Journal of Applied Microbiology 2003, **94**, 908–918

Molecular cloning, sequencing and characterization of *omp48*, the gene encoding for an antigenic outer membrane protein from *Aeromonas veronii*

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2002/386: received 15 October 2002, revised 7 January 2003 and accepted 5 February 2003

ABSTRACT

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Aims: To clone, sequence and characterize the gene encoding the Omp48, a major outer membrane protein from *Aeromonas veronii*.

Methods and Results: A genomic library of *Aer. veronii* was constructed and screened to detect *omp48* gene sequences, but no positive clones were identified, even under low stringency conditions. The cloned gene probably was toxic to the host *Escherichia coli* strain, so the cloning of *omp48* was achieved by inverse PCR. The nucleotide sequence of *omp48* consisted of an open reading frame of 1278 base pairs. The predicted primary protein is composed of 426 amino acids, with a 25-amino-acid signal peptide and common Ala-X-Ala cleavage site. The mature protein is composed of 401 amino acids with a molecular mass of 44 256 Da.

Conclusions: The *omp48* gene from *Aer. veronii* was cloned, sequenced and characterized in detail. BLAST analysis of Omp48 protein showed sequence similarity (over 50%) to the LamB porin family from other pathogenic Gram-negative bacteria.

Significance and Impact of the Study: Bacterial diseases are a major economic problem for the fish farming industry. Outer membrane proteins are potentially important vaccine components. The characterization of *omp48* gene will allow further investigation of the potential of Omp48 as recombinant or DNA vaccine component to prevent *Aer. veronii* and related species infections in reared fish.

Keywords: *Aeromonas veronii*, bacterial outer membrane proteins, LamB, cloning, adhesin, inverse PCR.

INTRODUCTION

The genus *Aeromonas* is widely distributed in aquatic environments and increasingly reported as a primary pathogen of human and lower vertebrates (Janda 1991; Janda and Abbott 1998). *Aeromonas veronii* is a Gram-negative bacterium that has been reported as an aetiological agent in a variety of human infections, including arthritis, gastroenteritis, meningitis, septicaemia and wound infections (Hickman-Brenner *et al.* 1987; Stelma *et al.* 1988; Neves *et al.* 1990; Joseph *et al.* 1991; Abbott *et al.* 1994; Kirov and Sanderson 1996; Hsueh *et al.* 1998; Janda and Abbott 1998; Steinfeld *et al.* 1998), and as a pathogen of amphibians, reptiles and fish (Trust *et al.* 1986; Krovacek *et al.* 1994; Simmaco *et al.* 1998). Members of the genus *Aeromonas* have been reported as primary pathogens of numerous cultured fish species (Trust *et al.* 1986; Austin and Adams 1996). *Aer. veronii* is the causative agent of bacterial haemorrhagic septicaemia (motile aeromonad

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septicaemia) of cultured warm-water fish and *Aer. hydrophila* is considered a major economic problem for the aquaculture industry (Austin and Austin 1999).

Previous studies have shown that *Aeromonas* strains, isolated from various sources, possess a strong lectin-like affinity for common mucosal constituents, such as mucin and lactoferrin (Chart and Trust 1983; Kishore *et al.* 1991; Ascencio *et al.* 1998). Recently, we identified a 48-kDa protein from *Aer. veronii* that shows the same mucosal constituents-binding characteristics (mucosal constituent-binding protein, MCBP48). In addition, its N-terminal amino acid sequence (VDFHGYMRS) indicates that this protein could be related to the LamB maltose-inducible porin family (unpublished data). The immunoprotective properties of this protein were demonstrated in fish (*Paralabrax maculatofasciatus*) challenged with pathogenic *Aer. veronii* cell suspensions. It was shown that oral immunization with MCBP48 protein, conjugated with the cholera toxin B subunit (immunogenic complex), protected almost 70% of infected fish, and that histological alterations of internal organs decreased (Merino-Contreras *et al.* 2001). Studies based on interactions between *Aer. veronii* and primary-culture epithelial cells of fish demonstrated that antibodies raised against MCBP48 prevent bacterial adhesion, suggesting the putative role of the cell surface protein as an adhesin (Guzman-Murillo *et al.* 2000).

Bacterial porins are specific (e.g. LamB) or non-specific (e.g. OmpC and OmpF) channel-forming transmembrane proteins found in the outer membrane of Gram-negative bacteria and function as molecular sieves to allow diffusion of hydrophilic molecules into periplasmic space (Jeanteur *et al.* 1991; Nikaido 1992). Porins are highly immunogenic because of exposed epitopes on bacterial surfaces. Considerable homology at their primary and secondary structures makes porins antigenically related (Jeanteur *et al.* 1991), so porins are suitable candidates for developing vaccines against Gram-negative bacterial infections (Gilleland *et al.* 1988; Matsui and Arai 1990; Nurminen *et al.* 1992; Dodsworth *et al.* 1993b; Tabaraie *et al.* 1994; Lutwyche *et al.* 1995).

