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Isolation and characterisation of the major outer membrane protein of *Erwinia carotovora*

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Abstract

The purified major outer membrane protein (37275 Da) from the psychrotrophic phytopathogen *Erwinia carotovora* MFCL0 was structurally characterised by MALDI-TOF mass spectrometry, N-terminal microsequencing and DNA sequence determinations, and secondary structure prediction analyses. The deduced amino acid sequence showed 76% and 72% of similarities with the *Serratia marcescens* and *Escherichia coli* OmpA proteins respectively. Dendrogram analysis allowed to point out that *E. carotovora* is close to the genus *Serratia*. After reconstitution into planar lipid bilayers, this major protein induced ion channels with a major conductance level of 630 pS in 1 M NaCl and a weak cationic selectivity. These functional and structural features allowed to identify this major outer membrane component of *E. carotovora* as an OmpA-like protein, i.e., a channel-forming protein which could be involved in the infection process of this phytopathogen agent.

Keywords: Porin; OmpA-like protein; Channel-forming property; DNA sequence; *Erwinia carotovora*

1. Introduction

Many bacteria possess the ability to produce tissue-macerating enzymes, but only a few have been associated with decay of living plant tissue. These bacteria include *Erwinia* spp., pectinolytic strains of *Pseudomonas* and apparently bacteria as *Ralstonia solanacearum*, *Burkolderia cepacia*, *Bacillus* spp., *Clostridium* spp. [1]. The soft-rot group *Erwinia* is the most important primary plant pathogen for which *E. chrysanthemi* and *E. carotovora* are the major members. These bacteria are responsible for important economic losses since they cause soft-rotting, wilting and dwarfing among other dysfunctions in range of plants [2]. Their pathogenicity is due to the secretion of spoilage enzymes occurring after their adhesion on the plant and which degrade the plant cell walls [1]. Other factors contribute to virulence in hosts plants and can be located on the bacterial surface such as pili, flagella or exopolysaccharides slime layers [2]. In other respects, in the outer membrane of Gram-negative bacteria, lipopolysaccharides (LPS) and porins are major toxic components [3,4]. Porins that are usually expressed at high levels, are channel-forming proteins that allow

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the passive diffusion of small molecules across the outer membrane [5]. They can be involved in the pathogenicity as it is the case for a *Pseudomonas aeruginosa* porin which is an apoptosis inductor of epithelial cells [6] or for the *Escherichia coli* major outer membrane protein OmpA, involved in the actin condensation of brain endothelial cells [7]. Porins could also play an important role during adhesion in the early steps of the infection process. Such a function has been already described for the major porin OprF of the psychrotrophic bacterium *Pseudomonas fluorescens* OE28.3 [8] and for the major porin of *Campylobacter jejuni* [9].

On the basis of these data, we report in this study the purification of the major outer membrane protein of the psychrotrophic phytopathogen *E. carotovora* strain MFCL0, its identification and characterisation at a functional and structural level.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Erwinia carotovora was extracted from celeriacs conserved at +1°C. Bacteria were grown in Nutrient Broth (NB, Difco) medium, used either as broth or solidified with agar (1.5% w/v), at 28°C under vigorous shaking.

2.2. Isolation of the major outer membrane protein

OM were isolated on discontinuous sucrose gradient as described by Hancock and Nikaido [10]. Briefly, after sonication (30 s, four passages at 100 W), membrane fractions were loaded onto a discontinuous sucrose gradient (50, 60 and 70%) and then centrifuged at $183\,000 \times g$ for 4 h. The OM were collected, suspended in water and stored at −80°C. Protein concentration was determined by Micro BCA kit (Pierce). The OM (150 μg) were analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (7–13% system) and stained by Coomassie brilliant blue G250. The major OM protein was then purified by preparative electrophoresis followed by electro-elution in SDS (0.05% SDS, 192 mM glycine, 25 mM Tris–HCl, Cerralabo system). Purity control was carried out by SDS–PAGE and silver staining [11].

2.3. Western immunoblotting and N-terminal sequence determination

After SDS–PAGE, purified protein was transferred to a nitrocellulose membrane (Hybond, 0.22 μm pore size, Amersham) as described by Towbin et al. [12] for immunodetection or to a polyvinylidene difluoride membrane (Millipore) for the N-terminal sequence analysis by automated Edman degradation (477A Protein Sequencer, Applied Biosystems). Polyclonal antibody directed against the *E. coli* OmpA protein was used for immunodetection [13].

