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Characterization of a Polysaccharide Capsular Antigen of Septicemic *Escherichia coli* O115:K"V165":F165 and Evaluation of Its Role in Pathogenicity

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Escherichia coli **strains of serogroup O115:K(−):F165 have been associated with septicemia in calves and piglets. These strains express a capsular antigen referred to as K"V165" which inhibits agglutination of the O antigen by anti-O115 serum. We used hybrid transposon Tn*phoA* mutants M48, 18b, and 2, and a spontaneous O-agglutinable mutant, 5131a, to evaluate the role of K"V165" in the pathogenicity of *E. coli* O115. Mutant M48 was as resistant to 90% rabbit serum and as virulent in day-old chickens as the parent strain 5131, mutants 18b and 5131a were less resistant to serum and less virulent in chickens, and mutant 2 was serum sensitive and avirulent. Analysis of outer membrane protein and lipopolysaccharide profiles failed to show any difference between the transposon mutants and the parent strain. In contrast, the spontaneous O-agglutinable mutant showed additional bands in the 16-kDa region of the polysaccharide ladder-like pattern. Mutants 2 and 5131a produced significantly less K"V165" capsular antigen than the parent strain, as demonstrated by a competitive enzyme-linked immunosorbent assay with adsorbed anti-K"V165" serum. In addition, electron microscopic analysis revealed that mutants 2 and 5131a had lost the capsular layer observed in the parent strain after fixation with glutaraldehyde-lysine. This capsule contained carbohydrate compounds and resembled an O-antigen capsule since it prevented O-antigen agglutination before the bacteria were heated at 100°C and induced bacterial serum resistance. The capsule-defective mutants colonized the intestinal epithelium of experimentally infected gnotobiotic pigs but failed to induce clinical signs of septicemia. We concluded that *E. coli* strains of serogroup O115 expressed a polysaccharide capsular antigen which induced serum resistance and consequently contributed to the pathogenicity of the bacteria.**

Escherichia coli is a frequent cause of intestinal and extraintestinal disease in humans and animals (32, 44, 56). Extraintestinal diseases include septicemia, newborn meningitis, polyserositis, and urinary tract infection (3, 24, 29, 56). Although the mechanisms involved in the development of these infections are not well understood, they are thought to include the abilities to colonize mucosal surfaces (37), to actively or passively traverse epithelial cell layers (34), to resist the bactericidal effect of complement (9, 26), to escape phagocytosis (9, 61), to survive and multiply in body fluids with low concentrations of free iron (25, 36), and to induce tissue damage by release of endotoxin or cytotoxins (24, 34, 56). Some virulence determinants, such as mannose-resistant fimbrial adhesins, motility, cytotoxins, aerobactin and ColV plasmids, outer membrane proteins, lipopolysaccharide (LPS), and capsule, have been associated with the ability of *E. coli* to induce extraintestinal infections (6, 7, 11, 24, 40).

E. coli may produce one of more than 74 different extracellular polysaccharidic capsules (45, 46), which are organized into distinct structures or excreted as extracellular slime (4). Capsular K antigens are serologically distinct from the LPS O antigens and are assumed to be present in an extracellular envelope covering the latter (45). Electron microscopy has revealed that many *E. coli* cells are indeed surrounded by a capsule; examination of this capsule at a higher resolution, however, requires certain stabilizing procedures (2).

Capsular antigens of *E. coli* are acidic polysaccharides which consist of oligosaccharide repeating units and differ in constituents, branching, and charge density (22). These capsules have been classified according to chemical composition, molecular weight, mode of expression, and genetic determination. Thus, *E. coli* acidic capsular polysaccharides have been divided into two groups: group I polysaccharide is similar to the capsule of *Klebsiella* spp., and group II polysaccharide is similar to the capsules of *Haemophilus influenzae* and *Neisseria meningitidis* (22, 23). Certain *E. coli* strains produce slime antigens described as viscous substances not anchored in the bacterial outer membrane, e.g., M antigen in members of the family *Enterobacteriaceae* (45, 46). Other strains produce long polysaccharide chains referred to as O-antigen capsule which cover the bacterial surface like a capsule (16, 45).

Acidic polysaccharide capsules have been the subject of intensive investigation because of their importance in the virulence of many bacteria and their usefulness as vaccines for the prevention of bacterial infection (1, 8, 31, 39). In fact, encapsulated bacteria are frequently associated with extraintestinal disease (3, 9). Strains of *E. coli* expressing the K1 capsule were associated with meningeal disease in newborns (51), whereas nonencapsulated derivatives of invasive K1 strains did not cause bacteremia in experimentally inoculated animals (48). The capsule is thought to allow the invading organisms to evade the immune system by a number of mechanisms, which include decrease of complement- and antibody-mediated opsonophagocytosis as well as resistance to the bactericidal activity of complement (9, 16, 26–28, 52, 61). However, the roles of other capsule-like

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structures, such as the O-antigen capsule, in pathogenesis have not been elucidated.

E. coli strains of serogroup O115 possessing the fimbrial antigen F165 and producing aerobactin and ColV plasmids induce septicemia and polyserositis in newborn pigs (11). Strains of this serogroup are nonenterotoxigenic and have been associated with severe enteritis and septicemia in calves and pigs (15, 38). Furthermore, they are negative for acidic polysaccharide capsule and are thus considered K(−) (43), but they produce a capsule-like structure which renders them nonagglutinable with anti-O115 serum and has been referred to as K"V165" (54). The purpose of the present study was to characterize the K"V165" antigen and to determine its role in the pathogenicity of *E. coli* O115 strains by using mutants obtained spontaneously or by Tn*phoA* mutagenesis.

(This study was presented in part at the 90th Annual Meeting of the American Society for Microbiology, Anaheim, Calif., 13 to 17 May 1990 [39a].)

