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Aeromonas salmonicida Possesses Two Genes Encoding Homologs of the Major Outer Membrane Protein, OmpA

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Two homologs of the outer membrane protein OmpA were identified in *Aeromonas salmonicida* by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunoblotting, and amino-terminal sequence analyses. An *A. salmonicida* genomic DNA library was constructed by using λGEM-11 and recombinant phage carrying both genes (*ompAI* and *ompAII*) selected by immunoscreening. A 5.0-kb *Bam*HI fragment containing the two genes in tandem was subcloned in pBluescript and used for further subcloning and sequencing of the genes. The encoded proteins (M_r = 33,564 and 32,536 for mature OmpAI and OmpAII, respectively) had only 64% identity with each other and otherwise had the highest level of homology to OmpA proteins from the members of the family *Enterobacteriaceae*. Based on the *Escherichia coli* OmpA model, an eight-stranded amphipathic β-barrel model for the membrane assembly of the N-terminal half of OmpAI and OmpAII was predicted. Most variation between the two proteins was localized to the predicted surface loops and periplasmic turns, while the transmembrane strands and C-terminal domains were highly conserved. Expression of *ompAI* and *ompAII* separately in *E. coli* indicated that both genes could be independently transcribed from their own promoters and that both gene products were assembled into the *E. coli* outer membrane. A survey of different *Aeromonas* spp. by PCR revealed that possession of two tandem *ompA* genes was widespread among this genus. This is the first report of any bacterial species possessing two genes for homologs of this major outer membrane protein.

The OmpA protein was initially identified as one of the major proteins of the outer membrane of *Escherichia coli*. Either a closely related homolog of this protein or a more distantly related relative has now been identified as a major protein of the outer membrane of a wide range of gram-negative bacteria (5, 12, 15, 16, 27). Major physiological functions attributed to the OmpA family include maintenance of the structural integrity and morphology of the cell (33) and porin activity (35), as well as a role in conjugation and bacteriophage binding (26). A role in bacterial virulence has been implicated for some of these proteins (9, 30, 37). The importance of the OmpA family of proteins has been further strengthened by the recent observation that amino acid (aa) sequence homology extends to certain proteins from gram-positive bacteria as well as lipoproteins from gram-negative bacteria, all with the common property of interacting with peptidoglycan (10).

In all cases thus far examined, cloning of the *ompA* (or *ompA*-like) gene has identified a single gene which appears to be constitutively expressed. The structure and assembly of the *E. coli* protein have been studied in detail. Current models (31, 36) propose that *E. coli* OmpA is assembled into the outer membrane with a bidomain structure: an N-terminal membrane domain (residues 1 to 170), comprising an eight-transmembrane-stranded amphipathic β-barrel, and a C-terminal periplasmic domain (residues 171 to 325). The relative resistance of the OmpA protein to complete denaturation in sodium dodecyl sulfate (SDS) is reflected in a characteristic heat-modifiable migration of the protein on SDS-polyacrylamide gel electrophoresis (PAGE) and provides a useful initial indicator of homologs of this protein (3) and a marker for assembly of the OmpA protein into the outer membrane (14).

As the causative agent of furunculosis, *Aeromonas salmonicida* is of major economic importance to the fish farming industry. Most attention has focused on the surface protein layer (A layer) and extracellular virulence factors of this bacterium. Little attention has been directed at the outer membrane itself, and only two genes encoding outer membrane proteins, a maltose-inducible *lamB* homolog (11) and the *exeD* gene, involved in extracellular protein secretion (20), from this bacterium have been cloned and sequenced. Two major outer membrane proteins of *A. salmonicida* have been shown to have pore-forming activity—a general 43-kDa protein (8) and more recently a protein of 28 kDa (22). An *A. salmonicida* 28-kDa protein was previously (24) shown to exhibit anomolous migration on SDS-PAGE similar to that of *E. coli* OmpA, with complete denaturation to a 32.8-kDa band being dependent on treatment with hot phenol. In this study, we identify this band as the *A. salmonicida* OmpA homolog and demonstrate that the 32.8-kDa band in fact comprises two OmpA homologs encoded by two distinct tandem genes. In view of the recent and still somewhat controversial demonstration of porin activity of *E. coli* OmpA (4, 35), this study provides interesting information on the structure of this new pore-forming protein and provides the basis for future comparative studies of two closely related potential porins.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *A. salmonicida* subsp. *salmonicida* NCIMB 1102 (National Collection of Industrial and Marine Bacteria) was used throughout the study except where otherwise indicated. The *E. coli* strains used as host strains for cloning were LE392 (F^- *supF supE hsdR galK trpR metB lacY tonA*) (Promega) and, for subcloning and expression, DH5α (*recA1 gyrA* Δ*lacU169 lacZ*Δ*M15*) (2) and UH203 (*ompA lac supF recA proA* or *proB rpsL*/F′ *lacI*^{q} *lacZ*Δ*M15 proAB*$^+$) (14). Liquid cultures were grown in LB broth (2) with shaking at 250 rpm, at 25°C for *Aeromonas* strains and 37°C for *E. coli*. For PCR, strains were grown on tryptic soy agar at 25°C. When required,

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ampicillin was added to a final concentration of 50 μg/ml for *E. coli* and 100 μg/ml for *A. salmonicida.*

Isolation of OM. Bacterial outer membranes (OM) were isolated from exponentially growing cells (optical density at 650 nm [OD_{650}], 0.8 to 1.0) by differential centrifugation at 27,100 × *g* for 30 min at 4°C following cell breakage by two consecutive passages through a French press (Aminco) at 16,000 lb/in^2. The pelleted OM were washed once, resuspended in an appropriate volume of deionized water or sample buffer (1 ml/200 ml of culture; 1.0 OD_{650} unit), and stored at −20°C.

