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TnphoA-Mediated Disruption of K54 Capsular Polysaccharide Genes in *Escherichia coli* Confers Serum Sensitivity

THOMAS A. RUSSO,[1]* M. CATHARINE MOFFITT,[2] CARL H. HAMMER,[3] AND MICHAEL M. FRANK[2]

Bacterial Pathogenesis Unit, Laboratory of Clinical Investigation,[1] and Biological Resource Branch,[3] National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892, and the Department of Pediatrics, Duke University Medical Center, Durham, North Carolina 27710[2]

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To assess whether non-K1, group 2 capsular serotypes are important in conferring serum resistance to extraintestinal isolates of *Escherichia coli*, a K54 blood isolate (CP9) was evaluated as a model pathogen. Transposon mutagenesis (Tn*phoA*) was used to generate isogenic capsule-negative mutants. CP9 was resistant to the bactericidal effects of serum, growing in 80% serum. In contrast, all of the capsule-negative mutants had an increased sensitivity to 80% normal human serum, undergoing a 2- to 3-log kill over 3 h when starting inocula of 10^4 to 10^7 CFU/ml were used. The killing of the capsule-negative strains was mediated through the alternative complement pathway and not by lysozyme or beta-lysins. The protective effect of the K54 capsule against the bactericidal activity of serum was not through inhibition of the complement cascade, nor did it appear to be through a difference in the binding of C3.

The extraintestinal isolates of *Escherichia coli* cause a variety of common infectious syndromes. Antimicrobial therapy has yet to minimize the significant morbidity and mortality caused by these pathogens (15, 33). One of the traits that characterizes this group of pathogens is their ability to survive a variety of host defense mechanisms. The strong association of resistance to the bactericidal effects of human serum in these blood and deep-tissue isolates of *E. coli* suggests that the bacterial factors responsible are important virulence traits (29, 41). The majority of adult, extraintestinal *E. coli* infections, including bacteremias, are caused by a variety of isolates that possess group 2, non-K1 capsular polysaccharides (4, 18, 22, 30). Although the *E. coli* group 2, K1 capsular polysaccharide appears to be a serum resistance determinant (1, 2, 5, 11, 21, 39), other group 2 capsules have not been established as being protective against the bactericidal effects of serum. Several epidemiologically based studies have supported (12, 17, 23) or refuted (3, 22, 38) such a role when correlating K antigens and serum resistance. However, such analyses may be unreliable because of the multiplicity of pathogenic factors involved, poorly defined or inappropriate bacterial strains, the influence of inoculum, and the variability of the underlying host. Other reports evaluated non-group 2 encapsulated strains when discounting a role for capsule in serum resistance (14, 35). A genetic approach is better suited to assessing the contribution of individual bacterial traits. Evaluations of non-K1, group 2 capsule-positive and -negative pairs are limited, and none of these studies evaluated proven isogenic pairs, leaving open the possibility that cryptic mutations may affect serum resistance. Nonetheless, these studies suggest that the K5 serotype is not protective (4, 11, 34) but that K23 may be protective against the bactericidal effects of serum (40). The capsular polysaccharide of veterinary isolate "V165" (K^-) appears to play a role in protecting against the bactericidal effects of serum; however, the relation of this capsule to group 1 and 2 capsular polysaccharides is unclear (24).

Therefore, to more precisely define the role of a non-K1, group 2 capsular polysaccharide as a complement resistance determinant we are studying a K54 blood isolate (CP9). We have generated proven isogenic capsule-negative derivatives through the insertion of the transposon Tn*phoA* into genes required for capsule production. Southern analysis and transduction experiments confirmed that a single transposon insertion is responsible for the capsule-negative phenotype of these strains (31). This paper details the utilization of these mutants to assess the role of the K54 capsular polysaccharide in protecting against the bactericidal effects of serum in vitro.