MATERIALS AND METHODS

Bacterial strains. The wild-type pathogenic *E. coli* strain 5131 (serotype O115:K"V165":H51:F165) and the O-agglutinable strain 862B [serogroup O115:K(−):F165(−)] were isolated at the Faculté de Médecine Vétérinaire, Université de Montréal, Saint-Hyacinthe, Québec, Canada, from the intestinal contents of pigs with diarrhea (11). Strain 5131a is a spontaneous O-agglutinable mutant of strain 5131 (11). Strains M48, 18b, and 2 are Tn*phoA* mutants of strain 5131.

Antisera. Anti-O115:K"V165" and anti-alkaline phosphatase (PhoA) type III-N (Sigma Chemical Company, St. Louis, Mo.) sera were produced in rabbits by standard techniques (10), and anti-O115 serum was similarly prepared in rabbits inoculated with strain 5131 which was heated at 121°C for 2 h. Antiserum against the K"V165" capsule was prepared by repeated adsorption of anti-O115:K"V165" serum with parent strain 5131 heated at 121°C for 2 h and *E. coli* HB101; standard techniques were used (10). Slide agglutination of cultures grown at 37°C for 18 h on tryptic soy agar (Difco Laboratories, Detroit, Mich.) was evaluated at room temperature (10).

Tn*phoA* mutagenesis. Mutations resulted from random insertion of the Tn*phoA* sequence into the chromosomal or plasmidic DNA of *E. coli* 5131. This was accomplished by using plasmid pRT733, which carries the Tn*phoA* insertion and the kanamycin resistance (Km^r) gene in *E. coli* SM10 lambda *pir* (59). Growth from the mating between *E. coli* SM10(pRT733) and *E. coli* 5131 was streaked onto Luria-Bertani agar (Difco) containing 40 μg of kanamycin ml^{-1} and the alkaline phosphatase substrate XP (5-bromo-4-chloro-3-indolylphosphate-*p*-toluidine) (Sigma). Km^r and streptomycin-resistant (Sm^r) blue colonies resulting from the transposition of Tn*phoA* into the genome of the recipient strain *E. coli* 5131 were selected and stored in glycerol at −70°C.

Aerobactin and colicin V production. Aerobactin and colicin V production were determined as described previously by a cross-feeding method (11) and by the agar overlay technique (14), respectively.

Serum bactericidal assay. Bacteria were grown overnight in tryptic soy broth (Difco) at 37°C. Cultures were resuspended in fresh medium at a 10-fold dilution, incubated under agitation at 37°C, and harvested in the logarithmic growth phase (after 2 h of incubation). Bacteria were washed at room temperature with gelatin-Veronal-buffered saline plus magnesium and calcium ions (GVB^{2+}; pH 7.35) (58) and were diluted in GVB^{2+} to a concentration of approximately 10^7 CFU ml^{-1}. A volume of 0.1 ml of the bacterial suspension was added to 0.9 ml of normal fresh rabbit serum and incubated at 37°C. Viable-cell counts were estimated at 0, 1, 2, and 3 h by plating 0.1 ml of this serum on brain heart infusion agar (Difco). A strain was considered serum resistant if the bacterial count increased or did not change, intermediate if a decrease of up to 2 orders of magnitude in the bacterial count was observed, and serum sensitive if a decrease of more than 2 orders of magnitude in the bacterial count was observed.

Virulence in chickens. An overnight tryptic soy broth culture was centrifuged and resuspended in phosphate-buffered saline (PBS), pH 7.4. A volume of 0.5 ml of bacterial suspension was inoculated subcutaneously into 1-day-old chickens obtained from a local hatchery. The 50% lethal dose of each strain was determined by inoculating groups of five chickens with doses of bacteria of between 10^8 and 10^6 CFU ml^{-1} in twofold dilution steps (49).

Extraction and electrophoretic analysis of LPS, total cell proteins, and K"V165" capsular antigen. Isolation and electrophoretic analysis of bacterial LPS, proteins, and capsular antigen and Western blot (immunoblot) analysis of bacterial LPS and protein were done as described previously (20, 30, 43, 55). Briefly, the bacteria were grown overnight on tryptic soy agar (Difco) at 37°C, collected in PBS (pH 7.4), and diluted to an A_{420} of 0.400 as measured with a Philips Pye Unicam PU 8600 UV/VIS spectrophotometer. A volume of 1.5 ml of the sample suspension was centrifuged for 1.5 min at 16,000 × *g* in an Eppendorf microcentrifuge (model 5415; Brinkmann Instruments, Inc., Westbury, N.Y.). The pellet was mixed and boiled in 50 μl of sample buffer for 10 min, and then 10 μl of total cell suspension was loaded onto a 12% polyacrylamide gel and examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with the buffer system of Laemmli (30). The remaining 40 μl of the sample was cooled and held at 60°C for 1 h in the presence of 8 μl of proteinase K (2.5 mg ml^{-1}; Sigma), and a volume of 5 μl of lysate was examined by SDS-PAGE as described above. Bands from the different gels were transferred onto 0.2-μm-pore-size nitrocellulose membranes (Bio-Rad Laboratories, Richmond, Calif.) at 400 mA for 1 h in Tris-HCl-glycine-Methanol electroblotting buffer (53). The nitrocellulose membranes were transferred to 5% skim milk (Carnation Inc., Don Mills, Ontario, Canada) for 1 h and washed twice, once in TBS-Tween (0.05% Tween 20 in Tris-buffered saline [TBS]) and once in TBS, at room temperature. The membranes were incubated in 2.5% skim milk containing rabbit anti-alkaline phosphatase (PhoA), anti-O115, or anti-K"V165" serum for 2 h, then in mouse anti-rabbit immunoglobulin G conjugated to biotin (Bio/Can Scientific Inc., Mississauga, Ontario, Canada), and finally in avidin-peroxidase (Sigma) for 1 h, with washings following each step as described above. The immunocomplexes were detected by incubating the membranes in 4-chloro-1-naphthol substrate solution (Sigma) for 5 to 20 min (53).

