INFECTION AND IMMUNITY, June 1996, p. 2019–2023 Vol. 64, No. 6
0019-9567/96/$04.00+0
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Identification of a Domain in Rck, a Product of the *Salmonella typhimurium* Virulence Plasmid, Required for Both Serum Resistance and Cell Invasion

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Received 27 December 1995/Returned for modification 16 February 1996/Accepted 12 March 1996

Rck is encoded on the *Salmonella typhimurium* virulence plasmid and is a member of a family of related 17- to 19-kDa outer membrane proteins of *Enterobacteriaceae*, including Ail (*Yersinia enterocolitica*) and PagC (*S. typhimurium*). Structural models for these proteins predict eight membrane-spanning domains alternating with hydrophilic inner and outer loops. When expressed in *Escherichia coli*, Rck and Ail, but not PagC, confer high-level resistance to the bactericidal activity of complement as well as the ability to adhere to and invade mammalian cell lines. To identify functional domains of Rck, we made and screened random mutations in Rck for decreased bioactivity. We found that a single amino acid substitution (glycine to aspartic acid) in the putative third outer loop greatly reduced Rck-mediated serum resistance and eukaryotic cell invasion. We then constructed two chimeric proteins between Rck and PagC. Substitution of the C-terminal half of Rck with the corresponding PagC fragment containing both the third and the fourth outer loops abolishes the Rck-mediated serum resistance and invasion phenotypes. Substitution of Rck with a smaller C-terminal portion of PagC containing the fourth outer loop did not affect the invasive phenotype or serum resistance. These data reveal that the third putative outer membrane loop region is important for the virulence-associated properties of the Rck protein and suggest a similarity between the mechanism of serum resistance and epithelial cell invasion involving the same domain of Rck.

The outer membrane proteins (OMPs) of invasive gram-negative pathogens provide specialized functions enabling the organisms to enter into and survive in the host environment. In pathogenic bacteria, a number of OMPs are specifically associated with the expression of virulence (18, 20).

Gram-negative bacteria causing systemic diseases are almost always resistant to the bactericidal activity of the serum (8, 23, 27). The outer membrane is the site of interaction between these bacteria and complement, and both long-chain lipopolysaccharide (LPS) and specific OMPs have been shown to be major determinants of serum resistance (9–11, 18, 30, 31). The *Salmonella typhimurium* virulence plasmid encodes the gene for Rck, an OMP that confers high-level serum resistance to *S. typhimurium* and *Escherichia coli* independent of the LPS structure (2–6, 22, 27). Expression of the *rck* gene in *E. coli* was also shown to confer the ability to invade cultured mammalian cell lines (7). The *rck* gene encodes a 19-kDa precursor protein which is inserted into the outer membrane after proteolytic cleavage of a leader sequence and inhibits complement lysis by preventing polymerization of C9 (5, 6).

DNA sequence analysis has shown that Rck is a member of a family of related 17- to 19-kDa OMPs of members of the family *Enterobacteriaceae* (5). Other members of this protein family are Ail (*Yersinia enterocolitica*), PagC (*S. typhimurium*), OmpX (*Enterobacter cloacae*), and Lom (bacteriophage lambda-lysogenic *E. coli*) (1, 14–17, 20, 21, 25, 26).

Structural models for these proteins predict several membrane-spanning domains alternating with hydrophilic inner and outer loops. The regions of greatest homology are the transmembrane segments, while putative outer membrane loops have little amino acid identity (5). Among these proteins, only Rck and Ail share the ability to induce both complement resistance and epithelial cell invasion (7, 19). Lom and OmpX are not involved in virulence, and PagC appears to be involved with survival of *S. typhimurium* in macrophages (14, 21).

In this study, we identify a domain of Rck that is crucial for both serum resistance and cell adhesion and invasion. We show that the third putative outer membrane loop is essential for the virulence-associated properties of the protein and suggest that the same domain is involved in conferring both serum resistance and the invasive phenotype.

