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Loss of the O4 Antigen Moiety from the Lipopolysaccharide of an Extraintestinal Isolate of *Escherichia coli* Has Only Minor Effects on Serum Sensitivity and Virulence In Vivo

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The O-specific antigen in extraintestinal isolates of *Escherichia coli* is believed to be an important virulence factor. To assess its role in the pathogenic process, proven isogenic derivatives with either a complete (CP921) or nearly complete (CP920) deficiency of the O4 antigen were obtained by Tn*phoA'1*-mediated transposon mutagenesis of an O4/K54/H5 blood isolate (CP9). By utilizing a previously reported isogenic K54 capsule-deficient derivative (CP9.137), additional isogenic derivatives deficient in both the K54 capsular antigen and either all (CP923) or nearly all (CP922) of the O4 antigen were also constructed. These strains and their wild-type parent were evaluated in vitro for serum sensitivity and in vivo by intraperitoneal challenge of outbred mice. The complete or nearly complete loss of the O4 antigen (CP920 and CP921) resulted in only a minor increase in serum sensitivity. In contrast, CP9.137 had a significant increase in serum sensitivity, and CP922 and CP923 were extremely serum sensitive. When tested in vivo, the complete or nearly complete loss of the O4 antigen resulted in a small but significant increase ($P \leq 0.05$), not the expected decrease, in virulence compared with its wild-type parent. In contrast, CP9.137 and CP922 were significantly less virulent ($P \leq 0.05$). These studies do not exclude a role for the O4 antigen moiety of lipopolysaccharide in the pathogenesis of extraintestinal *E. coli* infection; however, they demonstrate that the O4 antigen plays only a minor role in serum resistance in vitro and that its loss does not diminish and perhaps enhances the virulence of CP9 in vivo after intraperitoneal challenge.

Strains of *Escherichia coli* can be categorized into three independent epidemiologic groups. First, nonpathogenic isolates of *E. coli* compose a major portion of the aerobic bowel flora. Second, another group causes enteric disease but rarely, if ever, invades outside the gastrointestinal tract. The third group of *E. coli* causes extraintestinal disease, ranging from cystitis to sepsis. The clinical and economic impact of infectious syndromes caused by this latter cohort is enormous, despite the availability of efficacious antimicrobial therapy (11, 20). An increasing body of evidence suggests that extraintestinal isolates of *E. coli* possess unique virulence factors that enable them to cause systemic infection. We believe that an understanding of the pathogenic process utilized by these organisms is critical for the logical development of new preventative and/or therapeutic strategies.

Our laboratory is studying the clinical blood isolate CP9 (O4/K54/H5) as a model pathogen for infections caused by extraintestinal isolates of *E. coli*. We are evaluating putative virulence factors by creating proven isogenic mutants that differ from their wild-type parent in either a single or a combination of defined traits. In this way, their relative importance can be accurately assessed in appropriate in vitro and in vivo systems. We have previously shown that the K54 capsular polysaccharide is an important complement resistance determinant in vitro and that it increases the pathogenicity of CP9 in vivo (26, 27). However, this data demonstrated that the pathogenic process was clearly multifactorial in nature. The lipopolysaccharide (LPS) has long been believed to be another important virulence factor in *E. coli*. LPS consists of a lipid A moiety, an oligosaccharide core region, and an O antigen. It is likely that each of these LPS components has distinct functions in the pathogenic process. The O antigen is a long polysaccharide chain that confers serospecificity and is composed of a variable number of repeating oligosaccharide units. Epidemiologic studies have demonstrated that the majority of extraintestinal isolates possess an O-specific antigen (8, 14, 18). Further, of the >160 different serotypes recognized in *E. coli*, certain serotypes (e.g., O1, O2, O4, O6, O7, O16, O18, and O75) have been more frequently identified from clinical isolates (3, 14, 41). However, analyses like these that correlate serotypes with the ability to cause infection may be unreliable because of the influence of other bacterial factors, the size of the inoculum, and the variability of the underlying host. In vitro evaluations have supported LPS as playing an important role in protection against complement mediated lysis (4, 12, 18, 21, 22, 36) and phagocytosis (1, 15, 34). In vivo studies have also suggested that LPS is an important virulence factor in the development of pyelonephritis (5, 35, 38) and death in systemic infection (21, 33, 40). Unfortunately, none of these previous studies evaluated an appropriate pathogenic strain with a proven isogenic derivative lacking only the O-specific region of the LPS. In this study we describe the construction and initial characterization of isogenic CP9 derivatives that are deficient solely in the O4 antigen or in both the O4 and K54 antigens. These mutants were used to assess the relative role of the O4 antigen in protecting against the bactericidal effects of human serum and

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TABLE 1. *E. coli* strains used in this study

