on SDS-polyacrylamide gels, the proteinase K digestion-silver stain method of Hitchcock and Brown (21) was used.

Purification of protein. For pore determination, outer membranes were prepared as described above, and samples were mixed with an equal volume of sample buffer lacking bromphenol blue and electrophoresed on a 12.5% polyacrylamide gel. The unstained segment of gel corresponding to the approximate location of the 28-kDa protein (as judged by comparison with prestained standards) was cut out, and the protein was eluted into sterile H_2O overnight at 4°C. The protein was concentrated in a Microsep centrifuge concentrator with a 10-kDa cutoff. A portion of the sample was electrophoresed and stained to determine purity and concentration.

For purification of large amounts of the porin, preparative SDS-PAGE was carried out in a Bio-Rad preparative SDS-PAGE apparatus. Outer membrane preparations were solubilized in sample buffer (without heating) and applied to a 4.5% acrylamide stacking gel atop a 12.5% separating gel. Gels were run at 500 V for 16 h, and 5-ml fractions were collected every 10 min after the dye front had eluted in a Pharmacia fraction collector. An aliquot of each fraction (20 μl) was analyzed by minigel SDS-PAGE, and fractions containing the protein of interest were pooled and concentrated in Microsep centrifuge concentrators with 10-kDa cutoff filters (Filtron).

Heat modification and peptidoglycan association. To determine the heat modification behavior of outer membrane proteins, samples of outer membranes or of the purified 28-kDa protein were solubilized in loading buffer at a variety of temperatures, electrophoresed through SDS–12.5% polyacrylamide gels, and stained with Coomassie blue. The peptidoglycan association assay was performed essentially as described by Logan and Trust (30). Total membranes were suspended in extraction buffer containing 2% (wt/wt) SDS, 10 mM Tris (pH 7.4), and 10% (vol/vol) glycerol. The samples were then heated at 60°C for 30 min and centrifuged at 45,000 × *g* for 1 h. The supernatant was removed, and the pellet, containing proteins associated with the peptidoglycan, was resuspended in the same volume. Aliquots of this suspension were electrophoresed on SDS-polyacrylamide gels, which were stained with Coomassie blue.

Antiserum. An antiserum to the gel-purified protein was prepared in an adult New Zealand White rabbit by injection of homogenized gel fragments emulsified in Freund's complete adjuvent. Booster doses were given in Freund's incomplete adjuvent on days 15 and 30 after initial immunization. On day 45, the rabbit was bled, and the serum was collected and stored in aliquots at −20°C. The antiserum was preabsorbed with *E. coli* DH5α before use.

N-terminal amino acid sequence analysis. Outer membranes prepared as described above were electrophoresed through SDS–12.5% polyacrylamide gels. The proteins were electrophoretically transferred as described above onto Immobilon membranes on a Bio-Rad transfer apparatus. The membrane was stained with Coomassie blue for 1 s and destained with 10% acetic acid–40% methanol–50% H_2O. The membrane was dried, bands of interest were cut out, and the N-terminal sequences were determined by automated Edman degradation at the University of Victoria protein sequencing facility.

Immunoblotting. Proteins previously electrophoresed on polyacrylamide gels were transferred to nitrocellulose paper (NCP) by using a semidry transfer apparatus (Pharmacia/LKB). The NCP containing the immobilized proteins was blocked with a 5% (wt/wt) solution of skim milk in Tris-buffered saline (TBS). The NCP was then incubated with an appropriate dilution of antiserum in TBS–2% skim milk at room temperature for 3 h or overnight. After being washed three times in TBS, the NCP was incubated with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G for 2 h at room temperature, washed, and developed with 5-bromo-4-chloro-3-indolylphosphate (X-phosphate; Sigma) and nitroblue tetrazolium (Sigma) in 20 mM Tris-Cl (pH 9.6)–10 mM $MgCl_2$ buffer.