MATERIALS AND METHODS

Bacterial strains. *E. coli* HB101 [F$^-$ *mcrB mrr hsdS20 recA13 supE44 ara14 galK2 lacY1 proA2 rpsL20*(Smr) *xil-5* λ$^-$ *leu mtl-1*] was used as the host for the subcloning of all the constructs in pACYC184. DM1 [F$^-$ *dam-13*::Tn*9*(Cm^r) *dcm mcrB hsdR* M$^+$ *gal-1 gal-2 ara lac thr leu* Ton^r Tsx^r *sup*^{O} λ$^-$] strains were used for the digestion of *rck* at the *Cla*I site. Epicurian Coli XL-1 Red competent cells [*endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac mutD5 mutS mutD* Tn*10* (Tet^r)] (Stratagene) were used for the introduction of random mutations in the *rck* gene.

DNA isolation and manipulation. CsCl-purified plasmid DNA was prepared by the cleared-lysate procedure (28). Rapid isolation for plasmid clone analysis, cleavage of DNA with restriction endonucleases, gel purification of DNA fragments, use of Klenow fragment of DNA polymerase, and ligation, transformation, and amplification techniques were performed by standard methods (13).

Isolation of point mutants and methods of gene transfer. A 1.2-kb *Sal*I-*Eag*I fragment containing the *rck* gene and a 1.6-kb *Pst*I-*Sal*I fragment containing the *pagC* gene were cut out from previously created pBR322 constructs (7) and cloned in pACYC184 (Stratagene) by standard procedures (13).

For the random mutations, pBSK plasmid containing *rck* was transformed into an *E. coli* mutator strain (Stratagene). Plasmid DNA was harvested after two passages in Luria-Bertani broth with penicillin (200 μg/ml), and a *Eag*I-*Sal*I double digest was performed. The 1.2-kbp pooled mutated *rck* fragments were

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subcloned in pACYC184 and transformed into *E. coli* HB101. Clones expressing the mutated proteins and showing a decrease in serum resistance were sequenced by the dideoxynucleotide termination method of Sanger et al. (24) (Sequenase; U.S. Biochemicals Corp.).

Asp in position 43 was mutated into a Lys by site-specific mutagenesis with a synthetic oligonucleotide (5′-CAGGTTCACCCCGCGGATCTTCTTAAAATG CTCTATCCGGC-3′). The third mutant, containing both mutations (*rckD43K-G118D*), was constructed by substituting the *Kpn*I-*Eag*I fragment containing the G118D mutation in the plasmid containing the mutation in position 43.

Construction of *rck-pagC* hybrids. *rck-pagC1* was constructed by replacing the *Kpn*I-*Eag*I fragment of pACYC184::*rck* with a PCR fragment coding for the homologous region of *pagC*. The *Kpn*I site, absent in the *pagC* wild-type gene, was engineered by amplifying pACYC184::*pagC* with a mutated oligonucleotide containing the *Kpn*I site (5′-GCTGGCGGGTGTCGGTACCGTAAAGGCG-3′).

For the construction of *rck-pagC2*, pACYC184::*rck* was cloned in the DM1 bacterial strain and the methylation-sensitive *Cla*I site was digested. The *Cla*I-*Eag*I fragment of *rck* coding for the putative fourth extracellular loop of Rck was substituted in pACYC184::*rck* by a PCR fragment coding for the homologous region of *pagC*. The *Cla*I site, absent in the *pagC* wild-type gene, was engineered by PCR with a mutated oligonucleotide (5′-CCGCTGGAGAATATCGTCATC GATGTTGGG-3′). Both *rck-pagC1* and *rck-pagC2* were transformed into *E. coli* HB101 and verified by sequencing through the *pagC* regions.

