



The *Shigella flexneri* OmpA amino acid residues $_{188}\text{EVQ}_{190}$ are essential for the interaction with the virulence factor PhoN2



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ABSTRACT

Shigella flexneri is an intracellular pathogen that deploys an arsenal of virulence factors promoting host cell invasion, intracellular multiplication and intra- and inter-cellular dissemination. We have previously reported that the interaction between apyrase (PhoN2), a periplasmic ATP-diphosphohydrolase, and the C-terminal domain of the outer membrane (OM) protein OmpA is likely required for proper IcsA exposition at the old bacterial pole and thus for full virulence expression of *Shigella flexneri* (Scribano et al., 2014). OmpA, that is the major OM protein of Gram-negative bacteria, is a multifaceted protein that plays many different roles both in the OM structural integrity and in the virulence of several pathogens. Here, by using yeast two-hybrid technology and by constructing an *in silico* 3D model of OmpA from *S. flexneri* 5a strain M90T, we observed that the OmpA residues $_{188}\text{EVQ}_{190}$ are likely essential for PhoN2-OmpA interaction. The $_{188}\text{EVQ}_{190}$ amino acids are located within a flexible region of the OmpA protein that could represent a scaffold for protein-protein interaction.

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1. Introduction

Proper protein localization and protein–protein interaction are central to all cellular processes. Therefore, the unraveling of these complex phenomena is of fundamental importance for many areas of biochemical and biomedical research. *Shigella flexneri* is a Gram-negative facultative intracellular pathogen that causes bacillary dysentery by invading the epithelial cells of the large intestine of human hosts [2]. *S. flexneri* harbors a virulence plasmid that encodes a type III secretion system (T3SS) and the majority of T3SS-secreted effectors are responsible for *S. flexneri* pathogenesis [3]. After cell invasion, *S. flexneri* spreads intra- and intercellularly through the expression of the virulence factor IcsA. This protein is localized and exposed at the old bacterial pole and, by interacting with the eukaryotic proteins vinculin and neuronal Wiskott-Aldrich syndrome protein (N-WASP), induces actin polymerization with the formation of typical long F-actin comet tails [4].

Thus, proper protein localization and protein–protein interactions are central to the expression of *S. flexneri* virulence. In this respect, we have previously shown that *phoN2*, a gene located on a

highly conserved region of the virulence plasmid of *S. flexneri*, encoding a periplasmic ATP-diphosphohydrolase (apyrase), participates, at least indirectly, to proper localization of IcsA at the bacterial surface [1,5]. By generating *phoN2*:HA fusions in the wild-type *S. flexneri* 5a strain M90T, we have shown that periplasmic PhoN2 strictly localizes at the bacterial poles, with a strong preference for the old pole, just beneath IcsA [1]. To evaluate whether PhoN2 and IcsA may interact for the correct exposition of IcsA, we conducted two-hybrid assays in yeast and cross-linking experiments. Both experimental approaches failed to find evidences supporting the existence of a PhoN2-IcsA interaction. Surprisingly, a strong interaction of PhoN2 with the OM protein A (OmpA) was found. Sequence analysis indicated that OmpA binds PhoN2 through its periplasmic-exposed C-terminal domain (minimal interaction region, amino acid residues 166–250) [1]. OmpA is the major OM protein of Gram-negative bacteria principally involved in the structural integrity of the OM [6]. Moreover, it has been recently shown that OmpA represents an important virulence factor for several pathogens, including *S. flexneri* [7,8]. By using independent ΔompA mutants of the *S. flexneri* strain M90T we have shown that all invariably displayed IcsA exposed across the entire bacterial surface and that a functional OmpA protein is required and sufficient for proper IcsA exposition, plaque and protrusion formation [7]. Furthermore, we

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Fig. 1. Schematic representation of the OmpA regions of *S. flexneri* strain M90T selected for the first two-hybrid screen against the bait plasmid pGBKT7/*phoN2*. DNA fragments encoding the different OmpA domains were PCR-amplified and cloned individually into the polylinker site of the prey plasmid pGADT7. OmpA domains: residues 1–325, entire amino acids sequence of mature OmpA protein; residues 1–131, OmpA N-terminal region embedded into the OM, selected as negative control; residues 191–299, OmpA C-terminal region exposed into the periplasm, it includes part of the minimal interaction region previously identified (residues 166–250); residues 1–299, N-terminal and part of the C-terminal OmpA region and the linker sequence (residues 173–190). Residues 132–190, minimal interaction region identified in this study.

hypothesized that the PhoN2-OmpA interaction may be necessary for proper IcsA exposition and thus for full virulence expression of *S. flexneri* [1].