Porin characterization by using black lipid bilayers. Pore-forming ability was assessed in a planar lipid bilayer model membrane system as previously described (3). For single-channel conductance measurements, lipid bilayers made from 1.5% (wt/vol) oxidized cholesterol in *n*-decane were formed across a 0.2-mm^2 hole separating two compartments of a Teflon chamber which contained an aqueous salt solution. Approximately 10 ng of protein sample, solubilized in 0.1% Triton X-100, was added to one compartment, and 50 mV was applied across the lipid bilayer. Conductance increases were recorded for each salt solution used. Zero-current membrane potential experiments were performed in chambers with a 2.0-mm^2 hole separating the compartments, which each contained 6 ml of 0.1 M KCl. Fifty nanograms of protein (in 0.1% Triton X-100) was then added to one compartment, a voltage of 20 mV was applied, and the conductance across the bilayer was allowed to increase to 10^9 A, at which time the voltage was removed. An aliquot of 100 μl of 3.0 M KCl was added to one side of the membrane, while 100 μl of 0.1 M KCl was added to the other. Eight additions were made, and the zero-current membrane potential was measured after each addition.

Fish protection studies. Rainbow trout (*Onchorhyncus mykiss*) (weight, 10 to 12 g) were injected intraperitoneally with 20 μg of protein in phosphate-buffered saline emulsified with an equal volume of Microgen (Microtek International Ltd., Victoria, British Columbia, Canada). The fish were kept at 13°C (±1°C) in continually flowing dechlorinated city water for 5 weeks before challenge. The fish were fed ad libitum with fish feed until the time of challenge. Controls of nonhandled fish, fish injected with Microgen only, and fish immersed or injected with a preparation of commercial bacterin vaccine (formalin-killed *A. salmonicida* MT26; Microtek International Ltd.) were maintained under identical conditions. The fish were challenged by immersion in a suspension of the heterologous *A. salmonicida* strain MT16 (Microtek International Ltd.) (10^7 cell per ml, a concentration determined previously to kill approximately 70% of nonhandled fish) for 15 min with aeration. Seventy fish were used in each group. Mortalities were counted daily, and relative percent survival (RPS) was calculated as 1 − [(% vaccinated mortality/% control mortality) × 100].

RESULTS

Properties and purification of the 28-kDa protein. Figure 1A shows the SDS-PAGE outer membrane protein profile of *A. salmonicida* A440 at several different solubilization temperatures. A protein with an apparent M_r of 28,000 was a major species in the preparation, and its migration was unaffected by short exposure to solubilization temperatures from 20 to 100°C, whereas the 43-kDa protein, previously shown to be a porin (7), existed in a higher-molecular-weight form at the lower temperatures. As outer membrane proteins are often associated with PG, a PG association assay of the A440 outer membrane was performed. The resulting gel (Fig. 1B) showed that the 43-kDa porin was associated with the PG, whereas the 28-kDa protein was not.

The 28-kDa species was then purified from outer membrane preparations by using preparative-scale SDS-PAGE. An SDS-polyacrylamide gel of purified 28-kDa protein is shown in Fig. 1C. When the pure protein was heated at 100°C for long periods of time (Fig. 1C), a second band appeared at 33 kDa. This band is possibly due to a relaxing of the secondary structure of the protein, as demonstrated in other outer membrane proteins (8), and may represent the true molecular weight of the protein. To check that the protein was free of contaminating LPS, proteinase K-treated SDS-polyacrylamide gels of the protein preparation were silver stained with A440 whole-cell lysates as positive controls. Even at high concentrations of protein, no evidence of LPS contamination could be seen by