OMP preparation, SDS-PAGE, and immunoblotting. OMP preparation was performed by a small-scale cell fractionation protocol. Briefly, 1.5 ml from an overnight culture of *E. coli* HB101 expressing Rck, PagC, or the chimeric proteins was pelleted in an Eppendorf centrifuge for 5 min at 15,600 × *g* and 4°C. The pellet was resuspended in 150 μl of cold spheroplast buffer (100 mM Tris [pH 8.0], 0.5 mM EDTA, 0.5 mM sucrose, 20 μg of phenylmethylsulfonyl fluoride per ml), 100 μl of cold water was added, and the sample was incubated on ice for 1 min. Five microliters of cold 5 mM $MgCl_2$ was added, and the tube was centrifuged as described above. The pellet was resuspended in 150 μl of cold spheroplast buffer, 15 μl of lysozyme (2 mg/ml) and 150 μl of cold water were added, and the tube was incubated for 5 min on ice before centrifugation. The pellet was resuspended in 600 μl of cold 10 mM Tris (pH 8.0)-phenylmethylsulfonyl fluoride (20 μg/ml), subjected to four cycles of freeze-thawing (−80°C to 37°C), DNase treated, and centrifuged at low speed to remove the unbroken cells. The supernatant was then sedimented for 30 min at 15,600 × *g* in a microcentrifuge at 4°C. The pellet was solubilized in Laemmli buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Equal amounts of protein from total bacterial lysates were also analyzed by SDS-PAGE as described by Laemmli (12). The gels were stained with Coomassie blue or transferred to nitrocellulose for immunoblotting. The blots were probed with a rabbit polyclonal antibody which recognizes Rck. Alkaline phosphatase goat anti-rabbit conjugate was used as a secondary antibody.

Serum sensitivity studies. Serum sensitivity studies were performed by use of the Fothergill method. Normal human serum was collected from 10 healthy volunteers, pooled, and stored at −70°C until used (pooled normal human sera). Control sera were decomplemented by heating at 56°C for 30 min (heat-inactivated sera). Serial 10-fold dilutions of mid-log-phase bacterial suspensions in Dulbecco's phosphate-buffered saline were incubated for 60 min in 50% pooled normal human sera at 37°C and then plated on tryptic soy agar. Serum sensitivity was calculated as the difference between CFUs surviving incubation in pooled normal human sera and those in heat-inactivated sera, expressed in log base 10 and designated as log kill (7).

Cell adhesion studies. For the adhesion assays, 5×10^5 colony-forming bacteria were added to 2×10^5 Chinese hamster ovary (CHO) cells grown overnight on glass coverslips and incubated for 1 h at 37°C in a CO_2 incubator. Cells were washed six times in Hanks balanced salt solution to remove nonadherent bacteria, Giemsa stained, and read by a single investigator without prior knowledge of the identities of the samples (7). The number of cell-associated bacteria per 100 CHO cells was determined.

Cell invasion studies. For the invasion assays, 10^6 CHO cells were grown overnight in six-well Costar plates in Dulbecco minimal essential medium-F12 medium (50:50) supplemented with 2 mM L-glutamine and 7% fetal calf serum. Colony-forming bacteria (5×10^6) were added to the monolayer, and cells and bacteria were spun at 500 × *g* for 5 min and incubated for 1 h at 37°C in a 5% CO_2 incubator. At the end of the incubation, the cells were washed three times with Dulbecco minimal essential medium-F12 medium and exposed to gentamicin (50 μg/ml) for 1 h to kill the extracellular bacteria. The number of bacteria surviving the gentamicin treatment was determined by colony counts of serial dilutions from epithelial cell lysates (7).

RESULTS

Isolation of point mutations in *rck* and expression of the proteins. To identify functional domains in Rck, we screened randomly generated mutants for increased sensitivity to complement-mediated killing. From over a thousand mutated clones screened, 20 showed decreased serum resistance, but only one expressed Rck protein. This clone was sequenced to assess the type of mutation and the position of the mutated amino acid. We found that Gly in position 118 was mutated to Asp (G118D). This mutation mapped in the putative third extracellular loop of the Rck protein (Fig. 1A) in a residue conserved between Rck and Ail (Fig. 2).