SDS-PAGE and immunoblotting. Proteins were subjected to SDS-PAGE with the Laemmli discontinuous buffer system and, except where otherwise indicated, the Mini Protean II gel system (Bio-Rad Ltd.) running at 200 V for 1 h. When required, samples (approximately 200 μg of total protein in 400 μl) were extracted twice with an equal volume of 88% (wt/vol) phenol at 70°C for 10 min, and the proteins were precipitated with acetone prior to SDS-PAGE (24). All samples contained 5% 2-mercaptoethanol and were incubated at 100°C for 5 min prior to electrophoresis. Immunoblotting was performed following transfer to nitrocellulose basically as described before (2), with 5% skim milk powder as a blocking agent throughout and a 1:1,000 dilution of either rabbit anti-*E. coli* OmpA serum (17) or anti-*A. salmonicida* OmpA (see below) serum and a 1:10,000 dilution of goat anti-rabbit immunoglobulin G (IgG)-peroxidase conjugate.

For preparative SDS-PAGE of the OmpA proteins, phenol-extracted OM were solubilized in sample buffer at 100°C, applied to a Bio-Rad Prep Cell with an SDS–15% polyacrylamide gel, 37 mm in diameter, and separated with the Laemmli discontinuous buffer system at 40 mA for 10 h. From 700 μg of total OmpAI plus OmpAII (OmpAI + II) applied in one run, 300 μg of pure OmpAI+II was recovered over 25 fractions (2.5 ml each) at 5.25 to 6.33 h.

Proteolytic digestion of OM. OM (60 μl) were incubated with trypsin or proteinase K (final protease concentration, 75 μg/ml) or without protease (control) in 200 μl of 10 mM Tris-HCl, pH 8.0, at 37°C for 2 h, proteases were inhibited with trypsin inhibitor (150 μg/ml, final concentration) or phenylmethylsulfonyl fluoride (PMSF; 2 mM final concentration) for 30 min at room temperature. The membranes were recovered by centrifugation at 13,000 rpm for 30 min in an MSE MicroCentaur microcentrifuge at 4°C, washed once in 10 mM Tris-HCl, pH 8.0, and resuspended in 100 μl of distilled water. All samples were extracted with hot phenol, as described above, prior to SDS-PAGE.

Partial proteolysis of purified OmpA. Purified OmpA (15 μg; ninth OmpA fraction isolated by preparative SDS-PAGE [above]) was concentrated by precipitation with 10% (wt/vol) trichloroacetic acid, and the recovered pellet was washed twice in ethanol-ether (1:1) and twice in 10 mM Tris-HCl, pH 7.5, resuspended in 60 μl of Cleveland buffer (0.125 M Tris-HCl [pH 6.8], 0.5% SDS, 10% glycerol, 0.001% bromophenol blue), and boiled for 5 min. Partial proteolysis was performed for 2 h at 37°C with 1.5 μg of staphylococcal V8 protease and stopped by heating at 100°C for 5 min in the presence of SDS-PAGE sample loading buffer. C-terminal fragments were identified by immunoblotting. N-terminal sequencing of a 17.5-kDa fragment gave the internal aa sequence indicated in Fig. 3.

N-terminal aa sequence determination. For N-terminal protein sequencing, polypeptides (10 to 200 pmol) were electrophoretically transferred from an SDS–12.5% (OmpA) or 16% (OmpA fragments) polyacrylamide gel to a polyvinylidene fluoride membrane (Immobilon; Millipore) with 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid, pH 11 (CAPS buffer) and 10% methanol at 60 V for 2 h, briefly visualized with 0.1% Coomassie blue R-250 in 40% methanol–1% acetic acid, destained with 50% methanol, rinsed with distilled water, and dried, and the bands of interest were excised. Sequencing was performed by P. Barker (Microchemical Facility, Institute of Animal Physiology and Genetics Research, Cambridge Research Station) with an Applied Biosystems (Warrington, United Kingdom) 470A gas phase protein sequencer equipped with a 120-A on-line phenylthiohydantoin (PTH) analyzer.

Preparation of antibody against *A. salmonicida* OmpA. A New Zealand White rabbit was immunized with phenol-extracted, SDS-PAGE-purified (see above) OmpA protein in 0.0125 M Tris–0.096 M glycine–0.1% SDS (initial injection, 40 μg in complete Freund's adjuvant intramuscularly and subcutaneously; booster, 72 μg in incomplete Freund's adjuvant subcutaneously), and serum samples were collected post-booster injection.

DNA isolation. *A. salmonicida* chromosomal DNA was isolated essentially as described by Nakamura et al. (28) except that proteinase K treatment of the cell lysate was performed at 50°C instead of 37°C. Recombinant λ DNA was isolated as described before (2).

Construction of *A. salmonicida* λ library. A genomic library of *A. salmonicida* DNA was constructed by using the λGEM-11 *Xho*I half-site arms vector system (Promega). Partial digestion of 20 μg of chromosomal DNA with 20 U of *Sau*3AI for 2 min at 37°C gave maximum distribution of fragments in the required size range (9 to 23 kb), as determined by electrophoresis on a Tris-borate-EDTA (TBE)–agarose (0.4%) gel. The digested DNA was partially filled in with dATP, dGTP, and Klenow polymerase and ligated overnight at 4°C to λ half-site arms at the optimized ratio of approximately 2 μg of insert DNA to 1 μg of arms and 10 U of ligase (Promega) in a final volume of 10 μl. The ligation mix (5 μl) was packaged with 25 μl of the Packagene extract (Promega) according to the manufacturer's instructions. The titer of the library on *E. coli* LE392 was 3.8 × 10^3 PFU/μg of vector DNA.