CP9 is an *E. coli* blood isolate obtained from a patient hospitalized at the National Institutes of Health. It is characterized by growth in 80% human serum, beta-hemolysis, no known antibiotic resistance, O4/K54/H5 serotype, pyelonephritis-associated antigen positivity, and possession of a 36.2-kb cryptic plasmid (pJEG). CP9.171, CP9.C43, and CP9.C56 are CP9 capsule-negative derivatives and were constructed as previously described (31). Strains were maintained at −80°C in 50% Luria-Bertani medium–50% glycerol. Luria-Bertani medium (5 g of yeast extract, 10 g of tryptone, 10 g of NaCl per liter; Difco Laboratories, Detroit, Mich.) was used for all experiments. Kanamycin (40 μg/ml) (Amresco, Solon, Ohio) was added where appropriate. All cultures were incubated at 37°C unless otherwise stated. Human sera obtained from several donors were used fresh or stored at −80°C until required. Heat-inactivated serum was incubated at 56°C for 30 min (Δ56 normal human serum [NHS]). Human C8-deficient serum (C8D) was obtained from a National Institutes of Health patient in the form of plasma and had been maintained at −80°C prior to use. The plasma was reconstituted to serum by the addition of 2 M $CaCl_2$ (10 μl/ml) and 1 U of thrombin per ml. Human C2-depleted serum (C2D) was obtained from a National Institutes of Health patient, and guinea pig C4-deficient serum (C4D) was obtained from an animal maintained at the National Institutes of Health; both had been stored at −80°C before use. Bentonite-treated serum was obtained by addition of 5 mg of bentonite per ml to 100% NHS followed by incubation at 0°C for 5 min (42) or by addition of 10 mg/ml to

* Corresponding author.

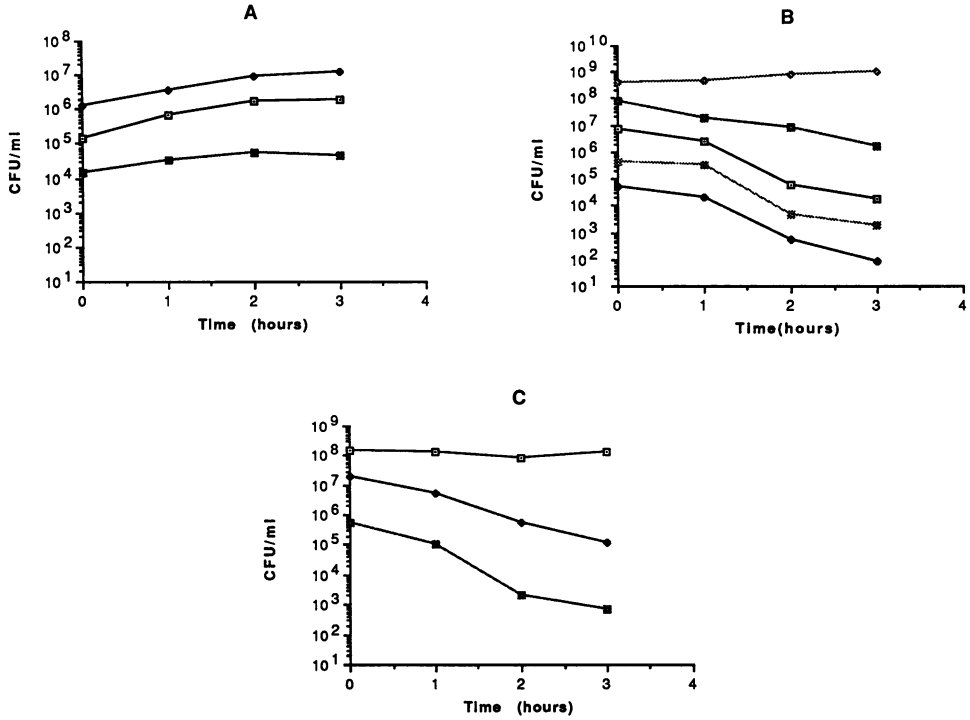

FIG. 1. Effects of 80% NHS in vitro on the viability of the wild-type isolate CP9 and its isogenic capsule-negative derivatives CP9.171 and CP9.C56. Each panel depicts three different starting inocula for the given strain. Kill curves are representative of at least two experiments for each strain at each starting inoculum. Assays were performed as described in Materials and Methods. (A) CP9; (B) CP9.171; (C) CP9.C56.

