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Cloning, Sequencing, and Role in Serum Susceptibility of Porin II from Mesophilic *Aeromonas hydrophila*

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We cloned and sequenced the structural gene for *Aeromonas hydrophila* porin II from strain AH-3 (serogroup O:34). The genetic position of this gene, like that of *ompF* in *Escherichia coli*, is adjacent to *aspC* and transcribed in the same direction. However, upstream of the porin II gene no similarities with *E. coli* were found. We obtained defined insertion mutants in porin II gene either in *A. hydrophila* (O:34) or *A. veronii sobria* (serogroup O:11) serum-resistant or -sensitive strains. Furthermore, we complemented these mutants with a plasmid harboring only the porin II gene, which allowed us to define the role of porin II as an important surface molecule involved in serum susceptibility and C1q binding in these strains.

The complement system plays a key role in humoral defense against microbial pathogens and has been reviewed extensively (33). Its importance is clearly seen in individuals with complement deficiencies because they are at increased risk to develop severe and recurrent microbial infections (7). Resistance to complement action is thus a requisite for pathogenic microorganisms, which have developed a variety of mechanisms to ensure survival in nonimmune serum (7). Gram-negative bacteria activate complement via the classical or alternative pathway (CPC or APC, respectively) which is required for the effective elimination of serum-sensitive strains (37). In previous studies (19, 22), we focused on defining the mechanisms of complement sensitivity in mesophilic *Aeromonas*. Only the CPC is effective in the elimination of *Aeromonas* serum-sensitive strains in nonimmune serum, as we previously reported (19, 22). Activation of the CPC by these strains led to the identification of a bacterial outer membrane (OM) protein, presumably porin II (13), that binds C1q and activates this pathway in nonimmune serum and in agammaglobulinemic serum in an antibody-independent manner (24).

Mesophilic aeromonads are increasingly being reported as important pathogens of humans and lower vertebrates including amphibians, reptiles, and fish (11). *Aeromonas* strains have been serogrouped on the basis of the O-antigen lipopolysaccharide (LPS) (30), the polysaccharide chains in the smooth LPS, also known as the somatic antigen. Recently, a group of virulent *Aeromonas hydrophila* and *A. veronii sobria* strains isolated from humans and fish have been described (12, 15), serologically related by their O-antigen LPS (serogroup O:11) with a known chemical structure and having a surface array protein of molecular weight of ca. 52,000 (termed S-layer) (26). The S-layer-expressing (S^+) strains from this serogroup are the most frequent isolates from septicemia caused by mesophilic *Aeromonas* sp. (12). Serogroup O:34 strains of mesophilic *Aeromonas*, recovered from moribund fish and from clinical specimens (21, 25), represent the single most common *Aeromonas* serogroup, accounting for 26.4% of all infections. Previous investigations have documented O:34 strains as an important cause of infections in humans (21, 25).

We cloned and sequenced the structural gene for *A. hydrophila* porin II, which allow us to obtain defined insertion mutants in this gene, and complemented these mutant strains with porin II. With all these strains, we were able to study the role of porin II in serum susceptibility and C1q binding to whole cells.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown on Luria-Bertani (LB) Miller broth and LB Miller agar, while *Aeromonas* strains were grown on tryptic soy broth or tryptic soy agar (5). Ampicillin (50 μg/ml), chloramphenicol (25 μg/ml), kanamycin (30 μg/ml), and tetracycline (20 μl/ml) were added to the different media when needed.

Cell surface isolation and analysis. Cell envelopes were prepared by lysis of whole cells in a French press at 16,000 lb/in^2. Unbroken cells were removed by centrifugation at 10,000 × *g* for 10 min, and the envelope fraction was collected by centrifugation at 100,000 × *g* for 2 h. Cytoplasmic membranes were solubilized with sodium *N*-laurylsarcosinate, and the outer membrane (OM) fraction was collected as describe previously (24). OM proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the Laemmli procedure (17). Protein gels were fixed and stained with Coomassie blue. LPS was purified by the method of Westphal and Jann (39). For screening purposes, LPS was obtained after proteinase K digestion of whole cells according to the procedure of Darveau and Hancock (6). SDS-PAGE was performed and LPS bands were detected by the silver staining method of Tsai and Frasch (36).

Antiserum. Antiserum against purified porin II was obtained as previously described (24).

Western immunoblotting. After SDS-PAGE, immunoblotting was carried out by transfer to polyvinylidine fluoride membranes (Millipore Corp., Bedford, Mass.) at 1.3 A for 1 h in the buffer of Towbin et al. (35). The membranes were then incubated sequentially with 1% bovine serum albumin, specific anti-porin II serum (1:500), alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G, and 5-bromo-4-chloro-indolylphosphate disodium–nitroblue tetrazolium. Incubations were carried out for 1 h, and washing steps with 0.05% Tween 20 in phosphate-buffered saline (PBS) were included after each incubation step. Colony blotting was performed with porin II antiserum as indicated above.