Owing to the importance of the 48-kDa protein (MCBP48) of *Aer. veronii* as a putative adhesin and its potential as a vaccine candidate, and to confirm its identity as an outer membrane protein, in the present study we report the molecular cloning and sequence analysis of the gene encoding this protein, which we designated *omp48* and the encoded protein Omp48.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

The *Aer. veronii* biotype *veronii* strain A186 used in the present study was obtained from the Microbial Culture Collection of the Hospital of the University of Lund, Sweden, and was kindly provided by Prof. T. Wadström. It was identified by its fatty acids profile (Sherlock System) at Auburn University, AL, USA (Bacterial Strain Identification and Mutant Analysis Service). *Escherichia coli* XL1-Blue MRF′ and pBluescript-SK phagemid were obtained from Stratagene (La Jolla, CA, USA). Boehringer Mannheim (Indianapolis, IN, USA) supplied pBR322 plasmid. *E. coli* and *Aer. veronii* strains were grown at 37°C in Luria-Bertani (LB) medium and ampicillin (50 or 100 μg ml^{-1}) or tetracycline (10 μg ml^{-1}) was added to the media when needed (Sambrook *et al.* 1989). To determine the effect of growth conditions on the outer membrane profile of *Aer. veronii,* the cells were grown to OD_{600} of 4–5 (mid- to late-exponential phase) in 100-ml volumes of LB medium using 1 l flasks at 22 or 37°C and in the presence or absence of 1% (w/v) maltose.

Isolation of outer membrane proteins from *Aeromonas veronii* and SDS-PAGE analysis

Outer membrane proteins (OMPs) of *Aer. veronii* were purified according to Jeanteur *et al.* (1992) and Luo *et al.* (1997). Bacteria were harvested in the mid- to late-exponential growth phase by centrifugation at 8000 × *g* for 20 min at 4°C, and washed twice with phosphate-buffered saline (PBS). The cell pellet was suspended in 5 ml of sonication buffer (20 mmol l^{-1} Tris, pH 7·4, containing 100 μg ml^{-1} of DNase and RNase). The cell suspension was sonicated eight times during 30 s. Unbroken cells were removed by centrifugation at 3000 × *g* for 20 min and the supernatant was centrifuged at 20 000 × *g* for 90 min to pellet cell envelopes. The supernatant was discarded and the pellet was dispersed by stirring in 5 ml of 20 mmol l^{-1} Tris (pH 7·4) containing 0·75% (w/v) sodium lauryl sulphate (SDS) at 37°C for 20 min. The suspension was centrifuged at 20 000 × *g* for 90 min to pellet the insoluble outer membranes. The outer membrane fraction was suspended in de-ionized water and protein concentration was determined according to Bradford (1976). Outer membrane proteins were separated electrophoretically on 12% SDS-polyacrylamide gels (SDS-PAGE) at 20 mA and stained with Coomassie blue R-250 (Laemmli 1970).

N-terminal and internal peptide amino acid sequencing of Omp48

Outer membrane proteins were electrophoresed by SDS-PAGE as mentioned above and Omp48 was purified by electro-elution (Model 422 electro-eluter, Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions. Purified Omp48 was sent to the Institut de Genetique et de Biologie Moleculaire et Cellulaire (IGBMC) peptide service

(France) and to the Instituto de Biotecnología, UNAM (Mexico) for amino acid sequencing.

DNA isolation

Genomic DNA was isolated as described by Dodd and Pemberton (1996), except that the final extract was not dialysed. A loopful of *Aer. veronii* culture, grown overnight, was suspended in 500-μl saline–EDTA buffer (150 mmol l^{-1} NaCl, 100 mmol l^{-1} EDTA) containing RNase (50 μg ml^{-1}) and lysozyme (25 μg ml^{-1}). Proteinase K (250 μg ml^{-1}) and 10 μl 25% (w/v) SDS were added after 15-min incubation at 65°C. Incubation continued for another 15 min, or until cell lysis was complete (clear suspension). The cell lysate was chilled on ice and then extracted twice with phenol–chloroform–isoamyl alcohol.

Oligonucleotide design and synthesis

Oligonucleotides used for PCR amplification were synthesized at the Instituto de Biotecnología, UNAM (Mexico) and are listed in Table 2. Degenerated primers were synthesized according to N-terminal amino acid sequence (Table 1) and highly conserved regions of the LamB family (Fig. 2). The lowest codon degeneracy of amino acids, and the preferential codon usage of *Aeromonas spp.* and of the *lamB* gene family were considered to decrease the degeneracy of the primers.

PCR amplification and cloning of the *omp48* gene

Partial regions of the *omp48* gene were amplified from genomic DNA of *Aer. veronii* as follows: forward primer MR (N-terminal) was used in combination with reverse primers AV1 (consensus 1), AV1A (consensus 2), AV3 (consensus 3) and AV4 (consensus 4) (Table 2 and Fig. 2). The PCR mix consisted of 200 ng of genomic DNA, 0·1 mmol l^{-1} dNTPs, 10 pmol of primers, 1 × Taq polymerase buffer and 1·25 U of Taq DNA polymerase (Boehringer Mannhein, Indianapolis, IN, USA) in a volume totalling 25 μl. PCR conditions consisted of initial incubation for denaturation at 95°C for 5 min, and then 35 cycles, each including a denaturation step at 95°C for 1 min, an annealing step at 53–55°C for 1 min and an extension step at 72°C for 2 min. Finally, extension incubation at 72°C for 10 min was performed. The structural gene encoding the primary and mature Omp48 was amplified with LP-AV7 and MR-AV7 primer pairs, respectively (Table 2). PWO DNA polymerase (Roche, Indianapolis, IN, USA) and a few amplification cycles were used to decrease the possibility of polymerization error during PCR amplification. All reactions were carried out on a Gene Amp PCR System 9600 (Perkin-Elmer, Foster City, CA, USA). Amplicons were purified with a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) and cloned into the pGEM-T vector (Promega, Madison, WI, USA).