100% NHS with incubation at 37°C for 10 min (13). Bentonite was removed by centrifugation, and the serum was filtered through a 45-μm Millipore filter. Adsorbed serum was generated by resuspension of a bacterial pellet containing 1.0×10^{10} CFU/ml in 100% NHS followed by incubation for 1 h at 4°C. The cells were removed by centrifugation, and this process was repeated twice. CP9 (capsule positive, lipopolysaccharide [LPS] positive), CP9.171 (capsule negative, LPS positive), and rough 1 (capsule negative, LPS negative) were the bacterial strains utilized for adsorption. Bactericidal assays were performed as follows. (i) For NHS, Δ56 NHS, and bentonite-treated NHS, cells were grown in Luria-Bertani broth to mid-logarithmic phase and diluted in gelatin-Veronal-buffered saline (GVBS) (37) so that 200 μl gave a starting inoculum of 10^4 to 10^7 CFU/ml when added to 800 μl of serum (80% final concentration). Aliquots were removed from this mixture at 0, 1, 2, and 3 h, and viable titers were determined by plating serial 10-fold dilutions in 1× phosphate-buffered saline (PBS) in duplicate on the appropriate media. (ii) For C8D, assays were performed as above with C8D, except that final volumes consisted of 300 μl. To ensure that the non-C8 complement components remained active, control assays measuring bactericidal activity were performed after adding back-purified C8 (Quidel, San Diego, Calif.). Functional activity was measured at 1.67×10^6 C8H50 U/ml, which represents 10× the normal serum activity; therefore, 1/10 of this concentration was added to reconstitute C8D. (iii) For C4D and C2D, the volumes of the mixture were adjusted as follows: 240 μl of C4D or C2D (final concentration, 80%)–30 μl of cells diluted in GVBS–30 μl of 1× PBS. (iv) For lysozyme, bacteria were diluted as before and exposed to final concentrations of 0, 2, and 10 μg of ultrapure (23,800 U/mg) lysozyme per ml (Amresco) diluted in 1× PBS. Serum was not utilized in this assay.

To perform C3 binding studies, log-phase bacterial cells were harvested, washed, and either resuspended in the same volume or concentrated 10-fold in GVBS–EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid]–Mg^{2+} (97.3 ml of GVBS, 2.5 ml of 0.2 M EGTA, 0.2 ml of 1 M $MgCl_2$). A 1-ml assay mixture, delivered into glass tubes (12 by 75 mm), consisted of an approximate bacterial titer of either 3.0×10^8 or 3.0×10^9 CFU/ml and 10 or 20% NHS in GVBS–EGTA–Mg^{2+}. Control tubes contained bacterial cells plus either complement EDTA (1 part NHS to 25 parts 0.02 M EDTA in GVBS, preincubated at 37°C for 15 min) or GVBS–EGTA–Mg^{2+}. Assays were performed in duplicate. After incubation in a 37°C shaking water bath for 30 min, cells were collected, washed in GVBS, and resuspended in 1 ml of 1× PBS at 4°C. Anti-C3 (Dako Corp., Carpinteria, Calif.) was labelled with ^{125}I with IODO-BEAD (Pierce, Rockford, Ill.) as per instructions of the manufacturer. ^{125}I-anti-C3 was added with continued incubation at 4°C for 1 h; the mixture was then washed twice in 1× PBS, and the bacterial pellet was counted (Cobra II Auto-Gamma Counting System, Packard Instrument Co., Meriden, Conn.). The highest mean control count was subtracted from the experimental count and divided by the optical density to give values as counts per minute per optical density value at 600 nm. The data were analyzed by StatWorks with Student's *t* statistic with 4 df.