Bacterial survival in human serum. Bacterial cells (10^8 CFU) in the logarithmic phase were suspended in 90% serum–PBS and incubated at 37°C. Viable counts were made at different times until 3 h by dilution and plating as previously described (19, 22). A pool of nonimmune human sera (NHS) was obtained from healthy volunteers. Control experiments using heat-decomplemented NHS were also performed (19, 22).

Binding of C1q to bacterial cells. C1q was purified from NHS and tested for purity in PAGE as previously described (2). Iodination of purified C1q was carried out with lactoperoxidase-glucose oxidase as described previously (34). Mid-logarithmic-phase bacterial cells were recovered by centrifugation, washed with PBS, and examined with radiolabeled C1q as described previously (2).

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TABLE 1. Bacterial strains, cosmids, and plasmids used

Strain, cosmid, or plasmid	Relevant characteristics	Reference or source
E. coli		
DH5α	F$^-$ *endA hsdR17* (r_k^- m_k^+) *supE44 thi-1 recA1 gyr-A96* ϕ80*lacZ*	9
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* (F′ *proAB lacIZΔM15* Tn*10*)	Stratagene
MC1061λ*pir*	*thi thr 1 leu6 proA2 his4 argE2 lacY1 galK2 ara14 xyl5 supE44* λ*pir*	27
SM10λ*pir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km^r λ*pir*	27
Aeromonas sp.		
AH-3	*A. hydrophila* wild type, serogroup O:34	20
AH-405	Rifampin-resistant mutant derived from AH-3	23
AH-53	Serum-sensitive rough mutant derived from AH-3	24
AH-330	Porin II insertion AH-405 mutant obtained with pFS-POR3	This work
AH-336	Porin II insertion AH-53 mutant obtained with pFS-POR3	This work
AH-334	Mutant AH-330 complemented by plasmid pLA-POR6	This work
AH-338	Mutant AH-336 complemented by plasmid pLA-POR6	This work
TF7	*A. veronii sobria* wild type, serogroup O:11	24
AH-408	Rifampin-resistant mutant derived from TF7	This work
AH-26	Rough S$^-$ serum-sensitive mutant derived from TF7	24
AH-331	Porin II insertion AH-408 mutant obtained with pFS-POR3	This work
AH-337	Porin II insertion AH-26 mutant obtained with pFS-POR3	This work
AH-335	Mutant AH-331 complemented by plasmid pLA-POR6	This work
AH-339	Mutant AH-337 complemented by plasmid pLA-POR6	This work
Plasmids		
pLA2917	Tc^r Km^r	3
COS-POR2	pLA2917 with 20-kb chromosomal AH-3 *Sau*3A insert (porin II gene)	This work
pLA-POR3	pLA2917 with 7.8-kb *Bgl*II insert from DNA insert of COS-POR2 (porin II gene)	This work
pLA-POR6	pLA2917 with the single porin II gene	This work
pFS100	pGP704 suicide plasmid, λ*pir*-dependent, Km^r	27
pFS-POR3	pFS100 with an internal fragment (996 bp) of porin II gene	This work

General DNA methods. DNA manipulations were carried out essentially as previously described (28). DNA restriction endonucleases, T4 DNA ligase, *E. coli* DNA polymerase (Klenow fragment), and alkaline phosphatase were used as recommended by the suppliers.

Construction of an *A. hydrophila* AH-3 genomic library. *A. hydrophila* AH-3 genomic DNA was isolated and partially digested with *Sau*3A as described by Sambrook et al. (28). Cosmid pLA2917 (3) was digested with *Bgl*II, dephosphorylated, and ligated to *Sau*3A genomic DNA fragments. DNA packaging by using Gigapack Gold III (Stratagene) and infection of *E. coli* DH5α were carried out as previously described (8). Recombinant clones were selected LB agar plates supplemented with tetracycline (20 μg/ml).

DNA sequencing. Primers used for DNA sequencing were purchased from Pharmacia LKB Biotechnology. Double-stranded DNA sequencing was performed by the Sanger dideoxy-chain termination method (29) with an ABI Prism dye terminator cycle sequencing kit (Perkin-Elmer).

DNA and protein sequence analysis. The DNA sequence was translated in all six frames, and all open reading frames (ORFs) greater than 100 bp were inspected. Deduced amino acid sequences were compared with those of DNA translated in all six frames from the nonredundant GenBank and EMBL databases using the BLAST network service at the National Center for Biotechnology Information (4). Multiple sequence alignments and determination of putative terminator sequences were done by using the PileUp and Terminator programs from the Genetics Computer Group (Madison, Wis.) package in a VAX 4300. Prediction of the secondary structure of the porin II sequence was performed using the H's program based on the prediction of beta strands of porins (31).