The K"V165" capsular antigen was extracted by heat treatment as described by Ørskov and Ørskov (43). Briefly, bacteria grown on tryptic soy agar were harvested in PBS (pH 7.4) to give a suspension of 10^{11} cells ml^{-1}. The cells were heated at 60°C and centrifuged at 30,000 × *g* for 20 min. Part of the supernatant was used as the 60°C extract, which contains capsular antigen, LPS, and proteins, whereas the other part was heated at 100°C for 1 h, resulting in an extract containing only LPS and referred to as the 60/100°C extract.

Analysis of outer membrane proteins. Bacterial membrane

extracts were analyzed by SDS-PAGE as described previously (5). Bacteria were grown under agitation in tryptic soy broth at 37°C overnight. Cells were collected by centrifugation and suspended in 50 mM Tris hydrochloride (pH 7.8)–1 mM EDTA. The suspension was vortexed vigorously for 2 min and was then centrifuged at 4,500 × *g* for 15 min. The pellet was suspended in 50 mM Tris hydrochloride (pH 7.8)–1 mM EDTA, and bacteria were lysed in a French pressure cell (SLM AMINCO; SLM Instruments, Inc., Urbana, Ill.). The resulting suspension was centrifuged at 1,200 × *g* for 20 min, the pellet was discarded, and the supernatant was centrifuged at 100,000 × *g* at 4°C for 1 h. The resulting pellet was mixed in sample buffer, boiled for 10 min, and examined by SDS-PAGE as described above.

Competitive ELISA. The K"V165" capsular antigen was examined by enzyme-linked immunosorbent assay (ELISA) as previously described (12), except that a 100-μl volume of 60°C or 60/100°C heat extract of the parent strain, 5131, diluted 1:2 in 0.1 carbonate buffer (pH 9.6), was coated overnight at 4°C onto the wells of polystyrene microtiter plates (Dynatech Laboratories Inc., Chantilly, Va.). The anti-K"V165" serum used in this assay was preincubated at 50°C for 4 h with the 60°C or 60/100°C extract; with the 60°C proteinase K (2.5 mg ml^{-1}; Sigma)-treated extract of parent strain 5131; with the 60°C extract of strain 5131a, 2, or 862B; or with saline as a positive control. These extracts were twofold serially diluted in carbonate buffer prior to the addition of antiserum. The ELISA optical density was measured at 415 nm 10 min after addition of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Sigma), with a model 3550 microplate reader (Bio-Rad). All tests were done in triplicate, and the mean A_{415} ± standard deviation was calculated.

Chemical analysis of capsular antigen. The total carbohydrate content of the heat-extracted capsular antigen (extracted as described above) was assessed with phenol reagent (18). Results are given as the mean absorbance value ± standard deviation, determined from a standard curve with anhydrous dextrose (Fisher Scientific Co., Fair Lawn, N.J.) by using a Philips Pye Unicam PU 8600 UV/VIS spectrophotometer.

Examination of the K"V165" capsule by transmission electron microscopy. (i) Fixation with glutaraldehyde-lysine. An overnight culture of bacteria grown on tryptic soy agar was suspended for 20 min at room temperature in freshly prepared fixative consisting of 1 volume of cacodylate buffer containing 5% glutaraldehyde and 0.15% (wt/vol) ruthenium red and 1 volume of cacodylate buffer containing 100 mM lysine (21). Cells were sedimented by centrifugation, and the fixation was continued for an additional 100 min in a solution containing 5% glutaraldehyde and 0.15% (wt/vol) ruthenium red in cacodylate buffer. Bacterial cells were immobilized in 4% agar, washed five times in cacodylate buffer, and post-fixed with 2% osmium tetroxide for 2 h. The washings were repeated as described above, and the samples were dehydrated in a graded series of acetone washes. Samples were then washed twice in propylene oxide and embedded in Spurr low-viscosity resin. Thin sections were poststained with uranyl acetate and lead citrate and examined with an electron microscope (Philips 201) at an accelerating voltage of 60 kV.

(ii) Polycationic ferritin labeling. Bacterial cells from an overnight culture on tryptic soy agar were fixed for 2 h at 20°C in cacodylate buffer containing 5% glutaraldehyde. Fixed bacteria were suspended in cacodylate buffer and allowed to react with polycationic ferritin (Sigma) (final concentration, 1.0 mg ml^{-1}) for 30 min at 20°C (21). The reaction was slowed by a 10-fold dilution with buffer, and the organisms were centrifuged and washed three times in cacodylate buffer. Bacterial cells were then immobilized in 4% agar and processed as described above.

(iii) Immunostabilization. Bacteria from an overnight culture on tryptic soy agar were harvested and washed once with phosphate-buffered saline (0.01 M, pH 7.2). The bacterial suspensions were further exposed to undiluted homologous anti-O115, anti-O115K"V165", or anti-K"V165" serum for 1 h at 4°C (21). They were suspended in cacodylate buffer (0.1 M, pH 7.0) containing 5% glutaraldehyde and 0.15% (wt/vol) ruthenium red and fixed for 2 h at 20°C. The bacterial cells were then immobilized in 4% agar and processed as described above.