Here to further characterize OmpA domains involved in the interaction with PhoN2 two two-hybrid screens in yeast were conducted. In the first screen we used four different PCR-generated *ompA* fragments. This screen allowed us to identify a new minimal region, encompassing amino acids 132–190, required for PhoN2-OmpA interaction. Furthermore, basing on the available sequences and resolved structures of OmpA of *Escherichia coli*, *Klebsiella pneumoniae* and *Salmonella enterica*, a 3D model of OmpA from *S. flexneri* strain M90T was built using the SWISS model workspace. The second two hybrid screen was conducted using this structural model in order to generate twelve independent *ompA* encoded peptides (from residue 136–325) in turn devoid of selected secondary structure elements. Our results indicated that the OmpA residues 188EVQ₁₉₀ are likely essential for PhoN2-OmpA interaction.

2. Materials and methods

2.1. Bacterial/yeast strains, plasmids and growth conditions

Bacterial/yeast strains and plasmids used in this study are listed in Table S1. The yeast *Saccharomyces cerevisiae* strain AH109 (Clontech) was used for two-hybrid assays as recommended by the manufacturer. *E. coli* and *S. flexneri* strains were grown at 37 °C either in Luria broth (LB) with aeration or on tryptic soy agar plates (TSA) containing 0.01% Congo red (Sigma), unless indicated otherwise. Antibiotics (Sigma), when appropriate, were added at the following concentrations: 100 µg ml⁻¹ ampicillin (Ap); 30 µg ml⁻¹ kanamycin (Km); 100 µg ml⁻¹ streptomycin (Sm).

2.2. DNA manipulations

DNA extraction, isolation of plasmids, restriction digestion, electrophoresis, purification of DNA fragments, construction of recombinant plasmids, transformation, and immunoblotting were performed by standard methods [9]. The primers used for PCR amplifications are listed in Table S2. Primers were designed to amplify *ompA* fragments based on the available *S. flexneri* genome sequence (Accession number CM001474). DNA sequence data were compared to known nucleotide sequences using the BLAST Server (National Centre for Biotechnology Information, Bethesda, Md).

2.3. Preparation of total protein extracts

Total protein extracts were prepared from yeast cells grown in

minimal medium ($OD_{600}=0.4\text{--}0.6$). Briefly, 20 ml of yeast cell cultures were centrifuged at 3,000 × g for 20 min at 4 °C and resuspended in 1 ml of a solution containing 2 M NaOH and 5% β-mercaptoethanol (lysis buffer). Proteins were concentrated by TCA (50%) precipitation. Precipitated proteins were washed with 90% acetone, centrifuged and resuspended in 2x Laemmli buffer (100 mM Tris-HCl pH 6.8, 4% SDS, 20 glycerol % and 5% β-mercaptoethanol) and stored at –20 °C. Equal amounts of proteins were separated on 12.5% SDS-PAGE, transferred to PVDF membranes (Hybond-P, Millipore) and analyzed by immunoblotting. A protein molecular weight marker (Pierce) was included in each electrophoresis run in order to determine the molecular weight of the proteins. Immunoblotting was carried out with mouse monoclonal anti-HA (Sigma) antibody. Anti-mouse antibody were used as secondary antibody and the signals were detected with enhanced chemiluminescence (GE Healthcare).