2.4. Mass spectrometry MALDI-TOF (matrix-assisted-induced desorption and ionisation time of flight)

Mass spectra were obtained with a time of flight mass spectrometer (Voyager Elite XL, Perseptive Biosystems, Framingham, MA, USA). All spectra were acquired in the positive-ion mode and the acceleration voltage was set to 20 000 V. Aliquots of 0.5 μl of the protein solution and 0.5 μl of 2.5 dihydroxybenzoic acid dissolved in a 50% (v/v) of acetonitrile/aqueous 0.1% TFA solution were mixed on the stainless plate and dried prior to analysis. External calibration was performed with bovine serum albumin (m/z 66 431).

2.5. Reconstitution in planar lipid bilayers

Virtually solvent-free planar lipid bilayers were formed by the method of Montal and Mueller [14]. Briefly, a 1% diphytanoylphosphatidylcholine (DPhPC, Avanti, Birmingham, USA) solution in hexane, lipid bilayers were formed by the apposition of two monolayers on 125-μm-diameter hole in thin Teflon film (10 μm) sandwiched between two half glass cells and pre-treated with hexadecane/hexane (1:40, v/v). The electrolyte solution was 1 M NaCl, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2 ethanesulfonic acid (HEPES) (pH 7.4). The major outer membrane protein was diluted in 0.3% octyl-PolyOxyEthylen (octyl-POE) and added to the measurement

compartments in a symmetric manner at a final concentration of 10^{-9} M.

2.6. General DNA procedures

Agarose gel electrophoresis and general DNA procedures were carried out according to Sambrook and Maniatis [15].

The polymerase chain reaction (PCR) was done with 10 ng purified chromosome: after a hot start at 94°C for 5 min, 30 cycles including denaturation at 94° for 1 min, primer annealing at 50°C for 2 min and elongation at 72°C for 2 min were performed. PCR reaction was concluded with a final 10 min elongation step.

2.7. Sequence comparison and secondary structure prediction

The OmpA sequence was compared to protein sequences using the Fasta software at the www.infobiogen.fr site. The three most related OmpA sequences (*E. carotovora*, *S. marcescens* and *E. coli*) were aligned by ClustalW, converted in MSF format and submitted to the PredictProtein server at EMBL (http : // www.embl.heidelberg.de/predictprotein/phd_pred.html) for consensus secondary-structure prediction by the PHD method [16]. In a final step, these three sequences were aligned in MACAW with the amphipathic residue pattern, the predicted secondary structure and the location of conserved sequences as guidelines.

2.8. Nucleotide sequence accession number

The nucleotide sequence of *ompA* was deposited in the EMBL nucleotide sequence database with accession no. CAB57308.

3. Results and discussion

The *Erwinia* genus is related genetically to *enterobacteria* like *E. coli* and *Salmonella typhimurium* that have served as model systems for genetic and physiological studies [17]. *E. carotovora* MFCL0 is a psychrotrophic bacteria involved in soft-rot of plant species [18]. As the *E. coli* major outer membrane protein has been described as a virulence factor [7], and to investigate the early steps of the infection process of celeriacs by this phytopathogen, we have purified its major outer membrane protein which could be involved in the adhesion process.

3.1. Purification of the major outer membrane protein of E. carotovora

The OM proteins were isolated by discontinuous sucrose gradient procedure [10] and analysed by SDS–PAGE (Fig. 1A). The electrophoretic profile shows a major proteic band of apparent molecular mass of 31 kDa (lane 1) which seemed to migrate at 37 kDa after heating the sample at 100°C (lane 2). This 31 kDa protein was purified by preparative gel electrophoresis and electro-elution, then analysed by SDS–PAGE. Fig. 1B (lane 1) shows the two bands of 31 and 37 kDa already observed and which could correspond respectively to the native and denatured forms of the protein. After heating of the purified sample (Fig. 1B, lane 2), the 31 kDa band has completely disappeared to give the 37 kDa band. This behaviour is generally observed with monomeric porins like *E. coli* OmpA [19] and OprFs from *P. aeruginosa* and *P. fluorescens* [20,21] but also with monomers of trimeric porins like MOMP from *C. jejuni* [22].

MALDI-TOF spectrometry experiments performed on the 31 and 37 kDa bands gave similar molecular mass values of $(37\,280 \pm 300)$ Da and $(37\,260 \pm 300)$ Da, respectively (Fig. 1C). This result shows that the heat modifiable behaviour is probably due to conformation modifications of the 31 kDa protein like the lost of the β-barrel structure [22] or an incomplete folding [23]. This behaviour is only observed for the native protein and can be abolished when the C-terminal domain of monomeric porins is cleaved [24].