Immunological screening of the λ library. Plaques (6 h) from the packaged library were transferred to nitrocellulose (Schleicher & Schuell) at 4°C for 1 h. The membrane was blocked with 20 mM Tris-HCl–0.5 M NaCl, pH 7.5 (TBS) plus 2% gelatin and 0.02% sodium azide and incubated sequentially at 25°C with (i) rabbit anti-*A. salmonicida* OmpA serum (see above) diluted 1:1,000 in TBS–1% gelatin–0.05% Tween for 2.5 h, (ii) TBS–0.05% Tween three times for 10 min each, (iii) a 1:3,000 dilution of peroxidase-conjugated goat anti-rabbit IgG in TBS-gelatin-Tween as in i, and (iv) TBS–0.05% Tween three times for 10 min each. Immunopositive plaques were detected by the standard 4-chloronaphthol procedure (2), and the corresponding plaques were picked into 1 ml of S-buffer (2% gelatin, 20 mM Tris-HCl, pH 7.4) and subjected to three rounds of purification with immunoscreening as above.

Oligonucleotides and Southern hybridizations. Degenerate oligonucleotides (synthesized by Genosys, Cambridge, UK) were derived as follows: SAR-2 (5′GC NGAYGAYATHTAYTTYGG) from ASOmpAI, Ala-1 to Gly-7; SAR-4 (5′GC NGCNCAYTTYAAYGGNYT) from ASOmpAI, Ala-12 to Leu-18; SAR-1 (5′GCNGAYGAYTGGTAYACVGG) from ASOmpAII, Ala-1 to Gly-7; and a degenerate guessmer, AS2 (5′ACYTCHTAYGTNGCBCCDGT), from the internal sequence TSYVAPV. Redundancies are indicated according to the International Union of Biology group codes; N = A + G + C + T, V = G + A + C, H = A + T + C, B = G + T + C, D = G + A + T, Y = C + T, and R = A + G. Oligonucleotides were end labelled with the ddUTP-DIG kit (Boehringer Mannheim). The *Acc*III 444-bp fragment from pRD87 (13) was labelled with dUTP-DIG by random hexanucleotide priming as described (DIG Kit; Boehringer Mannheim). Chromosomal or recombinant λ DNA was digested to completion with the appropriate restriction enzymes, separated on a TBE–agarose (0.8%) gel, and transferred to a Hybond N (Amersham) membrane by capillary action. Hybridization was done basically according to the DIG Kit. The hybridization temperature was 53°C for all probes. Washes were done at 61°C for ddUTP-DIG SAR-1 and at 58°C for all others.

Recombinant DNA techniques, nucleotide sequence determination, and analyses. Routine DNA manipulation techniques were performed as described before (2). Plasmid DNA for sequencing was isolated by the boiling method (1). Both strands were sequenced by cycle sequencing with an *fmol* DNA sequencing kit (Promega). Remaining compressions were resolved by using a Sequenase version 2.0 kit (United States Biochemicals) with dITP and inclusion of 40% formamide in the sequencing gel. Sequence assembly and analyses were performed with the Genetics Computer Group sequence analysis software package.

PCR. PCR was performed on lysed colonies with 200 ng each of primers 5′ATGGCAACCGTTACGAGAAC and 5′TTGACGTGGTTGAAGTAGGC (nucleotides [nt] 357 to 376 and complement nt 1904 to 1885, respectively [Fig. 3]), *Taq* polymerase (Amersham-USB), and 30 cycles of amplification (95°C for 1 min, 55°C for 1 min, and 72°C for 1 min) as described before (21). Products were separated on a TBE–agarose (2%) gel.

Nucleotide sequence accession number. The sequence data presented here have been deposited in the GenBank/EMBL database under accession number X91983.

RESULTS

Identification of two OmpA-like polypeptides in *A. salmonicida*. Two phenol-modifiable polypeptides migrating as a doublet both prior to extraction with hot phenol (apparent M_r, 27,000 and 28,000) and after phenol extraction (apparent M_r, 32,800 and 33,800) were identified in *A. salmonicida* OM preparations (Fig. 1A). Immunoblot analyses (not shown) with anti-*E. coli* OmpA serum identified the polypeptides as *A. salmonicida* OmpA equivalents. Unlike *E. coli* OmpA, the *A. salmonicida* OmpAs were not fully denatured by heating at 100°C for 5 min (Fig. 1A, lane 1). In OM, the *A. salmonicida* OmpAs were cleaved by trypsin and proteinase K to give fragments (trypsin, 23 and 22 kDa; proteinase K, 19.5 and 19 kDa, identified by immunoblotting) similar in size to those of the *E. coli* OmpA membrane protected proteolytic fragments (Fig. 1B).

Amino-terminal sequence analysis of the 33.8-kDa and 32.8-kDa polypeptides in phenol-extracted *A. salmonicida* OM confirmed the homology of these polypeptides with *E. coli* OmpA (see Fig. 3 and 4 below) but, more importantly, provided evidence that the OmpAI (33.8 kDa) and OmpAII (32.8 kDa) polypeptides were encoded by different but closely related genes.

Cloning and sequencing of the *A. salmonicida ompAI* and *ompAII* genes. Degenerate oligonucleotides SAR-1, SAR-2, and SAR-4, derived from the amino-terminal sequences of