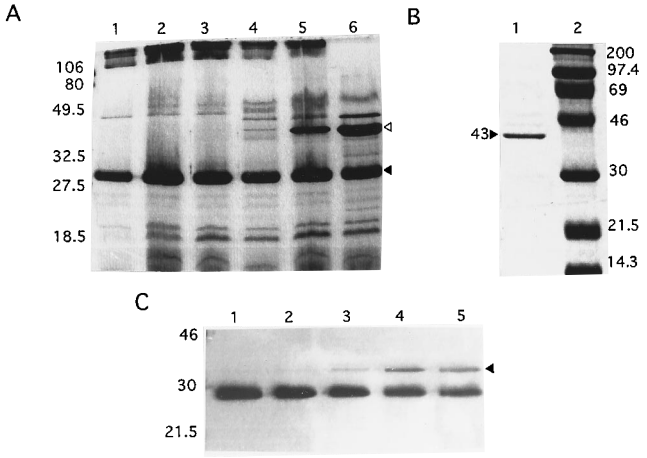

FIG. 1. (A) SDS-PAGE of *A. salmonicida* A440 outer membranes solubilized at different temperatures. Lanes: 1, 20°C; 2, 37°C; 3, 50°C; 4, 60°C; 5, 70°C; 6, 100°C. Protein standard molecular masses are shown in kilodaltons on the left. (B) SDS-PAGE of PG-associated outer membrane proteins (lane 1) and molecular mass standards (kilodaltons) (lane 2). (C) Heat modification of purified 28-kDa porin. Lanes: 1, unheated; 2, boiled for 5 min; 3, boiled for 20 min; 4, boiled for 1 h; 5, boiled for 2 h. Protein standard molecular masses are shown in kilodaltons on the left.

this method (data not shown). This protein was used to immunize a rabbit for the production of a polyclonal antiserum.

Conservation of protein among *A. salmonicida* strains. Western blots (immunoblots) of whole-cell lysates and outer membrane preparations of *A. salmonicida* A440 showed that the rabbit antiserum was specific for the 28-kDa protein. The antiserum also recognized a 33-kDa band in samples of the protein that had been boiled for extended periods (data not shown), indicating that this band is the same protein. Figure 2 is an immunoblot of this antiserum against whole-cell lysates of bacteria and shows that the protein was conserved among typical *A. salmonicida* strains from a wide variety of geographic areas. The protein also appeared to be conserved at the same molecular weight in atypical *A. salmonicida* strains, as well as *A. sobria* 430 and *A. hydrophila* TF7 and 250. The strength of the antibody reaction appeared less in these cases, possibly indicating epitopic differences or differences in levels of protein expression. An antigenically cross-reactive protein was not observed in *Vibrio anguillarum* or *Yersinia ruckeri* strains.

N-terminal amino acid sequence. Figure 3 shows the N-terminal sequence determined for 19 residues of the protein compared with the amino-terminal sequence determined for a 31-kDa *A. hydrophila* outer membrane protein reported by Jeanteur et al. (25). The sequences were 50% identical. Jeanteur et al. suggested that the 31-kDa protein was possibly an *Aeromonas* homolog of OmpA from *E. coli*, and the N-terminal portion of this protein is shown in Fig. 3 for comparison. There is little similarity between the N-terminal sequences of the *A. salmonicida* 28-kDa protein and *E. coli* OmpA (21% identity), indicating that the *A. salmonicida* protein may not be an OmpA homolog. The 28-kDa protein does share very limited identity with the OmpF, OmpC, and PhoE porins of *E. coli*, containing an N-terminal alanine and an IYXXXG motif.

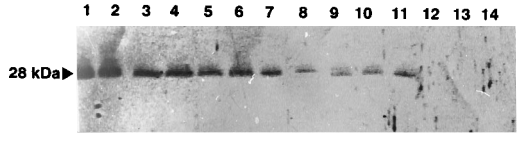

FIG. 2. Immunoblot of whole-cell lysates of various bacteria against a polyclonal antiserum raised against the 28-kDa protein. Lanes: 1, *A. salmonicida* A202; 2, *A. salmonicida* A362; 3, *A. salmonicida* A438; 4, *A. salmonicida* A440; 5, *A. salmonicida* A449; 6, *A. salmonicida* A500; 7, *A. salmonicida* A206; 8, *A. salmonicida* A419; 9, *A. hydrophila* A274; 10, *A. hydrophila* TF7; 11, *A. sobria* A225; 12, *V. anguillarum* PG75-834; 13, *Y. ruckeri* 2TC74-3kd; 14, *Y. ruckeri* 1ST75-3kd.

