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The *Salmonella typhimurium* Virulence Plasmid Complement Resistance Gene *rck* Is Homologous to a Family of Virulence-Related Outer Membrane Protein Genes, Including *pagC* and *ail*

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A fragment of the *Salmonella typhimurium* virulence plasmid containing the *rck* locus, when cloned in the recombinant cosmid pADE016, was shown previously to confer high-level complement resistance on both rough and smooth *Escherichia coli*, *Salmonella minnesota*, and *S. typhimurium* and was associated with the production of an outer membrane protein. We determined the nucleotide sequence of the fragment containing the *rck* locus. Mutations in the two major open reading frames confirmed that the complement resistance mediated by pADE016 was due to a single 555-bp *rck* gene encoding a 17-kDa outer membrane protein. Analysis of the *rck* gene revealed that the Rck outer membrane protein consisted of 185 amino acid residues, with a calculated postcleavage molecular mass of 17.4 kDa. Rck is homologous to a family of outer membrane proteins expressed in gram-negative bacteria, two of which have been associated with virulence-related phenotypes: PagC, required by *S. typhimurium* for survival in macrophages and for virulence in mice; and Ail, a product of the *Yersinia enterocolitica* chromosome capable of mediating bacterial adherence to and invasion of epithelial cell lines. Rck, most closely related to PagC, represents the third outer membrane protein in this five-member family with a distinct virulence-associated phenotype.

The outer membrane proteins (OMPs) of gram-negative bacteria provide specialized functions enabling the organisms to interact with the environment, promoting survival. For pathogenic bacteria, a number of specific proteins required for virulence are located in the outer membrane. Gram-negative organisms causing invasive disease must resist the bactericidal action of complement present in normal human serum (22, 46). The outer membrane is the initial site of interaction between complement and the bacterial cell, and both the structure of the lipopolysaccharide (LPS) and the presence of specific OMPs have been shown to be critical determinants of bacterial resistance to complement (22). In *Salmonella* spp., the number and length of the LPS side chains (O antigen) play a major role in complement resistance, since long-chain LPS leads to complement activation at a distance from the cell surface and precludes insertion of the membrane attack complex in the outer membrane (23, 24).

Several genes encoded by *Salmonella* virulence plasmids have been implicated in serum resistance. These plasmids, 50 to 100 kb in size, are present in several invasive *Salmonella* serotypes, and all appear to contain a common 8.4-kb region required for progressive infection of the reticuloendothelial system, leading to septicemia and death in experimental animals (14). Genetic loci specifying serum resistance map outside this core virulence region and include a *traT* allele (42), a determinant affecting LPS structure (26), and the *rsk* region, which enhances the ability of smooth *S. typhimurium* to grow in serum when the virulence plasmid is integrated in the chromosome (50, 51).

A distinct complement resistance locus was cloned from the *S. typhimurium* virulence plasmid by J. Hackett and colleagues (15). These workers showed that pADE016, containing a 2.4-kb *Cla*I-*Pst*I fragment in a multicopy cosmid vector, confers high-level complement resistance to a serum-sensitive *Escherichia coli* K-12 strain and to the rough, plasmid-cured *S. typhimurium* derivative of the wild-type parent strain (15). In addition, they found that expression of the serum resistance phenotype by pADE016 was associated with the production of an OMP. We have recently investigated the mechanisms by which this locus (designated *rck*, for resistance to complement killing) mediates serum resistance in gram-negative bacteria (17). These studies suggest that the *rck* gene product prevents complement-mediated bacteriolysis by inhibiting effective insertion of the terminal C5b-9 membrane attack complex into the gram-negative outer membrane, a mechanism of complement resistance similar to that noted in serum-resistant strains of *Neisseria gonorrhoeae* (25) but not previously described for *Salmonella* strains.

The present study reports the location and sequence of the *rck* gene, identifies the *rck* gene product, a 17-kDa outer membrane protein, and further confirms the association of *rck* with high-level serum resistance. Analysis of the predicted amino acid sequence of Rck reveals that it is homologous to a family of OMPs which includes PagC, an *S. typhimurium* chromosomal gene product required for in vitro macrophage survival and for virulence in mice (31, 41), and Ail, an OMP encoded by the *Yersinia enterocolitica* chromosome and associated with adherence to and invasion of epithelial cell lines (32, 33). The additional members of this family are the bacteriophage lambda OMP Lom expressed in *E. coli* (1) and OmpX of *Enterobacter cloacae* (44, 45).

MATERIALS AND METHODS

Bacterial strains. *E. coli* JA221 (*leuB trpE5 lacY recA hsdR hsdM*$^+$) (13) was used as the host for the subcloning of pADE016; *E. coli* DH5α [F-80d*lacZ*M15 Δ(*lacZYA-argF*)

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U169 recA1 endA1 hsdR17 supE44 thi-1 gyrA relA] served as the host for *rck* constructs in pUC18 and pUC19. For triparental matings, the helper plasmid pRK2073 (Tp^r Sm^r Tra^+) was maintained in *E. coli* MV12 (*trpE5 recA*) (18). For sequencing, *E. coli* JM101 (16) was used for the bacteriophages M13mp18 and M13mp19, and *E. coli* DH5α was used for pUC18 (53). *S. typhimurium* J42 (*recA1 srl*::Tn*5 leuB129 hisC527*), a rough derivative of LT2 strain *S. typhimurium* P9144 cured of the virulence plasmid, was used to test the serum sensitivity of pADE016 and pUC18rck derivatives and for OMP preparations. *E. coli* O111:B4, a wild-type, serum-sensitive strain (10), and *E. coli* JA221 were used to confirm the results obtained with *S. typhimurium* J42.