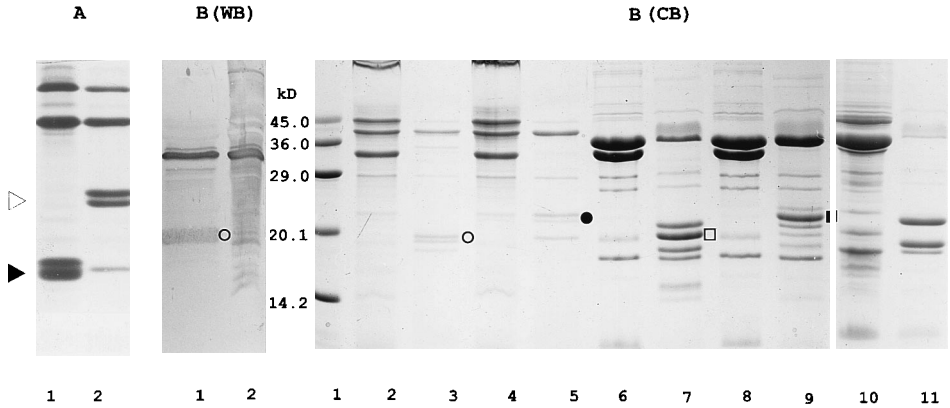

FIG. 1. Identification of *A. salmonicida* OM proteins OmpAI and OmpAII and their proteolytic fragments by SDS-PAGE. (A) *A. salmonicida* OM proteins following no extraction (lane 1) or extraction with hot phenol (lane 2) prior to analysis on an SDS–12.5% polyacrylamide gel overnight at 60 V and staining with Coomassie blue. Open arrowhead, fully denatured OmpAI (upper band) plus OmpAII (lower band); solid arrowhead, incompletely denatured OmpAI plus OmpAII. (B) Western blot (WB) of *A. salmonicida* and CB-stained *A. salmonicida* (lanes 2 to 5), *E. coli* DH5α (lanes 6 to 9), and *E. coli* UH203 (*ompA*) (lanes 10 and 11) OM proteins following digestion with proteinase K (WB, lane 1; CB, lanes 3, 7, and 11) or trypsin (CB, lanes 5 and 9) or in the absence of protease (WB, lane 2, and CB, lanes 2, 4, 6, 8, and 10) and separated on a 15% acrylamide gel. CB lane 1, molecular size standards. WB, immunoblot with anti-*A. salmonicida* OmpA serum. Open circle, proteinase K fragments, doublet of OmpAI and OmpAII; solid circle, tryptic fragments, doublet of OmpAI and OmpAII; open square, proteinase K fragment, *E. coli* OmpA; solid square, tryptic fragment *E. coli* OmpA.

OmpAII, OmpAI, and OmpAI, respectively, were used in Southern hybridization analyses of *A. salmonicida* genomic DNA. All three probes bound to the same restriction fragments—a 3.9-kb *Pst*I, 5-kb *Bam*HI, and 7-kb *Sma*I fragment—indicating that the two genes encoding these polypeptides must be located very close to each other on the chromosome. SAR-1 gave a particularly strong signal. Several different approaches to cloning these genes from *A. salmonicida* with the degenerate oligonucleotides were taken. These included screening a pLAFR3 *A. salmonicida* cosmid bank (20) with SAR-I; cloning purified 3.5- to 4.5-kb *Pst*I fragments of *A. salmonicida* genomic DNA into pUC19; and either screening by hybridization with SAR-I or PCR analyses with different combinations of the degenerate oligonucleotide probes and sequencing primers.

As all approaches with the degenerate primers failed, a λ bank was prepared from *A. salmonicida* genomic DNA and screened with antibody prepared against *A. salmonicida* OmpA. From 3×10^3 recombinant plaques screened, eight positive clones were identified. Analysis of the recombinant λ DNA isolated from these clones by Southern hybridization (not shown) with the oligonucleotide SAR-1 confirmed that all eight clones contained at least *ompAII* and that only clones 1 and 3 possessed similar-sized *Pst*I (3.9 kb), *Bam*HI (5 kb), and *Sma*I (7 kb) fragments hybridizing with SAR-1 in chromosomal DNA. In contrast, SAR-4 hybridized to clones 1 to 4 but not to clones 5 to 8. Thus, it was assumed that all recombinant phage selected contained at least part of *ompAII* and that clones 1 to 4 contained in addition all or part of *ompAI*. Further restriction analyses of the 5-kb *Bam*HI fragment of clone 1 inserted into pBluescript SK (pGC1) (Fig. 2) followed by Southern hybridization with selected probes (summarized in Fig. 2A) permitted localization of both genes to a 2.5-kb region within the 5-kb *Bam*HI fragment and development of a strategy for rapid subcloning and sequencing of both genes (Fig. 2B).

Analyses of the DNA sequences of *ompAI* and *ompAII*. Analyses of the complete 2.607-kb DNA sequence (Fig. 3) encompassing both genes revealed the two expected open reading frames (ORFs), of 1,020 and 993 bp. No ORF was encoded by the complementary DNA sequence. Both genes possess more

A.

ENZYME	SAR-1	SAR-4	C-TERM	AS2-D
*Cla*I	3.7	3.7	3.7	3.7
***Nco*I**	**3.4**	**1.3**	3.4, 1.3	3.4, 1.3
***Bst*XI**	**3.5**	**0.8**	3.5	3.5
***Bst*EII**	**1.4**	**1.0**	1.4, 1.0	*
*Bgl*I	4.5	4.5	*	*
***Bgl*I**	**2.0**	**0.4**	2.0	2.0
***Pvu*II**	**1.3**	**1.0**	1.0, 1.3	1.0, 1.3
***Eco*RV**	**1.8**	**1.1**	1.1, 1.8	1.1, 1.8
*Apo*I	3.9	3.9	3.9	3.9
*Sst*I	2.2	2.2	2.2	2.2
***Hin*cII**	**0.9**	**0.8**	0.8	*
*Sph*I	5.1	5.1	*	*
*Nru*I	5.1	5.1	5.1	5.1
*Mlu*I	2.6	2.6	2.6	2.6
*Kpn*I	2.3	2.3	2.3	2.3

B.