Strain	Genotype or other relevant characteristic(s)	Derivation	Source or reference
CP9	O4/K54/H5 clinical blood isolate, serum resistant		25
CP9.137	*cl1.137*::Tn*phoA* (K54 CL1 capsule gene Tn*phoA* fusion), serum sensitive		25
SG1039	Tn*10tet*::*pro*Δ*lacZ*		S. Gottesman
BW16948	pBW30::Tn*phoA′1* lambda *pir* RP4::MuKan *recA thr-1 leuB6 lacY1 tonA21 supE44*		44
TRR1	Spontaneous rough CP9 derivative ($O4^-$ $K54^-$)		28
TRR14	TRR1 *cps3*::ΔTn*10 tet*		28
TRR17	TRR1 Tn*10tet*::*pro*Δ*lacZ*	T4 (SG1039) × TRR1	This study
TR1377	*cl.137*::Tn*phoA′7*	Cm^r Kan^s swap with lambda::Tn*phoA′7*	This study
TR13737	*cl.137*::Tn*phoA′7 cps3*::ΔTn*10tet*	T4 (TRR14) × TR1377	This study
TR13777	*cl.137*::Tn*phoA′7 cps3*	Spontaneous Tet^s	This study
TR13787	*cl1.137*::Tn*phoA′7 cps3* Tn*10tet*::*pro*Δ*lacZ*	T4 (TRR17) × TR13777	This study
TRR20	*cl1.137*::Tn*phoA′7 cps3* Tn*10tet*::*pro*Δ*lacZ rfbO4*::Tn*phoA′1*	Kan^r exconjugant of BW16948 × TR13787	This study
TRR21	*cl1.137*::Tn*phoA′7 cps3* Tn*10tet*::*pro* Δ*lacZ rfbO4*::Tn*phoA′1*	Kan^r exconjugant of BW16948 × TR13787	This study
CP920	*rfbO4*::Tn*phoA′1*	T4 (TRR20) × CP9	This study
CP921	*rfbO4*::Tn*phoA′1*	T4 (TRR21) × CP9	This study
CP922	*rfbO4*::Tn*phoA′1 cl1.137*::Tn*phoA′7*	T4 (CP920) × TR1377	This study
CP923	*rfbO4*::Tn*phoA′1 cl1.137*::Tn*phoA′7*	T4 (CP921) × TR1377	This study

its contribution to virulence in mice challenged with intraperitoneal (i.p.) inoculation.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Bacteriophage, and Media. Strains and plasmids utilized in this study and their construction are listed in Table 1. K-12 strains (CS) with defined mutations in LPS production are described below. The parental strain, CP9, possesses the LPS serotype O4. It is a human blood isolate that grows in 80% normal human serum (NHS) and has been previously described (25). BW16948 used for transposon mutagenesis of CP9 and the lambda *P*am phage carrying the Tn*phoA′7* element were gifts from Barry Wanner (44); CS2057, CS2558, CS2334, and CS2772 were gifts from Carl Schnaitman (31); and SG1039 was a kind gift from Susan Gottesman. Strains were maintained at −80°C in 50% Luria-Bertani (LB) medium–50% glycerol. LB broth consisting of 5 g of yeast extract, 10 g of tryptone, and 10 g of NaCl per liter was used for growth of all strains in vitro, and incubations were performed at 37°C unless otherwise described. For plates, 15 g of Bacto Agar (Difco Laboratories, Detroit, Mich.) was added per liter, and kanamycin (40 μg/ml), chloramphenicol (25 μg/ml), and tetracycline (7.5 μg/ml) (Amresco, Solon, Ohio) were added where appropriate. Blood agar plates were obtained from Remel (Lenexa, Kans.).

Genetic manipulations. Transposon mutagenesis of CP9 with Tn*phoA* (Kan^r) and generalized transduction with bacteriophage T4 were performed as previously described (25). Recombinational switching of Tn*phoA* with the Tn*phoA* derivative Tn*phoA′7* (Cm^r) was achieved by infecting the host with lambdaPam phage carrying the Tn*phoA′7* element, selecting for chloramphenicol resistance, and screening transductants for loss of parental resistance (Kan^r) (44).

Detection of spontaneous Tet^s mutants derived from Tn*10*-promoted adjacent deletions utilized selection for fusaric acid resistance (13). Transposon mutagenesis of TR13787 was performed by the conjugal delivery of a Tn*phoA* derivative containing a *lacZ* transcriptional fusion (Tn*phoA′1*) on the suicide plasmid pBW30 (44). Despite the existence of homology between the Tn*phoA′7* contained in TR13787 and Tn*phoA′1* and the presumed presence of a functional RecA, transposition of Tn*phoA′1*, not homologous recombination with Tn*phoA′7*, was the usual genetic event observed (24a).

DNA manipulations and analyses. Whole-cell DNA was prepared as previously described (25). Restriction enzymes were obtained from New England Biolabs (Beverly, Mass.) and were used as suggested by the manufacturer. Standard DNA recombinant procedures and Southern hybridization were performed as described by Sambrook et al. (29). Probes for hybridization were generated by using a PCR radioactive labeling system (Gibco BRL, Gaithersburg, Md.). The probe used for detection of Tn*phoA′1* insertions was an internal fragment of Tn*phoA′1* generated with primers 63 (5′-GATCAAGAGACAGGATGA-3′) and 64 (5′-TGATCCTCGCCGTACTGC-3′) (Fig. 1A). The DNA sequence was determined by using the dideoxy chain termination method (30).

Construction of O4-specific antigen gene subclones p920.1 and p921.1. Subclones of the Tn*phoA′1* insertions in CP920 and CP921 were obtained by restricting whole-cell DNA with *Sal*I, which recognizes a site located to the right of the kanamycin resistance gene in Tn*phoA′1* (Fig. 1A), and with *Eco*RI, which does not cut within Tn*phoA′1*. Ligation of this restriction into pBS, electroporation into JM109, and selection of Kan^r transformants resulted in the identification of the subclones of interest, p920.1 and p921.1. Each contains the left 5.0 kb of Tn*phoA′1*, and p920.1 and p921.1 possess 7.0 and 1.15 kb, respectively, of chromosomal DNA upstream of the Tn*phoA′1* insertions. Sequence analysis initially involved the use of a Tn*phoA′1* fusion joint primer (1, 5′-AATATCGC CCTGAGC-3′) followed by primers derived from the deduced sequence.