Pathogenicity of mutants in pigs. Inoculation of colostrum-deprived newborn pigs was carried out under germfree conditions. Pigs were obtained by hysterotomy under maximal sterile conditions (11) and were subsequently transferred through an antiseptic polyvinylpyrrolidone-iodine (Proviodine) bath to stainless steel tub isolators containing up to four pigs per isolator, as described previously (35). These isolators were sterilized with 2% peracetic acid 24 h before housing the animals and were maintained at 30 to 35°C. The pigs were fed three times a day with evaporated milk (Carnation). When they were 2 days old, the pigs were inoculated intragastrically with 1 ml of an overnight culture in tryptic soy broth containing approximately 10^9 CFU of *E. coli* ml^{-1} diluted in 20 ml of 0.1% peptone (Sigma) water. The pigs were euthanized by an intracardiac injection of 0.5 ml of T61 (Hoechst Canada Inc., Montréal, Québec, Canada) when moribund or 4 or 8 days after inoculation.

Bacteriologic examination of inoculated pigs. Ileum, cecum, colon, lung, liver, spleen, kidney, and brain tissues were plated onto blood agar and MacConkey agar (Difco) (11). After overnight incubation at 37°C, lactose-positive colonies were identified as *E. coli* and confirmed as infecting bacteria by slide agglutination with anti-O115:K"V165" and anti-O115 serum.

Statistics. Student's *t* test for paired samples was used for statistical analysis (62). Differences were considered to be significant when *P* values of <0.05 were obtained.

RESULTS

Isolation of *E. coli* Km^r Sm^r Tn*phoA* mutants. *E. coli* 5131 was mated with *E. coli* SM10 lambda *pir* which carried the plasmid pRT733 and plated on Luria-Bertani agar containing kanamycin, streptomycin, and XP (alkaline phosphatase substrate) (40 μg of each ml^{-1}). Approximately 1% of the colonies observed were blue Sm^r and Km^r colonies. These colonies are presumed to incorporate Tn*phoA* within a gene which encodes an extracytoplasmic protein and thus provides the requisite signals for transport of the carboxy-terminal *phoA* fragment into the periplasmic space (19). Individual blue colonies were verified as mutants of the parent strain, *E. coli* 5131, by slide agglutination with anti-O115:K"V165" serum. A bank of blue mutant colonies was constituted and stored in glycerol at −70°C. Mutants were screened for aerobactin and colicin V production and serum resistance in 90% fresh rabbit serum. All of the mutants produced aerobactin and colicin V. Several mutants were less resistant to serum than strain 5131. Three mutants of 5131, each possessing a single insertion of the Tn*phoA* sequence either in the plasmid (strain 18b) or in the chromosome (strains M48 and 2), were chosen for this study.

TABLE 1. Presence of K"V165" capsule in *E. coli* O115 strain 5131 and mutants with decreased virulence

Strain	LD_{50}[a] (log_{10} CFU)	Serum resistance[b]	Agglutination with serum[c]	
			Anti-O115	Anti-K"V165"
5131	6.8	R	−	+
M48	6.7	R	−	+
18b	7.7	I	−	+
5131a	7.8	I	+	−
2	8.9	S	−	−
862B	9.2	S	+	−

[a] 50% lethal dose for day-old chickens.
[b] In 90% fresh rabbit serum. R, resistant; I, intermediate; S, sensitive.
[c] Slide agglutination test.

Mutants 18b and 2 showed a decrease in resistance to serum, whereas M48, which did not express F165 fimbriae, served as a serum-resistant Tn*phoA* mutant (Table 1) (19).

Serum resistance and virulence in chickens. The wild-type strain 5131, the three Tn*phoA* mutants, the O-agglutinable spontaneous mutant 5131a, and the O-agglutinable wild-type strain 862B were examined for survival in 90% rabbit serum and for virulence in day-old chickens. Strain 5131 multiplied in the serum during 3 h after inoculation and was virulent in chickens (Table 1 and Fig. 1). Mutant M48 survived but did not multiply in the serum and was virulent in chickens. Mutants 5131a and 18b both survived for at least 2 h and subsequently decreased in numbers but were not completely eliminated at 3 h after inoculation, and both were less virulent than strain 5131. Both mutant 2 and wild-type strain 862B were rapidly eliminated within 1 to 2 h after inoculation of serum and were nonvirulent in day-old chickens.

Screening for K"V165" capsule by slide agglutination. Strains were examined by slide agglutination with anti-O115: K"V165", anti-O115, and anti-K"V165" sera. All strains were agglutinated by the anti-O115:K"V165" antibodies. The anti-O115 antibodies agglutinated only mutant 5131a and wild-type strain 862B. The anti-K"V165" serum agglutinated strains 5131, M48, and 18b (Table 1).

Protein, LPS, and capsular extract electrophoretic analysis. All mutants produced smooth LPS chains similar to those of the parent strain 5131, in contrast to the semirough LPS of the O-agglutinable wild-type strain 862B (Fig. 2). However, the spontaneous O-agglutinable mutant 5131a showed additional low-molecular-mass bands (16-kDa region) in the ladder-like pattern of bands seen in the LPS profile of its wild-type parent strain.

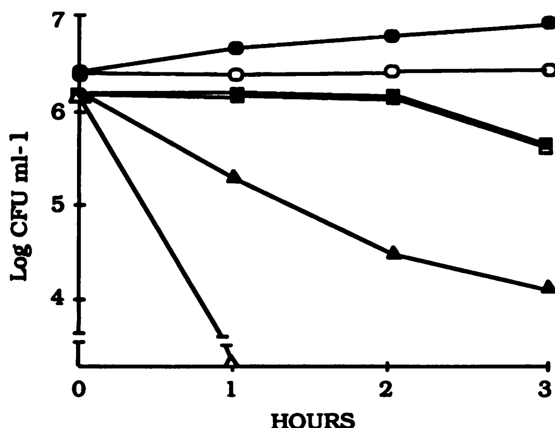

FIG. 1. Serum resistance. Survival and growth in 90% fresh rabbit serum of *E. coli* 5131 (●); spontaneous O-agglutinable mutant 5131a (■); transposon mutants M48 (○), 18b (□), and 2 (▲); and O-agglutinable wild-type strain 862B (△). Viable-cell counts were determined by plating the cells on brain heart infusion agar medium.