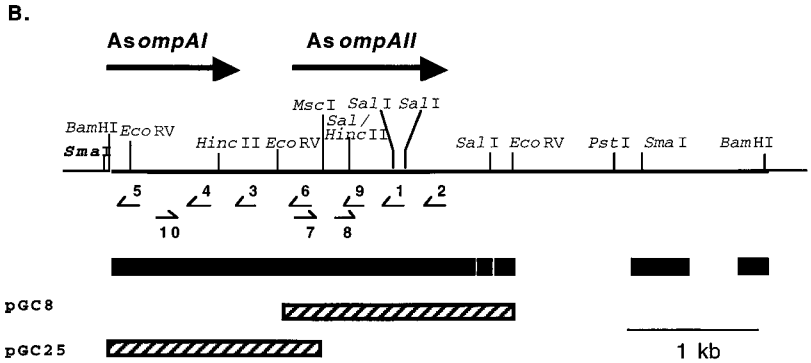

FIG. 2. Strategy for subcloning and sequencing *ompAI* and *ompAII*. (A) Southern analysis of pGC1, summary of restriction fragments (in kilobases) hybridizing with ddUTP-DIG SAR-1 (*ompAII* N terminus), ddUTP-DIG SAR-4 (*ompAI* N terminus), dUTP-DIG *Acc*I fragment of pRD87 (C terminus of *E. coli ompA*), and ddUTP-AS2-D (internal sequence). Enzymes distinguishing between the two genes are in boldface. (B) Partial restriction map of the 5-kb *Bam*HI fragment in pGC1. Arrows, positions of *ompAI* and *ompAII*, including promoters and terminators; thick line, insert *A. salmonicida* DNA; thin lines, pBluescript SK DNA (sites indicated are those used for subcloning small fragments into pBluescript SK or KS for sequencing); numbered half arrows, positions and directions of internal oligonucleotides used in sequencing; filled-in boxes, regions sequenced; cross-hatched boxes, *ompAI* and *ompAII* separately subcloned into pBluescript KS for expression.

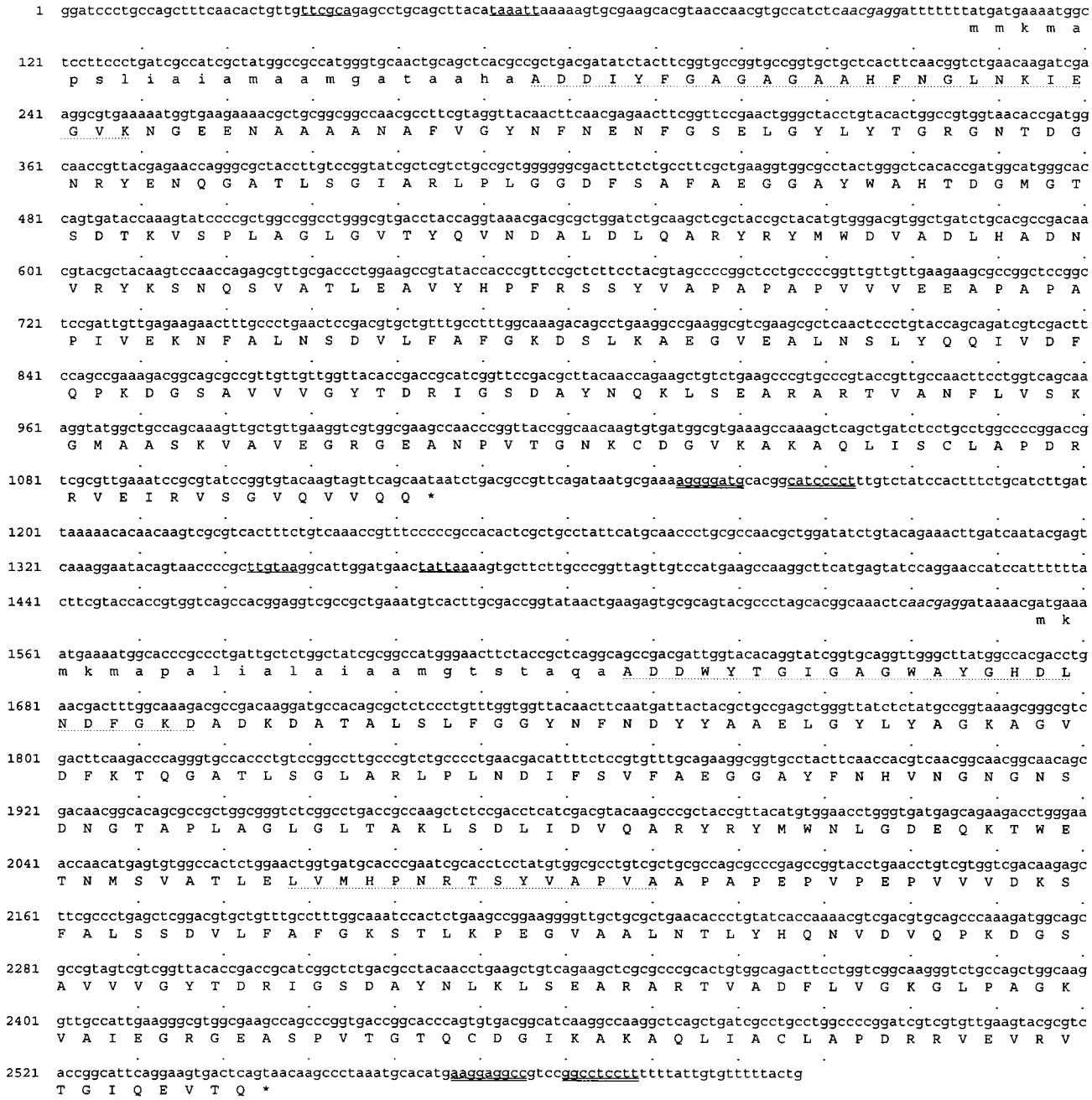

FIG. 3. DNA sequence of *ompAI* and *ompAII*. RBSs are in italics; proposed promoters are underlined; predicted terminator sequences are double underlined. Encoded aa sequences of the mature proteins are in uppercase letters, and those of the signal sequences are in lowercase letters. The aa sequences identified by N-terminal sequencing of intact proteins and an internal proteolytic fragment (see Materials and Methods) are dotted underlined.