Phenotypic analyses. (i) LPS. Whole-cell lysates were used for LPS analysis. Lysates were prepared as previously described (7), separated by using 14% polyacrylamide, and silver stained (39). The Western blot (immunoblot) assay was also performed as previously described (2), with unabsorbed polyclonal O4 antiserum that was generated by using an O4/K3/H5 *E. coli* strain (The Pennsylvania State University *E. coli* Reference Center, University Park). K-12 strains with defined mutations in LPS production were run concomitantly to establish which bands constituted the end of the core moiety of the LPS (Fig. 2). CS2334 has a disruption of *rfaL*, which is necessary for modification of the core so that the O antigen can be attached. CS2774 has a disruption of *rfaQ*, which is necessary for efficient O antigen attachment. CS2558 has a deletion in *rfaI*, and CS2057 has a deletion of *rfaGPSBI* (31).

(ii) Capsular polysaccharide. The ability to produce the group 1 capsule (colanic acid) was assessed by observing for mucoid colonies grown at 28°C after the introduction a multicopy plasmid containing the positive regulatory element *rcsA*. We have previously demonstrated that this phenotype in CP9 and its derivatives was due to colanic acid production (28). The group 2 capsule was extracted and analyzed as previously reported (25).

(iii) Total and outer membrane proteins. Cells (60 ml) were grown to log phase to an A_{600} of 0.5 to 0.6, washed in 1× phosphate-buffered saline (PBS), and resuspended in 11 ml of 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [pH 7.5]) containing 5 mM $MgCl_2$. Cells were disrupted by passage through a French press. Unbroken cells were removed by centrifugation. For total membrane protein preparation, the cell lysate was spun at 50,000 rpm (Beckman L8-60M ultracentrifuge, type 50 Ti rotor) for 1 h at 4°C, and the resulting pellet was resuspended in 200 μl of 50 mM HEPES–5 mM $MgCl_2$ (pH 7.5). For outer membrane protein preparation, 1.1 ml of 20% Triton X in 50 mM HEPES–5 mM $MgCl_2$ (pH 7.5) was added to the cell lysate, and the mixture was incubated at room temperature for 30 min. The lysate was spun at 50,000 rpm for 1 h at 16°C, and the pellet was resuspended in 200 μl of 50 mM HEPES (pH 7.5). Proteins were separated on 10% tricine gels by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Novex, San Diego, Calif.) and visualized with a 0.1% Coomassie brilliant blue R-250 stain.

(iv) Pyelonephritis associated pilus. Latex beads coupled with the P1 (α-Gal-1,4-β-Gal) disaccharide was used for detection of pyelonephritis-associated pilus (Chembiomed, Edmonton, Alberta, Canada). Strains were washed, resuspended in 1× PBS at a concentration of 1.0×10^9 CFU/ml, and mixed with 20 μl of the latex bead suspension. Agglutination of the latex beads was indicative of a positive reaction.

Serum sensitivity assays. Strains to be assayed for their sensitivity to the bactericidal effects of 80% NHS were grown at 37°C overnight. The next day, cells were diluted, grown to log phase (A_{600}, 0.4 to 0.5) at the same tempera-