The cloned PCR products (partial and complete *omp48* gene) were sequenced by the Keck/DNA sequencing service (Yale University) directly on a double-stranded DNA plasmid template with universal primers M13R and T7, by the dideoxy chain termination method of Sanger *et al.* (1977). The Abi Prism 310 Genetic Analyzer (Perkin-Elmer, USA) was used for sequencing.

Table 1 Amino acid sequence of Omp48 amino terminus (N-terminal) and internal peptides

Peptide	Sequence
Amino terminus	VDFHGYMR
Peptide IP-1	LGNEAETYGEVQLGQEAYNK
Peptide IP-2	DGSLELGYDYAR
Peptide IP-3	FINWGVLPVGDK
Peptide IP-4	VYASYLDSDGQFRPDANGVK

Table 2 List of primers

Primer	Sequence
MR	GT(G/T)GA(C/T)TTCCACGGCTA(C/T)ATGCG
AV1	TGGCG(C/T)TGGTAGAA(G/A)CGCTT(G/A) CCGGCCCA
AV1A	CC(A/T)GAGATGTCCCAGTAGTA(G/A)AA(G/A)TC
AV3	TACTGCACCACGAA(C/T)TTGTT(G/A)AA(G/A)CC
AV4	CCGGCCTGCCACTGCTG(G/T)GCCAG(G/A)GT
AV5	GAA GTA CCG ACA CCG GGA CGC
AV6	ATC CAT GCT GGG TGG CTT CAA C
AV7	TTA CCA CCA AGC TTC CGC TTG
LP	ATG AAA ATG AAA GCA AAG TGG C

Construction and screening of *Aeromonas veronii* genomic library

Aeromonas veronii genomic DNA was partially digested with *Sau*3AI. Restriction fragments were electrophoresed on agarose gel and fragments between 2 and 15 kb were isolated and ligated into *Bam*HI-digested and dephosphorylated pBluescript-SK phagemid. *E. coli* XL1-Blue MRF′ was transformed with the ligation products by the calcium chloride competent cells method (Sambrook *et al.* 1989). Transformants were plated on LB medium containing 100 μg ml^{-1} ampicillin. The library was screened by colony hybridization with a biotinylated MR-AV3 probe according to manufacturer's instructions. Partial genomic libraries were constructed both in pBluescript-SK phagemid (high copy number) and pBR322 (low copy number) with *Bam*HI, *Bgl*II, *Hin*dIII or *Pst*I-genomic DNA restriction fragments that hybridized with the MR-AV3 probe in a Southern blot assay. Screening of transformants was performed as previously described.

Southern blot hybridization

Southern blots were carried out under high stringency conditions according to Sambrook *et al.* (1989). The PCR product, amplified with primer combination MR-AV3, was used as a probe (MR-AV3 probe) and was biotin-labelled with a NEBlot phototope kit (New England Biolabs, Beverly, MA, USA). Hybridization of nylon membrane was conducted overnight at 65°C in 6× SSC containing 0·5% (w/v) SDS, followed by two washes for 10 min each at room temperature in 1× SSC containing 0·1% (w/v) SDS and two additional washes at 65°C with 0·1× SSC containing 0·1% (w/v) SDS. Chemiluminescent detection of the probe was performed with the Phototope-Star detection kit according to manufacturer's instructions (New England Biolabs, Beverly, MA, USA).

Inverse PCR

Primers AV5 and AV6 (Table 2) used for inverse PCR (IPCR) were designed based on the 5′ and 3′ ends of the MR-AV3 amplicon sequence. IPCR was performed as described by Ochman *et al.* (1988). Five micrograms of genomic DNA from *Aer. veronii* was totally digested with *Eco*RV and *Sph*I enzymes. The restriction enzymes were heat inactivated at 65°C for 20 min and digested DNA was diluted to 10 ng μl^{-1}. Self-ligations (intramolecular ligations) then were carried out at 16°C overnight. Self-ligated DNA was precipitated with absolute ethanol and suspended in 100 μl of de-ionized water. Finally, 5–10 μl was used for PCR amplification with primers AV5 and AV6 under amplification conditions as described above. PCR products were cloned and sequenced as previously mentioned.

Computer sequence analysis of *omp48*

The sequence of the *Aer. veronii omp48* gene was deposited in GenBank under accession no. AF538866. The searches for nucleotide and protein sequence similarities in the SwissProt, GenBank and EMBL databases were conducted with BLAST algorithm at the National Center for Biotechnology Information (Altschul *et al.* 1997, http://www.ncbi.nlm.nih.gov/blast/). Other online sequence analysis services, such as Clustal W, Expasy, were also used.

RESULTS

Analysis, purification and amino acid sequencing of Omp48 protein

Outer membrane proteins from *Aer. veronii* grown on LB medium were purified by detergent treatment of the cell surface. Dissolved OMPs were boiled at 100°C for 10 min in loading buffer and analysed by SDS-PAGE. Coomassie brilliant blue-gel stain revealed several proteins in the range of 20–80 kDa. Two major OMPs with apparent molecular weights of 38 and 48 kDa were defined (Fig. 1). There was no significant difference in relative expression level of Omp48 protein when *Aer. veronii* cells were grown in the presence or absence of 1% (w/v) maltose, or at different temperatures, on LB medium (Fig. 1).

