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Outer Membrane Protein A (OmpA) Contributes to Serum Resistance and Pathogenicity of *Escherichia coli* K-1

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We examined whether outer membrane protein A (OmpA) contributes to gram-negative pathogenesis by determining the effect of mutagenesis of *ompA* in a virulent *Escherichia coli* K-1 isolate. An OmpA mutant was generated by insertion of the transposon Tn*phoA*, which was genetically modified to increase the efficiency of its delivery by conjugation. The mutant was less virulent than its parent strain in two models of *E. coli* K-1 infection. Equal inocula of the OmpA$^+$ and OmpA$^-$ strains fed to neonatal rats resulted in a sevenfold-greater incidence of bacteremia at 72 h from the OmpA$^+$ strain. The lethal effect of the OmpA$^-$ mutant was significantly less than that of the OmpA$^+$ parent strain when inoculated onto the chorioallantoic membrane of 10-day embryonated chick eggs. There was, however, no difference between strains in growth characteristics under physiologic conditions, either in rat serum or in unembryonated chick eggs. In the presence of a 10-day chick embryo, there was a 10-fold increase in the survival and growth of the OmpA$^+$ strain. Correction of the mutation in *ompA* with an *E. coli* K-12 *ompA* gene restored a level of virulence equivalent to that of the parent strain. The *ompA* mutant was more sensitive to the bactericidal effect of pooled human serum by the classical pathway of complement activation. These results suggest that OmpA contributes to *E. coli* K-1 pathogenesis by a mechanism which may involve increased serum resistance.

Outer membrane protein A (OmpA) is one of the most abundant and most actively investigated proteins of the *Escherichia coli* K-12 cell envelope. Many functions have been attributed to the 35,000-Da, heat-modifiable, transmembrane protein in *E. coli* (reviewed in Nikaido and Vaara [18]). In addition to its nonphysiological role as a phage and colicin receptor, it serves as a mediator in F-dependent conjugation. Mutants lacking OmpA are viable and morphologically normal. However, mutants lacking both OmpA and murein lipoprotein acquire a spherical cell shape and an unstable outer membrane (31). The growth of mutants lacking OmpA is unaffected in nutrient media but defective under limiting growth conditions and at elevated temperatures (14). It remains controversial whether OmpA has a pore function.

A representative survey of gram-negative clinical isolates, including both enteric and nonenteric bacteria, showed that proteins with structures homologous to that of OmpA were present in all 17 genera tested (2). The corresponding genes from *Shigella dysenteriae* (5) and *Salmonella typhimurium* (9) have been cloned and sequenced. The deduced amino acid sequences differ from that of the *E. coli* protein by only 13 and 23 of 325 residues, respectively. It appears, therefore, that unlike other surface-exposed components of the bacterial cell envelope, OmpA has been highly conserved throughout evolution. However, none of the proposed functions for the *E. coli* OmpA would explain the unusual conservation of this class of proteins.

Previous investigations on the biological functions of OmpA have focused on laboratory strains studied exclusively in vitro. In this report we consider whether OmpA contributes to bacterial growth and pathogenesis in vivo by using a virulent isolate of *E. coli* K-1, an etiologic agent of neonatal septicemia and meningitis. An OmpA$^-$ mutant demonstrated normal growth under physiologic conditions but showed attenuated virulence in two different models of *E. coli* K-1 infection. Although not an absolute requirement for survival in vivo or the expression of virulence, the expression of OmpA appears to confer a significant selective advantage, which may explain its unusual conservation.

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. The bacterial strains, plasmids, and bacteriophages used in this study are described in Table 1. Chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise specified. Bacteria were grown in Luria-Bertani broth solidified with 1% agar. Minimal medium was prepared from M63 salts and 0.2% glucose with or without nicotinamide (5 μg/ml) (27). Antiserum agar medium contained tryptic soy broth (Difco Laboratories, Detroit, Mich.) with 1% agarose and 12% equine meningococcal B antiserum (Horse 46; provided by J. B. Robbins), which cross-reacts with the *E. coli* K-1 capsule (23). Antibiotics were added to the medium at the following concentrations unless otherwise noted; ampicillin, 100 μg/ml; tetracycline, 12.5 μg/ml; rifampin (U.S. Biochemical Co., Cleveland, Ohio), 100 μg/ml; and kanamycin, 100 μg/ml. Gene fusions to *phoA* were detected by the addition of the chromogenic substrate 5-bromo-4-chloro-3-indolyl-phosphate (XP) at 100 μg/ml to the medium.