Construction of porin II-defined insertion mutants. To obtain defined insertion mutants in the porin II gene, we used a method based on suicide plasmid pFS100 (27). Oligonucleotides 5′-TCTGGCTATTGCTATCCC-3′ (initial base 3283) and 5′-GCTAACACCGTTGATTTTG-3′ (initial base 4279) were used to amplify internal fragment from the porin II gene (996 bp). The amplified fragment was ligated to vector pGEM-T (Promega) and transformed into *E. coli* DH5α. The fragment was recovered by restriction digestion and was blunt ended with Klenow fragment; finally, it was ligated to *Eco*RV-digested, blunt-ended, and dephosphorylated pFS100 and transformed into *E. coli* MC1061(λ*pir*), selecting for kanamycin resistance (Km^r) to generate plasmid pFS-POR3. Plasmid pFS-POR3 was isolated and transformed on *E. coli* SM10(λ*pir*). Plasmid pFS-POR3 was transferred by conjugation to mesophilic *Aeromonas* sp. rifampin-resistant (Rif^r) strains to obtain defined insertion mutants in porin II gene, selecting for Rif^r and Km^r.

Nucleotide sequence accession number. The nucleotide sequence data presented here have been assigned GenBank accession no. AF183931.

RESULTS AND DISCUSSION

Cloning of *A. hydrophila* AH-3 genomic region encoding porin II. A cosmid-based genomic library of *A. hydrophila* AH-3 was constructed and introduced into *E. coli* DH5α as indicated in Materials and Methods. Tetracycline (20 μg/ml)-resistant (Tc^r) clones were immunoscreened by colony blotting using specific anti-porin II serum. We found several recombinant clones, a representative one being COS-POR2. Analyses of OM proteins by SDS-PAGE with Coomassie blue stain and Western blotting of OM proteins with specific anti-porin II serum revealed that *E. coli* DH5α harboring COS-POR2 showed an extra band of approximately 39 kDa, which reacted with specific anti-porin II serum, in comparison with *E. coli* DH5α or when cured of plasmid COS-POR2 (Fig. 1).

To localize the gene responsible for the production of porin II, *Bgl*II fragments of the recombinant cosmid COS-POR2 were subcloned into the *Bgl*II site of the same cosmid vector and transformed into *E. coli* DH5α. The recombinant transformants were immunoscreened as previously mentioned. It is important to point out that we use as plasmid vector in order to subclone the same cosmid vector (pLA2917) were initially was cloned the gene, because none of the usual plasmid vectors (pBR328, pACYC184, or pWSK) was able to maintain the DNA insert encoding the porin gene. The initial smallest stable recombinant plasmid (pLA-POR3) exhibiting porin II production (either in SDS-PAGE or Western blotting with anti-porin II serum) was found to harbor a 7.8-kb *Bgl*II insert fragment (Fig. 1).

Sequencing of the DNA conferring porin II production. The complete nucleotide sequence of the 7.8-kb DNA insert was determined in both directions. Because we could not use the typical vectors for reasons cited above, we started the sequenc-

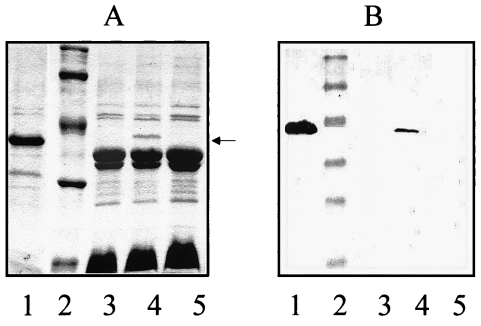

FIG. 1. Coomassie blue-stained gel (A) and Western immunoblot using specific anti-porin II serum (B) of OM proteins from different bacterial strains. Lanes: 1, *A. hydrophila* AH-3 (wild-type, serogroup O:34); 2, size standard (20, 31, 43, 62, and 97 kDa); 3, *E. coli* DHα5 harboring cosmid pLA2917; 4, *E. coli* DH5α harboring pLA-POR3 plasmid DNA (carrying the porin II gene); 5, the same strain as in lane 4 lacking the pLA-POR3 plasmid DNA. The arrow points to porin II (39 kDa).

ing on the same cosmid vector, pLA2917, with the oligonucleotides previously described for the *Bgl*II site of this cosmid; other sequence-derived oligonucleotides were used to complete the nucleotide sequence. Analyses of the deduced sequence of pLA-POR3 showed three complete ORFs (Fig. 2). ORF1 and ORF2 are divergently transcribed; between ORF2 and ORF3, a sequence with characteristic features of Rho-independent transcription termination signals was found.