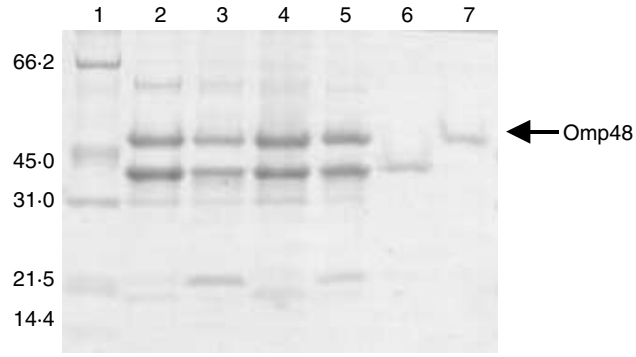

Fig. 1 SDS-PAGE of outer membrane proteins of *Aeromonas veronii* strain A186 grown under different conditions: lane 1, molecular mass markers expressed in kDa; lane 2, cells grown in LB (37°C); lane 3, cells grown in LB plus maltose 1% (37°C); lane 4, cells grown in LB (22°C); lane 5, cells grown in LB plus maltose 1% (22°C); lane 6, major OMP of 38 kDa (electro-eluted); lane 7, Omp48 (electro-eluted)

Purification of Omp48 from the OMP fraction by electro-elution rendered a single discrete band with corresponding molecular weight as visualized by SDS-PAGE (Fig. 1). Purified Omp48 was submitted to amino acid sequencing and its N-terminal sequence (VDFHGYMR) confirmed the previously determined sequence of the 48-kDa protein from the supernatant fraction. Further analysis generated four additional internal peptide sequences (IP1–IP4) from Omp48 (Table 1).

Cloning of the *omp48* gene

We subjected Omp48 to N-terminal sequencing to design degenerated oligonucleotides for PCR amplification of the *omp48* gene. Considering the N-terminal sequence and highly conserved regions of the LamB-related proteins, a set of internal primers for PCR amplification of the *omp48* gene was derived (Table 2 and Fig. 2). The combinations of primers MR with AV1, MR with AV1A, and MR with AV3 yielded unique PCR products of approximately 320, 350, and 680 bp, respectively, while that of primer MR with AV4 generated multiple products in the range of size expected. Single discrete amplicons were cloned in pGEM-T and sequenced. The open reading frame (ORF) deduced from the nucleotide sequence revealed significant homology to the LamB porin family, and three of the sequenced peptides

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lamBav          VDFHGYMRSGVGTSDNGSLQT----YSKAKVGRLGNEAETYGEVQLGQEAYNKDGKSFYV 56
lamBas          VDFHGYFRSGVGVSTDGSMQTGLSDNAKQKVGRLGNEADTYGEIQLGSEVFNKDGKTFYV 60
lamBec          VDFHGYARSGIGWTGSGGEQQCFQTTGAQSKYRLGNECETYAELKLGQEVWKEGDKSFYF 60
lamBst          VDFHGYARSGIGWTGSGGEQQCFQATGAQSKYRLGNECETYAELKLGQEVWKEGDKSFYF 60
lamBkp          VDFHGYARSGIGWTGSGGEQQCFKATGAQSKYRLGNECETYAELKLGQELWKEGDKSFYF 60
lamBye          -HFHGYARSGIGWTGSGGEQQCFKTTGAQSKYRLGNECETYAELKLGQELWKEGDKSFYL 59

lamBav          DSMFAMVSGQQGRDWESTSG------------------SDADFALRQFNVQAKGVLGFAP 98
lamBas          DSMVAMTS-NGSNDWESTESKFQCTSANGTALDGCENKEDATFALRQFNVQAKGLLGFAP 119
lamBec          DTNVAYSV-AQQNDWEATDP----------------------AFREANVQGKNLIEWLP 96
lamBst          DTNVAYSV-NQQNDWESTDP-----------------------AFREANVQGKNLIEWLP 96
lamBkp          DTNVAYSV-NQEDDWESTSP----------------------AFREANIQGKNLIDWLP 96
lamBye          DTNVAYSV-SQRDDWESTDP----------------------AFREANVQGKNLIESFP 95

lamBav          EATLWAGKRYYQRHDIHISDFYYWDVSGAGAGVENIQAGPGKLSFAWLRNDPWDDYDQFV 158
lamBas          EATLWAGKRYYQRHDVHISDFYYWNISGRGAGIEGIQAGPGKVSFAWVRND---RSGTNV 176
lamBec          GSTIWAGKRFYQRHDVHMIDFYYWDISGPGAGLENIDVGFGKLSLAATRSSEAGGSSSFA 156
lamBst          GSTIWAGKRFYQRHDVHMIDFYYWDISGPGAGIENIDLGFGKLSLAATRSTEAGGSYTFS 156
lamBkp          GSTLWAGKRFYQRHDVHMIDFYYWDISGPGAGLENVDLGFGKLSLAATRNSESGGSYTFS 156
lamBye          GSTMWAGKRFYQRHDVHMIDFYYWDISGPGAGLEAIDLGFGKLSVAATRNSEAGGSSAWI 155

lamBav          GGVGVDKRVDTD--VLDLRYAGIPLWKDGSLELGYDYARGNLTDEQK-AALGSKDYEKNG 215
lamBas          DGT-YNDEMNVN--TLDLRYAGIPLWQDGSLEVGVDYAIANPSDAQKDSANAQYKNAKDG 233
lamBec          SNNIYDYTNETANDVFDVRLAQMEINPGGTLELGVDYGRANLRDNYRLVDGAS----KDG 212
lamBst          SQNIYDEVKDTANDVFDVRLAGLQTNPDGVLELGVDYGRANTTDGYKLADGAS----KDG 212
lamBkp          SDDTKKYAAKTANDVFDIRLAGLETNPGGVLELGVDYGRANPQDDYRLEDGAS----KDG 212
lamBye          N-NQRKDADKTINDVYDIRLAGLETNPGGSLEFGVDYGRANTQDDYSLAPNAS----KDG 210