Construction of Tn*phoA'* for delivery by conjugation. The transposon Tn*phoA* was modified by replacing the 2,774-bp drug resistance region between IS*50* elements with a 1,466-bp fragment of pUC4-K, containing the kanamycin resistance gene of Tn*903* (19). Tn*phoA* was hopped onto pUC9, which has no undesired *Bcl*I sites, as follows. *E. coli* CC118 harboring pUC9 was infected with λTn*phoA*, and transduced cells were selected by growth at 30°C for 72 h in the presence of ampicillin and kanamycin (300 μg/ml). Plasmid DNA prepared from these colonies by the alkaline lysis method (24) was used to cotransform the ampicillin and

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TABLE 1. Bacterial strains, plasmids, and bacteriophages

Strain, plasmid, or phage	Genotype or characteristics	Reference or source
E. coli K-12 derivatives		
CC118	*araD139* Δ(*ara-leu*)*7697* Δ*lacX74* Δ*phoA20 galE galK thi rpsE rpoB argE*(Am) *recA1*	15
CC118(λ*pir*)	CC118 lysogenized with λ*pir*	Collection of R. Taylor
SM10	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Kan	29
SM10(λ*pir*)	SM10 lysogenized with λ*pir*	Collection of R. Taylor
BRE51	MC4100 Δ(*sulA-ompA*)*51*	6
LE392	F⁻ *supF supE hsdR galK trpR metB lacY tonA*	27
CAG18613	MG1655 *zcc-3112*::Tn*10kan*	30
DME558	MC4100 Δ*lamB106 zcb*::Tn*10*	Collection of S. Benson
GM2199	GM30 *dam-13*::Tn*9*	16
E. coli K-1 derivatives		
RS218	O18ac:K1:H7	Strain 101 (1)
E44	RS218 spontaneous Rif^r	This study
E58	E44 *ompA*::Tn*phoA'*	This study
E66	E44 capsule⁻ (Tn*phoA'*)	This study
E68	E58 OmpA$^+$ Tet^r Kan^s	P1 (DME558) × E58
E69	E68 OmpA$^+$ Kan^r Tet^s	P1 (CAG18613) × E68
Plasmids		
pUC9	Amp^r, *ori*ColE1	34
pUC4-K	Amp^r, *ori*ColE1, cloned Kan^r gene of Tn*903*	Pharmacia (Piscataway, N.J.)
pRT733	Amp^r, *ori*R6K, *mob*RP4 Tn*phoA*	32
pRT733'	Amp^r, *ori*R6K, *mob*RP4 Tn*phoA'*	This study
pRD87	Amp^r, *ori*ColE1, cloned *ompA*	10
Bacteriophages		
λTn*phoA*		11a
λ*pir*	*pir*R6K	13
P1*vir*		27

kanamycin resistance markers into *E. coli* DH5α (Bethesda Research Laboratories, Gaithersburg, Md.) with an electroporator according to a protocol supplied by the manufacturer (Bio-Rad, Richmond, Calif.). The pUC9::Tn*phoA* plasmid was transformed into the *dam* mutant strain GM2199 to allow digestion with *Bcl*I. The plasmid was partially digested with *Bcl*I (Boehringer Mannheim, Indianapolis, Ind.), which cleaves immediately adjacent to the termination codon of the IS*50*R transposase gene as well as the inside end of the IS*50*L, removing a 2,774-bp sequence (25). The *Bam*HI fragment of pUC4-K was ligated to the *Bcl*I ends, yielding pUC9::Tn*phoA'*. The 1,476-bp *Bgl*II (New England Bio-Labs, Beverly, Mass.) fragment of pUC9::Tn*phoA'* was then used to replace the corresponding *Bgl*II fragment on the transposon delivery vector pRT733, which requires *pir* in *trans* for replication (32). The resulting plasmid, pRT733', was propagated in CC118(λ*pir*). Finally, pRT733' was transformed into SM10(λ*pir*), which is able to mobilize resident plasmids containing *oriT* at high frequency.

