Extracellular Loops of the *Eschericia coli* Outer Membrane Protein A Contribute to the Pathogenesis of Meningitis

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Neonatal meningitis by *Eschericia coli* RS218 occurs due to bacteremia and its transmigration across the blood-brain barrier. Although the outer membrane protein A (OmpA), a molecule with extracellular loops has been shown to contribute to the above phenomenon, we do not know the exact the role of these individual loops. Using bacterial strains whose individual loops have been removed, we demonstrated that whereas Loops 1 and 2 contribute to 70%–80% bacterial survival in serum, bacterial entry into human brain microvascular endothelial cells (HBMEC) is governed by Loops 1, 2, and 3. Cellular invasion was shown to require activation of host cytosolic phospholipase A2 (cPLA2α) by Loops 1 and 2 but not 3. This suggests 2 distinct pathways for bacterial entry into host cells. Loop 4 played no role in either serum survival, cellular entry, or cPLA2α signaling. These findings demonstrate for the first time the different contributions of extracellular loops of OmpA to the pathogenesis of *E. coli* meningitis.

*Eschericia coli* is the most common cause of neonatal, Gram-negative bacillary meningitis with mortality rates approximating 10% and neurological sequelae in survivors ranging between 5% and 40% [1–5]. Our incomplete understanding of its pathogenesis is a major contributing factor to this mortality and morbidity [6, 7]. The disease outcome is primarily due to survival of bacteria in the bloodstream and their subsequent penetration of the blood-brain barrier. A number of microbial factors are known to play a role either in survival in the bloodstream or in invasion of the blood-brain barrier. Prominent among these factors are CNF1, FimH, FliC, IbeA, IbeB, IbeC, OmpA, and IroN [8–15]. While the K1 capsule does not play a role in invasion of the blood-brain barrier, it has been reported to maintain bacterial viability during the invasion [16].

OmpA, a 35 kDa protein, has been shown to contribute to both survival in serum and invasion of the blood-brain barrier [6, 7, 13, 17–20]. It is present as an 8-stranded and anti-parallel, β-barrel structure in the membrane, connected by large hydrophilic surface-exposed loops and short periplasmic turns [21, 22]. Computer generated models have shown its possible interaction with chitobiose but not with other sugars such as fucose or cellobiose [23, 24]. The structure and function of OmpA have been reviewed by Smith et al [25].

Although OmpA’s role in pathogenesis has been demonstrated, the role of individual extracellular loops of *E. coli* in meningitis is unknown. Hence, the objective of our present study was to identify the loops’ individual contribution to specific attributes of RS218 pathogenesis — its survival in host serum, its binding to and invasion of HBMEC, and the host signaling events underlying its entry into host cells.

**MATERIALS AND METHODS**

Reagents
Plasmids bearing deletions in individual extracellular loops of OmpA were a kind gift of Dr Ralf Koebnik from Germany [21]. Antibodies to cPLA2α and phospho-cPLA2α were obtained from Cell Signal Technologies.
(Danvers, MA) while OmpA antibodies were raised in-house, in rabbits. Gelatin veronal buffer (GVB), cellobiose, and 17-allylamino demethoxy-geldanamycin (17-AAG) came from Sigma (St. Louis, MO). Vectastain containing DAPI was obtained from Vector Laboratories (Burlingame, CA). Chitosugars were a gift from Dr Xibing Li of the Department of Biology, Johns Hopkins University.

**Bacterial Cultures**

*E. coli* K1 strain RS218 (Wt-RS218) came to us as an isolate from the cerebrospinal fluid of a neonate with meningitis, while ΔompA was derived from RS218 by deleting the ompA gene as described previously [19]. Deletions of individual loops were engineered by expressing the ΔompA strain with aforementioned plasmids (Table 1). CNF1, FimH, FlIC, IbeA, IbeB, or IbeC deletion mutants have been described previously [8–14]. Bacteria were cultured overnight at 37°C in the presence of antibiotics and used either for serum survival assays or HBMEC binding and invasion studies.