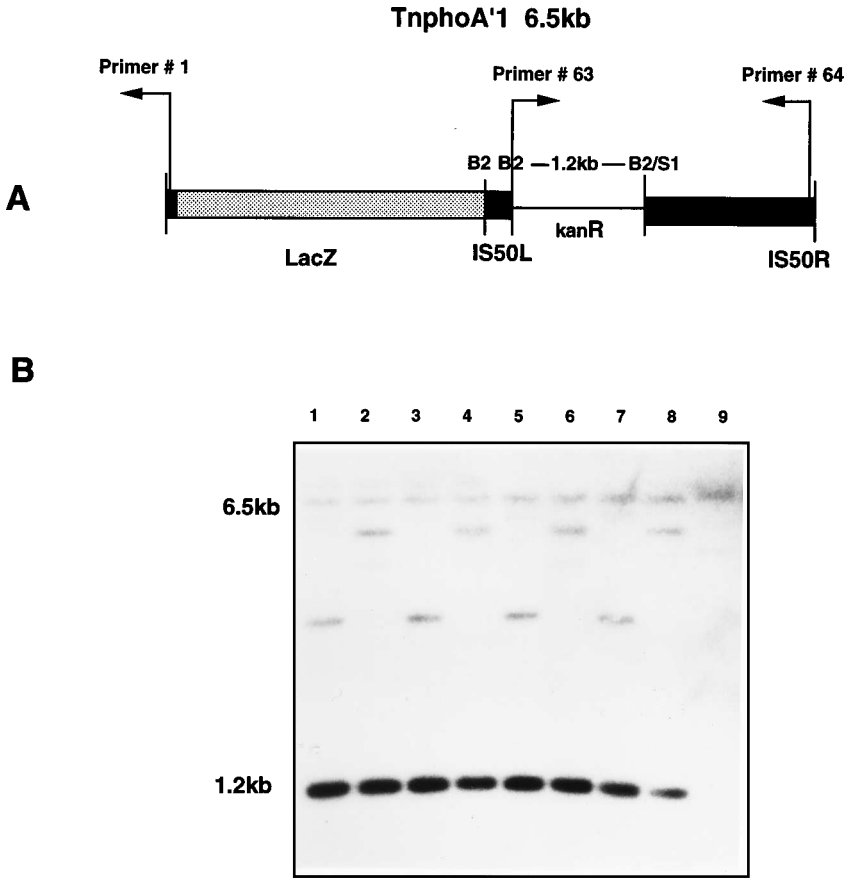

FIG. 1. (A) Schematic diagram of Tn*phoA'1*. The 2.4-kb fragment generated by PCR with primers 63 and 64 was used to probe for Tn*phoA'1* insertions. *Bgl*II-digested DNA containing Tn*phoA'1* produced a 1.2-kb internal fragment and a variably sized junction fragment. Primer 1 was the initial primer used for DNA sequencing of the subclones p920.1 and p921.1. B2, *Bgl*II; S1, *Sal*I. (B) Determination of number and locations of Tn*phoA'1* insertions in various mutants. The Southern blot of *Bgl*II-restricted whole-cell DNA was probed with the 2.4-kb fragment generated by PCR with primers 63 and 64 from Tn*phoA'1* under stringent conditions. Lanes: 1, TRR20; 2, TRR21; 3, TRR20T4; 4, TRR21T4; 5, CP920.17; 6, CP921.17; 7, CP920; 8, CP921; 9, CP9.

ture, and then diluted in isotonic gelatin-Veronal buffer with Ca^{2+} and Mg^{2+} (Isogever) (37) to give a starting inoculum of approximately 1.0×10^5 CFU/ml. A mixture containing 800 μl of NHS, 100 μl of cells, and 100 μl of 1× PBS (pH 7.4) was incubated at 37°C. Aliquots were removed at 0, 1, 2, and 3 h, and viable titers were determined by plating serial 10-fold dilutions in duplicate. Control serum was heated to 56°C for 30 min to inactivate serum-complement activity (Δ56°C for NHS). Serum was obtained from several donors. Each strain was assayed at least twice.

Mouse survival studies. Outbred Swiss mice (mean weight, 20 ± 2 g) were obtained through the National Institutes of Health animal breeding facility. Since outbred mice were utilized, they were challenged with the wild-type strain (CP9) in parallel with its mutant derivatives. The strains evaluated were grown overnight and diluted in 1× PBS such that an i.p. injection of 0.5 ml resulted in the delivery of 10^4 to 10^8 organisms. Bacterial titers were determined for dilutions to precisely determine the number of organisms inoculated. Animals were injected in groups of 10 with the same dilution. For data evaluation of a given strain, the initial titers for groups of animals with approximately the same starting inoculum were averaged to obtain a mean inoculum. Mice that expired within 5 min were excluded from the analysis, as this represented inadvertent damage to a vital organ and/or vessel. Mortality, the measured endpoint, was recorded at 24, 48, 72, 96, and 120 h. However, all mortalities were observed within the initial 24 h postinoculation (45).

Statistical analysis. The 50% lethal doses (LD_{50}s) generated from the mortality data of i.p. injection of live organisms were calculated by the Spearman-Karber method (16). A Dunnett multiple comparison test of the LD_{50}s was performed. CP9 was taken as the control and each LD_{50} was treated as a mean with two degrees of freedom. With these provisions, the allowance for the Dunnett test is as follows: $2.97 \times 0.10198 = 0.3029$ (alpha = 0.05, two-tailed test, 10 degrees of freedom).

RESULTS

Construction of isogenic O4 antigen-deficient mutants. Generalized transposon mutagenesis and subsequent screening on solid media for rough-appearing colonies were used to obtain O4 antigen-deficient mutants. Since the expression of either the group 1 (colanic acid) or group 2 capsular polysaccharide (K54) by CP9 confers a smooth colonial morphology, regardless of the state of the LPS, a group 1 and 2 capsular polysaccharide-deficient derivative of CP9 (TR13787) was constructed and utilized for the transposon mutagenesis (see Table 1 for construction). Derivatives of TR13787 with Tn*phoA'1* insertions were selected for on LB medium containing kanamycin, pooled, and plated on blood agar on which rough colonies were more readily visible. Two rough-appearing isolates, TRR20 and TRR21, were obtained. LPS analysis established that the rough colonial phenotype of TRR20 and TRR21 represented either loss of the O-specific antigen (TRR21) or nearly complete loss of the O4 antigen (TRR20) (data not shown). Confirmation that the transposon insertion (Tn*phoA'1*) was responsible for the rough phenotype observed for TRR20, and TRR21 was established by transducing the Tn*phoA'1* insertion back into TR13787. Transductants possessed a rough colonial morphology, and Southern analysis of TRR20, TRR21 and their transduction derivatives demonstrated that a single