Construction of OmpA and capsule mutants of *E. coli* K-1. RS218 is a well-characterized, pathogenic *E. coli* K-1 strain originally obtained from a case of neonatal meningitis (1). A spontaneous rifampin-resistant mutant of RS218, E44, was used as the recipient in conjugation experiments to allow selection of the *E. coli* K-1 strain. Transposon mutagenesis of the *E. coli* K-1 strain was carried out by mating E44 with SM10(λ*pir*)(pRT733'). A total of 10^8 cells of each strain from an overnight culture were mixed on a nonselective L-broth agar plate for 6 h at 37°C. The cells were scraped from the plate and resuspended in phosphate-buffered saline (PBS), and dilutions were plated on selective medium. *E. coli* K-1 recipients carrying the transposon were selected on L-broth agar containing kanamycin, rifampin, and XP. Only about 10% of these colonies retained the donor plasmid, as indicated by their resistance to ampicillin. Blue colonies were screened after 18 h at 37°C and then 24 h at 4°C. The OmpA mutant was identified by screening the kanamycin- and rifampin-resistant colonies by a colony immunoblotting procedure (35). Polyclonal rabbit antiserum to an *E. coli* K-12 OmpA (provided by U. Henning) was absorbed with OmpA⁻ strain BRE51 prior to use at a final dilution of 1:50,000.

The capsule-deficient mutant of *E. coli* K-1, E66, was obtained as described above except that following the mating, cells were plated on antiserum agar containing rifampin and kanamycin. Colonies were screened for lack of a halo reaction after 18 h at 37°C and then 24 h at 4°C.

Correction of the mutation in *ompA*. RS218 was chosen for this study because of its known susceptibility to bacteriophage P1 (4). Different drug resistance markers located on each side of the K-12 *ompA* gene were transduced to the E58 chromosome in two separate steps. A P1*vir* lysate of DME558 (28) was used to transduce a tetracycline resistance marker to E58 by a previously described procedure (4). Among the tetracycline-resistant colonies, approximately 50% had lost resistance to kanamycin. The loss of kanamycin resistance corresponded to the reacquisition of *ompA*, as determined by Western immunoblot analysis. To ensure that the transductant (E68) was not a result of intragenic recombination within *ompA*, a kanamycin resistance marker, located on the opposite side of *ompA* from the tetracycline resistance marker, was transduced from CAG18613 to the chromosome of E68. A transductant, E69, which had acquired the kanamycin resistance marker and lost the tetracycline resistance marker was obtained. Since *ompA* is located between these two markers, E69 would be expected to have the entire *ompA* of CAG18613, an *E. coli* K-12 strain.

Characterization of mutants. Mutants were confirmed to be derived from RS218 by their growth requirement for nicotinamide and the halo reaction on antiserum agar.

Preparation of total cellular DNA has been described before (12). Insertion of Tn*phoA'* in *ompA* was confirmed by Southern hybridization on endonuclease-digested total genomic DNA probed with nick-translated [α-^{32}P]dCTP-labeled cloned *ompA* (pRD87) or the Tn*903* kanamycin resistance gene (pUC4-K) (24). Restriction endonuclease maps of *ompA* in *E. coli* strains RS218, E58, and E69 were also obtained by Southern analysis with the labeled *ompA* hybridization probe.

Western blotting of whole-cell lysates was performed following sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (33). Antibody to bacterial alkaline phosphatase was purchased from 5'-3', Inc. (West Chester, Pa.).

The growth characteristics of E44 and E58 were compared in 100% pooled adult rat sera which had been heated to 56°C for 30 min to eliminate the effect of complement. Organisms were grown in serum at 37°C with vigorous shaking, and aliquots were removed for colony counting.

Bactericidal assays were performed in pooled fresh human sera from seven random donors at a final concentration of 50%. A 10-μl amount of a mixture containing 10^4 mid-log-phase organisms per ml, 40 μl of Hanks's balanced salt solution (GIBCO Laboratories, Grand Island, N.Y.), and 50 μl of serum was incubated with rotation for 60 min at 37°C and then placed on ice. Then, 50 μl was plated, and colony counts were compared with those in controls in which the serum was heated to 56°C for 30 min to determine the percent survival. Ca^{2+} was chelated to eliminate the effect of the classical pathway of complement activation by the addition of 0.05 M Mg^{2+} EGTA [ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid] (8).

Embryonic chicken model of bacterial virulence. Fertilized, antibiotic-free, White Leghorn chicken eggs (SPAFAS, Storrs, Conn.) were maintained in a humidified self-turning incubator at 38°C. On day 5, a window (1 by 1 cm) was made in the shell and covered with cellophane tape by the method of Scher et al. (26), and the eggs were returned to the incubator without turning. Approximately 70% of the eggs were still viable by day 10. On day 10, 50 μl of PBS-washed, mid-log-phase organisms adjusted to the desired concentrations was inoculated onto the chorioallantoic membrane. The eggs were examined by candlelight daily to assess the viability of the embryo. Growth in ovo was determined by homogenizing the contents of viable eggs, including the embryo, at various times after inoculation. Serial dilutions of the homogenate were plated to determine the number of organisms per milliliter of egg.