2.4. Yeast two-hybrid assay

OmpA domains interacting with PhoN2 were identified using the two-hybrid technique in yeast (Matchmaker TwoHybrid System Protocols; Clontech). The bait recombinant plasmid pGBKT/*phoN2* (full length) was constructed as previously described [1]. Four different *ompA* regions were PCR-amplified using M90T total DNA as template and the primers listed in Table S2. Each fragment was cloned into the polyclonal multiple cloning site of prey plasmid pGADT7 (Table S1 and Fig. 1). Each prey plasmid was transformed independently into the yeast strain AH109 carrying the bait plasmid pGBKT7/*phoN2* and plated on synthetic drop-out (SD) medium lacking Leu and Trp (SD/-Leu-Trp). The bait-prey interaction was detected by plating 250 well isolated colonies, obtained from each transformation, on solid SD medium lacking His, Ade, Leu, and Trp (SD/-His-Ade-Leu-Trp). The ability of the four different OmpA regions fused to the GAL4 AD domain to activate the reporter genes expression (the so-called self-activation) was tested. Each AH109 strain carrying one of the four prey plasmids was plated on solid SD/-Leu, SD/-His-Leu and SD/-Ade-Leu media. AH109 transformed with the empty vector pGADT7 was used as negative control. The toxic effect of the four different encoded OmpA regions was evaluated by comparing the growth rate of AH109 carrying the different prey plasmids. The AH109 strain carrying the void plasmid pGADT7 was used as control. Strains were grown in SD minimal broth medium without Leu for 18 h. Inserts were considered non-toxic if the yeast strains reached an $OD_{600}\geq 0.8$ after the incubation time. To determine the expression levels of the four encoded prey proteins, yeast cells were grown in SD minimal broth medium without Leu ($OD_{600}=0.4\text{--}0.6$) and used for total protein extraction preparations. Equal amount of whole cell extracts were subjected to Western blot analysis using the

monoclonal anti-HA antibody (Sigma).

For the identification of the OmpA amino acid residues involved in the interaction with PhoN2, twelve different forward primers were used in combination with a single reverse primer to generate twelve independent *ompA* amplicons (Table S2). Then, amplicons were purified from 1% agarose gel electrophoresis. Each amplicon was flanked by the specific sequences for homologous recombination with the *Sma*I linearized pGADT7 prey plasmid. AH109 pGBKT7/*phoN2* was transformed with a mixture of pooled amplicons, the linearized pGADT7 plasmid and plated as described above. DNA plasmid preparation from selected positive colonies was used to transform *E. coli* DH10b competent cells in order to determine the insert sequence.

2.5. Homology modeling of OmpA from *S. flexneri* strain M90T

Secondary structure of OmpA (GenBank EID61499.1) from *S. flexneri* strain M90T was built by using SWISS-MODEL workspace, applying default parameters [<http://swissmodel.expasy.org>; 10]. The OmpA sequence of *S. flexneri* strain M90T was compared via BLAST against non-redundant protein sequences database to determine the protein type. BLAST search was performed again against Protein Data Bank (PDB) to search suitable templates for the prediction of OmpA secondary structures. A total of 7 templates (PDB id: 2k0l [11], 1qjp [12], 2ge4 [13], 1g90 [14], 1bxv [15], 2jmm [16] and the deposited structure PDB id: 4erh; Table 1 and Fig. 2) were used to build the secondary structure.

3. Results

We have recently shown that the OM protein OmpA of the *S. flexneri* strain M90T is required for proper virulence expression and that the interaction with the virulence-associated periplasmic protein PhoN2 is likely required for proper IcsA exposition at bacterial surface [1]. A minimal DNA region of *ompA*, encoding residues 166–250, was identified to be involved in PhoN2-OmpA interaction. Residues 166–250 represent part of the OmpA C-terminal domain known to be exposed into the bacterial periplasm, the same bacterial compartment which hosts mature PhoN2 [1].

3.1. Identification of the OmpA region interacting with PhoN2

Here, to confirm and extend our previous results in order to precisely identify the OmpA region interacting with PhoN2, a first yeast two-hybrid screen was conducted by using four different PCR-generated *ompA* fragments encoding: i) the entire mature OmpA protein (residues 1–325) as positive control; ii) the OmpA N-terminal region known to be embedded into the OM (residues 1–131), as negative control; iii) the OmpA C-terminal region

exposed into the periplasm (residues 191–299), that includes part of the minimal region of interaction previously identified (residues 166–250); and iv) the OmpA region that includes the N- and part of the C-terminal OmpA domain (residues 1–299) and the linker sequence (residues 173–190) (Fig. 1). The four *ompA* DNA fragments were cloned individually into the pGADT7 prey vector thus generating prey plasmids pOA1–325, pOA1–131, pOA1–299, and pOA191–299, respectively (Table S1 and Fig. 1). Prey plasmids were then transformed separately into the *S. cerevisiae* strain AH109 harboring the bait plasmid pGBKT7/*phoN2* (Table S1). The ability of the four different OmpA derived domains to activate the reporter genes (self-activation), the toxicity, and their expression levels were evaluated. No evidence of self-activation, differences in the expression of the four different *ompA* fragments or toxicity was detected (Figs. S1, S2, and data not shown, respectively).