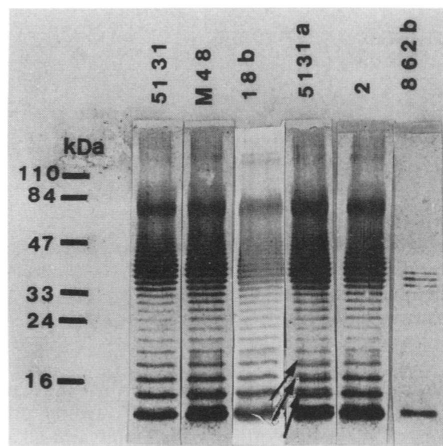

FIG. 2. Western blot analysis of LPS profiles with anti-O115 serum. Mutant 5131a shows additional bands (arrows) in the LPS ladder-like pattern. Mutants M48 (lane 2), 18b (lane 3), and 2 (lane 5) show the smooth LPS profile of the parent strain, 5131.

No bands were observed on the Western blot of the proteinase K-treated total cell preparation from strain 5131 when the blot was examined with anti-K"V165" serum, demonstrating that the anti-K"V165" serum did not contain antibody against the O115 O-polysaccharide chain which entered the polyacrylamide gel and that the K"V165" capsular antigen probably did not enter the gel (results not shown).

Analysis of outer membrane protein and total cell protein profiles did not reveal any differences between strain 5131 and the mutant strains (results not shown), whereas, on a Western blot, a 47-kDa band corresponding to *phoA* fusion was observed in mutant 2 but not in parent strain 5131 (Fig. 3).

Competitive ELISA. Pretreatment of the anti-K"V165" serum with either the 60°C extract from the capsule-negative strain 862B or with the 60/100°C extract from strain 5131 did not significantly change the reaction between the anti-K"V165" serum and the homologous 60°C extract from strain 5131 (Fig. 4), confirming that this antiserum did not contain anti-O115 antibody. Pretreatment of the anti-K"V165" serum with the 60°C extract from strain 5131 greatly reduced the reaction of this serum with its homologous 60°C extract, whereas pretreatment with 60°C extract from mutant 2 or 5131a reduced the homologous reaction but to a significantly lesser extent. Proteinase K treatment of the 60°C extract from strain 5131 did not significantly alter its ability to reduce the homologous reaction, suggesting that K"V165" capsule does not possess a major protein component.

Total carbohydrate analysis. The total carbohydrate content of the 60°C extract from strain 5131 (101.0 ± 1.0 μg ml^{-1}) did not significantly decrease after the extract was heated to 100°C (Table 2). The total carbohydrate content of the 60°C extract of mutant 2 was less than that of strain 5131 ($P < 0.02$), whereas the total carbohydrate content of the

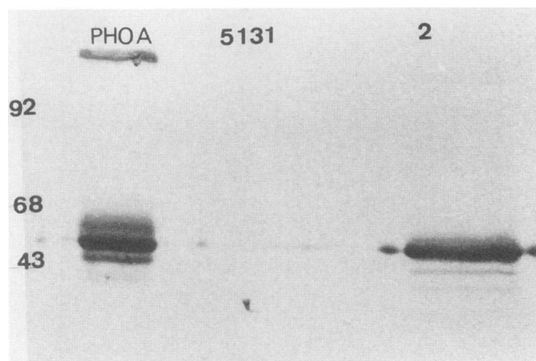

FIG. 3. Western blot analysis of total protein extracts with anti-alkaline phosphatase serum. The phoA fusion product was characterized by Western blotting with rabbit anti-alkaline phosphatase serum. This product was expressed in mutant 2 but not in parent strain 5131 and was similar in size to the alkaline phosphatase standard. Lanes PHOA, 5131, and 2 represent standard PhoA type III-N (diluted 1:1,000) and total protein extracts from strains 5131 and 2, respectively. The positions of molecular size markers are indicated on the left in kilodaltons.

60°C extract of mutant 5131a was significantly reduced ($P <$ 0.001) to the same level as that of the K(−) strain 862B.

Examination of K"V165" capsular antigen by electron microscopy. The presence of capsule in the various strains was evaluated by electron microscopy of thin sections of bacterial cells fixed with glutaraldehyde-lysine, labeled with polycationic ferritin, or exposed to homologous antisera. Anti-O115:K"V165" or anti-O115 serum failed to stabilize the K"V165" capsular layer in any of the strains (results not shown). However, examination of cells fixed with glutaraldehyde-lysine revealed that the parent strain, 5131, possessed a ruthenium red-stained capsular layer that was also observed in mutants M48 and 18b (results not shown) but was absent or seemed to be expressed as a dispersed conglomerate in the spontaneous mutant 5131a (Fig. 5). In contrast, cells of the Tn*phoA* mutant 2 had a reduced amount of capsular layer or did not appear to be capsulated. The O-agglutinable wild-type strain 862B did not express any capsular layer. Examination of polycationic ferritin-labeled cells showed that thin filaments were present on the bacterial cell wall of 5131 but were almost absent in mutants 5131a and 2 (results not shown), suggesting that the K"V165" capsule probably possesses a weak negative charge.