lamBav          HMLTAELTQSMLGGFNKTVVQYFADGYAAQAV-DYGSGSGSGL----------------S 258
lamBas          VMLTAELTQGILGGFNKTVLQYGTEGYSKTFA-FWGDRSWYG-----------------A 275
lamBec          WLFTAEHTQSVLKGFNKFVVQYATDSMTSQGK-GLSQGS--GVAFDNE-----KFAYN-- 262
lamBst          WMFTAEHTQSMLKGYNKFVVQYATDAMTTQGK-GQARGSDGSSSFTEELSDGTKINYANK 271
lamBkp          WMWTGEHTQSIWGGFNKFVVQYATDAMTSWNS-GHSQGTS-------------------- 251
lamBye          VLLTAEHTQSMMGGFNKFVVQYATDSMTSWNS-GHSQGTS-------------------- 249

lamBav          QSAAKGDGWRFINWGVLPVGDKVEFGHQLVYGT--------------ASDIDNDSADKDT 304
lamBas          EAKDGADGFRIINHGVIPMGNSWEMGHQLVYGV--------------GNDMWDTNDKWET 321
lamBec          -INNNGHMLRILDHGAISMGDNWDMMYVGMY----------------QDINWDNDNGTKW 305
lamBst          VINNNGNMWRILDHGAISLGDKWDLMYVGMY----------------QNIDWDNNLGTEW 315
lamBkp          -IDNNGSMIRVLDHGAMDFNDDWGLMYVAMY----------------QELDLDSKNGSTW 294
lamBye          -VNNNGHMLRVIDHGAINLAEKWDMMYVALY----------------QDTDWDNNNGTTW 292

lamBav          FSVVVRPMYKWN-DTMKTIAELGYHHDSYKPNNGTETSTIG-KKYTLAQAWSAGSSFWAR 362
lamBas          MSVVARPMYKWD-DFNKTIFEGGYFKDKNKSTNGTSEEDAG-YKLTLAQAWSAGSSFWAR 379
lamBec          WTVGIRPMYKWT-PIMSTVMEIGYDN---VESQRTGDKNNQ-YKITLAQQWQAGDSIWSR 360
lamBst          WTVGVRPMYKWT-PIMSTLLEVGYDN---VKSQQTGDRNNQ-YKITLAQQWQAGDSIWSR 370
lamBkp          YTVGVRPMYKWT-PIMSTQLEIGYDN---VKSQRTSENNNQ-YKITLAQQWQAGNSVWSR 349
lamBye          YSVGVRPMLQMELPIMSTLLEIGYNN---VKSQRTGKRNGQ-YKLTLAQQWQAGDSIWSR 348

lamBav          PEIRVYASYLDSDG-QFRPD--------------------ANG-VKQDDSWNFGVQAEAW 400
lamBas          PEIRVFASYLAQDKKEMKGN--------------------AFNNGTADDTWNFGVQAEAW 419
lamBec          PAIRVFATYAKWDEKWGYDYTGNADNNANFGKAVPADFNGGSFGRGDSDEWTFGAQMEIW 420
lamBst          PAIRIFATYAKWDEKWGYIKDG--DNISRYAAATNSGIS--TNSRGDSDEWTFGAQMEIW 426
lamBkp          PAIRIFATYAKWDENWGY------SNTSGLQTKDSSGSGAFTSSRGDDSEVTFGAQMEVW 403
lamBye          PATRVFATYANWDEKWGYNN---VDKSPDNGLAQNGTIG--TDSRGKSNECTFGAQF--- 400

lamBav          W 401
lamBas          W 420
lamBec          W 421
lamBst          W 427
lamBkp          W 404
lamBye          -
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Fig. 2 Multiple alignment of deduced amino acid sequence of Omp48 from *Aeromonas veronii* with members of the LamB porin family. Organism abbreviations are as follows: lamBav, *Aer. veronii*; lamBas, *Aer. salmonicida* (SwissProt accession no. Q44287); lamBec, *Escherichia coli* (SwissProt accession no. P02943); lamBst, *Salmonella typhimurium* (SwissProt accession no. P26466); lamBkp, *Klebsiella pneumoniae* (SwissProt accession no. P31242); lamBye, *Yersinia enterocolitica* (SwissProt accession no. Q56850). Consensus regions are shaded and amino acids specific to primer design are underlined

(N-terminal, IP1 and IP2) were localized in the deduced sequence, indicating that at least part of the *omp48* gene was cloned.

A genomic library was constructed with *Sau* 3A partially digested *Aer. veronii* genomic DNA, which was screened with the MR-AV3 amplicon probe, to clone the complete *omp48* structural gene. After repeated attempts, none of the screened colonies reacted with the probe under high stringency conditions and considerable background and unspecific hybridization were obtained when the stringency conditions were relaxed.

Southern blotting assays performed with totally digested genomic DNA from *Aer. veronii* strain A186, with MR-AV3 amplicon as probe, showed single-hybridization fragments. The approximate sizes of hybridized restriction fragments were as follows: *Bam*HI, 5·6 kb; *Bgl*II, 5·6 kb; *Eag*I, 6·8 kb; *Sma*I, 5·8 kb; *Eco*RV, 2·0 kb; *Hin*dIII, 8·0 kb; *Pst*I, 5·1 kb; *Sph*I, 3·0 kb; *Cla*I, 8·5 kb and *Bgl*I, 2·3 kb (data not shown).