A.

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H2N-ADDIYFGAGAGAAHFNGLN   A. salmonicida 28kDa protein
    A+D Y G GAG A+ + LN
H2N-ANDWYTGIGAGWAYGHDLN   A. hydrophila Omp V(25)
     N WYTG   GW   HD
H2N-DNTWYTGAKLGWSQYHDTG   E. coli OmpA
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B.

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H2N-AVIYDKDGTTFDVY        A. salmonicida 43kDa OMP
    AVIYDKDGTTFDVY
H2N-AVIYDKDGTTFDVY        A. hydrophila OMP II(25)
    A IY KDG   DVY
H2N-AEIYNKDGNKLDVY        E. coli PhoE
    AEIYNKDGNK D Y
H2N-AEIYNKDGNKVDLY        E. coli OmpF
    AE YNKDGNK DLY
H2N-AEVYNKDGNKLDLY        E. coli OmpC

    AEIYNKDGNK-DVY        Consensus
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FIG. 3. N-terminal sequence homologies for the 28-kDa protein (A) and 43-kDa protein (B). Crosses indicate conservative substitutions.

Figure 3 also shows the N-terminal amino acid sequence obtained for the predominant 43-kDa protein band present in outer membrane preparations. This protein has been previously shown to be a porin (7). The 43-kDa protein amino-terminal amino acid sequence was identical to that of the 39-kDa *A. hydrophila* outer membrane protein II (25), which has also been reported to be carbohydrate binding (40), and 57% identical to OmpF, 50% identical to OmpC, and 64% identical to PhoE from *E. coli*, which suggests that this protein is the *Aeromonas* analog of these well-characterized porins.

Pore-forming ability of 28-kDa protein. As the N-terminal sequence and the heat modification behavior of the protein suggested similarity to known porins, the pore-forming ability of the purified protein was tested in a planar lipid bilayer system. Stepwise increases in conductance across the lipid bilayer were observed only upon addition of solubilized 28-kDa protein to the model system (Fig. 4), not when detergent alone was added. This finding indicates that this protein does indeed form channels. Single-channel conductance measurements were fairly evenly distributed about a single mean for all salt solutions tested (Fig. 5), and the average single-channel conductance in 1.0 M KCl was 1.96 nS (Table 2). When larger anions and cations were used (CH_3COO^- and Li^+), the conductance was decreased. Zero-current membrane potential experiments showed a slight channel selectivity for cations. The permeability ratio Pc/Pa, as determined by the Goldman-Hodgkin-Katz equation, where Pc was the permeability of cations and Pa was the permeability of anions, was 2.91 ± 0.68.

Vaccine trial. To determine the ability of the purified porin to confer protective immunity on salmonids, a solution of the protein in adjuvant was injected into the peritoneal cavities of rainbow trout. The trout were maintained with feeding for 5 weeks and then challenged with a heterologous virulent *A. salmonicida* strain. Figure 6 shows the cumulative mortalities of experimental fish vaccinated with purified protein and those of control fish. The vaccinated fish had RPS values of 53