The Edman sequencing was carried out with the 31 kDa protein of *E. carotovora* MFCL0 and its N-terminal amino acid sequence was determined as APKDNTWYTGGKLGVSQFHDTG-FY. This sequence shows very strong identities with the OmpAs of *Serratia marcescens* (87%) [25] and *Enterobacter aerogenes* (87%) [26], indicating that this protein could be an OmpA like-protein. This hypothesis was confirmed in Western blotting experiments with

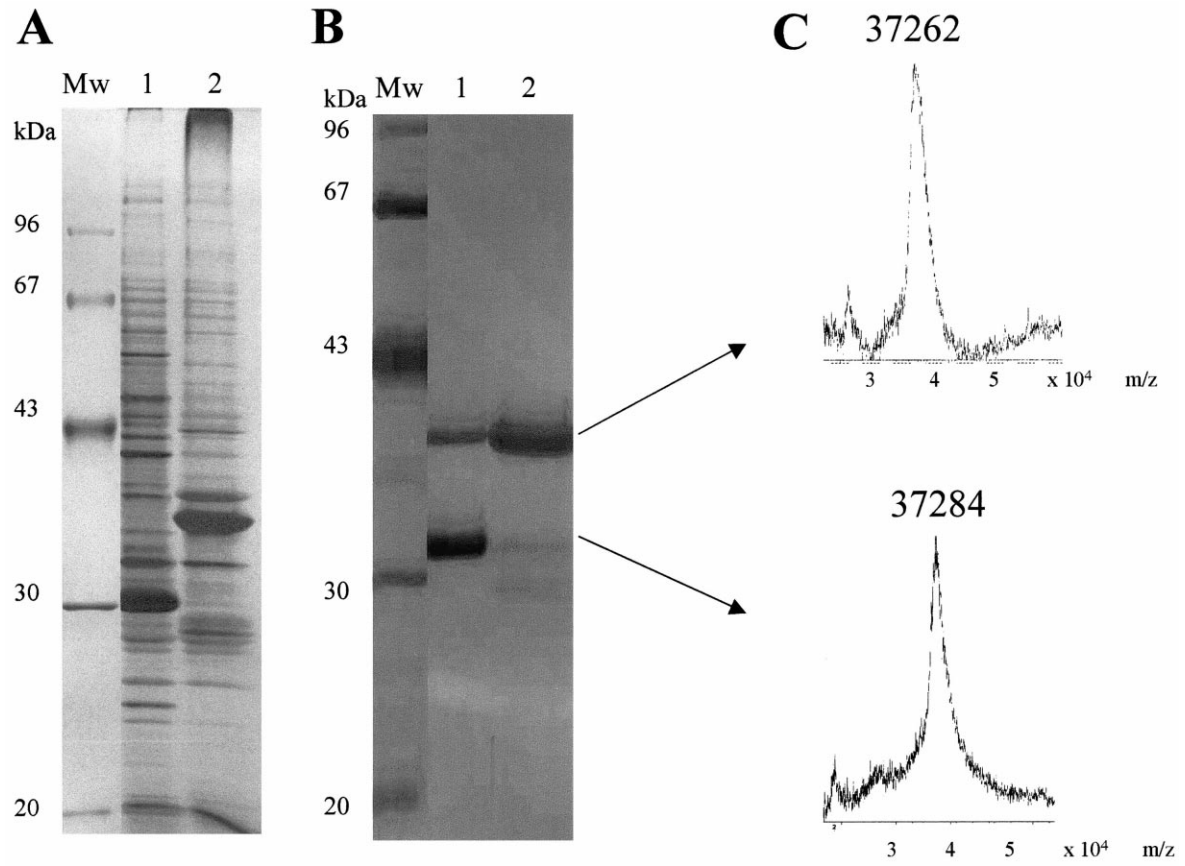

Fig. 1. (A) SDS–PAGE patterns of outer membrane (OMs) proteins of *E. carotovora*. Lane 1, OMs without heating; lane 2, OMs heated 3 min at 100°C. (B) SDS–PAGE (with silver staining) of the purified major outer membrane protein. Lane 1, purified protein without heating; lane 2, protein heated 3 min at 100°C. Gels were silver-stained. (C) MALDI-TOF spectra of purified proteins before or after heating.

an *E. coli* OmpA antiserum [13] that reacted positively with the 31 kDa protein and its heat modifiable form (data not shown).