Capsule-negative strains are sensitive to the bactericidal effects of NHS in vitro. The wild-type parent CP9 was highly resistant to killing by NHS, growing in 80% serum (Fig. 1A). In contrast, all of the K54 capsule-negative mutants were uniformly susceptible to the bactericidal activity of serum in vitro, incurring a 2- to 3-log kill in 80% serum over 3 h when starting inocula were less than 10^8 CFU/ml. The bactericidal activity of serum could be overwhelmed by starting inocula

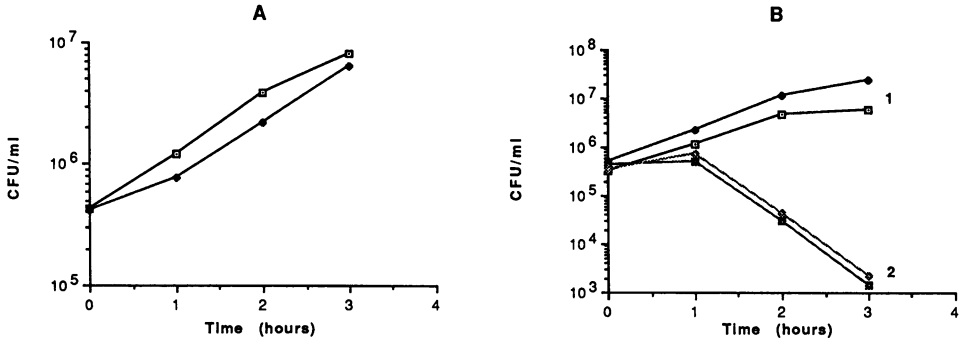

FIG. 2. Serum bactericidal effects are mediated via the complement cascade. Curves are representative of at least two experiments. (A) CP9.171 assayed in Δ56 NHS; (B) CP9.171 assayed in C8D (numeral 1) or C8D plus purified C8 (1.5×10^5 C8H50 U/ml) (numeral 2).

of greater than or equal to 10^8 CFU/ml (Fig. 1B and C). The strains CP9.171 and CP9.C56 shown in Fig. 1B and C were representative of the capsule-negative mutant cohort.

Serum killing of capsule-negative strains is mediated by the alternative complement pathway. It was established that complement was responsible for the bactericidal activity of serum against the capsule-negative strains. The complement cascade is inactivated by heating human serum to 56°C for 30 min (Δ56 NHS). When the capsule-negative strain CP9.171 was treated with 80% Δ56 NHS, no killing occurred (Fig. 2A). To confirm this finding, CP9.171 was treated with human serum deficient in C8 (C8D). As seen in Fig. 2B, no killing occurred in C8D; however, the serum's bactericidal activity was restored when purified C8 was added back into the C8D. Similar results were obtained when CP9.C43 was treated with Δ56 NHS and C8D (data not shown). The exposure of CP9.171 to 80% guinea pig serum deficient in C4 (C4D), human serum deficient in C2 (C2D), and adsorbed human serum demonstrated that complement killing was mediated through the alternative pathway. The classical pathway is inactive in serum deficient in C2 or C4. Nonetheless, killing of CP9.171 by these sera was equivalent to that seen with normal serum (Fig. 3A and B). Similarly, when natural antibodies were removed from normal serum by adsorption with either CP9 (capsule positive), CP9.171 (capsule negative), or rough 1 (capsule and O-polysaccharide negative), the killing of CP9.171 was again unaffected relative to the killing seen with unadsorbed serum, implying that