Analysis of deduced amino acid sequences. Proteins similar to each ORF gene product were analyzed to determine the levels of similarity and identity. As shown in Table 2, ORF1 was found to be similar to DING from both gram-positive and gram-negative bacteria. DING proteins have been proposed to be ATP-dependent helicases. ORF2 was found to be similar to several porin proteins of different enterobacteria and *Vibrionaceae*. As expected, the deduced amino acid sequence from ORF2 showed a putative signal sequence of 20 amino acid residues. On the other hand, the signal sequence characteristics and the first N-terminal amino acid of the putative mature protein strongly suggested that the AH-3 porin II is processed by a signal peptidase I. The sequence of the porin II gene predicted a protein product of 351 amino acids with a 20-residue signal peptide, whose sequence (1 to 20, MKKTILAI AIPALFASAANA) was previously confirmed by N-terminal sequencing of the purified porin (24). The mature protein deduced from the gene sequence consists of 331 amino acids with a molecular mass of 36,237 Da and has some features in common with other porins: a theoretical acidic pI (4.57), lack of cysteine residues, a regular peak and, as indicated by the Kyte-Doolittle hydropathy plot (16), a predicted structure with 16 beta strands, and a hydrophobic carboxy-terminal sequence with a final Phe that is crucial for the localization in the OM of OmpA and porin PhoE (14). Database comparisons showed that the highest scores of porin II were with porins OmpN, PhoE, and OmpF from *E. coli*, with porin PhoE from *Enterobacter cloacae*, with porins OmpK36 and OmpK37 from *Klebsiella pneumoniae*, and with the OmpL protein from *Photobacterium* sp., resulting in identity percentages of 27, 25, 24, 27, 26, 26, and 27, respectively. Detailed alignment of porin II amino acid sequence with those of enterobacterial porins with known three-dimensional structures is shown in Fig. 3. Given the low homology with other porins, many insertions and deletions were observed in the predicted loop regions, but the secondary structure of porin II could be predicted due to its beta strand content and to the presence of amino acids Lys-16, Arg-48, Glu-68, Arg-83, Asp-114, Glu-118, and Arg-129. These residues are well conserved in enterobacterial and nonenterobacterial porins (32) and in the known porin structures are distributed across the pore, resulting in a pronounced charge segregation: Asp-106, -113, -106, -114 (sequential PhoE, OmpF, OmpK36, porin II numbering); Glu-110, -117, -110, -118; main carbonyl groups from L3 on one side of the pore; and basic residues Lys-16, Arg-37, -42, -37, -48, Arg-75, -82, -75, -83, and Arg-126, -132, -125, -129 on the other side. These residues seem to be important for the function of porins, since they are also observed in the three-dimensional structure of the *Rhodobacter capsulatus* porin (38).

Finally, since the similarity and identity data strongly suggest that ORF3 encodes an aspartate aminotransferase, the gene was named *aspC*, consistent with its *E. coli* homolog. In *E. coli* the *ompF* gene is found between *asnS* and *aspC*, all three genes being transcribed in the same direction. The region described here showed the porin, II gene and *aspC* being adjacent and transcribed in the same direction (as in *E. coli*); however, no *asnS* was found upstream of the porin II gene.

Construction of defined porin II insertion mutants and complementation. Plasmid pFS-POR3, a replication *pir*-dependent construct carrying an internal fragment of the porin II gene, was transferred by mating independently to Rif^r mesophilic *Aeromonas* sp. strains AH-405 (serogroup O:34) and AH-408 (serogroup O:11), and Rif^r and Km^r colonies from both matings were selected. We obtained several mutants, AH-

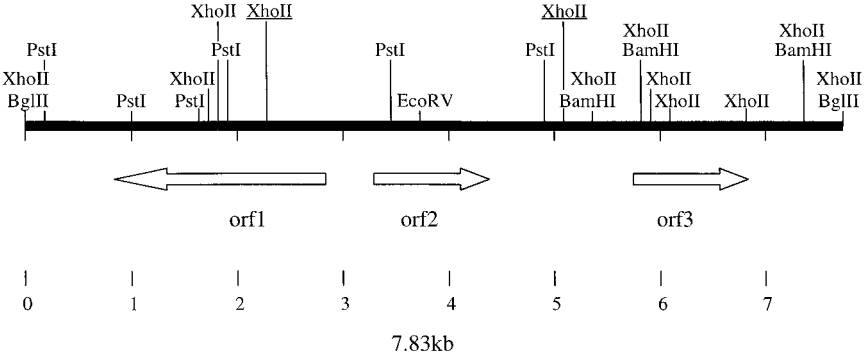

FIG. 2. Schematic of the organization of the three ORFs in the 7.83-kb DNA insert (from *A. hydrophila* AH-3) contained in pLA-POR3 plasmid DNA. *Bgl*II, *Pst*I, *Eco*RV, *Xho*II, and *Bam*HI restriction enzyme sites are indicated. The two *Xho*II restriction sites underlined were used to clone the complete porin II gene to construct pLA-POR6.