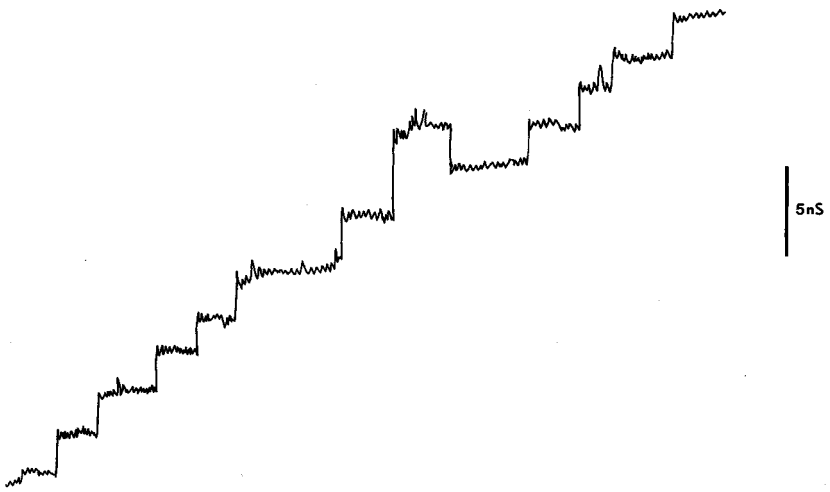

FIG. 4. Chart recorder tracing showing stepwise conductance increases which occur upon the addition of 10 ng of protein (diluted in 0.1% Triton X-100) to the model membrane system. No increases in conductance were observed when detergent alone was added.

compared to nonhandled control fish and 49 compared with the Microgen injection control (Table 3). Fish injected with adjuvant alone were protected from infection to a small degree (RPS = 8.4), a well-characterized phenomenon that probably results from nonspecific stimulation of the fishes immune system (34). Comparison with a commercial bacterin vaccine showed that the porin was more efficient than the bacterin administered by immersion (RPS = 19.4) but less efficient than intraperitoneally injected bacterin (RPS = 72.6). The purified porin injected intraperitoneally protected fish as well as a 15-min immersion in the live attenuated A440 parent strain (36a) (comparable results were obtained by Thornton et al. [44]).

DISCUSSION

This study describes the isolation and functional characterization of a major *A. salmonicida* porin and shows that this porin can provide significant protective immunity to *A. salmonicida* in rainbow trout. The porin has an apparent molecular mass of 28 kDa (non-heat modified), although its true mass is probably 33 kDa, as shown by heat modification experiments. This porin was different from the previously characterized 43-kDa porin of *A. salmonicida* in several respects. The 43-kDa porin exists as a multimeric unit that is dissociated when heated under denaturing conditions, whereas the 28-kDa porin described in this report appears to run as a monomer under all conditions, although it does display an increase in molecular mass when boiled for long periods of time, a feature common to classical monomeric porins. In contrast to the 43-kDa porin, which was peptidoglycan associated, the 28-kDa porin was not. The 43-kDa porin N-terminal sequence was very similar to that of the OmpC/OmpF/PhoE family of bacterial porins, known to exist as trimers, and it seems that the 43-kDa porin is a homolog of these systems. The *A. hydrophila* analog of this porin has been shown to bind sugar residues, leading to a hemagglutinating property (40), and results from binding assays using biotinylated yeast cell surface components performed in our laboratory suggest that this is also the case for the *A. salmonicida* protein (31a). This may account for the hemagglutination and yeast agglutination phenotypes of *A. salmonicida* strains that lack the A-layer.

The 28-kDa porin showed some N-terminal similarity to other well-known *E. coli* porins and also some similarity to the previously described (25) outer membrane protein V of *A. hydrophila*. Although it was not determined in the study of Jeanteur et al. whether protein V was a porin, it is thought to be a homolog of OmpA, a monomeric *E. coli* protein of 32 kDa that has been shown to be involved in the virulence mechanism of enterotoxigenic strains, increasing serum resistance (46), as well as being a porin (43).