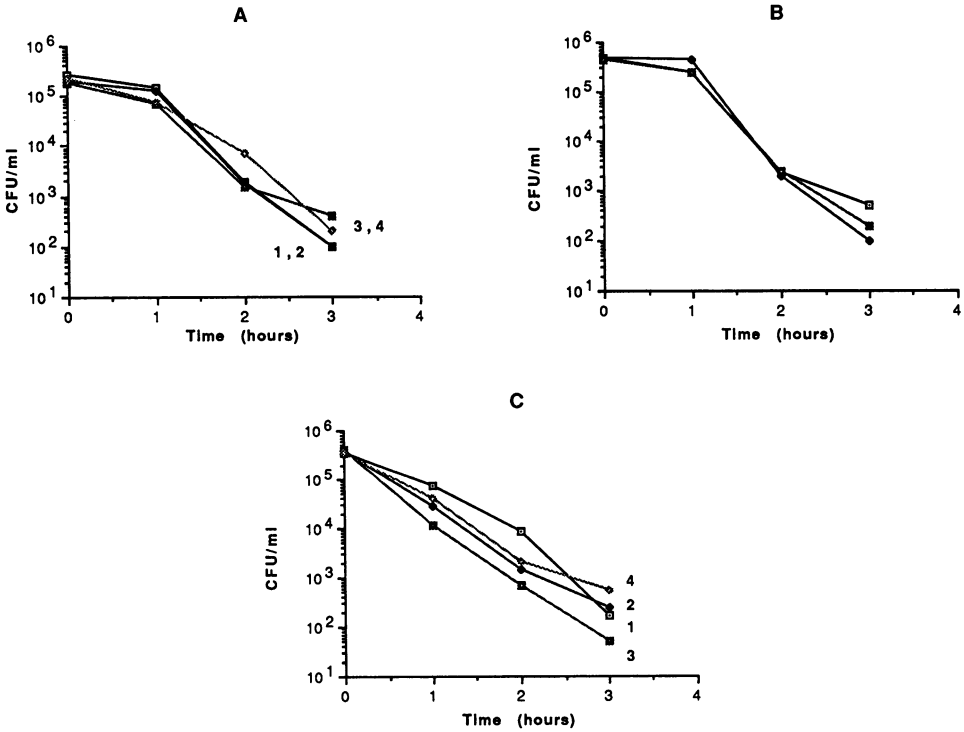

FIG. 3. Complement-dependent killing is directed through the alternative pathway. (A) CP9.171 assayed in 80% normal guinea pig serum (numerals 1 and 2) and 80% guinea pig C4D (numerals 3 and 4); (B) CP9.171 assayed in 80% C2D; (C) CP9.171 assayed in 80% NHS (numeral 1), 80% NHS adsorbed against CP9 (capsule and LPS positive) (numeral 2), 80% NHS adsorbed against CP9.171 (capsule negative and LPS positive) (numeral 3), and 80% NHS adsorbed against rough 1 (capsule and LPS negative) (numeral 4).

TABLE 1. Binding of C3 to CP9 and CP9.171

Expt no.	CFU/ml	% NHS	C3 binding with strain[a]:	
			CP9[b]	CP9.171[c]
1	~3.0 × 10^8	10	5,250	5,711
2	~4.0 × 10^8	10	2,557	3,704
3	~2.5 × 10^8	20	5,037	5,825
4	~3.0 × 10^9	10	1,024	497
5	~2.5 × 10^9	20	1,005	1,018

[a] Expressed as counts per minute per optical density value at 600 nm. P = 0.268 (comparison of CP9 and CP9.171 with Student's t statistic).
[b] Wild type, capsule positive.
[c] Capsule negative.

the serum's bactericidal activity was mediated through the alternative pathway (Fig. 3C).

Lysozyme and beta-lysins do not appear to play a primary role in the bactericidal activity of serum. As described above, the inactivation of complement by heating NHS to 56°C for 30 min resulted in the loss of bactericidal activity against the capsule-negative strains (Fig. 2A). Since the activity of beta-lysins and lysozyme is unaffected by such treatment, it appeared unlikely that they had a role in the in vitro killing of these strains (6, 7). Further experiments were performed to support this result. When the wild-type parent CP9 and its capsule-negative derivatives CP9.171 and CP9.C43 were treated with purified lysozyme at the physiologic concentration of 2 μg/ml as well as with 10 μg/ml, growth equivalent to the PBS control was observed for all strains (data not shown). The treatment of serum with bentonite results in the removal of lysozyme, beta-lysins, and, to a variable degree, properdin (42). In 80% bentonite-treated serum, CP9 grew, as was predicted, and CP9.171 and CP9.C43 were killed at the same rate and to the same degree as they were with 80% NHS (data not shown). These experimental results excluded a primary role for either lysozyme or beta-lysins in the serum-mediated killing of the K54 capsule-negative mutants.

