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Biochemical and functional characterization of the periplasmic domain of the outer membrane protein A from enterohemorrhagic *Escherichia coli*

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ABSTRACT

Outer membrane protein A (OmpA) plays multiple roles in the physiology and pathogenesis of the zoonotic pathogen enterohemorrhagic *Escherichia coli* (EHEC). The N-terminus of OmpA forms a transmembrane domain (OmpA^{TM}), and the roles of this domain in bacterial pathogenesis have been well studied. However, how its C-terminal domain (OmpAper), which is located at the periplasmic space in the bacterial membrane, contributes to virulence remains unclear. Herein, we report that OmpAper forms a dimer and binds to peptidoglycan *in vitro*. Furthermore, OmpAper is responsible for bacterial resistance to acidic conditions, high osmotic pressure and high SDS environments. In addition, OmpAper contributes to the adhesion of bacteria to HeLa cells *in vitro* and *ex vivo*. These results provide an additional understanding of the role of OmpA in EHEC physiology and pathogenesis.

1. Introduction

The Gram-negative bacillus enterohemorrhagic *Escherichia coli* (EHEC) can cause diarrhea, hemorrhagic enteritis, and even life-threatening hemolytic uremic syndrome (HUS) in humans and animals following colonization in the large intestine (Karch et al., 2005; Pennington, 2010). Unlike other bacterial infections, conventional use of antibiotics can enhance pathogenesis, and thus, prevention and treatment of EHEC infection is problematic (Tarr et al., 2005; Wong et al., 2000).

Outer membrane protein A (OmpA) plays multiple roles in EHEC physiology and pathogenesis and has been likened to a molecular Swiss army knife (Smith et al., 2007). To date, OmpA has been shown to help maintain the stability and integrity of the bacterial membrane (Koebnik, 1995). In addition, OmpA acts as a receptor for several bacteriophages to mediate F-factor-dependent conjugation (Schweizer and Henning, 1977). In addition to its physiological functions, EHEC OmpA is an active pathogenic component. Studies have shown that OmpA can mediate the initial adhesion of EHEC to host cells (Torres and Kaper, 2003). Moreover, this protein is able to stimulate the host immune response by recruiting dendritic cells to the sites of bacterial attachment and induce the upregulation of pro-inflammatory cytokines (Torres et al., 2006).

Bioinformatic analysis has shown that the 346 amino acids of OmpA from EHEC EDL933 form two domains: OmpATM (the transmembrane domain) and OmpAper (the periplasmic domain). OmpA^{TM} (approximate position: Met1-Pro198) shares complete identity with the transmembrane domain of OmpA from *E. coli* BL21 (DE3) and forms a structure of 8-stranded all-next-neighbor antiparallel β-barrel with long flexible loops at the external end as determined by X-ray diffraction (Pautsch and Schulz, 2000) and NMR (Cierpicki et al., 2006). In addition, OmpA^{TM} embeds in the outer membrane of *E. coli* and acts as the transmembrane component of OmpA. The four short protein loops emanating from the protein to the outside of the bacterium serve as the main basis of the function of OmpA (Smith et al., 2007).

The OmpAper domain extends from approximately Val199 to Ala346. However, unlike the OmpA^{TM} domain, little information regarding OmpAper and its contribution to bacterial pathogene-

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sis is available. Its homologues (OmpA-like proteins) from other Gram-negative bacteria have been shown to bind to peptidoglycan (Delcour, 2002). Therefore, it is reasonable to hypothesize that OmpAper of EHEC is able to bind to peptidoglycan and subsequently helps to maintain the stability and integrity of the cell wall. To test this hypothesis, soluble recombinant OmpAper was purified, and its peptidoglycan binding ability was analyzed. Additionally, the contribution of OmpAper to bacterial stress resistance and cell adhesion was determined.

2. Materials and methods

2.1. Preparation of recombinant OmpAper

The gene encoding OmpAper (Glu209-Ala346; accession number: NP_286832) was amplified from EHEC EDL933 (ATCC 700927) using primers P1 and P2 (Table S1) and cloned into pET-21a at the *Nde* I and *Xho* I restriction sites. The pET21a-*ompAper* was transformed into *E. coli* BL21 (DE3) and induced using 0.3 mM of isopropyl β-D-1- thiogalactopyranoside (IPTG) to express OmpAper. The cells were harvested and homogenized in buffer A (20 mM Tris, 50 mM NaCl, pH 8.0) by sonication. The soluble fraction was then bound to Ni^{2+}-NTA resin, and the His-tagged OmpAper was eluted with buffer A containing 250 mM imidazole. The eluates from the resin were then bound to a Resource Q column (GE Healthcare, Piscataway, NJ, USA) and eluted with buffer B (20 mM Tris, 50 mM NaCl, 1 M NaCl, pH 8.0). The eluted OmpAper was then concentrated and loaded on a Superdex75 16/60 column (GE Healthcare, Piscataway, NJ, USA) in buffer A. The OmpAper peak was collected and analyzed by SDS-PAGE. The concentration of purified OmpAper was measured using a BCA Protein Assay Kit (Pierce) according to the manufacturer's instructions.