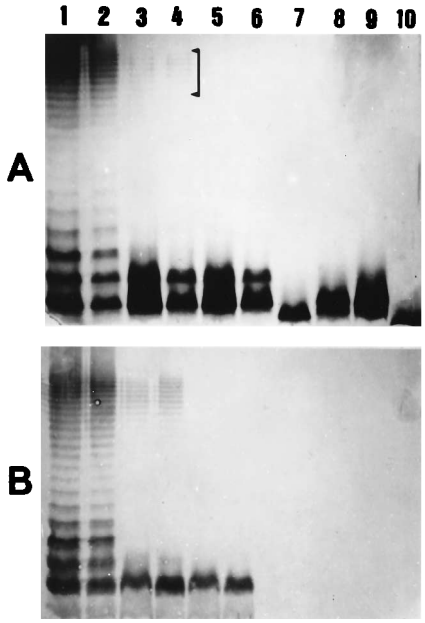

FIG. 2. Presence or absence of the LPS O4-specific antigen (LPS ladder) in CP9 and various derivatives. (A) Whole-cell extracts were separated on SDS–14% PAGE gel and silver stained. The arrowhead bracket marks the diminished amount of O4 antigen visible in CP920 and CP922. (B) Western blot analysis of gel described for panel A with unabsorbed polyclonal O4 antisera. Lanes: 1, CP9; 2, CP9.137; 3, CP920; 4, CP922; 5, CP921; 6, CP923; 7, CS2057; 8, CS2334; 9, CS2774; 10, CS2558.

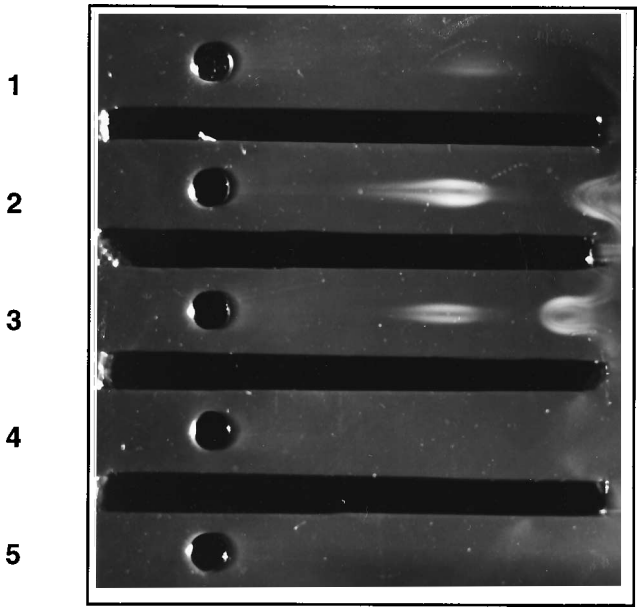

FIG. 3. Qualitative demonstration of presence or absence of capsular polysaccharide. Capsular extraction, immunoelectrophoresis, and precipitation by Cetavalon were done as described in Materials and Methods. Capsular extracts were not quantitated prior to being loaded. Rows: 1, CP9; 2, CP920; 3, CP921; 4, CP922; 5, CP923.

Tn*phoA′1* insertion was present and was physically in the same location (Fig. 1, lanes 1 and 3 and 2 and 4). The 6.5-kb band visualized in all lanes is an endogenous IS*50* homologous element. The faint band seen just above 6.5 kb in TRR20, TRR21, and their T4 derivatives is Tn*phoA′7* inserted into the *cl1.137* group 2 capsular locus. CP921 and CP920, the isogenic derivatives deficient solely in either the complete or nearly complete O4 antigen, were obtained by transducing the Tn*phoA′1* insertion from TRR21 and TRR20 into the wild-type isolate, CP9. CP923, the isogenic derivative deficient in both the K54 antigen and the O4 antigen, and CP922, deficient in the K54 antigen and nearly the complete O4 antigen, were constructed by transducing the Tn*phoA′1* insertions from CP921 and CP920, respectively, into TR1377 (*cl1.137*::Tn*phoA′7*). Southern analysis of CP920 (Fig. 1, lane 7) and CP922 (data not shown) established that Tn*phoA′1* was physically located in the same location as TRR20 (Fig. 1, lane 1); likewise, Tn*phoA′1* had an identical location in CP921 (Fig. 1, lane 8), CP923 (data not shown), and TRR21 (Fig. 1, lane 2). LPS analysis by both silver staining and Western blot established that CP921 and CP923 were O4 antigen-deficient (Fig. 2, lanes 5 and 6). Analysis of CP920 and CP922 revealed the presence of a faint LPS ladder (Fig. 2, lanes 3 and 4). The most rapidly migrating band in the lanes containing LPS from CP9 and its derivatives (Fig. 2A, lanes 1 to 6) has a migration distance similar to the LPS from CS2334 and CS2774 (Fig. 2A, lanes 8 and 9) (31). This suggests that this band consists of lipid A plus a core polysaccharide. These bands were not detected by polyclonal O4 antisera on Western blot analysis. On the basis of this finding, the lowest reactive LPS band by Western blot analysis from CP9 and derivatives (Fig. 2B, lanes 1 to 6) most likely represented lipid A, core polysaccharide, plus a single or a portion of an O4 antigen pentasaccharide. The location of the Tn*phoA′1* insertion in CP921 and CP923 results in disruption of the genes for rhamnose biosynthesis, consistent with the loss of the O4 antigen (see sequence analysis below). The Tn*phoA′1* insertion in CP920 and CP922 remained unclear after sequence analysis; therefore, the explanation for the small amount of O4 antigen produced by these strains has not yet been determined. Nonetheless, the small quantity of O antigen visualized in CP920 and CP922 does not appear to affect these mutants' phenotypes with regard to serum sensitivity testing or in vivo i.p. challenge of outbred mice since CP920 and CP921 behave in a similar fashion (see below). A qualitative group 2 capsule analysis performed on CP922 and CP923 confirmed the absence of the K54 antigen as expected (Fig. 3, lanes 4 and 5).