3.2. Sequencing of the gene, deduced amino acid sequence and dendrogram analyses

In order to compare the extent of the major OM protein structural homologies with other porins, the *ompA* gene of *E. carotovora* was amplified by PCR and sequenced (genomexpress, France). Complete DNA sequence was obtained using in the PCR experiments, as primer 5′-terminal GGAAAAATCGGCGAGTAA (placed upstream the ATG codon) and 3′-terminal, CCTTTAGTACGGCAGTAG (placed downstream the stop codon). These short sequences were quite well conserved as revealed by the alignment of the *ompA* nucleotide sequences of the ten best OmpA protein alignment scores (data not shown). A single 1.3 kb fragment was obtained, cloned and sequenced on both strands. An open reading frame of 1101 bp was identified in the nucleotide sequence, composed of 52% of (G+C), encoding a protein of 367 amino acid residues (Fig. 2). A potential ribosomal binding site (AAGAGG) was located 12 bases upstream of the ATG translation initiation codon, consistent with the general feature of ribosome-binding sites [27]. A search for the promoter region yielded two regions of the sequence, reminiscent of the suggested −10 and −35 promoter region (Fig. 2). At a distance of 25 bp downstream the stop codon, a (G+C) rich stemloop structure followed by a run of four thymidine residues could serve as a potential transcriptional terminator, similar to the Rho-independent stemloop structures described for *E. coli* [28,29].

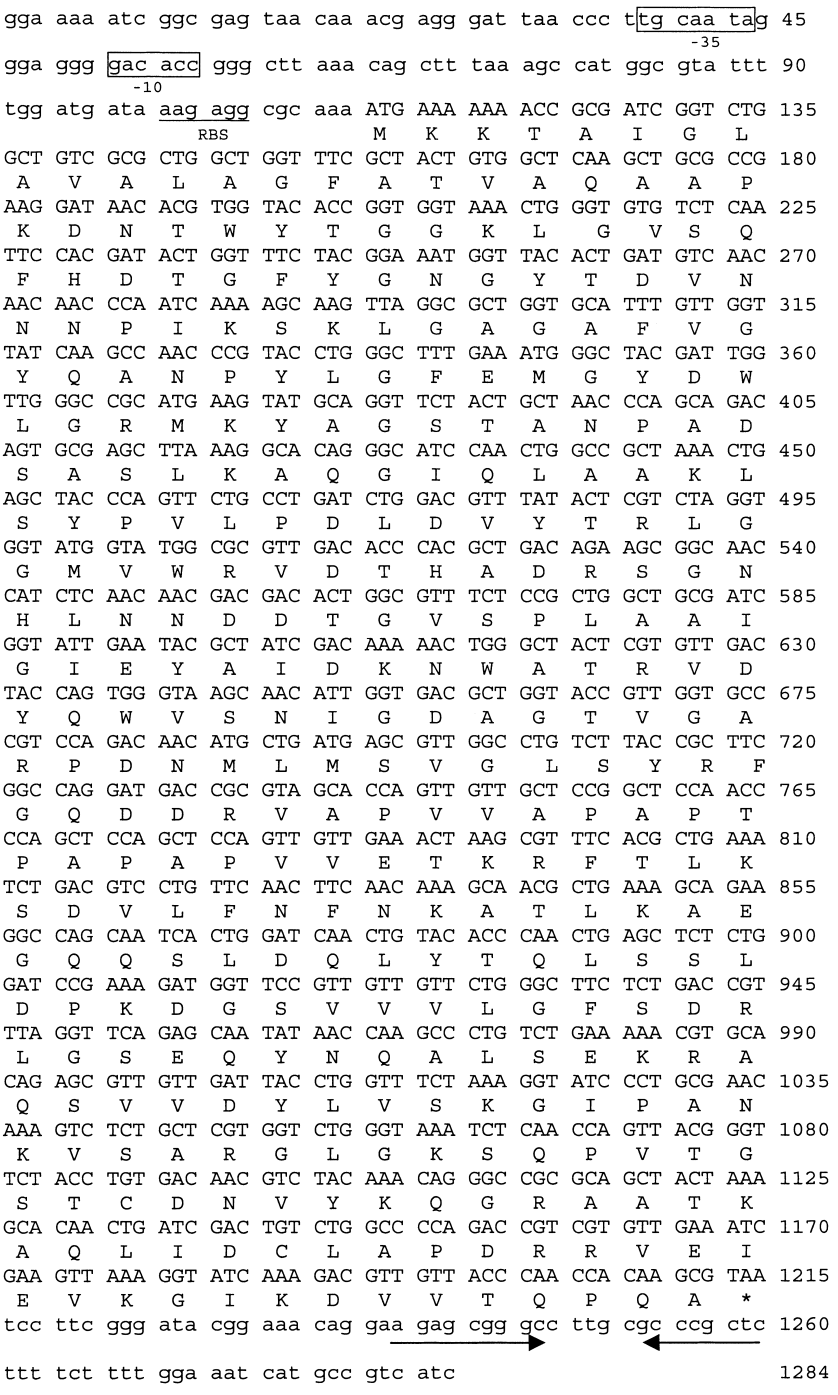

Fig. 2. Nucleotide and deduced amino-acids sequence of the *E. carotovora* OmpA protein. The putative −35 and −10 promoter are framed. Putative ribosomal binding site (RBS) is underlined. Transcription termination sites are represented with arrows.