Growth in unembryonated eggs was determined by inoculating 100 μl of PBS-washed mid-log-phase organisms at a concentration of 10^2 CFU/ml into the yolk of fertilized eggs which had not previously been incubated. The inoculated eggs were kept at 37°C, and the entire contents were homogenized for colony counts by serial dilutions of the homogenate.

Neonatal rat model for *E. coli* K-1 pathogenesis. Synchronized pregnant Sprague-Dawley rats were purchased from Taconic Farms, Germantown, N.Y. Neonatal rats, less than 24 h old were randomized between litters and then fed 10 μl of PBS-washed, mid-log-phase organisms adjusted to a density of 10^9 CFU/ml by the method of Bloch et al. (4). Animals receiving different strains were housed separately. At 72 h after the inoculation, the pups were killed by intraperitoneal injection of 5 mg of sodium pentobarbital. Blood was sampled by percutaneous cardiac puncture and spread on a selective agar plate (100 μl per plate) to detect and quantitate bacteremia. Cannibalized animals were not included in determination of the experimental results. The spleens of dead animals were excised and homogenized, and an aliquot was spread on selective plates. Dead animals with positive splenic cultures were considered to have been bacteremic.

RESULTS

Use of modified Tn*phoA* for transposon mutagenesis. Transposon mutagenesis was used to generate an insertion in the *ompA* gene that completely eliminated the expression of OmpA in a virulent isolate. The use of pRT733 to deliver Tn*phoA* was 100-fold less efficient with E44 as a recipient in conjugation experiments than with an *E. coli* K-12 recipient, such as LE392. We constructed a modified version of Tn*phoA* (Tn*phoA'*) which is smaller by 1,308 bp and contains a type I aminoglycoside 3'-phosphotransferase (APH type I) gene in place of a type II APH (19). It was originally designed for use in bacterial species in which the type II APH is not an effective selectable marker. Tn*phoA'* was delivered by pRT733' to E44 at a 20-fold-higher rate ($\sim 5.4 \times 10^{-6}$ kanamycin-resistant colonies per recipient cell) than Tn*phoA* was delivered by pRT733. Therefore, the modification of Tn*phoA* increased its efficiency as a tool in the mutagenesis of the *E. coli* genome.

A library of 400 independent gene fusions to *phoA* were obtained by screening for alkaline phosphatase activity (blue phenotype) in the presence of XP among 20,000 random insertions of Tn*phoA'* into the E44 genome. These colonies represent in-frame fusions of *phoA*, 3' to a signal that promotes protein secretion (15). The 400 isolates were screened for loss of reactivity with anti-OmpA serum by colony immunoblotting. No OmpA$^-$ mutants were identified among these isolates.

Three OmpA$^-$ mutants of E44 were obtained by screening 10,000 independent rifampin- and kanamycin-resistant colonies (without XP). None of the three OmpA$^-$ mutants showed increased alkaline phosphatase activity. The inability to identify an OmpA mutant among those with in-frame fusions to *phoA* suggests that 400 isolates were not adequately representative of mutations in exported proteins. Alternatively, the *phoA-ompA* fusion product may have been deleterious to the organism. A single OmpA$^-$ mutant of E44, strain E58, was used in all subsequent experiments.

Characterization of *E. coli* K-1 OmpA and the OmpA$^-$ mutant. E58 was compared with its parent strain, E44, by Western analysis with the polyclonal rabbit anti-OmpA serum to detect electrophoretically separated proteins from whole-cell lysates heat-treated for 10 min at 100°C (Fig. 1). E44 expresses a protein with an apparent molecular mass of 33,000 Da, which is indistinguishable from that of an *E. coli* K-12 strain (LE392). E58 lacks OmpA, as determined by Western analysis (Fig. 1) and by Coomassie blue staining of electrophoretically separated proteins (data not shown). In addition, no fusion product was detected in E58 by Western analysis with an antibody to PhoA (data not shown). Southern analysis provided direct physical evidence for insertion of Tn*phoA'* in the *ompA* gene (Fig. 2A). The labeled probe from a cloned *E. coli* K-12 *ompA* gene hybridized to 1.7- and 1.8-kb *Bam*HI fragments from total genomic DNA of E44. In E58, the 1.7-kb *Bam*HI fragment which hybridized to the *ompA* probe is replaced a fragment 6.4 kb larger, which was in accord with the increased size expected after insertion of

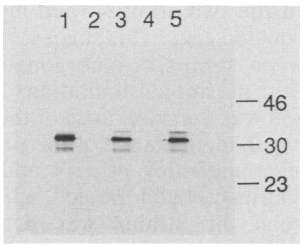

FIG. 1. Western blot of heat-treated, whole-cell lysates of *E. coli* reacted with anti-OmpA antibody. Positions of molecular size markers are shown (in kilodaltons). Lane 1, LE392; lane 2, BRE51; lane 3, E44; lane 4, E58; lane 5, E69.