TABLE 2. Chemical analysis of K"V165" capsular antigen extract of *E. coli* O115:K"V165" and its mutants

Strain	Total carbohydrate content (μg ml^{-1}, standard equivalent)[a] of indicated extract	
	60°C	60/100°C
5131	101.0 ± 1.0	93.3 ± 6.1
5131a	62.6 ± 5.0[b]	63.0 ± 7.5[b]
2	77.6 ± 7.5[c]	72.3 ± 7.7[c]
862B	63.3 ± 3.7[b]	60.0 ± 3.4[b]

[a] Micrograms of carbohydrate (as glucose) per milliliter; results are means ± standard deviations of three distinct tests.
[b] Significantly different ($P < 0.001$) from parent strain 5131 by the *t* test.
[c] Significantly different ($P < 0.02$) from parent strain 5131 by the *t* test.

Pathogenicity of mutants in pigs. Intragastric inoculation of gnotobiotic pigs with wild-type strain 5131 resulted in clinical signs of septicemia, i.e., anorexia, lameness, reluctance to move or lack of motor coordination, and death within 48 h. In contrast, pigs inoculated with the capsule-defective spontaneous O-agglutinable mutant 5131a, Tn*phoA* mutant 2, or O-agglutinable strain 862B did not develop clinical signs

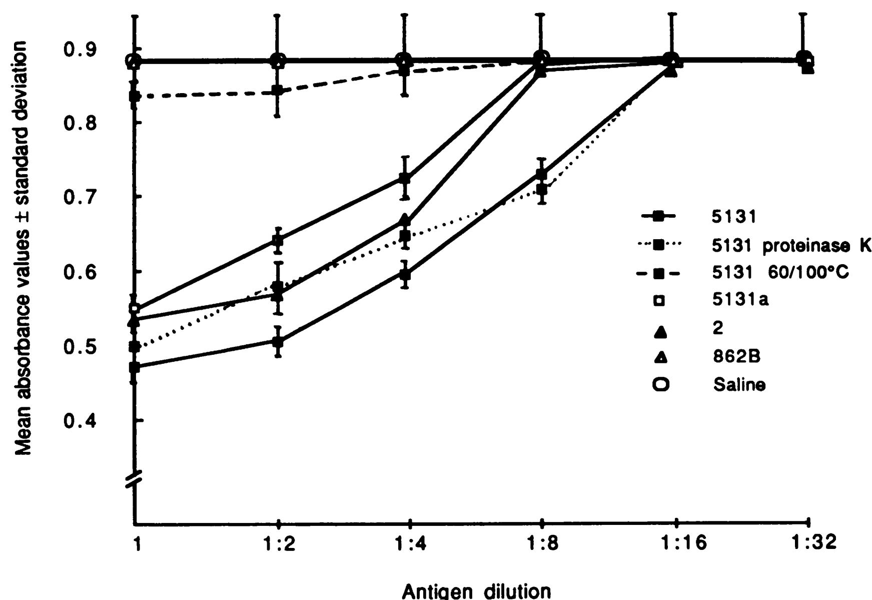

FIG. 4. Competitive ELISA. Anti-K"V165" serum incubated for 4 h at 50°C with the 60°C, 60/100°C, or proteinase K-treated 60°C extract of the parent strain 5131, with the 60°C extract of the O-agglutinable mutant 5131a, the Tn*phoA* mutant 2, or the wild-type strain 862B, or with saline was allowed to react with the 60°C extract of strain 5131. Mean absorbance values are from three tests. The symbols in the key refer to 60°C extracts unless otherwise stated.