TABLE 2. Amino acid sequence homology of ORF proteins from *A. hydrophila* AH-3 and other proteins

Protein, source	No. of amino acids	% Similarity[a]	% Identity[a]	Accession no.
ORF1 (helicase), *Aeromonas hydrophila* AH-3	690			AF183931
DING (probable ATP-dependent helicase)				
Escherichia coli	716	54.3	34.0	P27296
Mycobacterium tuberculosis	664	51.2	29.7	Q10640
Bacillus subtilis	931	49.8	28.5	P54394
Yom1 (putative ATP-dependent helicase in OmpH 5′ region, *Photobacterium* sp.	151	79.5	49.6	P29741
ORF2 (porin II), *A. hydrophila* AH-3	352			AF183931
PhoE, *Enterobacter cloacae*	350	52.7	30.5	Q47490
OmpF, *Serratia marcescens*	374	51.6	29.9	O33980
OmpN, *E. coli*	377	51.4	29.8	P77747
OmpK37, *Klebsiella pneumoniae*	374	50.4	29.2	e1325636
OmpL, *Photobacterium* sp.	341	48.7	28.9	Q52581
ORF3 (AspC), *A. hydrophila* AH-3	396			AF183931
AspC (aspartate aminotransferase)				
E. coli	396	92.4	63.6	P00509
Haemophilus influenzae	396	88.6	60.8	P44425
Moraxella sp.	397	67.8	49.7	O53137

[a] Obtained from pairwise comparisons using the Gap program (gap weight, 12; length weight, 12).

330 and AH-331 being representative of serogroups O:34 and O:11, respectively. The insertion of plasmid pFS-POR3 in these mutants was confirmed by Southern blotting using appropriate DNA probes. As shown in Fig. 4, no porin II could be detected either in SDS-PAGE of OM proteins or in Western blot analysis using OM proteins and antibodies against porin II.

Complementation of these mutants with the single gene for the porin II was accomplished as follows. pLA-POR3 was digested with *Xho*II; a band of 2,732 bp was recovered and ligated to pLA2917 previously digested with *Bgl*II and dephosphorylated, creating pLA-POR6. This plasmid was transferred by mating to mutants AH-330 and AH-331, selecting for Tc^r and Km^r, to obtain strains AH-334 and AH-335, respectively. Strains AH-334 and AH-335 exhibited porin II, as can be observed by SDS-PAGE of OM proteins or by Western blot analysis using OM proteins and antibodies against porin II (Fig. 4).

To study the role of porin II in previously obtained serum-sensitive mutants (AH-53 and AH-26 [24]), we performed the

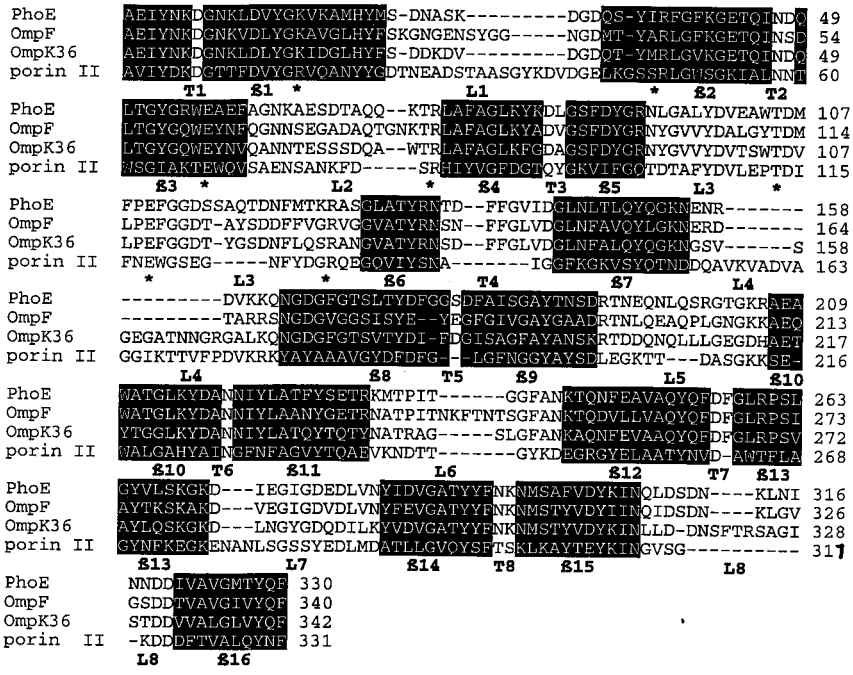

FIG. 3. Alignments of the *A. hydrophila* AH-3 porin II sequence with sequences of PhoE from *Enterobacter aerogenes*, OmpF from *E. coli*, and OmpK36 from *K. pneumoniae*. Protein sequences were derived from nucleotide sequences. Secondary structural motifs are those of OmpF structure. L1 to L8, loops 1 to 8; (in black letters); T1 to T8, turns 1 to 8; β1 to β6, beta strands (in black boxes); *, main basic residue conserved in enterobacterial or nonenterobacterial porin (32).