Tn*phoA'*. A single *Bam*HI fragment of 8.1 kb hybridized to the labeled kanamycin resistance gene found on Tn*phoA'*, indicating that E58 had only a single insertion of Tn*phoA'* (Fig. 2B).

Since the *ompA* gene of an *E. coli* K-1 strain has never been characterized and its similarity to the K-12 gene is unknown, a restriction endonuclease map of the RS218 *ompA* gene was determined by Southern analysis with the labeled *ompA* gene probe (Fig. 3). The relative sizes and positions of cleavage sites for several restriction endonucleases are identical to those in the published sequence of the *E. coli* K-12 *ompA* gene (17). Only a single restriction site polymorphism (*Bgl*II) near the 3' end of the gene was identified. It appears, therefore, that the K-1 and K-12 genes as well as gene products are highly homologous. The inserted Tn*phoA'* was mapped approximately 400 bp from the 3' end of *ompA*. The truncated gene product appears to be completely degraded.

To ensure that the observed effects of the mutation in E58 were due to the loss of OmpA and not to any unrecognized phenotypic differences, a strain was constructed in which the mutation was corrected by P1 transduction. The resulting *E. coli* K-1 strain, E69, contained the entire *ompA* gene of an *E. coli* K-12 isolate and expressed OmpA on Western analysis (Fig. 1). Southern analysis confirmed that the E69 *ompA* gene is no longer interrupted and contains a *Bgl*II site, as predicted for the *E. coli* K-12 gene (data not shown).

OmpA enhances virulence in an embryonic chicken model. The relative virulence of E44, E58, and E69 was tested by observing the lethal effect of organisms inoculated onto the chorioallantoic membrane of 10-day-old chick embryos. Initial experiments with E44 showed that only a few organisms were sufficient to kill an embryo. Higher inocula consistently caused death of a greater percentage of embryos. The majority of deaths occurred on the second day following inoculation, although in subsequent experiments observations were continued for 3 days. The lethal effect of the inoculation was shown to be due to the *E. coli* K-1 strain, since a sham inoculation with sterile PBS resulted in minimal mortality, most likely due to additional handling (Table 2).

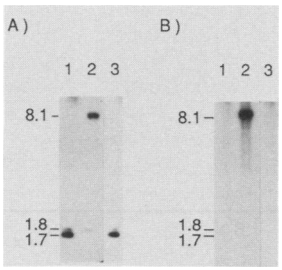

FIG. 2. Southern blot of chromosomal DNA digested with *Bam*HI. Molecular sizes are shown in kilobases. Lane 1, E44; lane 2, E58; lane 3, E69. Hybridization probes: (A) pRD87 (*ompA*); (B) pUC4-K (Tn*903* kanamycin resistance gene).

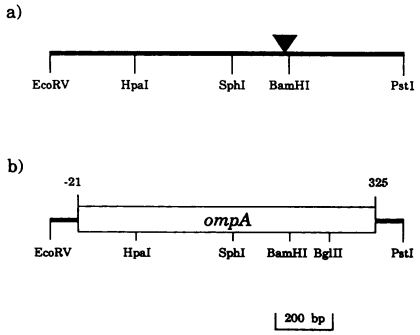

FIG. 3. (a) Restriction endonuclease map of the RS218 *ompA*, determined by Southern analysis with a cloned *ompA* as a hybridization probe. (b) Restriction endonuclease map of *ompA* based on a published nucleotide sequence (17). The position of the open reading frame is indicated (open box). The numbers of the first and last amino acids are indicated above the gene. The site of Tn*phoA'* insertion in E58 is indicated by the arrowhead in panel a.

The K-1 capsule has been shown to be important in pathogenesis in other models of infection (reviewed in Silver and Vimr [28]). A K-1 capsule-deficient mutant of E44, derived by transposon mutagenesis, was used to demonstrate the effect of a known determinant of virulence in the chick embryo model. The unencapsulated mutant, E66, had lower virulence than the encapsulated parent strain. This result demonstrated the usefulness of this model in defining virulence factors in near-isogenic *E. coli* K-1 strains.