**Serum Survival Assays**

For serum studies, pooled cord serum was mixed with GVB in a ratio of 2:3 to maintain complement activity. Bacteria were then incubated with serum at a concentration of 10⁵ colony forming units (CFUs) per 100 μL of reaction volume. At specific time points, 10 μL of the bacteria-containing solution were removed and quantified on blood agar plates. All procedures were done according to regulations of the Johns Hopkins Medical Institutions.

*E. coli binding and invasion assays in HBMEC*. HBMEC were cultured as mono-layers, in either 24-well tissue culture plates or 100-mm dishes at 37°C in 5% CO₂ in RPMI, with 20% FBS along with minimal essential vitamins, glutamine, sodium pyruvate, penicillin, and streptomycin, as previously described [26].

Bacterial invasion assays were performed by the well-established gentamicin protection assay (GPA) [8–14]. Briefly, HBMEC were incubated with bacteria at a multiplicity of infection (MOI) of 1:100 for 90 min, washed, and incubated with gentamicin (100 μg/mL) for 60 min to kill extracellular bacteria. Cells were then lysed and internalized bacteria enumerated on blood-agar plates. For HBMEC binding assays, the gentamicin step was omitted.

For inhibition studies, bacteria were first incubated with sugars on ice for 60 min and then added to the serum (for survival assays) or to HBMEC (binding/invasion assays) and analyzed.

**Treatment of HBMEC with 17-AAG**

HBMEC mono-layers were treated with either 1 μmol/L of 17-AAG or the vehicle control (DMSO) 1 day before the experiments. Initial pilot experiments showed that 18-hour treatment of HBMEC with 1 μmol/L of AAG had no effect on cell morphology. Hence, after overnight treatment with 17-AAG, the cells were washed and incubated with bacteria for either binding and invasion assays (preparing lysates for cell signaling studies) or immuno-cytochemical analysis.

**SDS-PAGE and Western Blotting**

Expression of OmpA in bacteria was examined after boiling them in SDS-Page buffer and subjecting them to electrophoresis by Western blotting. For analyzing cPLA2α activation in HBMEC, cells cultured in 100-mm dishes were incubated with bacteria and lysed in buffer containing 50 mM Tris (pH-7.4), 1% Triton X-100, 150 mM sodium chloride, sodium vanadate, and a protease inhibitor cocktail. Lysates were resolved by SDS-PAGE and analyzed for either phospho-cPLA2α or cPLA2α using specific antibodies in a Western blot assay. Bands obtained by Western blotting were quantified and graphically represented.

### Table 1. List of bacterial strains and characteristics

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Characteristics</th>
<th>Antibiotics</th>
<th>Ref</th>
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<tr>
<td>RS218</td>
<td>RS218 spontaneous streptomycin-resistant mutant</td>
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<td>Str, Km, Km</td>
<td>This study</td>
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<td>Str, Km, Km</td>
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using ImageJ software (available at the NIH Web site http://rsb.info.nih.gov/ij.)

**Immuino-Cytochemistry**

HBMEC, grown on 8-well chambers, were incubated with bacteria at 37°C for 30 min, fixed in 2% formalin for 10 min, blocked with 10% normal goat serum with 1% Triton X-100, and incubated with phospho-cPLA2α antibody. Cells were further incubated with anti-rabbit antibody conjugated to Alexa 488 and Alexa phalloidin 568, washed, and mounted in VECTASTAIN/DAPI reagent. Cells were visualized with a Nikon L100 inverted microscope using bright-field illumination for bacteria, with a GFP channel to visualize phospho-cPLA2α, a red channel for analyzing F-actin, and a blue channel for analyzing DAPI. In some experiments HBMEC were treated with 17-AAG and then processed as above.

**Statistical Analysis**

The means of bacterial counts obtained from 3 different experiments were calculated using Student t-test and graphically plotted along with standard deviation.