Then, the interaction between PhoN2 and the four different prey plasmids was evaluated by plating transformed yeast cells on minimal SD-His-Ade-Leu-Trp plates. As expected, AH109 pGBKT7/*phoN2* transformed with pOA1–325 (full length) was able to grow on SD-His-Ade-Leu-Trp medium, confirming the PhoN2-OmpA interaction. Conversely, AH109 pGBKT7/*phoN2* strain transformed with pOA1–131 did not grow on minimal medium. Plasmid pOA1–131 was selected as negative control since it encodes for the N-terminal β-barrel embedded into the OM (Fig. 1). Surprisingly, AH109 pGBKT7/*phoN2* transformed with pOA191–299 did not grow on minimal medium. The OmpA191–299 region was selected since it forms the C-terminal periplasmic OmpA domain and includes part of the minimal interaction region previously identified (residues 166–250) [1]. On the other hand, the OmpA1–299 fragment, containing the N-terminal, part of the C-terminal OmpA domains and the linker sequence, grew on SD-His-Ade-Leu-Trp- medium minimal medium, indicating that amino acid residues 132–190 are necessary for PhoN2-OmpA interaction (Fig. 1).

3.2. Secondary structure model of OmpA of *S. flexneri* strain M90T

To search for the OmpA critical amino acids involved in the PhoN2-OmpA interaction, a secondary structure prediction of OmpA of *S. flexneri* strain M90T was built using the SWISS-MODEL workspace. The sequence of the OmpA protein of M90T (accession number EID61499.1) was then blasted to search the database for suitable templates among Gram-negative bacteria containing OmpA homologues whose structure has been resolved (Fig. 2A and C). Among these, a total of 6 templates presenting 82–92% sequence identity (PDB id: 2k0l, 1qjp, 2ge4, 1g90, 1bxv, and 2jmm) were selected to model the N-terminal structure of OmpA of M90T (Table 1). OmpA of *S. enterica* (PDB id: 4erh), which presented 95% sequence identity with the OmpA C-terminal 152 residues of *S. flexneri* strain M90T, was selected as template for predicting the C-terminal structure (Table 1).

The structural model indicated that OmpA of M90T is a double domain protein presenting an N-terminal β-barrel domain (residues 1–172) composed by 8 β-strands connected by 4 extracellular and 3 periplasmic loops (Fig. 2A and B) while the C-terminal region is a globular domain (residues 191–325) which consists of 4 β-sheet, 5 α-helices and 6 connecting loops (Fig. 2C and D). These two domains are connected by the short linker sequence with no resolved structure (residues 173–190).

3.3. Identification of OmpA critical amino acids involved in the interaction with PhoN2

Basing on the structural model of OmpA of *S. flexneri* strain M90T, a set of twelve DNA fragments of different size were PCR-generated from the C-terminal domain of *ompA* (from encoded

Table 1

Templates from BLAST against PDB with OmpA from *S. flexneri* strain M90T.

PDB identification	Organism	Sequence/identity similarity %	Coverage (%)
2k0l	<i>Klebsiella pneumoniae</i>	82.80	57
1qjp	<i>Escherichia coli</i>	93.49	52
2ge4	<i>Escherichia coli</i>	92.40	53
1g90	<i>Escherichia coli</i>	92.40	53
1bxv	<i>Escherichia coli</i>	92.40	53
2jmm	<i>Escherichia coli</i>	84.83	45
4erh	<i>Salmonella enterica</i>	95.07	42