DNA isolation and manipulations. CsCl-purified plasmid DNA was prepared by the cleared-lysate procedure (47). Rapid isolation for plasmid clone analysis, cleavage of DNA with restriction endonucleases, gel purification of DNA fragments, use of the Klenow fragment of DNA polymerase, ligations, and transformations were performed by standard methods (28).

Plasmids and methods of gene transfer. pADE016 (Tc^r Km^r, pBR322 and R6K replicons), containing a 2.4-kb *Cla*I-*Pst*I fragment from the *S. typhimurium* virulence plasmid, was kindly provided by J. Hackett, University of Adelaide, Australia. This fragment contained the serum resistance locus *rck*, described elsewhere (15). Transformation of *E. coli* JA221 with pADE016 and of *E. coli* DH5α with pUC18 and pUC19 was accomplished by standard methods with calcium chloride preparations of competent cells (7); transformation of *S. typhimurium* J42 with pUC18 and pUC19 constructs was accomplished by the method of Dagert and Ehrlich (9). Transfer of pADE016 derivatives by conjugation from *E. coli* JA221 into *E. coli* O111:B4 and *S. typhimurium* J42 was accomplished by a triparental mating system with the helper plasmid pRK2073 (in *E. coli* MV12), which is capable of high-frequency mobilization, as described previously (3); transconjugants were shown not to contain the helper plasmid pRK2073 by confirming sensitivity to trimethoprim (100 μg/ml). The presence of the desired plasmid and the absence of the helper plasmid were confirmed in all transformants and transconjugants by restriction endonuclease digests of plasmid DNA.

Serum sensitivity studies. Normal human serum (NHS) was collected from 10 healthy volunteers, pooled, and stored at −70°C until use (pooled NHS [PNHS]). Control sera were depleted of complement by heating at 56°C for 30 min (heat-inactivated [HI] sera). Serum sensitivity studies were performed on *E. coli* JA221, *E. coli* O111:B4, and *S. typhimurium* J42 by the Fothergill method (29). Serial 10-fold dilutions of mid-log phase bacteria suspended in Dulbecco's phosphate-buffered saline were incubated at 37°C, at a final serum concentration of 50% (50% PNHS or 50% HI), for 60 min and plated on tryptic soy agar. Serum sensitivity was calculated as the difference in colony-forming units surviving incubation in PNHS and in HI sera, and this relative loss of viability is expressed as log kill.

Outer membrane protein preparations and SDS-PAGE. Triton X-100-insoluble outer membranes were isolated from *E. coli* and *Salmonella* strains by a modification of the method of Portnoy et al. (40). Briefly, 15 ml of stationary-phase broth cultures was centrifuged, and the bacterial sediment was suspended in 1.5 ml of 10 mM Tris (pH 7.5) with 5 mM $MgCl_2$, and 1 mM phenylmethylsulfonyl flouride (PMSF) added. The bacteria were lysed by sonication, and unbroken cells were removed by centrifugation for 1 min at 15,000 × *g* in an Eppendorf 5415C microcentrifuge. The supernatant fluid was sedimented for 30 min in the microcentrifuge at 4°C, and the supernatant was discarded. The sediment was resuspended in 300 μl of 10 mM Tris (pH 8.0) with 5 mM $MgCl_2$ and 2% Triton X-100; 1 mM PMSF was added, and the samples were incubated on ice for 20 min with frequent agitation. The samples were then sedimented for 30 min at 4°C in a microcentrifuge, the supernatant was discarded, and the sediment was resuspended in 40 μl of TBS (20 mM Tris-buffered saline [pH 7.6], 150 mM sodium chloride) with 1 mM PMSF, 1 μM *trans*-epoxy-succinyl-L-leucylamido(4-guanidino)butane, and 10 μM tosyl lysine chloromethyl ketone. Equal amounts of protein (determined by use of the Bio-Rad protein assay kit, with bovine serum albumin as the standard) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 7.5 to 15% polyacrylamide gradient gel under reducing conditions and stained with Coomassie blue R.

DNA sequencing. The *rck* fragment was cloned from pADE016 into M13mp18, M13mp19, and pUC18. The DNA sequence was determined by the dideoxynucleotide chain termination method of Sanger et al. (43) with [α-^{35}S]dATP and modified T7 DNA polymerase, provided in a kit (Sequenase; USB Corp.). The 17-base universal primer provided in the Sequenase kit and a set of *rck*-specific synthetic oligonucleotides synthesized on an Applied Biosystems DNA synthesizer were used as sequencing primers. The complementary strand was sequenced to confirm all sequences. DNA and protein sequences were analyzed with the DNASIS and PROSIS programs (Hitachi Software Engineering Company, Ltd.). The GenBank and EMBL data bases were searched for homologous amino acid sequences by computer with the FASTA program (39), and percent identity was also compared with this program. The MULTILIN multiple-alignment program (8) was used to assess the sequence similarities, and the RDF2 program (39) was used to calculate the comparison scores. Construction of phylogenetic trees was performed as described by Doolittle and Feng (11) and Feng and Doolittle (12).

N-terminal sequence analysis. Proteins transferred to polyvinylidene difluoride membranes were sequenced with an Applied Biosystems 470 A sequenator equipped with a Blott cartridge and a model 120 on-line high-pressure liquid chromatograph and the 03CBLT program provided by the manufacturer (19). Data resolution was done with a Perkin Elmer 7500 computer equipped with Chrom 3 software.

Nucleotide sequence accession number. The nucleotide sequence of the *rck* gene has been deposited in the GenBank data library under accession number M76130.