**RESULTS**

**Loops1 and 2 of OmpA Contribute to Serum Survival of E.coli**

OmpA has been shown to contribute to serum survival of RS218 [17, 20], but the role of individual loops is not known. Therefore, to examine the contribution of the loops, strains bearing individual loop deletions were first analyzed for expression of OmpA along with Wt-RS218 and ΔOmpA by Western blot analysis. Expression of OmpA was seen in Wt-RS218 as well as in all 4 loop mutants but not in ΔOmpA strains (Fig1A).

Next, deleted-loop mutants, along with Wt-RS218 and ΔompA strains (included as positive and negative controls, respectively), were incubated in pooled cord serum at 37°C for various time points. Bacteria surviving at indicated time points were determined by diluting aliquots and enumerating them on blood agar plates. Wt-RS218 (Wt) and the ΔompA strain were included as positive and negative controls, respectively. The data were a demonstration of 3 different experiments and presented as a percentage of surviving bacteria with the time point zero taken as a 100% (mean SD ± 1).

**C.** To analyze the effect of various chito-sugars on the survival of RS218 in serum, WT-RS218 was treated with 1 mM of chito-biose (C2), -triose (C3), -tetrose (C4), -pentose (C5) or -hexose (C6) for 60 minutes on ice and then incubated with pooled cord serum for various time points. Bacteria not incubated with sugars served as a positive control (C). An additional control was included where the bacteria were first treated with cellulbiose (CB) and then incubated with serum. The amounts of surviving bacteria were determined by diluting aliquots and plating them on blood agar plates and the mean (SD ± 1) of 3 experiments graphically plotted. The data was presented as a percentage of the surviving bacteria with the time point zero taken as a 100%. D. Wt-RS218 along with the OmpA loop deleted mutants (ΔLoop1, ΔLoop3 and ΔLoop4) were incubated with or without chito-hexose (C6) and then further incubated with pooled cord serum at 37°C for various time points. The numbers of surviving bacteria were enumerated and expressed as mean SD ± 1. The data was presented as a percentage of the surviving bacteria with the time point zero taken as a 100%.

**Figure 1.** Contributions of individual loops of RS218 OmpA to serum survival. A. RS218ΔOmpA was transfected with plasmids deleted in individual OmpA loops and lysates analyzed for the expression of OmpA along with Wt-RS218 and the ΔOmpA strain by Western Blotting (i). A part of the ponceau stained membrane is shown in (ii) to illustrate loading of the bacterial proteins. B. To analyze the contribution of OmpA of RS218 to serum survival, strains expressing individual loop deletions of OmpA were incubated in pooled cord serum at 37°C for various time points. Bacteria surviving at indicated time points were determined by diluting aliquots of the serum and enumerating them on blood agar plates. Wt-RS218 (Wt) and the ΔOmpA strain were included as positive and negative controls, respectively. The data were a demonstration of 3 different experiments and presented as a percentage of surviving bacteria with the time point zero taken as a 100% (mean SD ± 1).
While Wt-RS218 survived to almost 80% of the starting inoculum after 60 min of incubation, only 20% of ΔompA were recovered after 15 min of incubation. Interestingly, the loop-deletion mutants demonstrated various degrees of survival, suggesting their differential contributions to serum survival. While ΔLoop3 and ΔLoop4 strains decreased by approximately 50%, only 15% to 25% bacteria of ΔLoop1 and ΔLoop2 were recovered after 60 min (Figure 1B). Thus, Loops1 and 2 were evidently responsible for serum survival of RS218.

Since OmpA was shown to interact with chitobiose [23, 24], we examined the effect of these sugars in the survival of WT-RS218 in serum. Briefly, Wt-RS218 was incubated with polymers of C2–C6 chito-sugars (chito-biose, -triose, -tetrose, -pentose, and -hexose) and then further incubated with serum. Additionally, the bacteria incubated with either cellulose (since no interaction with OmpA was documented) or those without sugars served as positive controls.

While untreated or cellulose-treated bacteria showed little decrease in the CFUs after incubation with serum for 60 min, chito-sugars treated with RS218 showed an inverse relationship between survival and polymer size. Hence, while those incubated with chitobiose survived to approximately 70% of their starting inoculum, bacteria treated with chito-hexose decreased to almost 40% (Figure 1C). Bacteria incubated with chito-triose, chito-tetrose, or chito-pentose survived to approximately 50–60% of their starting inoculum.