than one in-frame potential ATG start codon. As bacterial signal sequences virtually always possess one or more N-terminal positively charged residue (23), it is most likely that the ATG codon at nt 1561 is the actual start codon of *ompAII*. Assignment of this ATG codon to the start codon as well as the ATG codon at nt 107 to the start codon of *ompAI* is further supported by the identification of a consensus ribosome-binding sequence (RBS) 9 bp upstream in both cases (Fig. 3). Although an RBS 13 bp upstream of the start codon has previously been proposed for the *A. salmonicida exeD* gene (20), in contrast to the *ompA* genes, this product is most likely expressed at very low levels. Interestingly, although the DNA sequence between the RBS and start codon and the upstream DNA sequence of both genes were quite different, a 12-bp sequence encompassing the consensus RBS was common to both genes, and the proposed RBS itself was identical to the *E. coli ompA* RBS (7). Putative promoter sites could be identified upstream of both genes, and the Terminator program predicted a potential terminator structure 27 and 20 nt downstream from the stop codons of *ompAI* and *ompAII*, respectively (Fig. 3). The second predicted terminator is particularly characteristic of a rho-independent terminator, with a 9-bp

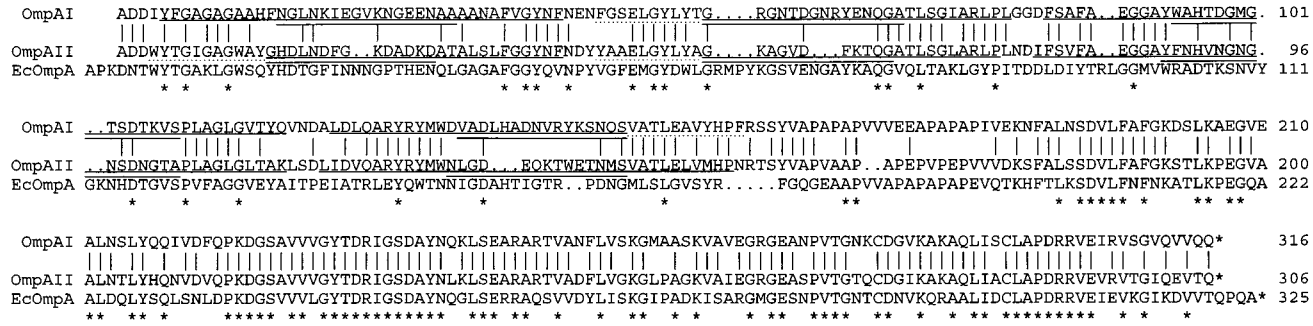

FIG. 4. Pileup alignment of the mature aa sequences of *A. salmonicida* OmpAI and OmpAII and *E. coli* OmpA. Vertical bars, residues identical between *A. salmonicida* OmpAI and OmpAII; asterisks, residues conserved among all three proteins. Predicted amphipathic β-strands are underlined; additionally assigned transmembrane strands are dotted underlined; and predicted surface loops are double underlined. Transmembrane strands and surface loops are referred to numerically in ascending order from the N terminus.

stem containing 6 G-C pairs followed by a poly(T) sequence. Although the efficiency with which the intervening stem-loop structure may function as an attenuator or terminator awaits experimental evidence, the existence of this plus a potential promoter upstream of *ompAII* suggests that *ompAII* has the potential to be independently transcribed.

Comparison of derived aa sequences. As expected, both polypeptides are evidently synthesized as precursors with classic cleavable signal sequences of 23 aa (OmpAI) and 24 aa (OmpAII). The cleavage site could be confirmed by comparison with the N-terminal aa sequences of the isolated proteins. In both cases, the extreme N terminus of the mature polypeptide was negatively charged, a characteristic of many secreted proteins and of OM proteins in particular (23). In accord with SDS-PAGE analyses, the molecular weights of the two derived mature polypeptides are 33,564 and 32,536, respectively. Comparison of the derived aa sequences with those of the two sequenced N termini and an internal aa sequence (Fig. 3) also confirmed (i) assignment of *ompAI* and *ompAII* as the genes encoding the OmpAI and OmpAII polypeptides with apparent molecular masses of 33.8 and 32.8 kDa, respectively, and (ii) identification of the correct reading frame for both polypeptides. The internal peptide sequence could be identified as Leu-148 to Ala-162 of mature OmpAII.

Bestfit analysis of the two mature aa sequences revealed 64% identity and 78% similarity. The level of homology was significantly more pronounced in the C-terminal region of both proteins: from Val-184 to the end of OmpAI and Val-177 to the end of OmpAII, the two proteins had 73.5% identity and 84.5% similarity. At the DNA level, the percent identity was found to be 70% over the entire length of both genes and 65 and 76% in the first and second halves, respectively.

A tFasta search of GenBank identified the highest level of homology with the enterobacterial OmpAs and gave comparable scores with both proteins for most members of the enterbacterial OmpAs sequenced, e.g., *Enterobacter aerogenes* and *E. coli* with pro-OmpAI, 37 and 39.5%, and with proOmpAII, 37.1 and 39.7%, respectively over the entire sequence. A Pileup alignment of the two *A. salmonicida* proteins together with *E. coli* OmpA revealed the overall homology within the C-terminal domain (Fig. 4). *A. salmonicida* OmpAI was 60% identical and 74% similar to the C terminus of *E. coli* OmpA, while the corresponding figures for OmpAII were 59% identity and 70% similarity.