Sequence analysis of disrupted genes in CP920 and CP921. Sequence analysis of subclones p920.1 and p921.1 was performed to identify the genes disrupted by the Tn*phoA′1* insertions in CP920 and CP921. The nucleotide sequence of 1.15 kb flanking the IS*50*L region of Tn*phoA′1* in CP921 was determined. A comparison of this sequence with entries in the GenBank database revealed a 91% degree of DNA homology with the rhamnose biosynthetic operon from the *rfb* locus (O antigen) of *Shigella flexneri* 2a (23) and an 81% nucleotide sequence homology with the *rfb* locus from *Salmonella typhimurium* B (strain LT2) (10). Rhamnose is a common component of the O antigen from each of these strains (9, 24, 32). The precise location of the transposon insertion corresponded to base 653 for the *Shigella* sequence (accession number L14842) and base 4053 for the *Salmonella* sequence (accession number X56793), which is downstream from the putative promoter region but proximal to the coding sequence of the first gene in the operon, *rfbB*. The equivalent region in *E. coli* has been cloned, but sequence data are not yet available (6). Nonetheless, our sequence data clearly establish the Tn*phoA′1* insertion in CP921 to be located in the 5′ untranslated region proximal to the start of the rhamnose biosynthetic operon in the *rfb* O antigen locus. The nucleotide sequence of 2.1 kb flanking the IS*50*L region of Tn*phoA′1* in CP920 was also

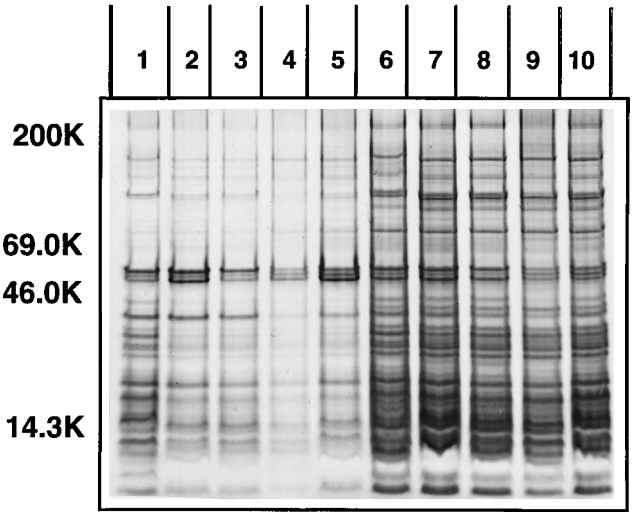

FIG. 4. Analysis of outer membrane (lanes 1 to 5) and total membrane (lanes 6 to 10) proteins. Proteins were purified as described in Materials and Methods, separated on a 10% tricine gel by SDS-PAGE, and stained with Coomassie blue. The positions of standard molecular mass markers are indicated on the left. Lanes: 1, CP9; 2, CP920; 3, CP921; 4, CP922; 5, CP923; 6, CP9; 7, CP920; 8, CP921; 9, CP922; 10, CP923.

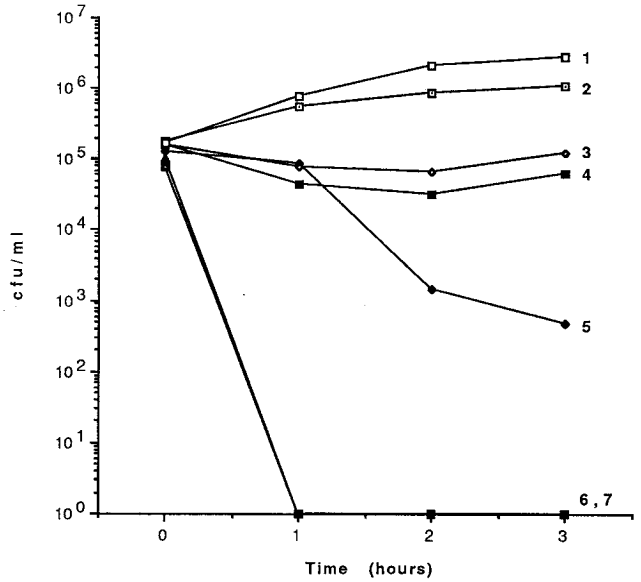

FIG. 5. In vitro effect of 80% NHS on viability of wild-type isolate CP9, its isogenic derivatives (CP920, CP921, CP922, CP923, and CP9.137). Curves are representative of the results for at least two experiments for each strain. Assays were performed as described in Materials and Methods. All strains were also assayed in the presence of 80% heat-inactivated NHS (Δ56°C). All strains grew in a similar fashion under these conditions; therefore, only the data for CP9 are depicted. Curves: 1, CP9 assayed in Δ56°C NHS; 2, CP9; 3, CP920; 4, CP921; 5, CP9.137; 6, CP922; 7, CP923.

determined. A comparison of this sequence with entries in the GenBank database did not reveal any matches with a high degree of homology.