We have then identified the related translated sequence in the current non redundant databases by using the softwares Blast, PsiBlast and sequence alignments have been performed using ClustalX (Fig. 3A). These searches yielded a large number of OmpA homologues, among them these from *Serratia marcescens* (76% similarity), *E. coli* (72%), *Shigella dysenteriae* (72%), *Salmonella typhimurium* (71%), *Klebsiella pneumoniae* (70%), *Enterobacter aerogenes* (70%), *Escherichia blatta* (69%), and *E. fergusoni*

A

E. carotovora	*S. dysenteriae*	*S. marcescens*	*S. thyphimurium*	*P. alcaligenes*	*K. pneumoniae*	*E. vulneris*	*E. fergusoni*	*E. coli*	*E. blattae*	*E. aerogenes*	*C. freundii*	*B. avium*	*E. hermanii*	% identity	Medline access number
100	72	76	71	19,1	70	47,7	68	72	69	70	47,7	23,2	49,9	*E. carotovora*	CAB57308
	100	76,2	91,7	22,8	81,7	60,2	68,7	96,6	59,3	82,8	62,2	24,1	49,4	*S. dysenteriae*	P02935
		100	75,3	20,9	75,6	50,1	50,4	77,1	51,2	75,9	50,3	23,4	61,8	*S. marcescens*	P04845
			100	22,2	80,1	61,6	63,2	92,6	59,7	82,2	63,2	24,6	48	*S. thyphimurium*	P02936
				100	21,7	25,2	25	23,4	24,4	22	25,4	27,2	25,2	*P. alcaligenes*	AAD40344
					100	61,4	61,3	82	60,5	84,3	60	23,8	52,2	*K. pneumoniae*	P24017
						100	87,3	59,9	83,6	58,1	88,5	31,6	69,5	*E. vulneris*	AAA24241
							100	66,4	86	57,2	90,6	28,5	72,5	*E. fergusoni*	P24747
								100	60,2	84,6	62,8	24,3	49,9	*E. coli*	P24754
									100	56,7	85,1	29,9	71,4	*E. blattae*	Q99124
										100	57,3	24,5	49,2	*E. aerogenes*	P09146
											100	29,2	69,4	*C. freundii*	P24016
												100	30,5	*B. avium*	Q05146
													100	*E. hermanii*	AAA24238

B

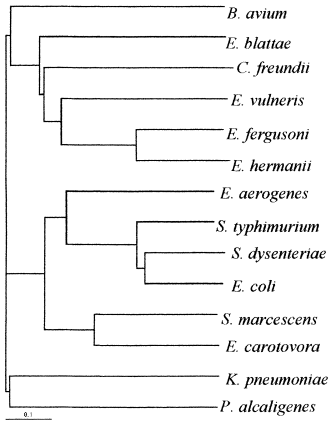

Fig. 3. (A) Matrix of the percentages of identical amino acids for 14 *Enterobacteriaceae* OmpA constructed by multiple alignment (ClustalX program) and (B) deduced dendrogram. Clustal is usually used in conjunction with NJplot, a simple program for tree reconstruction by the neighbour joining method. Medline identification numbers are given.

(68%). Sequence analyses of in vitro amplified genes encoding outer membrane proteins has already proven its value to pinpoint inter genius and inter-species differences, such as *Chlamydia* and *Neisseria* species [30,31]. Lawrence et al. [32] showed that such sequence data are quite useful for phylogenetic analyses of closely related bacteria such as enterics. Application of this approach to the *ompA* genes from several bacteria (Fig. 3A,B) revealed a close relationship of the phytopathogen *E. carotovora* MFCL0 to the *Serratia marcescens* genius, this sub cluster being related to another group including *Enterobacter aerogenes*, *Salmonella typhimurium*, *Shigella dysenteriae* and *E. coli* K12.

3.3. Structure of the OmpA-like protein

The 367-amino-acid sequence obtained by translation of the nucleotide sequence and the N-terminal sequence determined by microsequencing allowed us to find a 21-residue signal peptide. Thus, the OmpA-like protein possesses 346 residues and the calculated molecular mass is 37 278. This value is similar to the experimental one found by mass spectrometry (37 260 and 37 280). The cleavage site, localised between the amino acids Ala21 and Ala22 involves the action of a signal peptidase I for inducing the cleavage [33]. The presence of a signal peptide is consistent with the fact that this protein is exported across the cytoplasmic membrane.