Those Southern-hybridized restriction fragments potentially long enough to contain the entire *omp48* gene were cloned in pBS-SK. No positive clones were detected after several screening assays with the MR-AV3 probe. The repeated failure to detect a clone containing *omp48* gene suggested that heterologous porin was expressed from its own promoter and perhaps was toxic to the *E. coli* host strain.

We determined the flanking sequences of the cloned MR-AV3 amplicon (partial *omp48* gene) by IPCR to clone the complete *omp48* structural gene. Southern hybridization analysis prompted us to use *Eco*RV (2·0 kb) and *Sph*I (3·0 kb) digested genomic DNA fragments for IPCR. These fragments, of suitable size for PCR, were self-ligated separately, and AV5 and AV6 primers were used for PCR amplification (Table 2). Amplification products of the expected size were obtained: around 1350 bp for *Eco*RV self-ligated fragments and 2300 bp for *Sph*I self-ligated fragments. IPCR amplicons were cloned into pGEM-T vector and sequenced. Assembling the sequences from previous MR-AV3 PCR products and IPCR products allowed determination of the complete *omp48* coding sequence, including the upstream- and downstream-flanking sequences.

DNA sequence analysis of *omp48* gene

The complete nucleotide sequence of the *omp48* gene and the corresponding deduced amino acid sequence were determined (Fig. 3). Analysis of the sequence revealed an ORF of 1278 nucleotides encoding for a predicted primary protein of 426 amino acids. The N-terminal and internal peptide sequences of Omp48 determined by peptide sequencing matched perfectly with the corresponding DNA-deduced amino acid sequence (Table 1 and Fig. 3). As expected for an outer membrane protein, a signal peptide sequence consisting of the first 25 N-terminal amino acids was detected (Fig. 3). This sequence possessed the characteristic features commonly associated with signal peptide sequence: N-terminus basic amino acid residues, central hydrophobic region and the Ala-X-Ala (Ala-Phe-Ala) cleavage leader peptidase-recognition site at positions 23–25 (von Heijne 1983). A putative sugar binding motif, YYQRHD, at positions 133–138 of Omp48 was also detected (Fig. 3).

The mature protein consisted of 401 amino acids, starting with Val26 as the N-terminal sequence previously indicated. The predicted molecular mass of 44 256 Da was lower than the molecular mass determined by SDS-PAGE (48 kDa). The mature Omp48 protein possessed some characteristics that are typical of other bacterial porins, including a theoretical acidic pI (4·62) and high glycine content (12·2%). The lack of cysteine residues in Omp48 and in maltose-inducible porin (LamB) of *Aer. salmonicida* (Dodsworth *et al.* 1993a) is a remarkable feature, differing from the highly related LamB porins of *E. coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae* and *Yersinia enterocolitica* (Lang and Ferenci 1995), which contain two cysteines forming a disulphide bridge in Loop 1 (Schirmer and Cowan 1993; Schirmer *et al.* 1995). As Dodsworth *et al.* (1993a) reported for maltoporin from *Aer. salmonicida*, two sequences recognized as putative ribosome binding sites (Shine-Dalgarno sequence) were localized 3 and 9 bp upstream from the first ATG codon (Met1) and 9 and 15 bp upstream from the second ATG codon (Met3) (Fig. 3).

Genome database search showed considerable similarities to other bacterial porins. The percentages of identities/similarities shared with other LamB-like proteins of various Gram-negative bacteria were: *Aer. salmonicida*, 57/67%; *Salm. typhimurium*, 40/51%; *Vibrio cholerae*, 41/54%; *E. coli*, 38/50%; *Kl. pneumoniae*, 39/52%; *Y. enterocolitica*, 40/52% and *V. parahaemolyticus*, 39/51%.