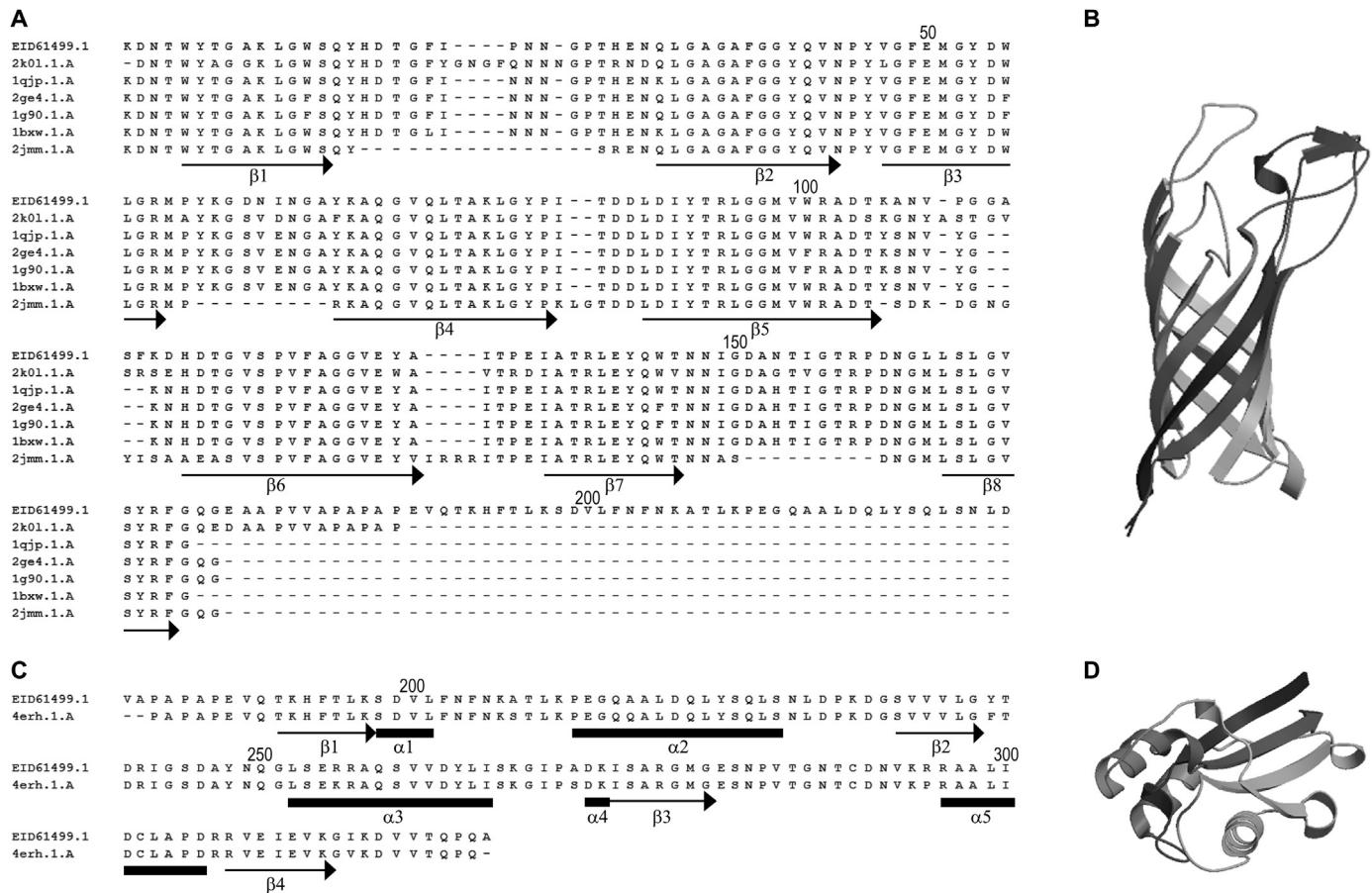


Fig. 2. Sequence alignment of OmpA homologues, and the predicted structure of OmpA from *S. flexneri* strain M90T. A) amino acid sequence alignment of the N-terminal OmpA domain (accession number EID61499.1) with the selected OmpA templates (PDB id: 2k0l, 1qjp, 2ge4, 1g90, 1bxw, 2jmm); B) structural model of the N-terminal OmpA domain based on PDB id 2k0l protein; C) amino acid sequence alignment of the C-terminal OmpA domain with the selected PDB id 4erh protein; D) Structural model of the C-terminal OmpA domain based on PDB id 4erh protein. Secondary structure elements are indicated under the alignment (arrows correspond to β -strands and black boxes correspond to α -helices). Residues numbers are for the OmpA of *S. flexneri* strain M90T.