OmpA amino acid sequences from *E. carotovora*, *E. coli* and *S. marcescens*, the most closely related sequences, were aligned in Fig. 4 using ClustalW, and secondary structure predictions were obtained with the PHD software [16] and compared with the structure determination of the OmpA N-terminal domain [23,34]. The OmpA structural family possesses several common features, in particular the presence of two domains, the N and C terminal parts [35]. The N-terminal domain, also called the membrane do-

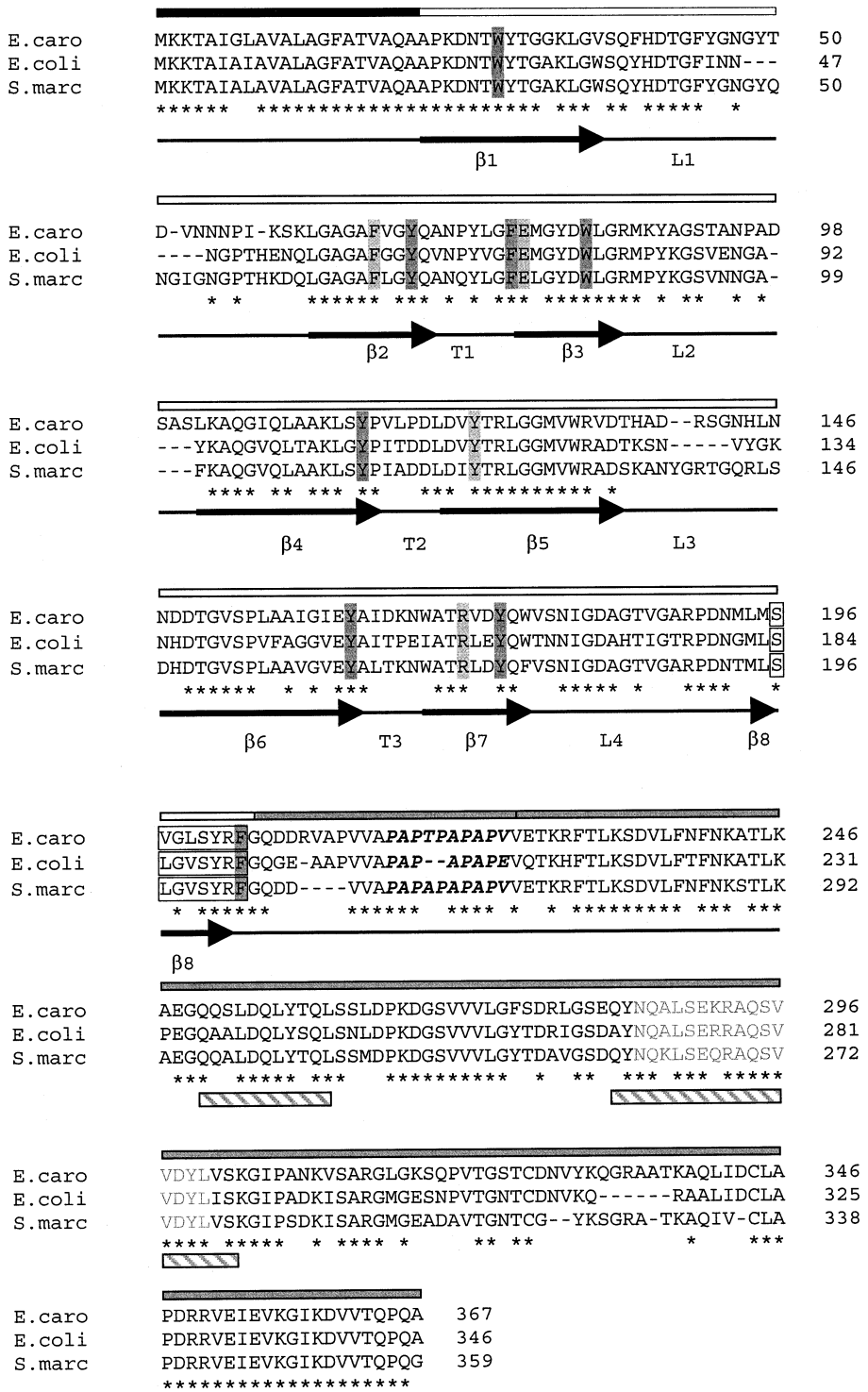

Fig. 4. Sequence alignment of the *E. carotovora* OmpA with two homologues: *Escherichia coli* (*E. coli*) and *Serratia marcescens* (*S. marc*). The three domains of the protein are indicated with a plain barrel: signal peptide is in black, the N-terminal region is in white and the C-terminal one is in grey. The β-strands are indicated by arrows. Aromatic amino acids flanking the β-sheets are shaded dark-grey. Conserved residues in the three sequences are indicated by an asterisk. Conserved residues in the major salt-bridge are shaded light-grey. The linker region is in italic. The region implicated in the protein folding is framed. The peptidoglycan binding site is indicated in grey letters. Hatched boxes represented the predicted α-helicals.