RESULTS

Map of the *Cla*I-*Pst*I *rck*-containing fragment and orientation of transcription. The cosmid clone pADE016 (Fig. 1A) contains a 2.4-kb *Cla*I-*Pst*I fragment carrying the *rck* locus, originally thought to consist entirely of DNA from the *S. typhimurium* virulence plasmid (15). DNA sequencing determined that the *Cla*I-*Pst*I fragment contained 2,384 bp but revealed that the first 358 bp, starting at the *Cla*I site, were in fact identical to bp 23 to 380 of plasmid pBR322, indicating that the pBR322-derived area of pcosEMBL2, from the *Eco*RI to the *Bam*HI site, had remained in pADE016 and was not eliminated as believed. This region contains the promoter for the tetracycline resistance gene in pBR322. Therefore, the actual *rck* fragment was found to contain 2,026 bp of DNA, extending from the *Bam*HI to the *Pst*I restriction sites, as shown in Fig. 1. The three significant

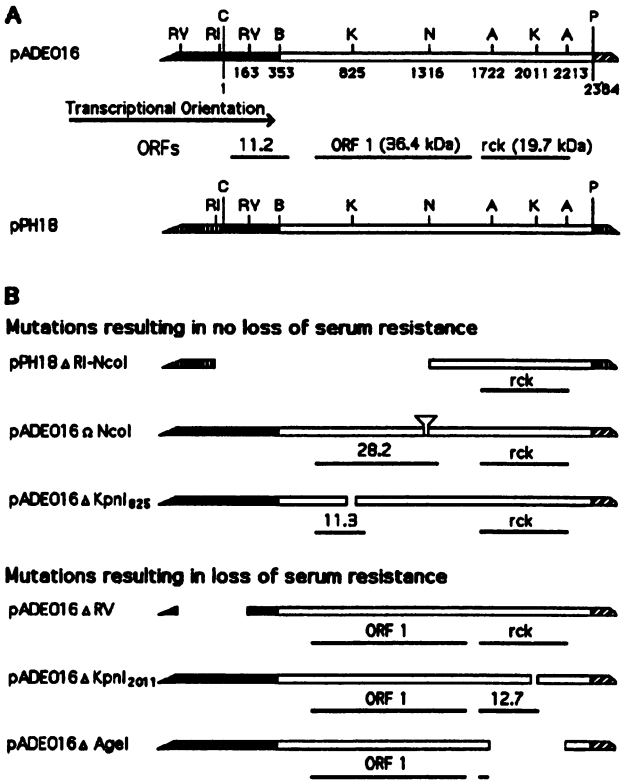

FIG. 1. Endonuclease restriction map of the *Cla*I-*Pst*I fragment from the *S. typhimurium* P9144 virulence plasmid. (A) Map of the 2,384-bp fragment in pADE016 and in pPH18, including transcriptional orientation and putative ORFs. Included are adjacent regions in pADE016 consisting of DNA from pcosEMBL2 (solid box) and pBR322 (diagonal stripes); pUC18 DNA in pPH18 is also shown (vertical stripes). The arrow below pADE016 shows the correct transcriptional orientation. Also shown are the three putative ORFs and calculated molecular masses of the encoded products. (B) Mutations in pADE016 and pPH18, classified by effects on serum resistance, showing the sizes of truncated ORFs. Endonuclease restriction sites: RV, *Eco*RV; RI, *Eco*RI; C, *Cla*I; B, *Bam*HI; K, *Kpn*I; N, *Nco*I; A, *Age*I; P, *Pst*I; H, *Hin*dIII. *Eco*RV restriction sites internal to the fragment are not shown.

open reading frames (ORFs), transcribed in the *Cla*I to *Pst*I direction, are shown in Fig. 1A as well.

Transcriptional orientation of *rck* and its dependence on external promoter activity in pADE016 and pPH18. Hackett et al. (15) presented evidence that expression of *rck* requires that transcription proceed in the *Cla*I to *Pst*I direction, as shown by cloning the 2.4-kb *Cla*I-*Pst*I fragment into both pUC8 and pUC9. We confirmed these observations by cloning the same fragment into pUC18 (construct designated pPH18) and pUC19 (pPH19); only transcription proceeding from the external *lac* promoter in the *Cla*I to *Pst*I direction (e.g., in pPH18) resulted in expression of serum resistance (Table 1).

These results suggest that transcription of the cloned *rck* locus is dependent on an external promoter, most likely supplied by the pBR322 tetracycline resistance promoter region in the case of pADE016. In support of this hypothesis, deletion of an *Eco*RV fragment containing this promoter (construct pADE016ΔRV) abolished the expression of serum resistance (Table 1). However, the *rck* phenotype was restored by cloning the *Bam*HI-*Pst*I fragment downstream from the *lac* promoter in pPH18 (construct pPH18Δ*Bam*HI; data not shown). These findings indicate that the pBR322 DNA present in pADE016 is not required for the expression of serum resistance if the *rck*-containing fragment is cloned downstream from an external promoter.

TABLE 1. Effect of pADE016 and pPH derivatives on serum sensitivity of *S. typhimurium* J42[a]

Plasmid present	Serum sensitivity (log_{10} CFU killed)
None	6.29 (0.87)
pADE016ΔRV	6.49 (0.36)
pADE016Ω*Nco*I-1316	0.03 (0.25)
pADE016Δ*Kpn*I$_{825}$	0.38 (0.26)
pADE016Δ*Kpn*I$_{2011}$	6.06 (0.66)
pADE016Δ*Age*I	6.63 (0.28)
pADE016	0.01 (0.18)
pUC18	6.39 (0.31)
pUC19	6.59 (0.30)
pPH18	0.29 (0.27)
pPH19	6.49 (0.11)
pPH18ΔRI-*Nco*I	0.27 (0.25)

[a] The values for *S. typhimurium* J42 with no plasmid and *S. typhimurium* with pADE016 represent the mean of nine and six experiments, respectively. All other values represent the mean of three experiments. The standard deviation for each value is given in parentheses.