Topology predictions of the membrane domain of OmpAI and OmpAII. Assignment of the membrane protected proteinase K fragments described above (Fig. 1B) to OmpAI (approximately 19.5 kDa) and OmpAII (approximately 19 kDa) was confirmed by sequence analyses of the first six aa for both fragments, providing evidence for a similar conformation of the N-terminal domains of the two proteins. The *Aeromonas* OmpAs showed the highest level of homology to *E. coli* OmpA, and the sizes of both the proteinase K and trypsin digest fragments from *A. salmonicida* were close to those from *E. coli* OmpA (Fig. 1B), suggesting that the *Aeromonas* and *E. coli* proteins are probably assembled in the membrane in a similar fashion. For these reasons, and because it has been shown that the N-terminal half of OmpA alone is sufficient for membrane assembly (31), we used the *E. coli* model of an eight-stranded amphipathic β-barrel along with computer predictions to identify potential transmembrane strands in the N-terminal half of both OmpAI and OmpAII.

Using the Amphi program (36), six amphipathic β-strands, 10 residues long, were predicted in the N-terminal half of both OmpAI and OmpAII (strands 1, 2, 4, 5, 6, and 7 and strands 2, 4, 5, 6, 7, and 8, respectively [Fig. 4]). Intervening loops and periplasmic turns were further identified by analyzing the sequence for β-turn prediction (6) and the location of β-turn promoters and blockers as described by Paul and Rosenbusch (29). The assigned transmembrane strands were all predicted, to some extent, as β-structure by Chou and Fasman prediction (6). Strands 8 of OmpAI and 1 of OmpAII, which were not strongly amphipathic, were also assigned as transmembrane β-strands because of their homology to the corresponding strand in OmpAII and -I, respectively, and the identification of a preceding (strand 8) and following (strand 1) surface loop. Phe has been identified as the last residue in the membrane domain of a number of OM proteins (34). The last residue of the assigned strand 8 of OmpAI is Phe, but not in the more readily predicted strand 8 of OmpAII (Fig. 4). Indeed, the closest aromatic aa was Tyr-157, but this was separated from the predicted end of strand 8 by a sequence with strong turn prediction, Pro-Asn-Arg-Thr-Ser-Tyr. It should be stressed that this sequence has been fortuitously confirmed, as it is part of the internal aa sequence obtained (see Fig. 3). No significant amphipathic β-strand was predicted in the region tentatively assigned as strand 3 in either OmpAI or OmpAII, but the existence of a transmembrane strand in this position in both proteins is supported by (i) the presence of both a clear periplasmic turn (Asn-43–Glu–Asn) following strand 2 and surface loop immediately prior to strand 4 in OmpAI and (ii) the reasonably high level of homology between assigned strands 3 of OmpAI and -II compared with the low level of the proposed loops. Indeed, on comparison of the homology be-

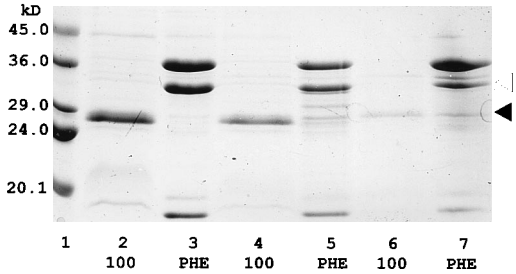

FIG. 5. Expression of *ompAI* and *ompAII* in *E. coli*. *E. coli* UH203 carrying pGC1 (*ompAI* plus *ompAII*) (lanes 2 and 3), pGC8 (*ompAII*) (lanes 4 and 5), or pGC25 (*ompAI*) (lanes 6 and 7) was incubated overnight at 37°C prior to preparation of OM. Samples in lanes 3, 5, and 7 (PHE) were extracted with hot phenol. All samples were heated at 100°C for 5 min in sample buffer prior to separation of proteins on an SDS–12.5% polyacrylamide gel and staining with Coomassie blue. Heating at 50°C for 15 min in sample buffer resulted in the same profile with respect to OmpAI and -II as heating at 100°C. Open and solid arrowheads, fully and incompletely denatured OmpAI/II, respectively. Lane 1, protein size standards.

tween these proteins and their predicted topological arrangement, the short regions of continuous similarity in the N-terminal half corresponded almost exactly to the predicted transmembrane strands and the intervening variable sequences to the surface loops or periplasmic turns (Fig. 4), providing additional support for the predictions.

Expression of OmpAI and OmpAII in *E. coli* and protease susceptibility. *ompAI* and *ompAII* were subcloned from pGC1 either on a 1.676-kb *Sma*I-*Msc*I fragment (OmpAI, pGC25) or on a 1.6-kb *Eco*RV fragment (OmpAII, pGC8) into *Eco*RV-digested pBluescript SK (Stratagene) (Fig. 2) and expressed in *E. coli* UH203 (OmpA deficient). Efficient expression of the respective *ompA* genes and assembly of the products were obtained from both plasmids as well as from pGC1 (Fig. 5). In both cases, production of the *A. salmonicida* OmpA polypeptide was independent of the presence of 0.6% glucose or 1 mM IPTG (isopropylthiogalactopyranoside) in the growth medium. Thus, it would appear that *ompAI* and *ompAII* were both expressed from their own promoters in *E. coli*. Although pGC1 (*ompAI* plus *ompAII*) and pGC25 (*ompAI*) had no visible effect on the growth of *E. coli*, a toxic effect on cells harboring pGC8 (*ompAII*) was evident. Colonies of *E. coli*/pGC8 were draftsman-like, and liquid cultures grew very slowly. The toxic effect was also observed in the presence of glucose. The reason for the toxicity only in the construct with *ompAII* alone is not clear. One possibility is that levels of expression of *ompAII* were higher and toxic from pGC8 than from pGC1.

In *E. coli*, both *Aeromonas* OmpA polypeptides retained the phenol-modifiable characteristic, i.e., they required treatment with hot phenol for full denaturation (Fig. 5). In *E. coli*, OmpA undergoes several conformational changes during its assembly, the final one being acquisition of the heat-modifiable conformation on assembly into the OM (14). This indicates that the *Aeromonas* OmpA polypeptides were correctly assembled into the *E. coli* OM.