DISCUSSION

Here, we describe the molecular cloning of *omp48,* the gene encoding for Omp48 protein, whose involvement in

```
 -24 ctcctctcagcgggaataggaaaatgaaaatgaaagcaaagtggctcccgatcgctgca 36
                                M  K  M  K  A  K  W  L  P  I  A  A
  37 gcagtaaccgcagccctggcttcccaagccgccttcgccgttgatttccacggctacatg 96
      A  V  T  A  A  L  A  S  Q  A  A  F  A  V  D  F  H  G  Y  M
  97 cgttccggtgtcggtacttccgataacggcagcctgcaaacctattccaaggcgaaagtc 156
      R  S  G  V  G  T  S  D  N  G  S  L  Q  T  Y  S  K  A  K  V
 157 ggtcgtctgggcaatgaagccgagacctacggcgaagtccagctaggtcaagaggcttac 216
      G  R  L  G  N  E  A  E  T  Y  G  E  V  Q  L  G  Q  E  A  Y
 217 aacaaggacggcaagtctttctacgtagactccatgtttgcaatggtttctggtcagcaa 276
      N  K  D  G  K  S  F  Y  V  D  S  M  F  A  M  V  S  G  Q  Q
 277 ggtcgcgactgggaaagcactagcggtagtgatgccgactttgccctgcgccaattcaac 336
      G  R  D  W  E  S  T  S  G  S  D  A  D  F  A  L  R  Q  F  N
 337 gtccaggccaaaggcgtgctgggcttcgcgccggaagccactctgtgggccggtaagcgt 396
      V  Q  A  K  G  V  L  G  F  A  P  E  A  T  L  W  A  G  K  R
 397 tactatcaacgtcacgacatccacatctccgacttctactactgggatgtgtctggtgcc 456
      Y  Y  Q  R  H  D  I  H  I  S  D  F  Y  Y  W  D  V  S  G  A
 457 ggtgctggtgtagaaaatatccaggctggtccgggcaagctctcttttgcatggctgcgt 516
      G  A  G  V  E  N  I  Q  A  G  P  G  K  L  S  F  A  W  L  R
 517 aacgacccgtgggatgattacgatcagtttgtaggtggtgttggtgtcgacaagcgtgtt 576
      N  D  P  W  D  D  Y  D  Q  F  V  G  G  V  G  V  D  K  R  V
 577 gatacggacgtactggacctgcgttatgccggtatcccgctgtggaaagacggctccctg 636
      D  T  D  V  L  D  L  R  Y  A  G  I  P  L  W  K  D  G  S  L
 637 gagctgggatatgactacgcccgcggtaacctgaccgatgagcagaaagcggctctgggc 696
      E  L  G  Y  D  Y  A  R  G  N  L  T  D  E  Q  K  A  A  L  G
 697 agcaaagattacgagaaaaacggccacatgctgaccgccgagctgacccaatccatgctg 756
      S  K  D  Y  E  K  N  G  H  M  L  T  A  E  L  T  Q  S  M  L
 757 ggtggcttcaacaagaccgtggttcagtatttcgccgacggttatgccgctcaggctgtt 816
      G  G  F  N  K  T  V  V  Q  Y  F  A  D  G  Y  A  A  Q  A  V
 817 gactacggtagcggttctggcagcggcctgagccaatctgccgccaagggcgatggctgg 876
      D  Y  G  S  G  S  G  S  G  L  S  Q  S  A  A  K  G  D  G  W
 877 cgcttcatcaactggggcgtgctgcccgttggcgataaggtcgagttcggtcatcagctg 936
      R  F  I  N  W  G  V  L  P  V  G  D  K  V  E  F  G  H  Q  L
 937 gtttacggtactgccagtgatatcgacaatgacagcgctgacaaagacaccttctccgtt 996
      V  Y  G  T  A  S  D  I  D  N  D  S  A  D  K  D  T  F  S  V
 997 gtcgttcgcccgatgtacaagtggaatgacaccatgaagaccatcgccgagcttggctat 1056
      V  V  R  P  M  Y  K  W  N  D  T  M  K  T  I  A  E  L  G  Y
1057 caccatgacagctacaagccgaacaatggcaccgaaaccagcaccatcggtaagaaatac 1116
      H  H  D  S  Y  K  P  N  N  G  T  E  T  S  T  I  G  K  K  Y
1117 accctggctcaggcctggtccgctggctccagcttctgggcccgcccggaaatccgcgta 1176
      T  L  A  Q  A  W  S  A  G  S  S  F  W  A  R  P  E  I  R  V
1177 tatgcttcctacctggattctgacggccagttccgcccggatgccaatggcgtgaagcag 1236
      Y  A  S  Y  L  D  S  D  G  Q  F  R  P  D  A  N  G  V  K  Q
1237 gacgactcctggaacttcggtgttcaagcggaagcttggtggtaa
      D  D  S  W  N  F  G  V  Q  A  E  A  W  W  *
```

Fig. 3 Nucleotide and predicted amino acid sequences of *omp48* gene from *Aeromonas veronii*. The N-terminal (N-ter) and internal peptides (IP1-4) are in bold italic. Putative ribosome binding sites (RBS), leader peptide and sugar binding motif sequences are underlined. The stop codon is marked by an asterisk

adherence of *Aer. veronii* to epithelial cells of fish (Guzman-Murillo *et al.* 2000) and immunoprotection attributes were recently detected (Merino-Contreras *et al.* 2001).

No significant difference was observed when *Aer. veronii* cells were grown in the presence or absence of 1% maltose on LB medium (Fig. 1). It seems that high production of Omp48

was not dependent on the presence of maltose in the medium. Jeanteur *et al.* (1992) reported that protein I (maltose-inducible protein) was a major component of *Aer. hydrophila* cells grown in LB broth in the absence of maltose and that induction was observed only on Riddle's defined medium supplemented with maltose. Hence, maltose induction of Omp48 in *Aer. veronii* cells remains to be proven.

No positive colonies were found when genomic and sub-genomic libraries of *Aer. veronii* were screened with the MR-AV3 probe. The complexity of cloning porin genes was previously documented for several Gram-negative bacteria, including *Neisseria gonorrhoeae, Haemophilus infuenzae, Aer. salmonicida, V. cholerae, Eikenella corrodens, Pasteurella multocida* and *V. vulnificus* (Carbonetti and Sparling 1987; Hansen *et al.* 1988; Costello *et al.* 1996; Sperandio *et al.* 1996; Yumoto *et al.* 1996; Luo *et al.* 1997; Litwin and Byrne 1998). The constructed genomic and sub-genomic libraries apparently consisted of restriction fragments potentially long enough to contain the entire *omp48* gene. Omp48 was likely expressed from its own promoter in unregulated fashion and recombinant porin was toxic to *E. coli* cells. The high copy number of pBS-SK plasmid could enhance this lethal effect because of high *omp48* copy number (gene dosage effect), but positive clones were not obtained even when we used the low copy number plasmid pBR322. The lethality of cloned porins remains unexplained. It is believed that when porin genes are highly expressed, the leader peptide targets recombinant porin to the outer membrane causing osmotic destabilization of *E. coli* cells, either by displacement of native *E. coli* porins or by altering the structural integrity of the outer membrane (Carbonetti and Sparling 1987; Luo *et al.* 1997).