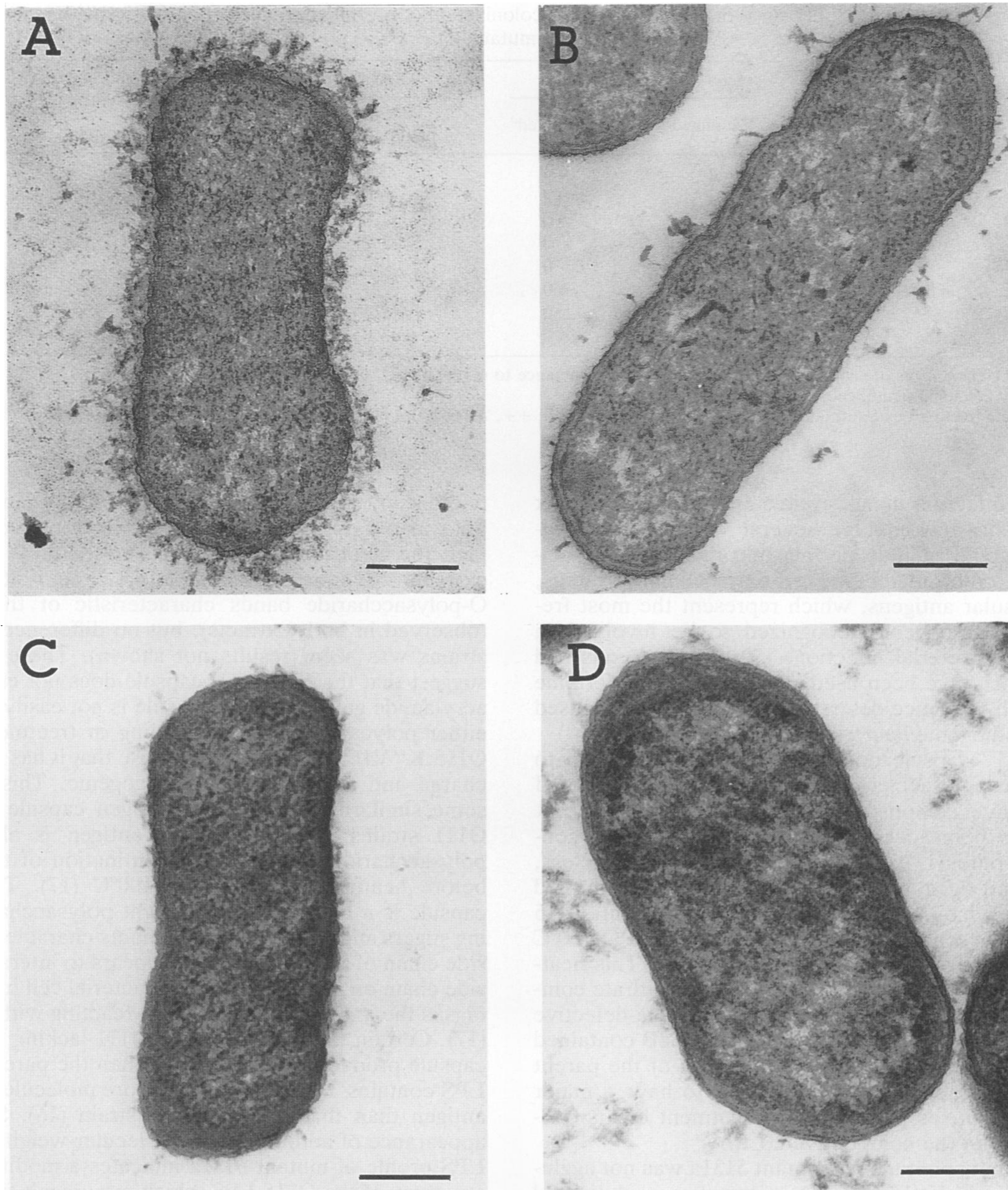

FIG. 5. Transmission electron micrographs of thin sections of *E. coli* wild-type strain 5131 (A) and the following capsule-defective strains: spontaneous mutant 5131a (B), Tn*phoA* mutant 2 (C), and O-agglutinable wild-type strain 862B (D). Strain 5131 possesses a ruthenium red-stained capsular layer which is absent from strain 862B. This capsular layer is absent from or expressed as a dispersed conglomerate in the O-agglutinable mutant 5131a. Mutant 2 appears nonencapsulated. Bars, 0.2 μm.

within 8 days after inoculation (Table 3). All strains colonized the intestinal mucosa equally well and translocated from the intestinal lumen to the mesenteric lymph nodes. Whereas the parent strain 5131 was found in extraintestinal organs of inoculated pigs, the capsule-defective mutants 5131a and 2 and the wild-type strain 862B were not found up to 8 days after infection.

At necropsy, pigs inoculated with 5131 had lesions of fibrinous polyserositis, i.e., serofibrinous exudate in the pericardial and peritoneal cavities, and congestion of the intestinal mucosa. No significant lesions were observed in pigs inoculated with strain 5131a, 2, or 862B.

DISCUSSION

Our results demonstrate that septicemic *E. coli* of serogroup O115 expresses a polysaccharide capsular antigen which is involved in resistance to the bactericidal effect of serum, virulence of the bacteria in day-old chickens, and pathogenicity in newborn pigs. We observed that both a spontaneous mutant and a Tn*phoA* capsule-defective mutant of this strain were less virulent in chickens and less pathogenic in pigs than their parent strain. *E. coli* O115:K"V165": F165 strain 5131 induces septicemia in experimentally infected piglets (11), and the ability of this strain to establish

TABLE 3. Appearance of clinical signs and colonization of organs after inoculation of pigs with capsule-defective mutants of *E. coli* 5131

Strain	Time of sacrifice (days postinoculation)	No. of pigs		Colony count in:			
		Examined	Affected[a]	Intestine[b] (10^9 CFU/g)	Mesenteric lymphnodes[c]	Lung[c]	Liver[c]
5131	1	4	4	1.8	+++	++	+
	2	2	2	6.0	++	+	+
5131a	4	2	0	1.0	+	−	−
	8	2	0	1.2	±	−	−
2	4	2	0	0.4	±	−	−
	8	2	0	1.1	±	−	−
862B	4	2	0	2.5	±	−	−
	8	2	0	3.0	±	−	−

[a] Moribund with clinical signs of septicemia: anorexia, lameness, reluctance to move, or lack of motor coordination.
[b] Mean of colony counts in ileum, cecum, and colon.
[c] ±, <5 colonies in the first quadrant; +, ≥5 colonies in the first quadrant; ++, ≥5 colonies in the second quadrant; +++, ≥5 colonies in the third quadrant.

such systemic infection in susceptible animals is a complex phenomenon and may involve several virulence determinants. These could include periplasmic membrane-associated proteins, outer membrane or extracellular proteins, LPS, and capsular antigens, which represent the most frequent surface components recognized to be involved in pathogenesis of bacterial infections (59). Spontaneous and Tn*phoA* mutants have been used successfully to determine the role of such virulence determinants in infections caused by different pathogenic bacteria (13, 33, 59, 60).

The K"V165" capsule does not seem to correspond to classical acidic polysaccharide or K antigen. Ørskov and Ørskov (43) have demonstrated that O115 strains do not produce acidic polysaccharide K capsule and have confirmed that strain 5131 is K(−) (42, 43). Nevertheless, these strains produce a not-well-defined capsule, termed K"V165" (54), which prevents agglutination with anti-O115 serum and can be removed by heating the bacteria at 100°C for 1 h, as observed in the competitive ELISA. This heat-extractable capsule appears to contain a carbohydrate component, since the 60°C extracts from the capsule-defective mutants and the acapsulate wild-type strain 862B contained significantly less total carbohydrate than that of the parent strain 5131. This capsule does not appear to have a major protein component, as proteinase K treatment did not reduce its activity in the competitive ELISA.