Phenotypic assessment of non-LPS surface structures in O4 antigen-deficient isogenic mutants. Analyses of capsular polysaccharides, pyelonephritis-associated pilus, and membrane proteins were performed to exclude the possibility that the loss of the O4 antigen (CP920 and CP921) or both the K54 and O4 antigens (CP922 and CP923) affected their assembly onto the cell's surface. The group 1 (colanic acid) capsular polysaccharide (data not shown), the group 2 (K54) capsular polysaccharide in CP920 and CP921 (Fig. 3), the total membrane and outer membrane protein profiles (Fig. 4) and the PAP (data not shown) were evaluated. No differences in any of these structures were found compared with that of the parental wild-type strain, CP9, other than some minor variations in band intensity in the membrane protein analysis.

Effect of O4 antigen, K54 antigen, and O4 and K54 antigen on serum sensitivity. The wild-type parent CP9 was resistant to killing by 80% NHS, with growth of approximately 0.5 log in 3 h (Fig. 5, curve 2). CP920 and CP921 showed a slight increase in serum sensitivity, undergoing transient 0.2- and 0.5-log losses in viability over the first hour but nearly recovering to their initial titers by 3 h. As previously observed (27), the K54 capsule-deficient mutant, CP9.137, had a significant increase in susceptibility to the bactericidal activity of serum in vitro, incurring a 2-log kill during this period (Fig. 5, curve 5). The double K54-O4 antigen mutants CP922 and CP923 were extremely serum sensitive (Fig. 5, curves 6 and 7), with 100% kill from an initial titer of 1.0×10^5 CFU/ml within an hour.

Survival of Swiss outbred mice after i.p. inoculation of live strains. Mice were challenged in parallel by i.p. inoculation with various titers of the wild-type strain CP9 and its isogenic derivatives CP920, CP921, CP9.137, and CP922. The results are summarized in Table 2. CP9 had an LD_{50} of 1.4×10^7 CFU (log 7.1 ± 0.0683). Contrary to our expectation, infection with CP920 and CP921 (LD_{50}s of 3.2×10^6 CFU [log 6.5 ± 0.1325] and 5.2×10^6 CFU [log 6.7 ± 0.1198], respectively) resulted in significantly increased mortality ($P \leq 0.05$). Significantly fewer mortalities occurred with the capsule-deficient strain CP9.137 (LD_{50} of 2.9×10^7 cfu [log 7.46 ± 0.0915]), confirming previous observations (26), and even fewer mortalities occurred with the double K54-O4 antigen mutant, CP922 (LD_{50} of 6.4×10^7 CFU [log 7.8 ± 0.0840]). These differences in mortality were significant compared with that for CP9 ($P \leq 0.05$). Although there also appears to be a significant difference between CP9.137 and CP922, the Dunnett test utilized for statistical analysis does not enable such a comparison. Bacteria were recovered from the spleens of three animals infected and killed by CP9.137, CP920, CP921, and CP922. Capsule and LPS analyses of these strains did not reveal any alterations in their capsule and LPS profiles (data not shown).

DISCUSSION

In this study the role of the O4 antigen in the pathogenesis of extraintestinal *E. coli* infection was evaluated. Our usual approach in identifying virulence factors of importance is to perform random mutagenesis on our wild-type model pathogen CP9 followed by a screen or enrichment designed to isolate mutants of interest. Previous studies performed in this manner identified the K54 antigen as an important serum resistance determinant and virulence factor in vivo (25–27). Because LPS is also believed to be an important virulence trait in extraintestinal isolates of *E. coli*, we specifically constructed proven isogenic mutants deficient in the O4 antigen and the O4 and K54 antigens. Our results, however, are in contrast to those of previous reports. The O4 antigen appears to play only a minor role in protecting against complement-mediated killing since two independent mutants deficient in either all or nearly all of the O4 antigen had only a minor increase in their sensitivity to 80% NHS. Further, when mice were challenged i.p. with these strains, a small but statistically significant increase in the LD_{50} was observed, not the expected decrease.

TABLE 2. Survival of mice infected i.p. with live CP9, CP9.137, CP920, CP921, and CP922

Strain	Mean inoculum	No. of mice killed/no. injected (%)[a]	LD_{50} (CFU)
CP9 ($K54^+$)	4.0×10^6	0/10 (0)	1.7×10^7 (log 7.1 ± 0.0683)
	4.5×10^6	2/10 (20)	
	8.0×10^6	4/10 (40)	
	8.0×10^7	10/10 (100)	
CP920 ($O4^-$)	6.0×10^5	0/10 (0)	3.2×10^{6b} (log 6.5 ± 0.1325)
	1.7×10^6	2/5 (40)	
	4.0×10^6	15/25 (60)	
	6.0×10^6	20/20 (100)	
	5.0×10^7	9/10 (90)	
CP921 ($O4^-$)	3.5×10^5	0/10 (0)	5.2×10^{6b} (log 6.7 ± 0.1198)
	1.7×10^6	1/5 (20)	
	3.3×10^6	9/15 (60)	
	3.5×10^6	7/20 (35)	
	3.5×10^7	10/10 (100)	
CP9.137 ($K54^-$)	9.0×10^5	0/10 (0)	2.9×10^{7b} (log 7.5 ± 0.0915)
	9.0×10^6	0/20 (0)	
	6.0×10^7	9/10 (90)	
CP922 ($O4^-$ $K54^-$)	3.5×10^6	0/10 (0)	6.4×10^{7b} (log 7.8 ± 0.0840)
	3.5×10^7	2/10 (20)	
	4.2×10^7	2/20 (10)	
	5.5×10^7	10/10 (100)	

[a] Mortality was determined at 24 h; no mortalities occurred after this time point.
[b] Significantly different compared with CP9 (wild type) at an overall level of significance of P of <0.05 (Dunnett test).