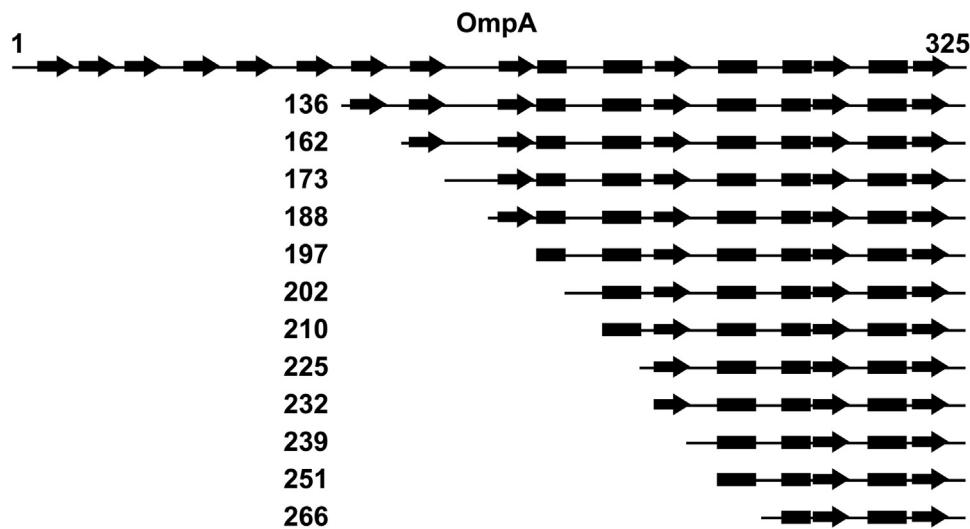


Fig. 3. Schematic representation of the twelve OmpA fragments selected for the two-hybrid screen. Representation of the full length OmpA of *S. flexneri* strain M90T (residues 1-325) and the twelve OmpA fragments (starting from residue 136). The position of the secondary structure elements is shown as in Fig. 2.

residue 136–325) (Fig. 3 and Table S2). The twelve *ompA* encoded peptides were challenged for their ability to interact with PhoN2 by the two-hybrid assay. Each fragment was generated in order to exclude in turn secondary structure elements, namely β -strands, α -helices or loops (Fig. 3). Competent cells of *S. cerevisiae* strain AH109 carrying the bait plasmid pGBT7/phoN2 were

simultaneously transformed with the pooled DNA fragments and the *Sma*I linearized pGADT7 prey plasmid. The interactions between PhoN2 and the different OmpA peptides was screened by plating the transformation mixture on SD-His-Ade-Leu-Trp minimal medium. Positive colonies were streaked again on the same medium and the prey plasmids DNA was extracted from

positive colonies, and used to transform *E. coli* DH10b competent cells to determine the inserts sequence. Each sequence was identified by BLAST analysis. Remarkably, the great majority (79%) of peptides encoded by the prey plasmids corresponded to the OmpA residues 188–325. A low number of sequenced inserts (21%) corresponded to the 136–325, 162–325 and 173–325 OmpA peptides. The OmpA_{188–325} amino acid sequence contains part of the minimal region (residues 166–250) that we have previously identified as responsible of the PhoN2-OmpA interaction [1].

Comparing the results obtained, we evidenced an amino acid stretch, ₁₈₈EVQ₁₉₀, which is included in the OmpA_{188–325} interacting peptide and absent in the OmpA_{191–299} domain. Since we did not observe interaction between the OmpA_{191–299} domain and PhoN2 we concluded that the amino acid stretch ₁₈₈EVQ₁₉₀ is required for PhoN2-OmpA interaction.

We hypothesized that the low efficiency of interaction detected for the 136–325, 162–325 and 173–325 peptides could be due to the characteristic of the fused peptides. Indeed, it is known that the GAL4 AD domain of the prey plasmid and the prey protein are separated by a linker region that is critical to ensure proper flexibility of the fused protein in order to facilitate protein-protein interaction [17]. Thus it might be possible to envisage that the amino acids ₁₈₈EVQ₁₉₀ directly fused to the GAL4 AD linker are accessible for the binding to PhoN2.