Although a majority of porins identified to date are active in vivo as trimers, the pore-forming abilities of a number of monomeric pore-forming proteins, including OmpA and its ho-

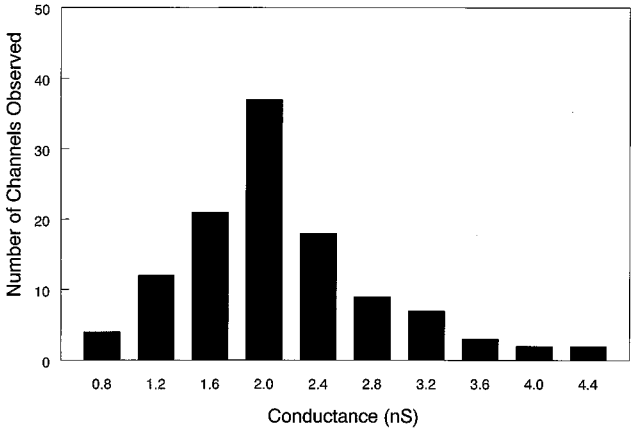

FIG. 5. Distribution of single-channel conductance measurements for the 28-kDa porin in 1.0 M KCl. The histogram shows a distribution about a single mean, which is typical for most porin proteins.

TABLE 2. *A. salmonicida* single-channel conductance measurements

Salt	No. of channels observed	Avg single-channel conductance (nS)
0.3 M KCl	111	0.68
1.0 M KCl	112	1.96
3.0 M KCl	107	5.55
1.0 M CH_3COOK	92	1.18
1.0 M LiCl	100	1.17

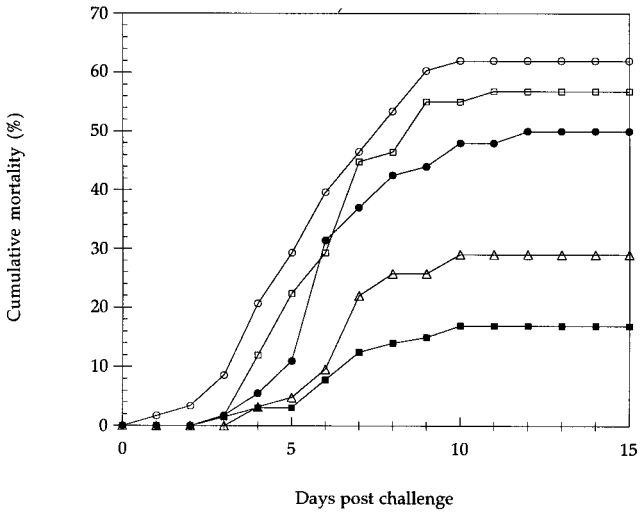

FIG. 6. Fish protection study. The graph shows percentage cumulative mortality against time for vaccinated and control fish. Open circles, nonhandled control; open squares, Microgen injection control; filled circles, bacterin immersion; open triangles, 28-kDa porin injection; filled squares, bacterin injection.

mologs, have been characterized (3, 38, 42). The 28-kDa *A. salmonicida* porin appeared to be of the latter class of proteins, as purified monomers were shown to exhibit pore-forming activity in lipid bilayers. These monomers produced larger channels than did other monomer porins, such as those found in *Campylobacter* and *Helicobacter* species (e.g., *Campylobacter coli*, *C. jejuni*, *C. rectus*, and *Helicobacter pylori*, all of which have pore sizes in the range of 0.24 to 0.82 nS in 1.0 M KCl [14, 22, 28, 38]). This also holds true when the channel sizes of monomer subunits of typical trimeric porins are compared. For example, *E. coli* OmpC, which is a trimeric porin, has a single-channel conductance of 1.5 nS in 1.0 M KCl (4), indicating that each monomer has a conductance of 0.5 nS, compared with the 1.96-nS conductance observed with the 28-kDa monomer porin. The trimeric 43-kDa porin from *A. salmonicida* has a conductance of 1.6 nS in 1.0 M KCl (7), very similar to that of OmpC. Although the N-terminal amino acid sequence of the 28-kDa porin has little homology with that of OmpA, OmpA displays the same heat-induced SDS-PAGE molecular mass increase, from approximately 27 kDa to 33 kDa (8), as the *A. salmonicida* protein. Furthermore, OmpA also exists as a monomer and has been shown to form quite large pores in liposome swelling assays (42). Similar observations have been made with another OmpA homolog, OprF from *Pseudomonas aeruginosa*. OprF alternatively forms either very large (5.6 nS) (3) or quite small (0.34 nS) (47) channels in 1.0 M KCl. Thus, the channels formed by the 28-kDa *A. salmonicida* porin are not unique in their large size, which may indicate a functional relationship to the OmpA family of proteins.