Sequence of the *Cla*I-*Pst*I fragment and location of the *rck* gene. The DNA sequence of the *Cla*I-*Pst*I 2,384-bp fragment revealed three putative ORFs, consistent with the *Cla*I to *Pst*I transcriptional orientation required for *rck* expression; these ORFs were calculated to encode proteins of 11.2, 36.4, and 19.7 kDa (Fig. 1A). The 11.2-kDa product was a potential fusion protein consisting almost entirely of the N-terminal region of the pBR322 tetracycline resistance inner membrane protein. This potential protein was shown not to be required for the *rck* phenotype, since its elimination in pPH18Δ*Bam*HI resulted in no significant diminution of serum resistance.

The two ORFs in the remaining 2.0-kb fragment (ORF 1 and *rck*; Fig. 1) were evaluated as potential candidates for the *rck* gene. The unique *Nco*I site at bp 1316 in ORF 1 was cleaved, and the overhanging 5′ ends were filled in with deoxynucleotides by use of the Klenow fragment of DNA polymerase and religated; the expected 4-bp insertion was confirmed by DNA sequencing, and the derivative was designated pADE016Ω*Nco*I-1316 (Fig. 1B). Analysis of this altered DNA sequence indicated that the 36.4-kDa protein would be truncated to 28.2 kDa owing to a frameshift-induced translational stop. Despite the alteration in this putative protein, no effect on serum resistance was noted (Table 1), suggesting that the C terminus of the 36.4-kDa ORF 1 product was not required for *rck* expression.

To determine whether sequences in ORF 1 proximal to the *Nco*I site might still be capable of expressing serum resistance, the region extending from the *Eco*RI site in the pUC18 multiple cloning site in pPH18 to the unique *Nco*I site in the *rck* fragment was deleted (construct designated pPH18ΔRI-*Nco*I) (Fig. 1B). This deletion still expressed complete serum resistance when transformed into *S. typhimurium* J42 (Table 1) and in *E. coli* JA221 (data not shown), indicating that DNA remaining in the region defined by the *Nco*I and *Pst*I restriction sites was sufficient for full expression of the *rck* phenotype.

These results indicated that the ORF specifying the 19.7-

kDa product (19.7-kDa ORF) was the most likely candidate for the *rck* gene. Further deletion analysis was carried out in pADE016, since we found that strains containing pPH18 and its derivatives, although capable of expressing high-level serum resistance, grew slowly, making comparison of serum resistance between strains difficult. Two unique *Age*I restriction sites were used to construct pADE016Δ*Age*I by deleting the internal 492-bp *Age*I fragment within the 555 bp encoding the 19.7-kDa ORF (Fig. 1A); this deletion led to complete loss of serum resistance when placed in *S. typhimurium* J42 (Table 1) and in *E. coli* JA221 (data not shown).

To further confirm that the 19.7-kDa ORF was responsible for the *rck* serum resistance phenotype, two separate 4-bp deletions were introduced in the ORFs predicted to encode proteins of 36.4 and 19.7 kDa. pADE016 was linearized by partial digestion with *Kpn*I, and the 3′ overhanging ends of the cleaved restriction sites were removed and blunted by the exonuclease activity of the Klenow fragment; these were ligated and transformed. This resulted in two plasmids, one containing a 4-bp deletion at the former *Kpn*I site at bp 825 in the 36.4-kDa ORF (designated pADE016Δ*Kpn*I$_{825}$), the other with a similar 4-bp deletion at the former *Kpn*I site at bp 2011 in the 19.7-kDa ORF (designated pADE016Δ*Kpn*I$_{2011}$) (Fig. 1A). In both cases, the ORFs underwent frameshift mutations, leading to ORFs predicted to encode truncated proteins of 11.3 and 12.7 kDa, respectively (confirmed by nucleotide sequencing). This marked truncation of ORF 1 in pADE016Δ*Kpn*I$_{825}$ resulted in no loss of serum resistance, whereas in pADE016Δ*Kpn*I$_{2011}$, a similar truncation in the ORF predicted to encode the 19.7-kDa protein resulted in complete loss of serum resistance (Table 1), confirming that the 19.7-kDa ORF was required for expression of the *rck* phenotype.

Effect of deletion mutations on expression of a 17-kDa OMP. Hackett et al. (15) found an OMP, reported as 11 kDa, associated with the *rck*-encoded serum resistance phenotype. Using a different OMP preparation based on Triton X-100 insolubility, we found that the only OMP uniquely associated with *E. coli* and *Salmonella* strains containing pADE016 was approximately 17 kDa in size. To determine whether the 19.7-kDa ORF encoded this 17-kDa OMP, OMP fractions of *S. typhimurium* J42, with and without the plasmids listed in Table 1, were prepared as described above and electrophoresed on an SDS-PAGE gradient gel (7.5 to 15% polyacrylamide) under reducing conditions.