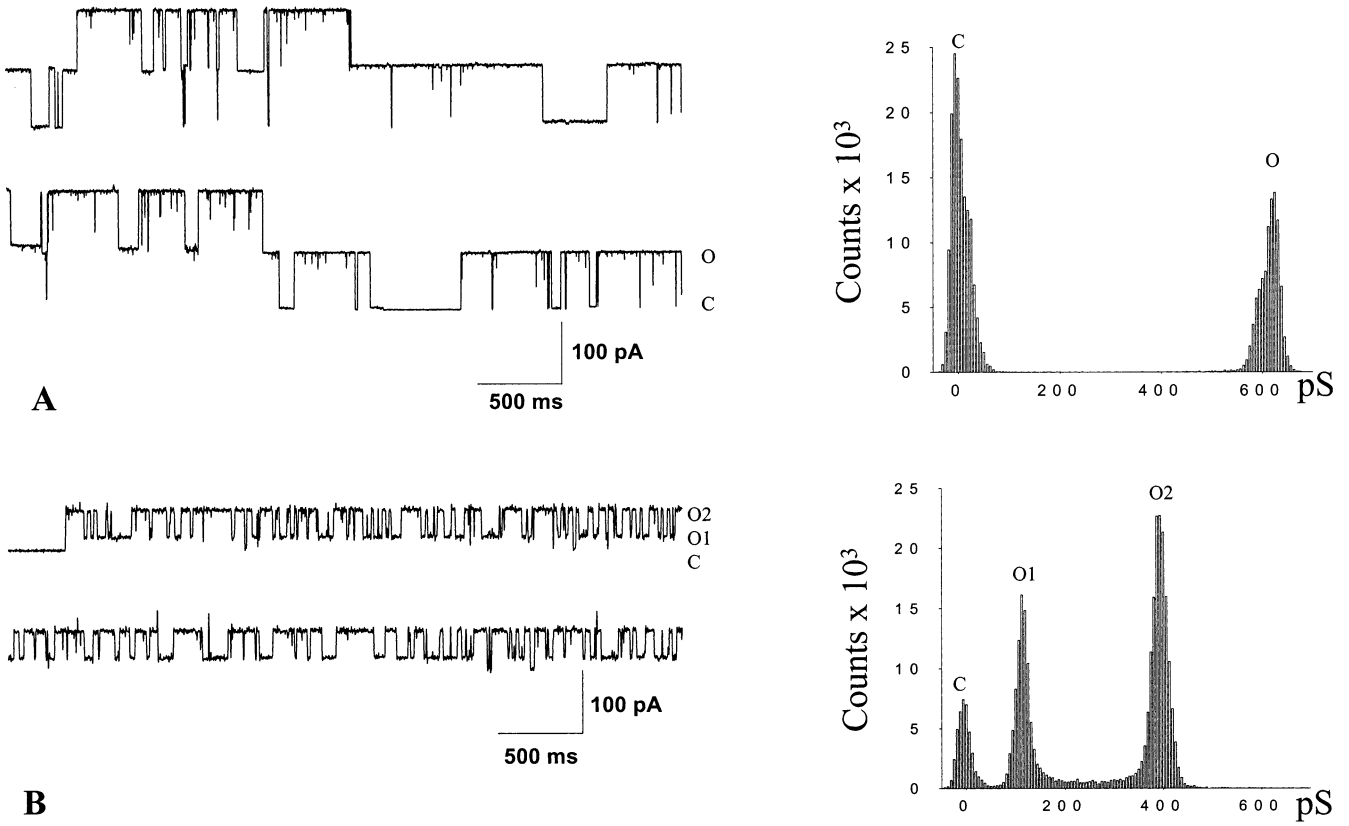

Fig. 5. Selected recordings of OmpA-like protein ion channels and associated amplitude histograms. OmpA-like protein was reincorporated into planar lipid bilayers formed with DPhPC. Recordings were performed in 1 M NaCl, 10 mM HEPES, pH 7.4. (A) Major channels of OmpA-like corresponding to (630 ± 10) pS at +150 mV and (B) minor channels of (113 ± 13) pS and (280 ± 16) pS at +160 mV. An associated histogram showing the current distribution is presented in each panel. 'C' and 'O' denote the closed and open states, respectively.

main, forms a regular eight-stranded β-barrel with long external loops and short connection turns located at its periplasmic side [23,34,36]. The β-sheets are flanked by aromatic residues pointing towards the membrane. They form belts of intermediate polarity interacting with the polar moiety of lipids (Fig. 4, shaded residues) and are characteristic of membrane porins [37,38]. The barrel interior may contain several salt bridges and water-filled cavities, in particular a very prominent barrier including four residues: Phe, Glu, Tyr, Arg which are conserved in the *E. carotovora* OmpA sequence (Fig. 4, hatched residues). In β_8, the sequence $_{196}$SVGLSYRF$_{203}$, corresponding to a consensus one, may be implicated in the protein folding and insertion into the OM as suggested by Stoorvogel et al. [39].