The reasons for variance between our study and previous studies are not completely clear; however, several potential explanations exist. We established that our O4- and K54-deficient derivatives were truly isogenic. None of the earlier reports evaluated proven isogenic derivatives of a clinically relevant extraintestinal isolate. Some assessed enteric isolates which do not possess a group 2 capsule (4, 36). Others studied spontaneous mutants with either loss or alterations in the LPS (5, 22). Unfortunately, the possibility of a cryptic mutation(s) being responsible for the observed differences in such strains can not be excluded. Further, regardless of the means of obtaining an LPS-deficient mutant, it is possible that concomitant alterations in other surface structures may occur. Therefore, we qualitatively assessed and found no differences in the group 1 and 2 capsular polysaccharides, the pyelonephritis-associated pilus, or the total and outer membrane protein profiles from our O4 antigen isogenic derivatives. Such evaluations were not always performed for some of the previously described mutants. In addition, it is essential to define the precise nature of the LPS mutant being evaluated. Although our study did not support a major role for the O4-specific antigen in serum resistance, it remains possible that the core region may confer a greater degree of protection. If some of the previously evaluated strains had lost various degrees of the core region in addition to their O antigen, then the protective effects of the O antigen may have been mistakenly overemphasized.

Another potential reason for the discrepancy between our results and others is the possibility that not all O antigens serve identical biologic functions. In our model pathogen, it is clear that the K54 capsule is a major complement resistance determinant and that the O4 antigen is not. However, it is possible that such roles could be reversed or that both antigens serve such a function depending on the serotype. One report supports this hypothesis, although nonisogenic derivatives were evaluated (3).

It is important to emphasize that the results of this study do not exclude the O4 antigen as being an important virulence factor. Its specific role will undoubtedly be clarified with further in vitro and in vivo evaluations. A number of previous studies have suggested that the LPS is an important factor in protecting against the effects of bactericidal/permeability-increasing protein (42, 43). Studies with our isogenic O4 antigen mutants support this contention (24a). Therefore, despite the fact that the O4 antigen did not seem to contribute to the virulence of our strain in a mouse systemic infection model, studies of other in vitro and in vivo model systems will likely establish its role in the pathogenesis of extraintestinal *E. coli* infection.

The results of the in vitro serum sensitivity testing expand the understanding of complement resistance mechanisms in *E. coli* (12). A previous study established that in CP9, the K54 antigen does not prevent the activation of complement, that C3 is equally deposited on K54 positive and negative derivatives, and that the loss of the K54 antigen alone significantly increases serum sensitivity (27). Findings from this study established that the loss or nearly complete loss of the O4 antigen alone results in only a minor increase in serum sensitivity. However, the loss of both the K54 capsule and either all or most of the O4 antigen together results in a synergistic increase in serum sensitivity which significantly exceeds that observed with the loss of the K54 capsule alone, both in magnitude and rate of killing. It will be necessary to perform studies with labelled complement components to establish the mechanism of this observation at the molecular level. These results suggest that the group 2 capsular polysaccharide is the primary bacterial structure that impedes either the formation of a C5b-9 membrane-attack complex or prevents it from reaching its active site, a role previously suggested for the LPS in *E. coli* (12). When the K54 capsule is absent, the O4 antigen would appear to only slow this process, not prevent it, since a 2- to 3-log kill occurs over 3 h. When both the K54 O4 antigens are absent, a 5-log kill occurs in less than an hour, suggesting that the formation and effective insertion of the membrane-attack complex is now minimally impeded by any remaining bacterial component in CP9. Whether these roles established for the K54 group 2 capsule and the O4 antigen in CP9 can be extrapolated to other serotypes of extraintestinal isolates of *E. coli* awaits evaluation of additional strains.

The small, but statistically significant increase in LD_{50} observed with the K54-positive, O4 antigen mutants (CP920 and CP921) after i.p. challenge in mice was surprising but unequivocal. Because outbred mice were used, they were challenged concomitantly with CP9 and its mutant derivatives. The importance of this approach was reinforced when the calculated LD_{50} for CP9 in this study was approximately 0.5 log higher than that in earlier studies (26). There are several possible explanations for the O4 antigen-deficient strains having lower LD_{50}s than CP9. The first is that while the K54 capsule protects the strain from host-mediated killing, the loss of the O antigen moiety results in either a greater release of lipid A or increases the accessibility of the bacterially bound lipid A to the host. This proinflammatory mediator is a well-defined factor that can incite the cascade of events that leads to septic shock (17, 19). Another explanation may be that the host processing and/or ensuing responses may be different for LPS with and without the O4 antigen moiety. In contrast, when the K54 antigen-deficient and nearly complete O4 antigen-defi-

cient mutant (CP922) was tested in this model, it was the least virulent. This result suggests that the additional loss of the K54 group 2 capsule may permit a more successful eradication of the pathogen, thereby minimizing the host's exposure to lipid A and circumventing its deleterious effects. It should be noted that differences seen in LD_{50}s with the loss of the K54 or O4 antigen alone or in combination are significant but modest at best. This suggests either that several virulence factors need to be absent for profound differences in virulence to be discerned or that a factor(s) other than the K54 or O4 antigen is quantitatively more significant but remains to be tested or identified.

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