A representative Coomassie-stained gel is shown in Fig. 2. Lane 1, containing an OMP preparation from the plasmid-cured strain *S. typhimurium* J42, reveals a faint band in the 17-kDa range, suggesting a small amount of a native, chromosomally encoded OMP in this range. Lanes 8 and 10, with similar preparations from *S. typhimurium* J42(pUC18) and *S. typhimurium* J42(pPH19) (with the 2.4-kb *rck*-containing fragment in the incorrect transcriptional orientation), show similar findings. All three of these strains were highly serum sensitive (Table 1). Lane 2 contains an OMP preparation from *S. typhimurium* J42(pADE016ΔRV), lacking 1.0 kb of DNA from the cosmid vector pcosEMBL2, including the putative external promoter from the tetracycline resistance gene, as discussed above. This construct was also serum sensitive and lacked a significant 17-kDa protein band, whereas the pPH18ΔRI-*Nco*I-containing strain was serum resistant and showed a heavy 17-kDa protein band (lane 11), indicating that the region deleted from pcosEMBL2 in pADE016ΔRV was unnecessary for expression of the *rck* phenotype and the 17-kDa OMP when another external

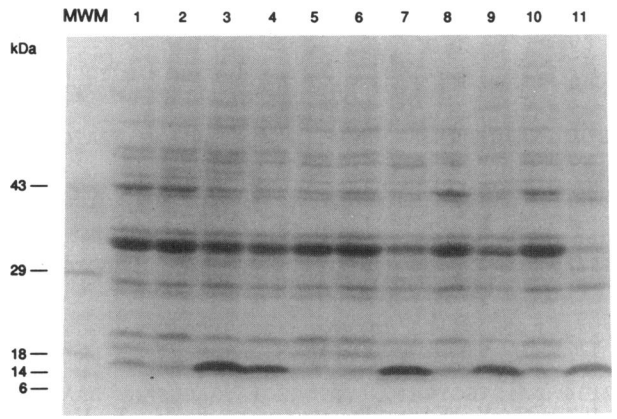

FIG. 2. Effect of mutations in pADE016 and pPH18 on expression of a 17-kDa OMP. SDS-PAGE (7.5 to 15% gradient) was performed on Triton X-100-insoluble outer membrane fractions of *S. typhimurium* with and without plasmids and stained with Coomassie blue R. Lane 1, *S. typhimurium* J42 with no plasmid; lane 2, with pADE016ΔRV; lane 3, with pADE016Ω*Nco*I-1316; lane 4, with pADE016Δ*Kpn*I$_{825}$; lane 5, with pADE016Δ*Kpn*I$_{2011}$; lane 6, with pADE016Δ*Age*I; lane 7, with pADE016; lane 8, with pUC18; lane 9, with pPH18; lane 10, with pPH19; lane 11, with pPH18ΔRI-*Nco*I. Low-molecular-mass markers are shown at the left of the figure. Each lane was loaded with 30 mg of protein.

promoter was available, such as the *lac* promoter in pPH18ΔRI-*Nco*I.

Lanes 3, 4, 7, and 9 (OMP preparations from strains containing pADE016Ω*Nco*I, pADE016Δ*Kpn*I$_{825}$, pADE016, and pPH18) also all show evidence of an intensely staining OMP band approximately 17 kDa in size; these strains were all associated with high-level serum resistance (Table 1), and all contained intact 19.7-kDa ORFs. However, OMP preparations from strains containing pADE016Δ*Kpn*I$_{2011}$ and pADE016Δ*Age*I (lanes 5 and 6), with deletion mutations resulting in truncation of the 19.7-kDa ORF, revealed loss of this 17-kDa OMP, with only faint bands remaining, comparable to those seen in the control strains. Confirmation of these results was obtained with *E. coli* O111:B4 and *E. coli* JA221 containing pADE016Δ*Kpn*I$_{825}$ and pADE016Δ*Kpn*I$_{2011}$ (data not shown). Mutations in the 19.7-kDa ORF are uniquely associated with loss of both the *rck* serum resistance phenotype and the 17-kDa OMP, indicating that the 17-kDa OMP is most likely the product of the *rck* gene (the 19.7-kDa ORF) and the probable mediator of *rck*-encoded serum resistance.

Separation of the *rck*-associated 17-kDa band from the faint band seen in the same range in strains lacking *rck* was observed in 15% polyacrylamide SDS-PAGE gels (data not shown), suggesting that the latter band is not likely to represent a chromosomally encoded Rck protein.

Sequence analysis of the *rck* gene. The nucleotide sequence from bp 1577 to 2384 of the original 2,384-bp *Cla*I-*Pst*I fragment is shown in Fig. 3. The proposed ATG start is preceded by a ribosome-binding site (GGAG at bp −8), and the predicted protein sequence contains 24 N-terminal amino acid residues consistent with established signal sequences found in prokaryotic OMPs (52). Cleavage of these 24 residues would result in a final OMP of approximately 17.4 kDa, consistent with the *rck*-encoded 17-kDa OMP found by SDS-PAGE. Comparison of the *rck* nucleotide sequences and the Rck amino acid sequences revealed no significant homology to any of the known *Salmonella* virulence plas-