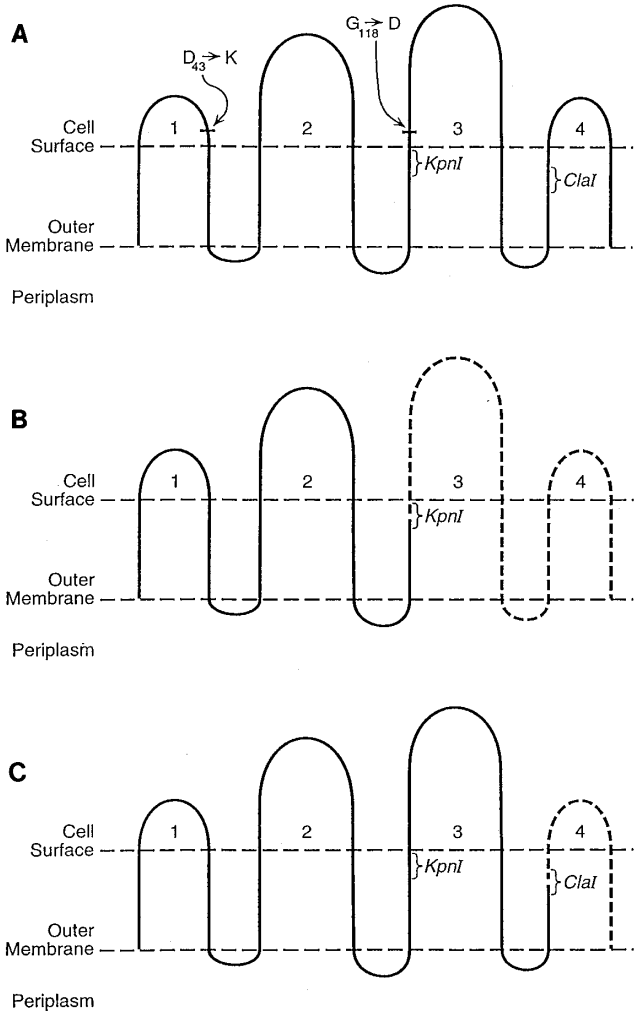

FIG. 1. Structural model of Rck and Rck-PagC hybrids within the outer membrane, based on a similar model presented for Ail (15) and OmpX (25, 26). (A) Rck mutants; (B) Rck-PagC1; (C) Rck-PagC2. The arrows indicate the positions of the D43K and G118D mutations. Rck is represented by a solid line; PagC is represented by a dotted line. The positions of the *Kpn*I and *Cla*I restriction sites are also indicated.

Independently, we introduced a site-specific mutation at amino acid position 43, substituting lysine for aspartic acid (D43K) in the putative first extracellular loop (Fig. 1A). This mutation was chosen because both Rck and Ail contain aspartic acid at this site, while PagC, OmpX, and Lom contain a neutral or basic residue (Fig. 2). A third mutant Rck, containing both substitutions (*rckD43K-G118D*), was constructed by fragment exchange with the central *Kpn*I site. Protein products of these constructs were analyzed by SDS-PAGE and immunoblot probed with polyclonal Rck antibodies (Fig. 3). Comparable levels of expression were achieved for mutant and wild-type proteins.

Serum sensitivity of point and double mutants. *E. coli* HB101 cells transformed with the constructs expressing wild-type *rck*, *rckG118D*, *rckD43K*, and *rckD43K-G118D* were as-

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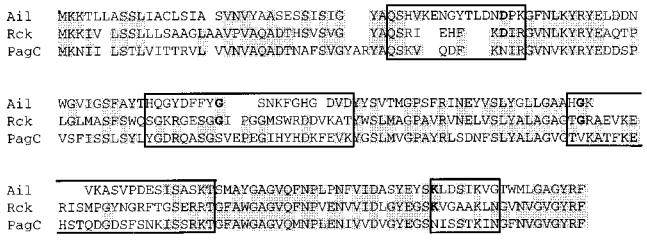

FIG. 2. Homology among OMPs Rck, Ail, and PagC. Shading indicates conserved amino acid residues. Amino acid residues located in the putative extracellular loops (boxes) (15) and conserved in Rck and Ail but not PagC are shown in boldface.