4. Discussion

OmpA is a 35 kDa protein embedded in the bacterial OM as a β-barrel protein, highly conserved among Gram-negative bacteria [18,19]. Recent studies have suggested that OmpA has to be considered a pleiotropic protein that plays different roles in the mechanism of virulence of different bacterial pathogens [8]. Furthermore, OmpA of *S. flexneri* 2a has been identified as a novel molecule coordinating the innate and adaptive immune response, indicating that OmpA may also represent a promising antigen for vaccine development [20,21]. In spite of its relevance in the immune response the secondary structure of OmpA of *S. flexneri* has not been determined yet and, to date, few data are available on the role of OmpA on the virulence of this bacterial pathogen [7]. In this context, we have recently shown that the virulence-associated periplasmic protein PhoN2 interacts with the C-terminal domain of OmpA of *S. flexneri* strain M90T and that null-mutants of either gene influence bacterial virulence by altering proper IcsA exposition at the bacterial surface [1,7]. To further characterize the OmpA domains essential for PhoN2-OmpA interaction, we built a secondary structural model of OmpA of *S. flexneri* strain M90T by using the SWISS-MODEL workspace (Fig. 2B and D). By *in silico* analysis we compared the secondary structure of several OmpA proteins (Fig. 2A and C). The overall architecture of the built model shows that *S. flexneri* OmpA is a double-domain protein where the C-terminal globular domain (the OmpA-like domain) represents a conserved structural motif in several OmpA-related proteins [22–24]. It has been reported that the OmpA-like domain interacts with the peptidoglycan through two conserved amino acid residues that bind diaminopimelate acid [25] and that is essential for dimer formation [24,26]. Although, the biological function of dimerization is not well understood yet, it has been proposed that it could be involved in OmpA-mediated bacterial virulence. It was also shown that the periplasmic domain of OmpA might increase folding efficiency functioning as a chaperone-like structure, important for proper OmpA insertion into the OM [27].

We have previously shown that the C-terminal domain of OmpA of *S. flexneri* strain M90T interacts with the PhoN2 protein (OmpA minimal interaction region residues 166–250; [1]). To confirm and extend our previous results, we performed

two-hybrid experiments using prey plasmids pOA1–325, pOA1–131, pOA191–299 and pOA1–299 (Table S1 and Fig. 1) and the bait plasmid pGBT7/phoN2 (Table S1) [1] (Fig. 1). As expected, the results of the two-hybrid assay confirmed that the full length OmpA (plasmid pOA1–325), interacted with PhoN2, while the OmpA N-terminal region that forms the β-barrel, (plasmid pOA1–131) did not (Fig. 1). Interestingly, the C-terminal OmpA region, (plasmid pOA191–299), that forms part of the periplasmic domain of the protein, did not interact with PhoN2, while the 1–299 fragment, (plasmid pOA1–299), that includes both the N- and part of the C-terminal domains of OmpA and a short flexible linker sequence, interacted with PhoN2. Overall, these results indicated residues 132–190 as the minimal region essential for the interaction with PhoN2 (Fig. 1). To refine our analysis we used the predicted secondary structure of OmpA of *S. flexneri* to generate a set of OmpA fragments. As outlined above, the overall architecture of the built model suggests that OmpA of *S. flexneri* strain M90T is organized into two domains. In particular, the N-terminal beta-barrel domain (residues 1–172) is composed of 8 anti-parallel beta strands (Fig. 2B), while the C-terminal OmpA-like globular domain (residues 191–325) is composed of 4 beta sheets, 5 alpha helices and 6 connecting loops (Fig. 2D). The N- and the C-terminal domains of OmpA are connected by a short region (residues 173–190) with unresolved structure that forms a flexible linker [28]. Basing on our tridimensional model, a set of twelve *ompA* fragments were PCR-generated (Fig. 3). Thus, each fragment was designed to exclude in turn selected secondary structure elements encompassing the C-terminal domain of OmpA (from residue 136–325; Fig. 3). The interaction between PhoN2 and the different *ompA* fragments was assayed using the two-hybrid assay. In this second screening, the *ompA* encoded 188–325 peptide was detected as a major PhoN2 interactor. Comparing the results obtained by the two screening, we evidenced a specific amino acid sequence, ₁₈₈EVQ₁₉₀, that is present in the PhoN2 interaction-positive 188–325 peptide and absent in the interaction-negative 191–299 peptide (Figs. 1 and 3). These results led us to conclude that the three amino acids ₁₈₈EVQ₁₉₀ are likely essential for the PhoN2-OmpA interaction. The ₁₈₈EVQ₁₉₀ amino acids are located within the linker flexible region of OmpA, that could represent a scaffold for protein-protein interaction.

In conclusion our results extend our previous results on the PhoN2-OmpA interaction [1]. Experiments are underway to introduce point mutations into the OmpA ₁₈₈EVQ₁₉₀ stretch in order to precisely establish the role of the three amino acids in the PhoN2-OmpA interaction.

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Transparency document Supporting information

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Appendix A. Supplementary material

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