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                                                                                 Age I
1576 AGGATGAAGCGGCGTTACGGGTGTGCAGGAGCAGAAAGCACACCTTCTGTCATAACACAATGAACTTAACTGTGTTCAGGGAGTTTTATCATG
   1                                                                                                M
1669 AAAAAAATCGTTCTGTCCTCACTGCTGCTGTCCGCAGCCGGGCTGGCTGCCGTACCGGTGGCACAGGCTGACACCCATTCCGTGTCGGTGGGA
   2  K  K  I  V  L  S  S  L  L  L  S  A  A  G  L  A  A  V  P  V  A  Q  A  D  T  H  S  V  S  V  G
1762 TATGCCCAGAGCCGGATAGAGCATTTTAAGGATATCCGTGGGGTGAACCTGAAATACCGCTATGAGGCTCAGACGCCGCTGGGACTGATGGCG
  33  Y  A  Q  S  R  I  E  H  F  K  D  I  R  G  V  N  L  K  Y  R  Y  E  A  Q  T  P  L  G  L  M  A
1855 TCGTTCAGTTGGCAGTCAGGTAAGCGCGGAGAGTCCGGTGGCATTCCTGGCGGAATGAGCTGGCGTGATGATGTGAAGGCGACGTACTGGTCG
  64  S  F  S  W  Q  S  G  K  R  G  E  S  G  G  I  P  G  G  M  S  W  R  D  D  V  K  A  T  Y  W  S
                                                                                 Kpn I
1948 CTGATGGCGGGTCCGGCTGTCCGTGTGAACGAGCTGGTATCTCTGTATGCACTGGCCGGTGCCGGTACCGGCAGGGCTGAAGTGAAAGAGCGT
  95  L  M  A  G  P  A  V  R  V  N  E  L  V  S  L  Y  A  L  A  G  A  G  T  G  R  A  E  V  K  E  R
2041 ATCAGCATGCCGGGATACAACGGGCGGTTCACGGGTTCGGAGCGCAGAACGGGGTTTGCCTGGGGAGCCGGCGTACAGTTTAATCCGGTGGAA
 126  I  S  M  P  G  Y  N  G  R  F  T  G  S  E  R  R  T  G  F  A  W  G  A  G  V  Q  F  N  P  V  E
                                                                                       Age I
2134 AATGTGGTCATCGATCTGGGCTATGAGGGAAGTAAAGTTGGCGCAGCGAAACTGAACGGCGTTAACGTTGGTGTCGGTTACCGGTTCTGATAA
 157  N  V  V  I  D  L  G  Y  E  G  S  K  V  G  A  A  K  L  N  G  V  N  V  G  V  G  Y  R  F
2227 CGGAGAGCAGGAAAGGGAGCGGAGCCGCAGGCTTCCGCTCCCGGTCAGATATGATGAAAAACATAACATTCAGCGCATCTTTTTTCAGGGAAT
2314 TATTCTGGTTTGTGGTGATGGCAGGAAGCTGGTTTGTGGTGTGGTACATGCCCGGGCTGCTGCAG
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FIG. 3. Sequence analysis of the *rck* gene. The nucleotide sequence of the original *Cla*I-*Pst*I fragment is shown from bp 1576 through 2384. A potential ribosome-binding site is underlined, and nucleotides identical to the consensus sequence are in boldface letters. The *rck* gene begins at bp 1666 with the methionine start codon; included in the sequence are internal *Age*I and *Kpn*I endonuclease restriction sites. The amino acid sequence is 185 residues long; the shaded area represents the signal sequence, ending at the probable cleavage site. There are no consensus −10 or −35 boxes indicative of prokaryotic promoter sequences between ORF 1 (terminating at bp 1575) and the beginning of *rck*.

mid-related serum resistance loci (*traT*, *rsk*) or to the eukaryotic proteins, such as homologous restriction factor 20 and decay-accelerating factor, known to be associated with control of complement-mediated lysis (30, 37, 42, 50, 51).

N-terminal amino acid sequence of Rck. The eight N-terminal amino acid residues of the 17-kDa protein present in *rck*-containing strains were determined as described in Materials and Methods. This sequence, D-T-H-S-V-S-V-G, was identical to that predicted from the *rck* DNA sequence if the 185-amino-acid polypeptide was cleaved after residue 24 (Fig. 3). These data confirmed that the 17-kDa protein associated with *rck*-containing strains is the product of the *rck* gene and further suggest that the precursor polypeptide is processed in a manner consistent with export of this protein to the outer membrane.

Homology between Rck and outer membrane proteins PagC, Ail, OmpX, and Lom. Computer searches for homologous amino acid sequences in the GenBank and EMBL data bases revealed homology between Rck and four other OMPs: PagC, an 18-kDa OMP encoded by an *S. typhimurium* chromosomal gene regulated by the two-component transcriptional activator *phoP* and associated with survival in macrophages and with virulence in mice (31, 41); Ail, an OMP with a precleavage calculated molecular mass of 19.5 kDa, encoded by a *Y. enterocolitica* chromosomal locus and associated with epithelial cell adherence and invasion in vitro (32, 33); OmpX, an 18-kDa OMP encoded by a chromosomal gene of *Enterobacter cloacae*, the function of which is not yet known (44, 45); and Lom, an OMP with a precleavage calculated molecular mass of 21.8 kDa, encoded by lysogenic bacteriophage lambda, the function of which is also unknown (1). These five OMPs and their regions of amino acid identity with Rck are shown in Fig. 4. The relative degree of homology between pairs of these five prokaryotic outer membrane proteins is expressed as percent identity in Table 2. Notable is that, of the five OMPs, Rck and PagC are most similar (53.3% identity). Lom, on the other hand, is the least similar to the other four (28.1 to 32.1% identity). A proposed phylogenetic tree, constructed by the method of Feng and Doolittle (12), is shown in Fig. 5. Rck and PagC, both encoded by loci found in *S. typhimurium*, form a clustered pair, as do Ail and OmpX, encoded by chromosomal loci of the enterobacteria *Y. enterocolitica* and *Enterobacter cloacae*, respectively. The greatest evolutionary divergence appears to be between Lom, encoded on lysogenic bacteriophage lambda, and the Ail-OmpX clus-

```
Rck   MKKIV LSSLLLSAAGLAAVPVAQADTHSVSVG    YAQSRI     EHF    KDIRGVNLKYRYEAQ TP
PagC  MKNII LSTLVITTRVL VVNVAQADTNAFSVGYARYAQSKV     QDF    KNIRGVNVKYRYEDD SP
OmpX  MKKIACLSALAAVLAV   SAGTAVAATSTVTGG    YAQSDM     QG VM NKTNGFNLKYRYEQDNNP
Ail   MKKTLLASSLIACLSIA SVNVYAASESSISIG    YAQSHVK   ENGYTLDNDPKGFNLKYRYELD DN
Lom   MRNVCIAVAVFAALAVTVTPARAEGGHGTFTVGYFQVKPGTLPSLSGGDTGVSHLKGINVKYRYELTDZS

Rck   LGLMASFSWQSGKRGESGGI PGGMSWRDDVKATYWSLMAGPAVRVNELVSLYALAGAGTGR       AEV
PagC  VSFISSLSYLYGDRQASGSVEPEGIHYHDKFEVKYGSLMVGPAYRLSDNFSLYALAGVGTVK       ATF
OmpX  LGVIGSFTYTEKDRTENGS          YN    KGQYYGITAGPAYRLNDWASIYGVVGVGYGK         F
Ail   WGVIGSFAYTHQGYDFFYG     SNKFGHG DVDYYSVTMGPSFRINEYVSLYGLLGAAHGK
Lom   VGVMASLGFAASKKSSTVMTGEDTFHY ESLRGRYVSVMAGPVLQISKQVSAYAMAGVAHSRWSGSTMDY

Rck   KERISMPGY          NGRFTGSERRTGFAWGAGVQFNPVENVVIDLGYEGSKVGAAKLNGVNVGVGYRF
PagC  KEHSTQDGD          SFSNKISSRKTGFAWGAGVQMNPLENIVVDVGYEGSNISSTKINGFNVGVGYRF
OmpX  QQ    TENQ         GLNRTASNSDYGFSYGAGMQFNPIENVALDFSYEQSRIRNVDVGTWIAGVGYRF
Ail         VKAS         VFDESISASKTSMAYGAGVQFNPLPNFVIDASYEYSKLDSIKVGTWMLGAGYRF
Lom   RKTEITPGYMKETTTARDESAMRHTSVAWSAGIQINPAASVVVDIAYEGSGSGDWRTDGFIVGVGYKF
```