2.2. Gel-filtration and dynamic light scattering analysis

The purified OmpAper and standard proteins (blue dextran 2000, aldolase, conalbumin, ovalbumin, carbonic anhydrase and ribonuclease A) were diluted to 10 mg/ml (GE Healthcare, Piscataway, NJ, USA). A Superdex75/300 gel-filtration column was equilibrated with buffer (20 mM Tris, 50 mM NaCl, pH 8.0). A total of 100 μl of each diluted protein was loaded into the column, and the exclusion peak was recorded. The gel phase-distribution coefficient, K_{aV}, was calculated using the formula $K_{aV} = (V_e - V_0)/(V_t - V_0)$, where V_e, V_0 and V_t are the elution volume, volume of the column vacuum and the total volume, respectively. SPSS 13.0 was used to determine the linear regression of K_{aV} and the log molecular weight. The sample was purified and diluted to 0.5 mg/ml and loaded into a dynamic light-scattering instrument (Zetasizer) equipped with an argon ion laser; the analysis was then performed three times at 25 °C.

2.3. Chemical cross-linking analysis

Glutaraldehyde and ethylene glycol bis (EGS) were used for the cross-linking reaction (Fadouloglou et al., 2008). Purified OmpAper was diluted to 30 ng/ml and incubated with glutaraldehyde at 37 °C for 30 min. The final concentration of glutaraldehyde in each reaction was adjusted to 0.01%, 0.05%, 0.1%, 0.2%, 0.3%, 0.4%, and 0.5%. For EGS cross linkage, a gradient concentration (0.1 mM, 0.2 mM, 0.4 mM, 0.6 mM, 0.8 mM, 1 mM, and 5 mM) of EGS was incubated with purified OmpAper on ice for 60 min. The cross-linking reaction was then terminated by the addition of loading buffer containing SDS and Glycine. The protein samples were then analyzed by SDS-PAGE.

2.4. Peptidoglycan binding assay

The peptidoglycan binding assay was performed as describe previously with slight modifications (Leone et al., 2008). OmpAper (0.5 mg/ml) was centrifuged for 30 min at 400,000 × *g*. The same concentration of bovine serum albumin (BSA) was used as the negative control. Subsequently, 50, 100, 150 and 200 μg of commercial insoluble peptidoglycan (InvivoGen) were incubated with OmpAper or BSA separately in 10 mM sodium phosphate buffer with 50 mM NaCl and pH 8.0 for 1 h at room temperature. After the mixtures had been centrifuged for 30 min at 400,000 × *g*, the supernatants were collected, and the precipitates were washed with 500 ml 10 mM sodium phosphate buffer (with 500 mM NaCl, pH 8.0). Finally, the precipitates were resuspended in 40 ml of Laemmli buffer and heated for 10 min at 96 °C. The supernatant and precipitate fractions were analyzed by western blotting using rabbit anti-OmpAper or anti-BSA polyclonal antibodies as primary antibodies. The density of each band was quantified by ImageJ software (Schneider et al., 2012).

2.5. Construction of EHEC O157 isogenic mutants

The EHEC O157:H7 Δ*ompA* (*ompA* knockout) strain was constructed using the λ Red recombinase method (Datsenko and Wanner, 2000). The primers were designed to amplify the chloramphenicol resistance gene from plasmid pKD3 (Primers P3 and P4 in Table S1). The purified PCR product was electroporated into EHEC O157:H7 carrying the λ Red recombinase expression plasmid pKD46. Primers flanking the deleted region and primers within the chloramphenicol cassette were used in primer-specific PCR to verify the deletion of *ompA* (primers P5, P6, P7, P8, P9 and P10). The EHEC O157 mutant strains were grown on LB (with chloramphenicol added) to select for chloramphenicol resistance. The plasmid pKD46 was cured by growing the bacteria at 42 °C.

To construct EHEC strains complemented with *ompA* or *ompA*^{TM}, genes encoding OmpA or OmpA^{TM} and the promoter were amplified using primer pairs P11–P12 and P11–P13 (Table S1), respectively. EHEC O157:H7 Δ*ompA* were transformed by these plasmids, which generated *EHEC CompA* and *EHEC CompA*^{TM} (Table 1). The expression of OmpA and OmpA^{TM} was detected by western blotting using rabbit anti-OmpA polyclonal antibodies.

2.6. Analysis of EHEC O157 mutant resistance to environmental stress

The growth rate of these 3 EHEC mutants and a wild-type strain was recorded in advance. For acid survival analysis, bacteria at exponential growth phase were diluted 30-fold in PBS (Wang, 2002). One-tenth of the bacterial suspension was mixed with LB containing 60 mM acetic acid (pH 4.0) and incubated for 20 min at 37 °C. For the high osmotic pressure resistance assay, the bacteria were grown in liquid LB culture containing 5 M NaCl for 300 min. For the high SDS resistance assay, LB-grown bacteria ($OD_{600} \approx 0.6$) were diluted in 0.9% NaCl to ~10^4 CFU/ml and plated on LB agar containing 0.5%, 1.0% or 2% SDS. The plates were incubated at 37 °C overnight, and the number of viable bacteria were counted. The relative growth index refers to the ratio of the number of bacteria after growing in different conditions relative to their starting point.

2.7. Cell adhesion assay

The HeLa cell attachment assay was performed as previously described by Wang et al. (2010). In brief, HeLa cells were grown in DMEM supplemented with 10% fetal calf serum. The cells were seeded onto glass coverslips at ~5×10^4 per coverslip at 24 h before infection. Approximately 1×10^6 CFU of EHEC mutants were added