Existence of two *ompA* genes in a variety of *Aeromonas* spp. To ascertain if possession of two *ompA* genes was unique to *A. salmonicida*, *A. hydrophila* ATCC7966, *A. caviae* ATCC15468, and *A. veronii* biogroup veronii ATCC 35624 were tested by PCR for the presence of *ompAI* and *ompAII* with one primer homologous to *ompAI* and the other homologous to *ompAII*. PCR fragments of approximately 1.6 kb were obtained with all three strains, indicating that possession of the tandem genes *ompAI* and *ompAII* is at least common among *Aeromonas* spp.

DISCUSSION

We have demonstrated here for the first time the presence of two *ompA* genes in a single bacterial species. Possession of both genes is evidently not unique to *A. salmonicida* but is widespread at least among *Aeromonas* spp. It remains to be seen if this arrangement also exists in nonaeromonad species. The genes are arranged tandemly and have presumably arisen by some form of gene duplication. The level of homology between the two proteins (64% identical and 78% similar) is lower than that between the OmpA protein encoded by the genes sequenced from *E. coli* and those of the closely related strains *Salmonella typhimurium* (93% identity and 97% similarity) and *Enterobacter aerogenes* (85% identity and 93% similarity). It is also much lower than that expected for the same protein between *A. salmonicida* and the closely related species *A. hydrophila*; for example, the OM ExeD proteins of these species have 90% aa identity. Indeed, the level of homology between OmpAI and OmpAII is on a par with that between the different general pore formers OmpF and PhoE, which have 63% sequence identity (25). In contrast, it is likely that at least the corresponding *ompAII* genes from different *Aeromonas* species are highly conserved. This is indicated from the positive PCR results, as primers were designed against variable loop-encoding regions, and supported from a comparison of the N-terminal sequence of OmpAII with that of protein V of *A. hydrophila* OM. The first 21 N-terminal aa of *A. hydrophila* protein V have been reported (19), and with the exception of an Asn residue at position 2, the sequence is identical to the N terminus of OmpAII. For the above reasons, one can conclude that (i) acquisition of two *ompA* genes presumably occurred relatively far back along the evolutionary time scale and (ii) there is a good possibility that the two proteins differ to some degree in function.

As with OmpA proteins from different bacterial species, the C termini of OmpAI and OmpAII were more highly conserved than the N termini, and within the N termini, most of the variation was localized in the proposed surface loops and periplasmic β-turns. It has recently been noted that sequence homology within the C termini of OmpA proteins extends to some peptidoglycan-interacting proteins (10). This may then implicate the highly conserved C terminus as an important part of OmpA in the role of this protein (33) in maintenance of structural integrity of the OM and cell morphology. As the C termini of OmpAI and OmpAII have 73.5% identity and 84.5% similarity, there is a lower probability of significant differences in function between the C-terminal domains of OmpAI and OmpAII than between the N-terminal domains.

Numerous different functions have been attributed to the OmpA family of proteins; therefore, elucidation of functional differences between OmpAI and OmpAII will be an interesting challenge. As a recent report (22) has described the ability of a 28-kDa protein of *A. salmonicida* OM to form nonselective channels in planar bilayers, similar in size to those of OmpF and OmpC, and we can now say conclusively that this 28-kDa protein is in fact one of the *A. salmonicida* OmpA homologs (the reported sequence of the 19 N-terminal aa of this protein was identical to that of the N terminus of OmpAI), comparative analysis of the pore-forming activities of OmpAI and OmpAII is one potential function which will be analyzed in detail. A range of selective and nonselective porin activities may be important to *A. salmonicida*, which is found primarily in association with fish but may also survive for some time in water (18). Such studies may be facilitated by the demonstration that both OmpAI and OmpAII apparently assemble correctly into the *E. coli* outer membrane (Fig. 5). Interestingly,

the purified *A. salmonicida* 28 kDa protein (OmpAI) was also shown to confer some protection against furunculosis (22), a disease of salmonid fish. A direct role of other members of the OmpA family in bacterial virulence has been reported. *Neisseria gonorrhoeae* protein III contributes to serum resistance by binding blocking antibody (30), and *E. coli* OmpA similarly enhances the pathogenicity of *E. coli* K-1 (37). Other OmpA homologs have been shown to interact directly with eucaryotic cells by functioning as adhesins (9, 32). Differences in the surface loops of OmpAI and OmpAII provide the opportunity for distinct roles for each of these proteins in interaction with the host organism during pathogenesis of this major fish pathogen.

The folded structure of the *A. salmonicida* OmpA proteins appears to be even more stable than that of *E. coli* OmpA; the *Aeromonas* proteins require extraction with hot phenol for complete denaturation to the 32.5- and 33.5-kDa forms, whereas *E. coli* OmpA is fully denatured following heating at 70°C in SDS. Should this be related to interaction with lipopolysaccharide (LPS), it is clearly not specific to *A. salmonicida* LPS, as the same behavior was seen with both genes when expressed in *E. coli*. Nor can the extreme stability of these proteins be attributed to high amphipathic β-strand formation of the terminal strands in the β-barrel, as strand 1 of OmpAII, like that of *E. coli* OmpA and strand 8 of OmpAI, gives poor amphipathic predictions.

Finally, should the two *Aeromonas* OmpA proteins have different functions, one might expect them to be differentially expressed. From the limited analyses which we have performed here, both genes appeared to be expressed at high levels under normal laboratory conditions (Fig. 1A). However, the potential for differential expression of OmpAI and OmpAII evidently exists. A potential promoter site was identified upstream of both genes, the two genes were separated by a long intergenic region within which lay a strong stem-loop structure likely to have attenuator if not terminator function, and when cloned separately in *E. coli*, both clones were apparently expressed from their own promoters.

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