FIG. 4. Homology between OMPs Rck, PagC, OmpX, Ail, and Lom. Shading indicates amino acid residues identical to those in Rck.

TABLE 2. Sequence comparisons of the OMPs Rck, PagC, Ail, OmpX, and Lom by percent identity[a]

OMP	% Identity			
	PagC	Ail	OmpX	Lom
Rck	53.3 (51)	42.7 (34)	38.3 (29)	32.1 (31)
PagC		39.2 (26)	37.1 (24)	31.6 (28)
Ail			43.5 (31)	29.2 (5)
OmpX				28.1 (5)

[a] Values in parentheses denote the number of standard deviations higher than those obtained with 50 comparisons of randomized sequences of these protein segments. Percent identities with higher numbers of these standard deviations are of greater significance.

tered pair. Computerized hydropathy plots (27) for all five protein sequences revealed similar, slightly hydrophilic profiles, marked by alternating hydrophobic and hydrophilic regions, a pattern shared by other gram-negative OMPs, including porins (49).

DISCUSSION

A fragment of the *S. typhimurium* virulence plasmid containing the *rck* locus, when cloned in the recombinant cosmid pADE016, was shown to confer high-level complement resistance on both rough and smooth *E. coli* and *Salmonella* strains and was associated with the production of an OMP (15). By using the entire 2.4-kb fragment containing *rck* as a probe, homologous sequences were found in a variety of *Salmonella* virulence plasmids other than that of *S. typhimurium*, including *S. dublin* and *S. enteritidis*, but not on those of *S. cholerae-suis* (17).

We determined the nucleotide sequence of the fragment of the *S. typhimurium* virulence plasmid containing the *rck* locus, finding two candidate ORFs, ultimately designated ORF 1 and *rck*. Mutations in the *rck* ORF were associated with loss of the serum resistance phenotype as well as loss of a 17-kDa OMP, whereas mutations in ORF 1 had no effect. Sequence analysis of the 555-bp *rck* gene revealed that the Rck OMP consists of 185 amino acid residues, with a calculated molecular mass of 19.7 kDa. Cleavage of a 24-amino-acid N-terminal prokaryotic signal sequence was projected to reduce the final membrane protein to 17.4 kDa, consistent with the 17-kDa OMP demonstrated by electrophoresis. N-terminal sequencing of the 17-kDa protein confirmed that the N terminus of the mature Rck protein was identical to that predicted from the *rck* nucleotide sequence following cleavage of the signal sequence. We conclude that the complement resistance mediated by pADE016 is due to a single gene encoding the Rck OMP.

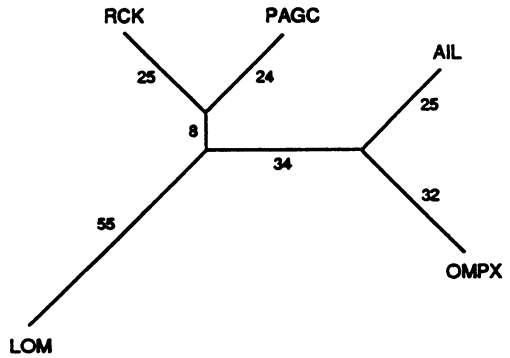

FIG. 5. Proposed phylogenetic tree for the five members of the OMP family, based on computer analysis of probable branching order and clustering. Relative numerical values for branch lengths are shown; the sums of branch lengths represent calculated distance scores between protein sequences.

Although a major determinant of serum resistance in gram-negative bacteria appears to be the presence of long-chain LPS (23, 24), a variety of protein products, including OMPs, have also been correlated with expression of serum resistance (4, 25, 35, 36, 48). The *S. typhimurium* virulence plasmid *traT* allele, associated with serum resistance when introduced into rough, cured *S. typhimurium*, encodes a 27-kDa OMP (42). In addition, it has recently been determined that bacteriophage lambda contains a gene, designated *bor*, that is highly homologous to the *iss* serum resistance locus of the ColV2-K94 plasmid (1, 6). During lysogeny in *E. coli* K-12 strains, *bor* encodes a 10.4-kDa OMP associated with serum resistance. Our sequence analysis of *rck* reveals no significant homology with published sequences of either the *traT*, *bor*, or *iss* genes and proteins. However, a second bacteriophage lambda gene, *lom*, described concurrently with *bor*, was found to express an OMP (calculated precleavage molecular mass of 21.8 kDa) during lysogeny in *E. coli* K-12 (1). No phenotype was associated with the Lom protein; unlike the *bor* gene product, it did not mediate serum resistance.