We turned to IPCR to overcome this inconvenience. IPCR allowed us to determine the upstream- and down-stream-flanking sequences of the previous MR-AV3 PCR fragment and eventually to clone the complete *omp48* gene, including sequence encoding for the signal peptide. IPCR is effective for particular genes that are hard to clone and for determination of neighbouring regions from known sequences (Ochman *et al.* 1988; Triglia *et al.* 1988; Huang *et al.* 2000).

The high degree of homology with LamB from *E. coli* and other related maltose-inducible porins suggests strongly that Omp48 is the corresponding LamB porin of *Aer. veronii,* although no maltose induction of Omp48 was found in the present study. Because of the importance of cysteine in the formation of disulphide bridges, it should be noted that Omp48 lacks these residues, which might suggest that the structure of Omp48 differs from that of LamB, at least at Loop 1 where cysteine residues form the bridge (Schirmer *et al.* 1995). The molecular mass predicted for mature Omp48 (44 256 Da without leader peptide) was lower than that determined by SDS-PAGE. A possible explanation is that porins are strongly associated with LPS, which are difficult to eliminate during purification. Therefore, associated LPS might be affecting electrophoretic migration of Omp48 on SDS-PAGE, resulting in indicated molecular mass higher than actual.

Omp48 was localized extracellulary (data not shown) and its presence in supernatants of exponentially growing *Aer. veronii* cultures is consistent with the fact that many Gram-negative bacterial cells growing in liquid media shed fragments of their outer membranes as vesicles (OMV) whose protein composition resembles that of OMP preparations (Hoekstra *et al.* 1976; Loeb and Kilner 1978; Gamazo and Moriyon 1987). These OMVs are believed to be formed and released during exponential growth, when the outer membrane of bacteria is synthesized faster than the peptidoglycan layer, resulting in the formation of surface blebs (Loeb and Kilner 1978). Extracellular localization of OMPs was also reported for *Aer. hydrophila* and *Aer. salmonicida* (MacIntyre *et al.* 1980). Electron microscopy of gastric biopsies reveals that *Helicobacter pylori* release OMV into gastric mucosa *in vivo* (Fiocca *et al.* 1999; Keenan *et al.* 2000), as they do *in vitro* (Cao *et al.* 1998; Fiocca *et al.* 1999). From the fact that VacA cytotoxin was found on *H. pylori* -OMV, the hypothesis arises that release of OMV might be a method for delivery of *H. pylori* virulence factors, apart from classical secretion pathways (Fiocca *et al.* 1999). This might also be the case for other Gram-negative bacterial pathogens including *Aer. veronii,* although this mechanism remains to be demonstrated.

The adhesive properties of porins that might play a role in interaction between bacteria and host in the colonization process were also reported (Sengupta *et al.* 1992; Sperandio *et al.* 1995; Achouak *et al.* 1998). Production of surface-associated adhesins seems to be a feature common to *Aermonas* species, as demonstrated by particle agglutination of latex beads coated with the glycoproteins collagen, fibronectin, laminin, lactoferrin, mucin, fibrinogen and transferrin (Ascencio *et al.* 1990, 1991, 1992, 1998). Quinn *et al.* (1993, 1994) stated that carbohydrate-reactive outer membrane proteins (CROMPs) of 40 and 43 kDa from *Aer. hydrophila* might be responsible for this agglutinin property. As agglutination can be inhibited specifically by sugars (Atkinson and Trust 1980), bacterial surface adhesins likely interact with the carbohydrate component of glycoproteins in a lectin-like fashion. Although the carbohydrate affinity of Omp48 from *Aer. veronii* (unpublished data) and CROMPs from *Aer. hydrophila* was determined (Quinn *et al.* 1993, 1994), the specific protein motifs for this binding remain to be identified. Whereas heparin-binding hemagglutinin from *Mycobacterium tuberculosis* contains Lys/Pro-rich repeated motifs, directly associated to interaction with sulphated carbohydrates (Menozzi *et al.* 1998) no repeated motifs were

localized in the deduced amino acid sequence of Omp48 from *Aer. veronii*. Omp48 contains the sequence YYQRHD instead, which is one of four conserved sequences that form the maltodextrin-binding sites in amylases (Vihinen and Mantsala 1989; Schneider *et al.* 1992). The sequence FYQRHD was established as part of the sugar-binding site in LamB from *E.coli* by X-ray crystallography (Dutzler *et al.* 1996). This sugar binding motif is located theoretically in the Loop 3, based on the secondary structure as predicted by the Kyte–Doolittle hydropathy plot (Kyte and Doolittle 1982), and compared by alignment to the structure of LamB (Schirmer and Cowan 1993; Schirmer *et al.* 1995). This motif might be involved in the lectin-like interactions of *Aer. veronii*.

As Omp48 has been associated with adherence properties of *Aer. veronii* (Guzman-Murillo *et al.* 2000), its cloning and sequencing will elucidate these properties through generation of specific mutants and testing of their ability to adhere to epithelial surfaces *in vivo* and *in vitro*. Immunoprotective attributes of Omp48 demonstrated in fish (Merino-Contreras *et al.* 2001) make the *omp48* gene a suitable model for testing the potential of bacterial porins in DNA vaccine generation, using aquacultured fish (Heppell and Davis 2000).

ACKNOWLEDGEMENTS

This work was supported by CIBNOR, project PAC11. R.C.V.J. is grateful to the Mexican Council of Science and Technology (CONACyT) for financial support (grant no. 121100). We thank Dr L.D. Possani (IBT, UNAM, Mexico) for N-terminal peptide sequencing and Dr J.M. Egly (IGBMC, France) for internal peptide sequencing. The help of the CIBNOR editing staff is also appreciated.

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