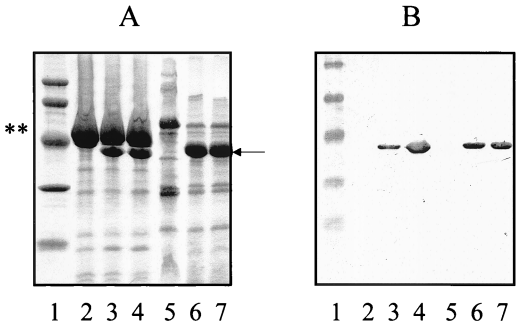

FIG. 4. Coomassie blue-stained gel (A) and Western immunoblot using specific anti-porin II serum (B) of OM proteins from different mesophilic *Aeromonas* sp. strains. Lanes: 1, size standard (20, 31, 43, 62, and 97 kDa); 2, AH-331 (porin II-deficient mutant from strain AH-408); 3, AH-408 (TF7 Rif^r, serogroup O:11); 4, AH-335 (AH-331 complemented with pLA-POR6 plasmid DNA [porin II gene]); 5, AH-330 (porin II-deficient mutant from strain AH-405); 6, AH-405 (AH-3 Rif^r, serogroup O:34); 6, AH-334 (AH-330 complemented with pLA-POR6 plasmid DNA [porin II gene]). The arrow points to porin II; ** denotes protein S of the S-layer characteristic of strains from serogroup O:11.

same experiments to obtain defined insertion mutants in the porin II gene (AH-336 and AH-337, respectively) and complementation studies with pLA-POR6 on these mutants AH-336 and AH-337 (strains AH-338 and AH-339, respectively).

Serum susceptibility and C1q binding. Porin II-defined insertion mutants (AH-330 and AH-331) from the serum-resistant wild-type strains (AH-3 and AH-1, respectively) showed similar resistance to complement-mediated killing. This result suggested that when porin II was lost but other surface molecules like O:34 antigen LPS in AH-330 (smooth strain) and O:11 antigen LPS and S-layer in AH-331 (smooth S^+ strain) remained, no changes in serum susceptibility occurred (Table 3). However, strain AH-330 cultivated under conditions where no O:34 antigen LPS is expressed (rough) (37°C and low osmolarity [1, 20]) was resistant to serum, while the wild-type strain (AH-3) and the Rif^r mutant (AH-405) were serum sensitive under the same growth conditions (1, 20). Complementation of AH-330 with pLA-POR6 (porin II gene) renders this strain (AH-334) serum sensitive under the growth conditions mentioned above. This finding prompted us to examine the porin II-defined insertion mutants (AH-336 and AH-337) from serum-sensitive strains (AH-53 and AH-26, respectively) which showed resistance to complement-mediated killing similar to the initial wild-type strains (AH-3 and AH-1). However, when we reintroduced the porin II gene in these strains (AH-336 and AH-337) by complementation with plasmid pLA-POR6 (strains AH-338 and AH-339, respectively), they became as serum sensitive as strains AH-53 and AH-26 (Table 3). These results indicate that when mutants lacking the O-antigen LPS or the O:34 strains not expressing this antigen (serum sensitive) are devoid of porin II, they became serum resistant, and reintroduction of the porin II gene renders the strains again serum sensitive. Because we previously showed that porin II is able to bind C1q (the initial component of the CPC pathway), we studied the C1q binding of whole cells in these strains. As shown in Table 3, porin II-defined insertion mutants (AH-336 and AH-337) from serum-sensitive strains showed a large reduction in C1q-bound molecules in comparison with AH-53 and AH-26 and became serum resistant. Complementation of these defined insertion mutants (AH-336 and AH-337) with pLA-POR6 (carrying the porin II gene), i.e., reintroduction of porin II, renders these strains (AH-338 and AH-339) serum sensitive because there are numerous of C1q molecules bound to their bacterial surface. From these results seems clear that porin II is the major C1q binding surface on these mesophilic *Aeromonas* sp. strains.

A confirmation of this point is that porin II-defined insertion mutants from serum-resistant wild-type strains showed no difference, in either serum susceptibility or C1q-bound molecules, because of the interference of the O-antigen LPS and also the S-layer in serogroup O:11 strains. However, when porin II-defined insertion mutant AH-330 of serogroup O:34 showed a rough LPS (growing conditions of 37°C and low osmolarity [1, 20]), it was resistant to serum killing and exhibited a great reduction of C1q-bound molecules in comparison with the wild-type strains or the same mutant complemented by porin II gene (pLA-POR6) in the same growth conditions (Table 3).