Interestingly, we found that the Rck 17-kDa serum resistance protein was similar to Lom, with 32.1% amino acid identity (1). In addition, Rck was homologous to a family of OMPs to which Lom belongs. Two of these have virulence-associated phenotypes distinct from serum resistance. PagC is the 18-kDa OMP product of the chromosomal *S. typhimurium* gene *pagC*, regulated by the two-component transcriptional activator *phoP*. The expression of *pagC* has been shown to be required for *S. typhimurium* survival in cultured macrophages and for virulence in mice (31). Sequence comparison of Rck with PagC revealed that these two proteins are very similar, with 53.3% identity at the amino acid level, and that they are the most closely related of the five OMPs in the family (41).

The *Y. enterocolitica* gene *ail* is one of two chromosomal loci (the other is the invasin gene *inv*) associated with adherence and invasion of cultured epithelial cells in vitro (33). When cloned into a recombinant pBR322 plasmid, *ail* mediates the adhesion and invasion of *E. coli* HB101 into certain cultured epithelial cell lines. In addition, the presence of *ail* has been closely associated with disease-causing *Y. enterocolitica* serotypes (34). The *ail* gene encodes an OMP with a molecular mass of 17 kDa (32). The Ail protein is 42.7 and 39.2% identical to Rck and PagC, respectively, based on aligned sequences.

Finally, homology searches revealed that a recently described OMP, OmpX, encoded by an *Enterobacter cloacae* chromosomal gene, is the fifth member of this protein family (44, 45). OmpX overproduction on a multicopy plasmid in *E. coli* led to a diminution in production of the porins OmpC and OmpF, apparently mediated by decreased transcription, and led to an increase in resistance to beta-lactam antibiotics, presumably due to a loss of porin channels. However, the phenotype associated with *ompX* in the native state remains to be elucidated, since deletion of the *ompX* gene from the *Enterobacter cloacae* chromosome did not alter cell growth, porin production, or beta-lactam susceptibility. The effects of OmpX on serum resistance or other virulence phenotypes were not evaluated.

We noted that high-level Rck expression, analogous to

high-level OmpX expression, was associated with a decrease in certain other OMPs greater than that expected by normalization of protein concentrations; in particular, the 43-kDa OMP appears to be decreased in Rck-containing strains (Fig. 2, lanes 3, 4, 7, 9, and 11). However, this 43-kDa protein band is also diminished in strains with mutated *rck* genes (lanes 5 and 6); these strains are as serum sensitive as J42 and J42(pADE016ΔRV) (lanes 1 and 2), which lack Rck but have large amounts of the 43-kDa protein. Therefore, decreases in the amounts of OMPs are unlikely to entirely account for the Rck-associated increase in serum resistance.

The homologies between PagC, Ail, and Lom have been noted previously, and certain characteristics of the deduced amino acid sequences have been discussed (41). Our examination and computer analyses of the five related OMP sequences confirmed the presence of a highly conserved "signature" sequence. The proposed signature sequence, G[VIHLF]N[LVIH]KYRYE (in which the brackets indicate the presence of any one of the enclosed amino acid residues), represented by residues 46 to 54 in Rck, may be of use in future identification of other members of this protein family. This sequence is present in all five proteins but is absent from all other proteins present in the current Protein Identification Resource protein data bank.

Stoorvogel et al. (44), in their analysis of OmpX, noted that the C-terminal consensus sequence X-Z-X-Z-X-Z-X-Tyr-X-Phe (where X is any amino acid residue and Z is any hydrophobic residue) is seen in many gram-negative OMPs. A similar C-terminal region is present among all five members of the OMP family and is homologous to a variety of other OMPs, including the major OmpA proteins of *E. coli* and other enterobacteria (2, 5) and the C-terminal portion of the *E. coli* porin proteins OmpF and PhoE (20, 38), which may constitute the final membrane-spanning segment in these folded OMPs (49).

All five protein sequences, when subjected to hydropathy analysis, revealed the presence of similar alternating hydrophobic and hydrophilic regions, with the hydrophobic regions generally no more than 10 residues long. This arrangement of residues has been discussed by Stoorvogel et al. (44). A topological model, based largely on work with porins, has been proposed in which OMPs are folded across the outer membrane so that the hydrophilic maxima are exposed at the cell surface, while the membrane is spanned by 9- or 10-residue amphipathic B sheets (49). Applying this model to the five-member family of proteins, the regions most highly conserved would be predominantly membrane-spanning segments, with variations between protein sequences occurring largely in the hydrophilic regions exposed at the cell surface. However, there is uncertainty concerning how accurately this model reflects porin topology (21), and it remains to be experimentally verified in these proteins.

The variability in phenotypes expressed by the members of this protein family may be due to the differences in the hydrophilic regions exposed on the cell surface. Such a location for crucial residues seems logical for proteins involved in interaction with extrabacterial proteins, such as complement (Rck), and with eukaryotic epithelial cells (Ail) and macrophages (PagC). Mutational analysis of these regions and studies comparing the abilities of these proteins to encode the various virulence-associated phenotypes should offer further insight into the common molecular strategies employed by microorganisms in promoting invasion of host tissues and in evading defense mechanisms.

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