TABLE 3. Rainbow trout protection

Treatment	% mortality	RPS[a]
Nonhandled control	62	0
Microgen injection	57	8.4
Bacterin immersion	50	19.4
Bacterin injection	17	72.6
A440 immersion[b]	29	53.2
Porin injection	29	53.2 (relative to nonhandled control) 49.0 (relative to emulsigen injection)

[a] Defined as 1 − [% vaccinated mortality/% control mortality] × 100. Seventy fish were used in each group.
[b] Data from Noonan and Trust (36a).

Polyclonal antibodies that were raised to purified 28-kDa porin recognized a band of approximately the same molecular mass when immunoblots were performed with a variety of typical and atypical *A. salmonicida* strains and a variety of other species of the genus *Aeromonas* but did not recognize any proteins from the related genera *Vibrio* and *Yersinia*. This finding indicates that this porin is *Aeromonas* specific.

Experiments with rainbow trout showed that the purified porin when intraperitoneally injected with Microgen afforded a significant degree of protection from experimental *A. salmonicida* infection. The challenge was with a heterologous *A. salmonicida* strain, and therefore the applicability of the protective agent is likely to be broad. Immunization with purified porins has been correlated with protection against other bacteria, such as *Haemophilus influenzae* (17) and *Neisseria gonorrhoeae* (19), but this is the first report of a purified outer membrane protein providing protection against furunculosis. Portions of the 43-kDa porin recombinantly expressed in *E. coli* have, however, been shown to protect against *A. salmonicida* (6), and an extracellular protease has been reported by Ellis et al. (12) to be a protective antigen. Ellis et al. have also reported that two other purified antigens from *A. salmonicida* are capable of eliciting protection (11), but the nature of these antigens was not disclosed. Active immunization of Atlantic salmon with outer membrane preparations of *A. salmonicida* grown in iron-restricted conditions has been shown to be more effective than immunization with regular outer membrane preparations, suggesting that iron-repressible outer membrane proteins also have protective properties (20). Although studies have shown that isolated *A. salmonicida* A-layer (5, 31) and LPS (1, 31) induce a significant immune response in fish, the protective effects of these components have not been demonstrated. Protection has been shown not to correlate well with antibody titer (18), suggesting that cell-mediated immunity is most significant in providing protection. Although earlier studies suggest that A-protein may be a protective antigen (33), recently Thornton et al. (44) have shown that the vaccination of rainbow trout with attenuated live *A. salmonicida* strains that lack the A-layer and LPS O antigens can still significantly protect the fish from experimental challenge. This result correlates with the work described here, indicating that other outer membrane components are probably important in eliciting protective immunity in salmonids. These results also suggest either that the outer membrane is partially exposed to the components of the fish's immune system during infection or that the integrity of the A-layer is compromised at some stage.

The A-layer is vital for virulence of *A. salmonicida*, but the exact mechanism of pathogenicity remains unclear. *A. salmonicida* has been shown by Garduño and Kay (16) to be a facultative intracellular pathogen capable of surviving and replicating within macrophages and able to penetrate fish epithelial cells (epithelioma papulosum cyprini from common carp) in an A-layer-independent manner. Garduño (15) speculates that the A-layer mediates attachment of bacteria to host cells, which leads to the promotion of secondary interactions that trigger internalization of the bacterium. These secondary interactions could be mediated by outer membrane proteins, such as the putative 43-kDa hemagglutinin recognizing cell surface carbohydrate moieties, if they are exposed and may require the partial shedding of the protective surface array.

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