It is important to point out that rough *Aeromonas* sp. strains (serum sensitive, easily eliminated by complement), if they lose

TABLE 3. Serum susceptibility and binding of ^{125}I-labeled C1q to mesophilic *Aeromonas* sp. whole cells

Strain	Relevant characteristic(s)	% Survival after 3 h in NHS[a]	Bound C1q (molecules/bacterial cell)[a]
AH-3	O:34 (smooth)[b]	101 ± 4	89 ± 17
AH-3	O:34 (rough)[c]	<0.1	304 ± 37
AH-405	AH-3 Rif^r (smooth)	103 ± 3	87 ± 18
AH-405	AH-3 Rif^r (rough)	<0.1	309 ± 40
AH-53	O^- derived from AH-3 (20)	<0.1	378 ± 42
AH-330	Porin II mutant of AH-405 (smooth)	108 ± 6	<30
AH-330	Porin II mutant of AH-405 (rough)	105 ± 4	<30
AH-334	AH-330 + pLA-POR6 (smooth)	99 ± 3	89 ± 15
AH-334	AH-330 + pLA-POR6 (rough)	<0.1	317 ± 33
AH-336	Porin II mutant of AH-53	98 ± 5	<30
AH-338	AH-336 + pLA-POR6	<0.1	369 ± 36
TF7	O:11, S^+	145 ± 12	<10
AH-408	TF7 Rif^r	139 ± 8	<10
AH-26	O^- S^-, derived from TF7 (22)	<0.1	389 ± 38
AH-331	Porin II mutant of AH-408	141 ± 6	<10
AH-335	AH-331 + pLA-POR6	143 ± 7	<10
AH-337	Porin II mutant of AH-26	129 ± 8	<30
AH-339	AH-337 + pLA-POR6	<0.1	376 ± 35

[a] Average of at least three independent experiments ± standard deviation. Some of the data have been previously published (24).
[b] Strains of serogroup O:34 showed O:34 antigen LPS (smooth) when grown at 20°C (20).
[c] Strains of serogroup O:34 are unable to show O:34 antigen LPS (rough) when grown at 37°C and in low-osmolarity medium (1).

porin II by different mechanisms (for instance, by insertion sequence interruption of the gene as happens in other gram-negative bacteria [10, 18]), could became serum resistant, develop antibiotic resistance, and emerge as a serious clinical problem.