To further analyze the interaction of chito-sugars with individual loops, mutants along with the Wt-RS218 were incubated with chito-hexose and subjected to serum-killing assays (Figure 1D). While no further decrease of ΔLoop1 and ΔLoop2 — data not shown) bacteria were observed, the CFUs of ΔLoop3 and ΔLoop4 strains decreased to levels of ΔLoop1, suggesting interaction of the sugars occurred with either Loop1 or 2.

The above results provide strong evidence that Loops1 and 2 are primarily responsible for serum survival of RS218.

Loops1, 2, and 3 of OmpA Orchestrate Binding and Invasion into HBMEC

Previous studies have shown that OmpA contribute to binding and invasion of RS218 into HBMEC [13, 24]. However, the precise role of individual loops in such events is not known. Hence, HBMEC mono-layers were incubated with various loop-deletion mutants along with Wt-RS218, ΔompA as well as a ΔompA strain transformed with a plasmid deleted in all extracellular loops (ΔALL). The bacteria were incubated for 90 min and analyzed by the gentamicin protection assay mentioned above. Internalized bacteria were enumerated after cultivating on blood agar plates. For estimating total cell-bound bacteria, the gentamicin step was omitted.

In comparison to Wt-RS218, reduced binding of approximately 10–20% was observed in all mutant strains (Figure 2A). However, while the number of internalized bacteria for either ΔAll strain or ΔompA was approximately 20% that of Wt-RS218, ΔLoop1, ΔLoop2, and ΔLoop3 strains showed decreased invasion by approximately 60%–65%. The ΔLoop4 mutant did not exhibit significant decrease in invasion of HBMEC. Thus, Loops1, 2, and 3 appear to be responsible for invasion of RS218 into HBMEC.

Since chito-sugars were observed to interact with OmpA, we examined the binding and invasion of RS218 into HBMEC in the presence of sugars. Briefly, Wt-RS218 was treated with either of the C2 to C6 sugars or cellulose at a concentration of 1 mM for 60 min and then added to the HBMEC mono-layers. Untreated bacteria served as positive controls.

Whereas we saw a decrease of approximately 20% in adhesion to HBMEC in the presence of either C5 or C6 sugars with respect to controls, no significant effect with other sugars was observed (Figure 2B). While chitobiose-treated bacteria behaved similarly to those treated without sugars, bacteria incubated with either C3 or C6 sugars decreased significantly by ~30% - 45%, with C6 sugar demonstrating maximum inhibition by internalization.

Since bacteria incubated with C6 sugar showed least invasion, chito-hexose was further tested on individual-loop mutants. Briefly, loop mutants along with Wt-RS218 were incubated with C6 sugar and then analyzed for HBMEC binding and invasion. While binding was not affected significantly, the percentage of internalized Wt-RS218 or ΔLoop4 strains decreased considerably in comparison to ΔLoop1 (and ΔLoop2, data not shown) strain (Figure 2C). Of significant interest, ΔLoop3 showed only a 10% decrease in HBMEC invasion, while no further decrease in ΔLoop1 (and ΔLoop2) was observed.

Taken together, the above data demonstrate that OmpA Loops1, 2, and 3 contribute to binding and invasion of RS218 into HBMEC, while Loop4 does not play a significant role. Additionally, inhibition of ΔLoop3 with C6 sugar but not of ΔLoop1 and 2 suggests differences in interaction of the loops with HBMEC.

OmpA Activates cPLA2α of HBMEC for Internalization

Host cPLA2α has been shown to be important in the entry of RS218 into HBMEC [27]. It also affects cytoskeletal rearrangements, an event that is crucial for RS218 entry into host cells [28, 29]. Since cPLA2α activation on bacterial incubation of HBMEC has not been demonstrated, host cells were incubated with Wt-RS218 for various times and lysates examined for enzyme activation. Western blot analysis using specific antibodies revealed a time-dependent increase in activation of cPLA2α on bacterial incubation (Figure 3A).