The finding that spontaneous mutant 5131a was not agglutinated by the anti-K"V165" serum but was agglutinated with the anti-O115 serum indicates that this mutant has almost totally lost the polysaccharide K"V165" capsule. This capsule was observed on this mutant as dispersed conglomerates under an electron microscope after glutaraldehyde-lysine treatment of the bacteria and was still detected by the highly sensitive competitive ELISA. The 60°C extract of Tn*phoA* mutant 2 contained less carbohydrate than the parent strain but more than the capsule-negative strain 862B or mutant 5131a, suggesting that this mutant still produces a certain amount of capsular material which masks its O agglutinability (Table 1). This was confirmed by electron microscopy after glutaraldehyde-lysine treatment and by the competitive ELISA.

This capsule is antigenically different from the O115 LPS, since the anti-K"V165" serum did not react either with O115 LPS bands in the Western blot or with the 60/100°C extract of strain 5131, which contained mostly O115 antigen, in the competitive ELISA.

The K"V165" capsule was easily observed by electron microscopy after glutaraldehyde-lysine fixation of bacteria but was not observed in SDS-PAGE after silver staining. In fact, the electrophoretic analysis of the 60°C and 60/100°C extracts of strains 5131, 5131a, 2, and 862B revealed O-polysaccharide bands characteristic of the O115 LPS (observed in both extracts), but no difference between the strains was seen (results not shown). These observations suggest that the K"V165" capsule does not enter the polyacrylamide gel. Since this capsule is not easily stabilized by either polycationic ferritin labeling or treatment with anti-O115:K"V165" serum, we suppose that it has little negative charge and is not highly immunogenic. This capsule has some similarities with the O-antigen capsule observed on O111 strains, since the latter antigen is also a neutral polysaccharide and inhibits agglutination of the O antigen before heating of bacteria to 100°C (17). The O-antigen capsule is a high-molecular-weight polysaccharide containing sugars and antigenic determinants characteristic of the O side chain of *E. coli* O111 and appears to interact with the O side chain on the surface of the bacterial cell in a way which masks the antigenic determinants reacting with the O serum (17). Certain mutants of *E. coli* O111 lacking the O-antigen capsule produce 50% more LPS than the parent strain; this LPS contains 30% fewer lipid A core molecules devoid of O antigen than that of the parent strain (16). Similarly, the appearance of additional low-molecular-weight bands in the LPS profile of mutant 5131a indicates a modification in the structure of the O115 LPS which may result from cessation of production of an O-antigen polysaccharide capsule. In contrast, the mutation in strain 2 is due to a single insertion of Tn*phoA* into the DNA of strain 5131 (19), which has resulted in a decrease in or modification of, but not the complete absence of, the K"V165" capsule, since this mutant is not agglutinated by the anti-O115 serum. However, this mutation has not resulted in any change in the LPS profile.

The role of the K"V165" capsule in pathogenicity was assessed by intragastric inoculation of capsule-defective mutants 5131a and 2 into newborn gnotobiotic pigs. We observed that both mutants failed to induce clinical signs of septicemia (Table 3). Although they colonized the intestinal mucosa of inoculated pigs and traversed the intestinal mucosa to localize in the mesenteric lymph nodes, they did not persist in other extraintestinal organs. These results suggest that, by losing their capsular antigen, the capsule-defective mutants had also lost their capacity to survive and multiply in the bloodstream, possibly by resisting phagocytosis

and/or the bactericidal effects of the classical or alternate complement pathway (8, 47, 61). The decrease in serum resistance of these mutants in the absence of specific antibodies indicates a role for K"V165" antigen in resistance to the alternate complement pathway and subsequently in virulence for newborn chickens. It is possible that the K"V165" capsular antigen masks sites for binding of C3b and initiation of the complement pathway. However, it is surprising that mutant 5131a, which almost totally lacks the capsule, is more resistant to the effects of serum than mutant 2, which appears to produce some capsular material still covering the O115 LPS. The specific carbohydrate composition of the lipid A-linked O antigen is known to play an important role in complement fixation, and increased O-antigen chain length could promote resistance to the bactericidal effects of serum (16, 50, 57). In addition, LPS-defective mutants of various bacterial species have been associated with decreased virulence (33, 41). However, our experiments revealed that the less virulent and avirulent mutants had the same smooth-pattern LPS as the parent strain. It is more likely that the K"V165" capsule mediates binding of C3b to sites far enough away from the cell surface to prevent insertion of the terminal membrane attack complex and subsequent lethality (26–28). Thus, modification in the capsule of mutant 2 would allow insertion of C3b closer to the cell surface and subsequent lethality. Modifications in the polysaccharide chains of the LPS of mutant 5131a could inhibit insertion of the terminal membrane attack complex. Similarly, certain O111 mutants lacking O-antigen capsule but possessing 50% more LPS than the parent were resistant to complement killing in the presence of specific antibody, probably because of limited access of the terminal membrane attack complex to the cell surface (16, 26, 28).

It is interesting that the spontaneous O-agglutinable mutant 5131a was moderately virulent in day-old chickens but did not induce clinical signs of septicemia in pigs. These results could signify a profound difference in the nonspecific host defense mechanisms of the immune systems of chickens and pigs which would allow the bacteria to more readily spread systemically in the chickens and kill them. Alternatively, the results may reflect the importance of the route of entry in bacterial infection. The subcutaneous route of inoculation in chickens would allow the bacteria to easily reach the bloodstream and induce systemic infection and death, whereas after intragastric inoculation of pigs, bacteria must survive passage through the gastrointestinal tract and subsequent translocation through the epithelial cells before entering the system. Similarly, Nnalue and Lindberg (41) observed that *Salmonella choleraesuis* mutants deficient in O antigen remained virulent for mice after parenteral inoculation but were avirulent after oral administration.

In conclusion, we found that *E. coli* O115:K"V165":F165 produces a polysaccharide capsule that is quite similar to the O-antigen capsule described by Goldman et al. (17) and is an important determinant in the development of septicemia and polyserositis in newborn pigs, at least in part because of its role in resistance to the bactericidal effects of the alternate complement pathway.

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