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REFERENCES

1. **Aguilar, A., S. Merino, X. Rubires, and J. M. Tomás.** 1997. The influence of osmolarity on lipopolysaccharide and virulence of *Aeromonas hydrophila* strains serotype O:34 grown at 37°C. Infect. Immun. **65:**1245–1250.
2. **Albertí, S., G. Marqués, S. Camprubí, S. Merino, J. M. Tomás, F. Vivanco, and V. J. Benedí.** 1993. C1q binding and activation of the complement classical pathway by *Klebsiella pneumoniae* outer membrane proteins. Infect. Immun. **61:**852–860.
3. **Allen, L. N., and R. S. Hanson.** 1985. Construction of broad-host-range cosmid cloning vector: identification of genes necessary for growth of *Methylobacterium organophilum* on methanol. J. Bacteriol. **161:**955–962.
4. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
5. **Atlas, R. M., and L. C. Parks (ed.).** 1993. Handbook of microbiological media. CRC Press Inc., Boca Raton, Fla.
6. **Darveau, R. P., and R. E. W. Hancock.** 1983. Procedure for the isolation of bacterial lipopolysaccharides from both smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains. J. Bacteriol. **155:**831–838.
7. **Figueroa, J. E., and P. Densen.** 1991. Infectious diseases associated with complement deficiencies. Clin. Microbiol. Rev. **4:**359–395.
8. **Guasch, J. F., N. Piqué, N. Climent, S. Ferrer, S. Merino, X. Rubirés, J. M. Tomás, and M. Regué.** 1996. Cloning and characterization of two *Serratia marcescens* genes involved in core lipopolysaccharide biosynthesis. J. Bacteriol. **178:**5741–57475.
9. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**577–580.
10. **Hernández-Allés, S., V. J. Benedí, L. Martinez-Martinez, A. Pascual, A. Aguilar, J. M. Tomás, and S. Albertí.** 1999. Development of resistance during antimicrobial therapy caused by insertion sequence interruption of porin genes. Antimicrob. Agents Chemother. **43:**937–939.
11. **Janda, J. M., and R. P. Kokka.** 1991. The pathogenicity of *Aeromonas* strains relative to genospecies and phenospecies identification. FEMS Microbiol. Lett. **90:**29–34.
12. **Janda, J. M., L. S. Guthertz, R. P. Kokka, and T. Shimada.** 1994. *Aeromonas* species in septicemia: laboratory characteristics and clinical observations. Clin. Infect. Dis. **19:**77–83.
13. **Jeanteur, D., N. Gletsu, F. Pattus, and J. T. Buckley.** 1992. Purification of *Aeromonas hydrophila* major outer-membrane proteins: *N*-terminal sequence analysis and channel-forming properties. Mol. Microbiol. **6:**3355–3363.
14. **Jeanteur, D., J. H. Lakey, and F. Pattus.** 1994. The porin superfamily: diversity and common features. *In* J. M. Ghuysen and R. Hakenbeck (ed.), Bacterial cell wall. Elsevier, Amsterdam, The Netherlands.
15. **Kokka, R. P., and J. M. Janda.** 1990. Isolation and identification of autoagglutinating serogroup O:11 *Aeromonas* strains in the clinical laboratory. J. Clin. Microbiol. **28:**1297–1299.
16. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157:**105–132.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
18. **Martinez-Martinez, L., S. Hernández-Allés, S. Albertí, J. M. Tomás, V. J. Benedí, and G. A. Jacoby.** 1996. In vivo selection of porin deficient mutants of *Klebsiella pneumoniae* with increased resistance to cefoxitin and expanded-spectrum cephalosporins. Antimicrob. Agents Chemother. **40:**342–348.
19. **Merino, S., S. Camprubí, and J. M. Tomás.** 1991. The role of lipopolysaccharide in complement-killing of *Aeromonas hydrophila* strains of serotype O:34. J. Gen. Microbiol. **137:**1583–1590.
20. **Merino, S., S. Camprubí, and J. M. Tomás.** 1992. Effect of the growth temperature on outer membrane components and virulence of *Aeromonas hydrophila* strains of serotype O:34. Infect. Immun. **60:**4343–4349.
21. **Merino, S., S. Camprubí, and J. M. Tomás.** 1993. Incidence of *Aeromonas* sp. serotypes O:34 and O:11 among clinical isolates. Med. Microbiol. Lett. **2:**48–55.
22. **Merino, S., X. Rubires, A. Aguilar, S. Albertí, S. Hernández-Allés, V. J. Benedí, and J. M. Tomás.** 1996. Mesophilic *Aeromonas* sp. serogroup O:11 resistance to complement-mediated killing. Infect. Immun. **64:**5302–5309.
23. **Merino, S., X. Rubirés, A. Aguilar, and J. M. Tomás.** 1997. The role of flagella and motility in the adherence and invasion to fish cell lines by *Aeromonas hydrophila* serogroup O:34 strains. FEMS Microbiol. Lett. **151:** 213–217.
24. **Merino, S., M. M. Nogueras, A. Aguilar, X. Rubires, S. Albertí, V. J. Benedí, and J. M. Tomás.** 1998. Activation of the complement classical pathway (C1q binding) by mesophilic *Aeromonas hydrophila* outer membrane protein. Infect. Immun. **66:**3825–3831.
25. **Misra, S. K., T. Shimada, R. K. Bhadra, S. C. Pal, and G. B. Nair.** 1989. Serogroups of *Aeromonas* species from clinical and environmental sources in Calcutta, India. J. Diarrhoeal Dis. Res. **7:**8–12.
26. **Murray, R. G. E., J. S. G. Dooley, P. W. Whippey, and T. J. Trust.** 1988. Structure of an S-layer on a pathogenic strain of *Aeromonas hydrophila*. J. Bacteriol. **170:**2625–2636.
27. **Rubirés, X., F. Saigi, N. Piqué, N. Climent, S. Merino, S. Albertí, J. M. Tomás, and M. Regué.** 1997. A gene (*wbbL*) from *Serratia marcescens* N28b (O4) complements the *rfb-50* mutation of *Escherichia coli* K-12 derivatives. J. Bacteriol. **179:**7581–7586.
28. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
29. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
30. **Sakazaki, R., and T. Shimada.** 1984. O-serogrouping scheme for mesophilic *Aeromonas* strains. Jpn. J. Med. Sci. Biol. **37:**247–255.
31. **Schirmer, T., and S. Cowan.** 1993. Prediction of membrane-spanning beta strands and its application to maltoporin. Protein Sci. **2:**1361–1363.
32. **Struyvé, M., M. Moons, and J. Tommassen.** 1991. Carboxy-terminal phenylalanine is essential for the correct assembly of a bacterial outer membrane protein. J. Biol. Chem. **218:**141–148.
33. **Taylor, P. W.** 1988. Bacterial resistance to complement, p. 107–120. *In* J. A. Roth (ed.), Virulence mechanisms of bacterial pathogens. American Society for Microbiology, Washington, D.C.
34. **Tenner, A. J., P. H. Lesavre, and N. R. Cooper.** 1981. Purification and radiolabeling of human C1q. J. Immunol. **127:**648–653.
35. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
36. **Tsai, C. M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharide in polyacrylamide gels. Anal. Biochem. **119:**115–119.
37. **Vukajlovich, S. W., J. Hoffman, and D. C. Morrison.** 1987. Activation of human serum complement by bacterial lipopolysaccharides: structural requirements for antibody independent activation of the classical and alternative pathways. Mol. Immunol. **24:**319–331.
38. **Weiss, M. S., A. Kreusch, E. Schiltz, U. Nestel, W. Welte, J. Weckesser, and G. E. Schulz.** 1991. The structure of porin from *Rhodobacter capsulatus* at 1.8 A resolution. FEBS Lett. **280:**379–382.
39. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides: extraction with phenol-water and further applications of the procedure. Carbohydr. Chem. **5:**83–91.

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