Next, the bacterial determinant responsible for activation of the enzyme was examined. Briefly, HBMEC were incubated with isogenic mutants of RS218 (such as cnf1, fimH, fliC, fbeA, fbeB, ibeC, or ompA) that were observed to be deficient in either adhesion or invasion in earlier studies. Lysates were then examined for either p-cPLA2α or cPLA2α by Western blotting.
While lysates of cells incubated with ΔfliC, ΔibeB, and ΔibeC revealed lower enzyme activation as compared to Wt-RS218, the decrease was most pronounced in cells incubated with ΔompA strain (Figure 3B). Densitometric quantitation of band intensities calculated by ImageJ software and represented as a fold change of the ratio of p-cPLA2α to cPLA2α. The quantitation showed an approximately 5-fold decrease in enzyme activation in HBMEC incubated with ΔompA as those compared to Wt-RS218 (Figure 3C).

Since chito-hexose decreased Wt-RS218 invasion by interacting with OmpA, therefore, enzyme activation was examined in the presence of C6. Briefly, HBMEC, incubated with Wt-RS218 that were previously mixed with various concentrations of C6 (0–500 μM) for 30 min were lysed and analyzed for either p-cPLA2α or cPLA2α by Western blotting. Untreated bacteria, or those incubated with 500 μM Cellobiose, served as a positive control.

While lower concentrations of chito-hexose did not affect enzyme activation, 100 or 500 μM/C6 concentrations of C6 showed significant decrease in the cPLA2α activation in comparison to either Wt-RS218 alone or with Cellobiose (Figure 3D). Densitometric analysis revealed approximately 2–3 fold decrease in enzyme activation when the bacteria were pretreated with either 100 μM/L and 500 μM/L of C6 (Figure 3E).

Taken together, this study demonstrates for the first time the novel role of OmpA in the activation of host cPLA2α.

Loops1 and 2 of OmpA Trigger cPLA2α Activation in HBMEC

Host cPLA2α is important for bacterial entry as well as cytoskeletal rearrangements. Since demonstrated that Loops1, 2, and 3 of OmpA are important for the invasion of HBMEC by RS218 and that OmpA activates host cPLA2α hence the role of individual loops in activating host cPLA2α was examined. We incubated HBMEC mono-layers were incubated with either individual loop-deleted strains, Wt-RS218 or ΔompA (positive and controls, respectively) for various time points and analyzed lysates by Western blotting.

While strong activation of cPLA2α was observed in cells incubated with either Wt-RS218, ΔLoop3, or ΔLoop4 strains, those incubated with ΔLoop1 or ΔLoop2 strains showed minimal activation and profiles were similar to ΔompA (Figure 4A). Densitometric estimations showed an approximately 2.5 to 3 fold decrease in cPLA2α activation with ΔLoop1, ΔLoop2, or ΔompA as compared to either Wt-RS218, ΔLoop3, or ΔLoop4 (Figure 4B).

Since ΔLoops1, 2, and 3 showed decreased invasion, but only ΔLoop1 and 2 but not ΔLoop3 activated cPLA2α, actin condensation in response to the mutants was examined by immuno-cytochemistry. Briefly, HBMEC incubated with either Wt-RS218, ΔLoop1, or ΔLoop3 strains for 30 min were analyzed for phospho-cPLA2α with specific antibody followed by Alexa488-coupled conjugate while actin condensation was
examined by staining with Alexa phalloidin. While activation of cPLA2α along with actin condensation was observed in HBMEC incubated with either Wt-RS218 or ΔLoop3 strains, neither cPLA2α activation nor actin condensation were seen with ΔLoop1 strains (Figure 4C).

These findings suggest that Loop1 (and Loop2, data not shown) of OmpA trigger actin condensation in HBMEC through cPLA2α-dependent pathways, while Loop3 moderates actin polymerization through a cPLA2α-independent pathway.