Binding of C3 via the alternative pathway is equivalent in K54 capsule-positive and -negative strains. Studies were performed to assess whether the K54 capsular polysaccharide modified the binding of the third complement component (C3) to the bacterial cell. The classical complement pathway was selectively inhibited by EGTA and Mg^{2+} (10), allowing for C3 deposition to be mediated through the alternative pathway. This arm of the complement cascade had been established as mediating the killing of the capsule-negative mutants. No difference was found between the amount of C3 bound in a comparison of the capsule-positive wild-type strain (CP9) with the isogenic capsule-negative derivative CP9.171 (P = 0.268). The binding of C3 was assayed at 10 and 20% NHS and at bacterial titers of approximately 2.5 × 10^8 to 4.0 × 10^8 and 2.5 × 10^9 to 3.0 × 10^9 CFU/ml, the results of which are summarized in Table 1. Specific C3 binding was diminished in experiments 4 and 5, in which higher concentrations of bacteria were utilized, suggesting that C3 may be limiting. While the gross counts were higher than those for experiments 1 to 3, maximum binding appeared to occur when bacterial titers were less than 2.5 × 10^9 CFU/ml.

We have previously described the creation of isogenic capsule-negative mutants derived from a K54 blood isolate of *E. coli* by transposon mutagenesis (31). In this paper, we utilized these strains to evaluate the role a non-K1, group 2 capsular polysaccharide (K54) plays in protecting against the bactericidal effects of NHS. Previous studies examining this question have been inconclusive or suggested that non-K1 capsules were unimportant (8, 18–20, 36). Our findings have clearly demonstrated that the K54 capsular polysaccharide plays a major protective role in vitro and have unequivocally established that at least one non-K1, group 2 serotype is a complement resistance determinant. If other non-K1, group 2 capsular polysaccharides also prove to be important in the pathogenic process, then this group of capsules may become a target for therapeutic intervention.

Another important aspect of this study was the use of proven isogenic capsule-negative mutants. If a nonspecific method for mutagenesis or selection for a desired phenotype is utilized (e.g., phage resistance or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine), multiple cryptic mutations may occur, making it impossible to unequivocally determine what is truly responsible for an observed effect (21, 27, 28). Furthermore, confirmation that a transposon insertion is responsible for the observed phenotype is mandatory, and this was done for the mutants evaluated in this study. We have found a class of capsule-negative mutants generated by transposon mutagenesis that contained only a single complete copy of Tn*phoA*; however, it was not the Tn*phoA* insertions, but probably the secondary IS50 transpositions, that were responsible for their capsule-negative phenotype (31).

We are presently in the process of evaluating the precise mechanism by which the K54 capsular polysaccharide affords protection against the bactericidal activity of serum. The K1 antigen, an α-2-8-linked homopolymer of sialic acid, however, has been studied in detail. The sialic acid residues appear to resist the activation of the alternative complement pathway, resulting in serum resistance (9, 25–27, 32). The K54 antigen is composed of 3)-β-D-glucosyluronic acid-(1-3)-α-L-rhamnosyl-(1- repeating units (16). We have established that the alternative complement pathway is activated in the presence of the K54 capsule and that the amount of C3 binding is similar in both capsule-positive and capsule-negative derivatives. Therefore, the mechanism of complement resistance mediated by the K54 capsule is distinct from K1. Further studies comparing CP9 with our isogenic capsule-negative mutants, which include an assessment of C3 degradation and formation of C5b-9 membrane attack complex, are under way. Such evaluations of our isogenic capsule-negative strains should clarify the mechanism of complement resistance mediated by the K54 capsular polysaccharide.

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