TABLE 1. Effects of plasmid constructs on serum sensitivity of *E. coli* HB101

Construct	Log kill[a]
pACYC184	6.1
pACYC184::*rck*	0.3
pACYC184::*rckG118D*	1.6[b]
pACYC184::*rckD43K*	0.24
pACYC184::*rckD43K-G118D*	2.4[b]
pACYC184::*rck-pagC1*	5.8
pACYC184::*rck-pagC2*	0.6

[a] Each value represents the mean of 10 experiments.
[b] These values were shown to be statistically different from each other by paired *t*-test analysis, $P < 0.01$.

sayed for resistance to complement-mediated killing by the Fothergill method (Table 1). Control HB101 transformed with the vector alone exhibited greater than 6 logs of killing and did not survive in 50% pooled human serum, while HB101 transformed with pACYC184::*rck* showed a high level of resistance (killing, <1 log). The single mutation of Gly-118 to Asp (G118D) was found to reduce Rck-mediated serum resistance by more than 1 order of magnitude (killing, 1.6 log). While the single mutation of Asp-43 to Lys (D43K) did not show any detectable effect (killing, <1 log), the construct combining the two mutations (G118D-D43K) showed a further reduction in serum resistance compared with that of G118D (killing, 2.4 log).

Invasiveness of point mutants. We compared the ability of *E. coli* HB101 transformed with the vector alone, *rck*, and the mutant *rck* constructs to invade CHO cells in culture by determining survival of cell-associated bacteria exposed to gentamicin for 1 h (Table 2). The *rck*-containing strain invaded CHO cells 100 times more efficiently than did the control *E. coli*. The G118D mutant Rck showed decreased invasion of CHO cells compared with the wild-type protein, while invasion by the D43K mutant was the same as that by the wild type. The construct combining the two mutations (G118D-D43K) was similar to the single G118D mutation.

Construction of *rck-pagC* hybrids. To further assess the role of putative extracellular domains of Rck, we constructed two chimeric proteins between Rck and PagC, since PagC expresses neither the serum resistance nor the cell invasion phenotype (7). One chimeric protein (Rck-PagC1) was constructed by substituting the C-terminal half of Rck, containing the third and the fourth outer loops, with the homologous region of PagC (Fig. 1B). The *Kpn*I-*Eag*I fragment coding for the putative third and fourth loops of Rck from pACYC184::*rck* was replaced by a PCR fragment coding for the putative third and fourth loops of PagC. The *Kpn*I site, absent in the *pagC* wild-type gene, was introduced by PCR mutagenesis to facilitate construction of an in-frame fusion.

In a second chimeric protein (Rck-PagC2), the fragment *Cla*I-*Eag*I coding for the putative fourth extracellular loop of Rck was substituted in pACYC184::*rck* by a PCR fragment coding for the fourth loop of PagC (Fig. 1C). The *Cla*I site, absent in the *pagC* wild-type gene, was engineered by PCR with a mutagenic oligonucleotide. Both *rck-pagC1* and *rck-pagC2* were transformed into *E. coli* HB101. The clones were screened for expression of the chimeric proteins by SDS-PAGE of total bacterial extracts (Fig. 3A). The identity of the chimeric proteins was confirmed by sequencing of the clones and by immunoblot probed with a polyclonal Rck antiserum (Fig. 3B). A third chimeric protein, containing the N-terminal half of PagC fused to the C-terminal half of Rck, was also constructed, but it was not expressed at a level comparable to that of the wild type or the other two chimeras and therefore was not suitable for phenotypic analysis.