Inoculation of E44 resulted in a dose-related increase in mortality from 27% (10 CFU) to 62% (10^5 CFU). In contrast, the $OmpA^-$ mutant E58 killed from 10 to 18% of embryos over the same dose range, a three- to fivefold reduction in virulence over the background rate of mortality caused by the procedure alone. Correction of the *ompA* mutation with an *E. coli* K-12 *ompA* gene restored a level of virulence equivalent to that of E44. This confirmed that the loss of OmpA was responsible for the observed effect. In addition, the restoration of virulence in E69 shows that the OmpA proteins in *E. coli* K-1 and K-12 are equivalent as virulence factors in this model.

Effect of OmpA on *E. coli* K-1 pathogenesis in neonatal rats. A second in vivo model was used to compare directly the

TABLE 2. Lethal effect of genetically defined *E. coli* K-1 mutants on 10-day chick embryos

Strain (phenotype)	No. dead/no. inoculated[a] (% dead) at inoculum (CFU):		
	10^1	10^3	10^5
E44 (parent)	6/22 (27)	8/23 (35)	13/21 (62)
E58 ($OmpA^-$)	2/21 (10)	2/23 (9)	4/22[b] (18)
E69[c] ($OmpA^+$)	4/13 (31)	3/13 (23)	11/14 (79)
E66 (capsule deficient)	0/12 (0)	0/12 (0)	2/12[d] (17)

[a] One (3%) of 32 sham (PBS)-inoculated mice died.
[b] $P < 0.01$ versus $OmpA^+$ strains for equivalent inoculum (chi-squared).
[c] Strain E58 with corrected mutation.
[d] $P < 0.05$ versus parent strain for equivalent inoculum (chi-squared).

TABLE 3. Relative effect of loss of OmpA expression on incidence of bacteremia in neonatal rats

Strains inoculated	No. bacteremic/no. inoculated (% bacteremic)			
	E44	E58	E69	Total
E44 + E58	15/38 (39)[a]	2/38 (5)		17/38 (44)
E44 + E69	6/39 (15)		9/39 (23)	15/39 (38)

[a] $P < 0.001$ versus E58 (chi-squared).

relative virulence of E58 and its OmpA$^+$ parent strain. The original isolate, RS218, has been shown to cause a high incidence of bacteremia following oral feeding of large inocula to infant rats (4). Rat pups were fed 10^7 organisms consisting of equal mixtures of E44 and E58, and the incidence of bacteremia due to each strain was determined 72 h later. Of 38 rats inoculated, 17 were bacteremic; only 2 of 17 had kanamycin-resistant organisms (E58), compared with 15 of 17 with kanamycin-sensitive organisms (E44) (Table 3). Therefore, the relative incidence of bacteremia was about sevenfold less with the OmpA$^-$ mutant ($P < 0.001$). In a parallel experiment, mixed inocula of E44 and E69 resulted in 15 of 39 rats becoming bacteremic. There was no significant difference between strains expressing the K-1 (E44) or K-12 (E69) OmpA. Correction of the *ompA* mutation correlated with an increase in virulence to equal that of the parent strain. There were no animals with mixed infections. These results, which were consistent with those of the chick embryo experiments, provided evidence for the selective advantage conferred by OmpA in *E. coli* K-1 infection.

Growth characteristics of the OmpA$^-$ mutant. The relationship between virulence and growth in ovo was assessed by determining viable bacterial counts on the total contents of 10-day chicken eggs after chorioallantoic membrane inoculation. The growth of E44 was 10-fold greater than that of E58 by 20 h postinoculation (Fig. 4). This difference was maintained over the 120-h observation period ($P < 0.05$ at 20 and 120 h). Nonviable eggs were excluded from these determinations, which would be expected to diminish the differences between strains, since a far greater percentage of eggs receiving E44 died. It was concluded that differences in virulence correlated with the ability to survive and multiply in ovo. In contrast, there was no difference in the growth characteristics of the two strains when inoculated into the yolk of unembryonated eggs. This suggests that the observed differences between strains in embryonated eggs was unlikely to be due to nutritional limitations in chick eggs. A further implication was that clearance of the OmpA$^-$ mutant was more efficient than clearance of the parent strain.

The growth characteristics of E44 and E58 were also compared in heat-inactivated pooled adult rat sera. No difference between the strains was detected (data not shown). Differences in the incidence of bacteremia in neonatal rats could not be attributed to differences in the ability of the two strains to grow in serum.