As usually observed [40], the major differences between the OmpAs structures from *E. coli* and *E. carotovora* reside in the loops (39% identity), in contrast to the β strands which are more conserved (83% identity). For *E. carotovora*, L1, L2 and L3 are larger (six, four and three extra amino acids, respectively). L3 undergoes the more drastic modifications since all amino acids are different from those present in the OmpA *E. coli* sequence. However, mutations on loops agree with their numerous functions like evading an immune system [41,42], conjugation [43,36] or bacteriophage recognition for the loop L3 of the *E. coli* OmpA [44,45].

Following the N-terminal domain, the linker region constituted of a proline-rich sequence (5 PX motif), forms a hinge, typical of the Gram-negative bacteria OmpAs [46]. The C-terminal domain of the OmpA is less well characterised. It has been proposed to either fold in eight transmembrane segments [47], or more commonly to be a total periplasmic

domain providing a binding site for the periplasmic peptidoglycan [44,48–50]. This binding site could correspond to an α helix with a consensus sequence

$(NX_2LSX_2RAX_2VX_3L)$ [50],

i.e., $_{285}$NQALSEKRAQSVVDYL$_{300}$

in the case of *E. carotovora* (Fig. 4).

3.4. Channel-forming properties

In order to characterise the channel-forming behaviour of the OmpA-like protein from *E. carotovora*, the purified protein was re-incorporated into planar lipid bilayers of DPhPC. At a protein concentration of 10^{-9} M in the electrolytic compartment, the OmpA-like porin induced fast fluctuations of current when a voltage of −150 mV is applied (Fig. 5A). The average conductance value was (630 ± 10) pS in 1 M NaCl buffered with 10 mM HEPES, pH 7.4 (associated amplitude histogram in Fig. 5A). Occasionally, we observed smaller current fluctuations corresponding to conductance values of (280 ± 16) pS and (113 ± 13) pS (Fig. 5B and associated amplitude histogram). These latter conductance values, though minor ones, are in good agreement with the *E. coli* OmpA ionophore properties already described: this protein may form small (50–80 pS) and large (260–320 pS) channels, the small ones being associated with the N-terminal transmembrane domain of the molecule when the large ones required both domains of the protein [51,52]. However, in the case of OmpA from *E. carotovora*, major conductance correspond to the 630 pS value with a step-like incorporation of the conducting unit. For OmpA from *E. coli*, same measurements (600 pS) were made by Saint et al. [53] with an identical reconstitution technique, lipids and electrolyte. One can notice also that another OmpA-like protein, OmpATb from *Mycobacterium tuberculosis*, exhibits similar stepwise behaviour and conductance value of 700 pS in 1 M NaCl [54]. This larger conductance value may result from the incorporation or opening of a rather stable oligomer, like a dimer of the OmpA protein.

Ionic selectivity experiments were performed after installation of a NaCl gradient (0.1 M:1 M, *cis:trans*). The resulting reversal potential allowed the estimation, from the application of the Goldman–Hodgkin–Katz equation [55], of a P_{Na}/P_{Cl} ratio 2.3, indicating a slight cationic selectivity of this porin.

In conclusion, the major outer membrane protein from *E. carotovora* shows channel forming properties that correlate well with those of the *E. coli* OmpA porin [51,53]. From these data and from the nucleotidic and deduced amino acids sequences, we can conclude that this protein belongs to the OmpA-like family. Widely speaking, this protein could be structurally related to the family of the eight stranded β-sheet proteins, as proposed by Baldermann et al. [24] from larger secondary structure prediction alignments. It is noteworthy that this family includes virulence proteins such as the *S. typhimurium* Rck and *Yersinia enterocolitica* Ail, involved in adhesion to and invasion of epithelial cells [56,57]. Moreover, *E. coli* OmpA protein, itself, would be implicated in the invasion of brain endothelial cells [42] and contributes to the pathogenicity of *E. coli* [41]. Several bacterial strains like *C. jejuni* or even so a psychrotrophic *P. fluorescens*, have been demonstrated to use major porins as adhesion agents [8,9]. In this context, the major outer membrane porin OmpA of *E. carotovora* could also contribute to the virulence of this phytopathogen strain.

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