Loops 1 and 2 of OmpA Require HSP90 to Invade HBMEC

OmpA of E. coli has been shown to bind HSP90 homologues such as gp96 on the surface of HBMEC to gain entry [30, 31]. Although gp96 has been shown to interact with OmpA, HSP90 has not been investigated for its contributions to bacterial invasion. To identify the role of HSP90 in invasion of RS218 to HBMEC, either untreated host cells or those treated with 17-AAG — a specific inhibitor of HSP90 — were incubated with Wt-RS218 or ΔompA and then analyzed for binding and invasion.
While binding of Wt-RS218 decreased by ~20% in 17-AAG treated HBMEC as compared to control cells, ~60%–65% decrease in invasion was observed. No significant change was observed in either binding or invasion by DompA (Figure 5A).

Next, we examined the effect of 17-AAG on invasion using strains deleted of individual loops. Briefly, 17-AAG treated or control HBMEC were incubated with either of the 4 deleted-loop strains along with Wt-RS218 and analyzed for adhesion or invasion. While no further decrease in the CFUs of internalized ΔLoop1 (or ΔLoop2 - data not shown) strain was observed, invasion of Wt-RS218 and ΔLoop4 strains decreased ~70–75% in 17-AAG treated HBMEC as compared to control cells (Figure 5B). It is notable that ΔLoop3 showed a further decrease of ~20% in 17-AAG treated cells, suggesting that the inhibitor further affected invasion in addition to already low frequencies demonstrated by the strain.

HSP90 has been shown to activate cPLA2α in macrophages, although no such activation has yet been demonstrated in endothelial cells [32]. Since Loops1 and 2 were shown to activate cPLA2α, we hypothesized that activation occurs through HSP90. To examine the role of HSP90 in activating cPLA2α, monolayers of either untreated or 17-AAG treated HBMEC were incubated with Wt-RS218, ΔLoop1, or ΔLoop3 and analyzed along with the condensation of F-actin by immunocytochemistry. Briefly, HBMEC mono-layers were incubated with either Wt-RS218 or loop mutants. The HBMEC were fixed in formalin and then stained for either p-cPLA2α using Alexa-488 (green) or F-actin using Alexa phalloidin (red). Magnification 20x.

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To further examine the role of HSP90 in the activation of cPLA2α and actin condensation in HBMEC in response to Wt-RS218, HBMEC treated with 17-AAG and followed by bacterial incubation for 30 min. were analyzed by immunocytochemistry. Phase contrast microscopy showed the presence of bacteria on both treated and untreated cells (Figure 5D). However, activation of cPLA2α (green) along with F-actin condensation was observed only in untreated HBMEC, while neither cPLA2α activation nor actin condensation was observed in 17-AAG treated cells. This suggested that HSP90 orchestrated cPLA2α activation and actin rearrangements in HBMEC in response to Wt-RS218.
Figure 5. Effect of OmpA receptor analogues on E. coli invasion and host cPLA2α activation. A. To examine the role of the HSP90 (a homologue of the OmpA receptor, gp96) in E. coli interaction with HBMEC, host cells were cultured in 24-well tissue clusters and treated overnight with 1 μmol/L of 17-AAG, an inhibitor of HSP90. Adhesion and invasion assays were then carried out with either Wt-RS218 or the ΔompA strain and compared with the results of untreated cells. The amounts of bound or internalized bacteria were determined and expressed as relative % binding and invasion frequency (mean SD±) compared to that of Wt-RS218 (Wt) taken as 100%. B. To demonstrate the role of OmpA loops and their interaction with HSP90 in HBMEC binding and invasion, HBMEC treated with 17-AAG were incubated with ΔLoop1, Δloop3 or Δloop4 strains or Wt-RS218 and examined for binding and invasion frequency and compared with binding and invasion frequency in untreated cells. The results were expressed as relative % binding and invasion frequency (mean SD±) compared to binding and invasion frequency of Wt-RS218 (Wt) in untreated cells taken as 100%. C. To examine the effect of HSP90 on host cell cPLA2α activation in response to Wt-RS218, HBMEC treated with or without 1 μmol/L of 17-AAG overnight were incubated with RS218 for various time points and the HBMEC lysates analyzed for the amounts of phospho-cPLA2α and cPLA2α using specific antibodies in Western Blot assays. D. To establish the role of HSP90 on cPLA2α activation and actin condensation, HBMEC mono-layers treated with or without 17-AAG were incubated with Wt-RS218 for 30 min. The cells were then fixed and stained for either p-cPLA2α using Alexa 488 (green) or F-actin using Alex phalloidin (red). Bacteria were visualized using brightfield illumination (arrows). Magnification 40x.
Overall, our data presents for the first time that Loops 1 and 2 of OmpA contribute to serum survival, HBMEC invasion, and cPLA2α signaling. Although Loop 3 plays a role in invasion, its mechanism of action differs from Loops 1 and 2. Taken together, Loops 1 and 2 contribute to HBMEC invasion by activating cPLA2α through HSP90.