Effect of OmpA on serum bactericidal activity. E44 is relatively resistant to the bactericidal activity of normal human serum. Greater than 50% of E44 organisms survived in 50% serum after incubation for 60 min at 37°C. Loss of expression of OmpA correlated with increased sensitivity to serum killing (Fig. 5). The magnitude of the decrease in serum resistance caused by loss of OmpA expression and by loss of the K-1 capsule was similar. The addition of Mg^{2+} EGTA, to remove the contribution of the classical pathway of complement activation, had no effect on the capsule-deficient mutant but eliminated the serum killing of the OmpA$^-$ mutant. Therefore, as has been reported previously, the polysialic acid capsule protects the organism from activation of the alternative pathway (20). Expression of OmpA, in contrast, increased resistance to serum killing via the classical pathway. Correction of the mutation in *ompA* restored a level of serum resistance equivalent to that of the parent strain.

DISCUSSION

The unusual conservation of OmpA-like proteins across gram-negative species suggests that they serve an important cellular function (2). However, no essential role for OmpA-like proteins has yet been identified. In this study, we considered whether OmpA is an important factor in the host-parasite interaction. The function of OmpA in vivo as a potential determinant of virulence has not previously been investigated.

E. coli OmpA has been shown to have sequence homology with two major surface antigens of pathogenic *Neisseria* species: the membrane-associated portion of OmpA (amino acids 41 to 171) with protein II (PII), and the periplasmic

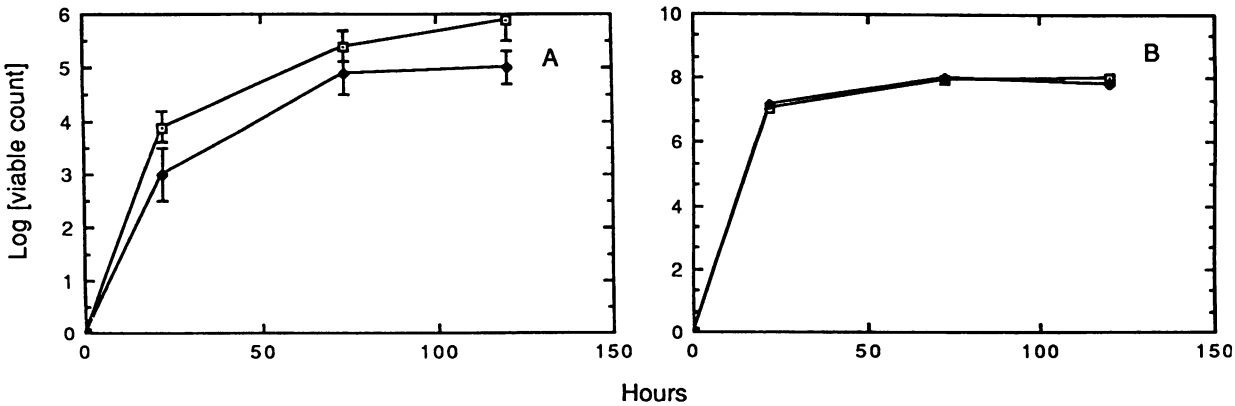

FIG. 4. Comparison of the growth of E44 (□) and E58 (●) in chick eggs. Values represent the mean for eight eggs, expressed as counts of viable organisms per milliliter of total egg homogenate. (A) Ten-day embryonated eggs inoculated on the chorioallantoic membrane. Error bars indicate standard deviation. (B) Unembryonated eggs inoculated in the yolk.