To verify the localization of the two chimeric proteins on the outer membrane, a small-scale cell fractionation was performed. Both Rck-PagC1 and Rck-PagC2 were preferentially

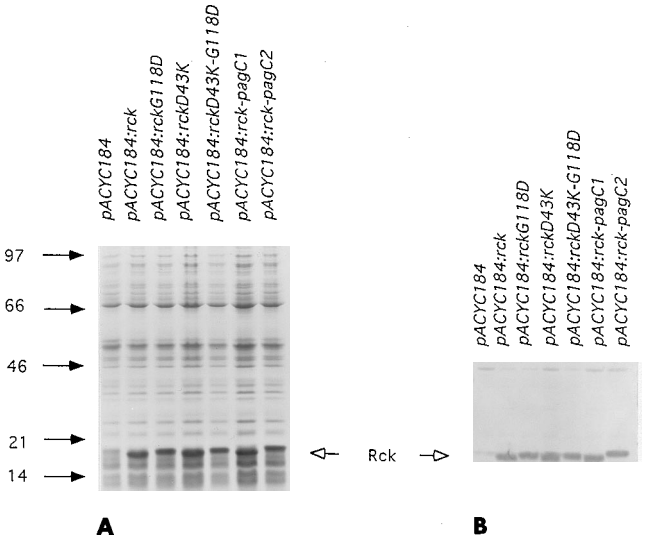

FIG. 3. Expression of wild-type Rck and mutant proteins in *E. coli* HB101. (A) Bacteria were solubilized in Laemmli buffer, and the proteins were separated in an SDS–12% polyacrylamide gel and stained with Coomassie blue R. Thirty micrograms of protein was loaded in each lane. Molecular mass markers, expressed in kilodaltons, are shown to the left of the figure. (B) Immunoblot reacted with polyclonal anti-Rck rabbit serum.

TABLE 2. Effects of plasmid constructs on invasion of CHO cells by *E. coli* HB101

Construct	% Invasion[a]
pACYC184	0.004
pACYC184::*rck*	0.6
pACYC184::*rckG118D*	0.09
pACYC184::*rckD43K*	0.5
pACYC184::*rckD43K-G118D*	0.08
pACYC184::*rck-pagC1*	0.004
pACYC184::*rck-pagC2*	0.6

[a] Percentage of invasion represents the number of viable bacteria surviving gentamicin treatment times 100 divided by the original inoculum as described in Materials and Methods. Results represent the mean of five independent experiments.

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localized in the OMP fraction as was the Rck wild type. These results were confirmed by immunoblot of the OMP fractions probed with polyclonal Rck antiserum (data not shown).

Serum sensitivity of chimeric proteins. *E. coli* HB101 transformed with constructs expressing *rck* wild type, *rck-pagC1*, and *rck-pagC2* were tested for resistance to complement-mediated killing by the Fothergill method (Table 1). *E. coli* HB101 expressing Rck-PagC1 did not survive in 50% serum, showing a killing of >6 logs. In contrast, the expression of Rck-PagC2 conferred the same level of serum resistance as did the Rck wild type.

Cell adherence and invasion of Rck-PagC1 and Rck-PagC2. *E. coli* HB101 transformed with the *rck* wild type, the plasmid vector, and the two chimeric constructs were tested for adhesion to mammalian cells in culture by microscopic examination of Giemsa-stained cells. Bacteria transformed with the chimera containing the C-terminal half of PagC (Rck-PagC1) showed a low adhesion to CHO cells (0.06 bacterium per cell), while the chimera with only the putative fourth extracellular loop of PagC (Rck-PagC2) showed the same level of adhesion (1.0 bacterium per cell) as that of wild-type Rck (1.1 bacteria per cell). (The plasmid vector alone adhered at 0.03 bacterium per cell.) We next compared the ability of the chimeric constructs to invade eukaryotic cells in culture by determining the survival of bacteria after exposure to gentamicin for 1 h. We found that the bacteria expressing Rck-PagC2 were 100-fold more invasive than the bacteria expressing Rck-PagC1 (Table 2). The level of CHO cell association and invasion mediated by Rck-PagC1 was equivalent to that of the control *E. coli*, while Rck-PagC2 was functionally equivalent to wild-type Rck.

DISCUSSION

These studies identify a region of the Rck protein involved in both resistance to complement and invasion of eukaryotic cells. *rck*, encoded by the virulence plasmid of *S. typhimurium*, is homologous to the chromosomal *pagC* gene also present in this organism (2, 4, 5). However, these loci are functionally distinct. *pagC* is a *phoP*-controlled gene required for survival in mouse macrophages and virulence for Ity^s mice (14). PagC possesses neither the serum resistance nor the invasive phenotype, even when expressed at levels comparable to Rck (7). However, Rck shares both phenotypes with Ail, a chromosomal gene product of *Yersinia* spp. (7, 15, 16, 19). Two other members of this family of OMPs, OmpX from *E. cloacae* and Lom from bacteriophage lambda, do not possess any known virulence-related phenotypes (1, 7, 25, 26).