**DISCUSSION**

OmpA’s structure has been well characterized with respect to detailed amino acid compositions of the 8-stranded, transmembrane protein [33], along with its insertion in the membrane. Functional analysis such as bacteriophage binding has been reported through deletion studies [20, 21]. The OmpA molecule is conserved across many Gram-negative species and characterized by 4 surface-exposed loops [21, 22].

Previous studies showed the importance of OmpA to *E. coli* survival in the bloodstream using strains with the complete molecule deleted [17, 20]. However, this is the first report where individual loops have been examined for their contributions to serum survival. Our study demonstrated the differential contributions of extracellular loops to serum-mediated killing with Loop 1 and 2, contributing predominantly to serum resistance. The construction of mutants whose individual loops were deleted has provided a unique way to study their contributions to the pathogenesis of *E. coli* meningitis [21].

Although OmpA has been shown to bind complement regulators, this study did not address such binding.

Earlier studies have shown that chitobiose may interact with OmpA and influence invasion into HBMEC [23, 24]. Our study, however, differs because it demonstrates that higher polymers such as chito-hexose but not chitobiose affect serum survival as well as invasion into HBMEC. This suggests that sugar polymer size is important in the interactions of Wt-RS218 with host factors.

*E. coli* K1 invasion of HBMEC has been shown to be a prerequisite for its penetration into the brain [6, 7]. The low levels of invasion with Loop 1, 2, and 3 further establish their importance in *E. coli* entry into HBMEC. The involvement of 3 loops in HBMEC invasion instead of the first 2, as seen in serum survival, demarcates the different roles of individual loops in various steps of *E. coli* pathogenesis in meningitis.

The importance of OmpA in triggering signaling events as well as the significance of host-cell cPLA2α in *E. coli* K1 invasion of HBMEC been demonstrated earlier [27, 31]. Our study shows that among the various RS218 mutants defective in either HBMEC have binding or invasion, ΔompA exhibits negligible or no activation of cPLA2α. Also, Loops 1 and 2 but not Loops 3 and 4 are important in cPLA2α activation. The way Loops 1 and 2 differ from Loop 3 suggests the variability that OmpA has generated to interact with various receptors on host cells to elicit different signaling patterns. Decreased invasion with ΔLoop 1, ΔLoop 2 and ΔLoop 3, taken together with cPLA2α activation by Loops 1 and 2 but not Loop 3, suggests 2 different pathways of RS218 entry into HBMEC. Additional studies are needed to further characterize the seemingly diverse mechanisms involved with *E. coli* invasion of HBMEC.

cPLA2α is triggered by clustering of membrane receptors, including HSP90 at the site of bacterial interaction with HBMEC [28, 29]. Inhibition of HSP90 results in decreased invasion of bacteria and reduced cPLA2α activation, both events orchestrated by Loops 1 and 2. Data expressed in this study should help in the development of therapeutics against *E. coli*. Our current hypothesis of *E. coli* RS218 survival in serum as well as invasion of HBMEC through interactions of OmpA loops with host components is depicted diagrammatically in Figure 6.

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**References**