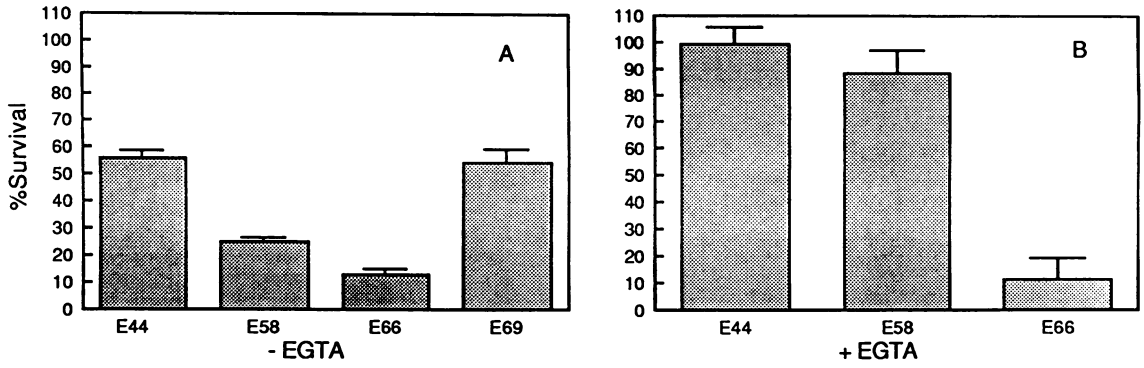

FIG. 5. Bactericidal effect of pooled normal human sera on *E. coli* K-1 mutants. The percent survival was calculated by the ratio of colonies in active serum to those in inactivated serum after 60 min in 50% serum. Values are means of four experiments; error bars show standard deviation. (A) Bactericidal assay without 0.05 M Mg^{2+} EGTA. (B) Bactericidal assay in the presence of 0.05 M Mg^{2+} EGTA.

portion of OmpA (amino acids 176 to 316) with protein III (PIII) (11). Both PII and PIII have been implicated as contributing to the pathogenicity of *Neisseria* species, although there are no adequate in vivo models of *Neisseria* infection with which this can be directly addressed. PII appears to be important in the interaction and attachment of neisseriae to host cells (3). It has been proposed that expression of PIII increases the resistance of *N. gonorrhoeae* to complement-mediated killing. Specific antibodies of the immunoglobulin G class directed against PIII block the bactericidal effect of immune serum (22). The structural similarities with *E. coli* OmpA suggest that OmpA may have a role in gram-negative pathogenesis.

For this study, an *E. coli* K-1 isolate was chosen, because animal models which mimic the natural infection are well characterized. *E. coli* is a leading cause of invasive neonatal infection in humans, and 80% of these isolates have a polysialic acid capsule (K-1) (28). Furthermore, it was expected that the structure of the K-1 OmpA would resemble that of the K-12 OmpA, which has been the subject of much previous investigation. We found no significant phenotypic or structural difference between the K-1 and K-12 OmpA proteins.

The $OmpA^-$ mutant was compared with its parent strain in two different models of infection. In both cases, elimination of OmpA expression correlated with attenuation but not total loss of virulence. Correction of the mutation in *ompA* restored a level of virulence equivalent to that of the parent strain, confirming that the observed effects were due to loss of OmpA expression. It was possible to demonstrate, in competition experiments with neonatal rats in which bacteremia was an endpoint of infection, that the $OmpA^+$ strain had a selective advantage in vivo over the $OmpA^-$ mutant. These results demonstrated the relative importance of this highly conserved protein as a determinant of virulence.

Previous studies have shown that the pathogenicity of *E. coli* isolates for embryonated chick eggs inoculated onto the chorioallantoic membrane is highly variable from isolate to isolate (21). To our knowledge, this convenient model of virulence has not been used to compare the effect of defined mutations in genetically manipulated *E. coli* strains. Single mutations (eliminating expression of the K-1 capsule or OmpA) could be shown to alter significantly the virulence of an *E. coli* strain for chick embryos. The embryos that died after exposure to the bacteria had superficial hemorrhages and large bilateral cranial sinus collections containing leukocytes and abundant organisms when Gram stained. These resembled the gross pathologic findings after experimental *N. meningitidis* infection of chick embryos (7).

There is evidence that OmpA has structural and metabolic functions in *E. coli*. It was considered whether the diminished virulence of the OmpA mutant might be due to differences in its ability to multiply under physiologic conditions. Differentiating between virulence and growth in vivo is not straightforward. One advantage of the embryonated egg model for this study was that growth and virulence could be compared under similar conditions. It was possible to show that the growth of the $OmpA^-$ mutant was unaffected in unembryonated eggs, suggesting that its decreased virulence in this model was not likely to be the result of diminished growth due to metabolic limitations. Furthermore, there was no difference between $OmpA^+$ and $OmpA^-$ strains in the rate of growth in rat serum in which the effect of complement was eliminated.

In addition to the other functions attributed to OmpA, our results indicate that OmpA is a factor in determining resistance to complement-mediated serum killing. The increased resistance of $OmpA^+$ strains to normal serum may account for their greater virulence in vivo, as shown in this study. OmpA may serve to stabilize the outer membrane, making it more resistant to the effects of complement. Alternatively, *E. coli* OmpA may bind antibodies that block serum killing, as has been proposed for PIII in *N. gonorrhoeae*.

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