Comparative analysis of these protein sequences shows the presence of alternating hydrophobic and hydrophilic regions in all the members of this family (5, 25). In the proposed structural model, based on work with porins (29), the membrane proteins are folded across the outer membrane with the hydrophilic stretches exposed at the cell surface and the hydrophobic segments spanning the membrane. The amino acid identity is high within the proposed hydrophobic membrane-spanning domains, while the identity in the putative extracellular domains is much lower (5, 15). A possible basis for the specific phenotypes may be the differences in the hydrophilic regions exposed on the cell surface. Crucial amino acid residues in the outer membrane loops of these proteins could be responsible for the interaction with host factors such as complement proteins and/or receptors on eukaryotic cells. We investigated this hypothesis by mutational analysis of these regions. Substitution of aspartic acid for glycine in the third extracellular loop greatly reduced Rck-mediated serum resistance and decreased adhesion and invasion in CHO cells compared with that of the wild-type protein. The Ail protein also contains a glycine at this position (Fig. 2).

We also mutated a second amino acid conserved among Ail and Rck. The Asp in position 43 was mutated to Lys (D43K) in the first extracellular loop, and the mutant was screened for serum resistance and cell adhesion. Unexpectedly, this mutation did not affect the virulence-associated phenotypes, showing that not all of the residues exposed on the extracellular loop and conserved between Ail and Rck are equally important for the expression of serum resistance and cell association.

Interestly, we found that the mutant combining the two single amino acid substitutions showed a further reduction in serum resistance compared with that of the construct bearing only the G118D mutation. The point mutants demonstrate that a specific amino acid sequence in the third extracellular loop is critical for the interaction of the protein with both complement factors and a putative membrane receptor on the cell membrane. In the presence of the third loop mutation, a substitution in the first loop further reduces the function of the protein, suggesting a secondary role for this region in complement resistance. The possibility of an indirect effect on the Rck functions due to the amino acid substitution, although unlikely, could not be excluded.

Thus, a different approach, to prove the importance of the third extracellular domain, was employed. We constructed two chimeric proteins between Rck and PagC. Rck-PagC1 was constructed, substituting in *rck* the sequence coding for the putative third and fourth extracellular loops of PagC. In Rck-PagC2, only the sequence coding for the fourth loop of Rck was replaced with the C-terminal half of *pagC*. As expected, the virulent phenotype was knocked out in the mutant expressing the chimeric protein with the third extracellular loop of PagC (Rck-PagC1), while the expression of the chimera with only the fourth loop of PagC conferred the same level of serum resistance and invasion properties as that of the wild-type Rck. Comparable protein expression and correct localization of the chimeric proteins in the outer membrane was assessed by SDS-PAGE and immunoblot of membrane fractions from bacteria expressing the different constructs.

Our findings show that a single amino acid residue in the proposed third outer loop of Rck is important for the expression of the serum resistance and eukaryotic cell association phenotypes. We also prove that the region of Rck containing this third outer membrane loop is essential for these virulence-associated properties of the protein and suggest that the same domain is involved in conferring both serum resistance and invasive phenotypes.

ACKNOWLEDGMENTS

We thank S. Okamoto for technical advice and C. Chen, P. Grob, V. Water, and S. Libby for helpful discussion and encouragement.

This work was supported by grants from the National Institutes of Health (AI-32178 to D.G.G. and DK-35108 to J.F.) and the U.S. Department of Agriculture (93-37204 to D.G.G.). D.M.C., on leave from the Ospedale S. Giovanni Battista, Torino, Italy, is the recipient of a fellowship from the Italian Association for Biotechnology.

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Editor: P. J. Sansonetti

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