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Characterization of an Avirulent Mutant of a Virulent Avian Escherichia coli Isolate

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SUMMARY. A virulent, complement-resistant avian *Escherichia coli* isolate and its avirulent, complement-sensitive, transposon-insertion mutant were compared for the purpose of revealing structures associated with complement resistance. Both had a smooth lipopolysaccharide layer, contained *traT*, and lacked a capsule, but the mutant possessed a 16.2-kilodalton outer-membrane protein (OMP) not present in the wild-type. This protein may be the product of a coding region interrupted by transposon insertion. Such results suggest that an OMP greater than 16.2 kilodaltons in size may be responsible for the complement resistance and virulence of this wild-type *E. coli*.

RESUMEN. Caracterización de una mutante avirulenta originada de una cepa virulenta de Escherichia coli aviar.

Con el objeto de revelar las estructuras asociadas con la resistencia al complemento, se comparó una cepa de *Escherichia coli* virulenta, resistente al complemento, con su mutante avirulenta producida por inserción de un transposón, sensible al complemento. Ambas cepas tenían una cubierta lipopolisacárida lisa, contenían traT, no tenían cápsula, pero la mutante poseía una proteína en la membrana externa de 16.2 kilodaltons que no estaba presente en la cepa original. Esta proteína puede ser el producto de una región codificante interrumpida por la inserción del transposón. Estos resultados sugieren que una proteína de la membrana externa mayor de 16.2 kilodaltons en tamaño puede ser responsable de la resistencia al complemento y la virulencia de esta cepa original de *E. coli*.

In a previous study undertaken to assess the role of complement resistance in the virulence of an avian *Escherichia coli*, a mutant, characterized by its reduced complement resistance, was generated from a complement-resistant, virulent avian *E. coli* isolate using transposon mutagenesis (16). This mutant, which contained an insertion in a small plasmid and an insertion in the chromosome, was also shown to be less virulent than the wild-type in chicken embryos. These data suggested that complement resistance is important in the virulence of this avian *E. coli* isolate (16).

In the present study, the mutant was compared with the wild-type *E. coli* in an attempt to identify those factors changed in the mutant that contribute to the complement resistance and virulence of the wild-type organism. These phenotypic changes may be due to alterations in the mutant's elaboration of a K1 capsule, long-chain lipopolysaccharides (LPS), or outer-membrane proteins (OMP) such as TraT (1,2,5,6,8,14,15,17,22). Growth differences between the mutant and wild-type might also produce differences in the organisms' complement resistance and virulence.

MATERIALS AND METHODS

Test organisms. *E. coli* used were a virulent, complement-resistant wild-type isolate cultured from a chicken with systemic colibacillosis and a transposon-insertion mutant of the virulent isolate that was less complement-resistant and virulent than the wild-type (16).

Media. Bacterial isolates were maintained in Luria-Bertani (LB) broth or on LB agar with tetracycline (10 μg/ml) added as required (19).

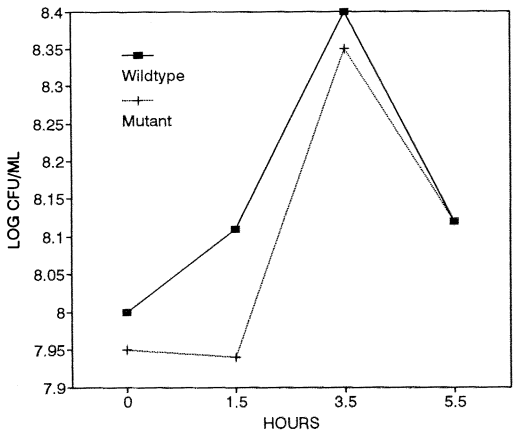

Fig. 1. Wild-type and mutant *E. coli* growth curves.

Growth curve analysis. Growth curves of the wild-type isolate and its mutant were determined (13). Bacteria were grown overnight in LB broth at 37 C with shaking. Ten-ml samples of both cultures were adjusted to similar optical densities. Each sample was used to inoculate 200 ml of fresh LB broth and incubated at 37 C with shaking until saturation. An aliquot from each culture was removed at 2-hour intervals for 6 hours, diluted in phosphate-buffered saline, and plated to determine viable counts.

Capsule detection. Capsule production was identified using the acid fuchsin staining technique of Maneval (12). Isolates were also examined for possession of K1 antigen with a commercially available K1 antigen detection kit from Wellcome Diagnostics (Dartford, England).

Outer-membrane-protein analysis. Outer-membrane proteins were isolated by the method described by Dassouli-Mrani-Belkebir *et al.* (4), and the samples were subjected to gel analysis run according to the method of Laemmli (9). Samples were loaded into wells of 10% polyacrylamide gels (Amresco Inc., Solon, Ohio) in a Hoefer SE 600 Vertical Slab Electrophoresis unit (Hoefer Scientific Instruments, San Francisco, Calif.). The electrophoresis was run at 20 mA using a constant current power source (Model 452; E-C Apparatus Corp., St. Petersburg, Fla.). Proteins of known size were included on the gel as molecular-weight standards (Bio-Rad Laboratories, Richmond, Calif.). Gels were stained with Coomassie Blue R-250 (Bio-Rad) (17) and photographed with Polaroid type 55 film (Polaroid Corp., Cambridge, Mass.) on a Polaroid MP4 Land camera.

LPS isolation. Overnight cultures, in amounts sufficient to yield 1 g of wet pellet each when centrifuged, were pelleted by centrifugation (4,000 × *g* for 15 minutes), and the pellets were subjected to the Darveau and Hancock method of LPS isolation (3). The LPS samples were suspended in pyrogen-free water (McGaw, Inc., Irvine, Calif.) and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Samples were mixed with equal volumes of a solubilizing agent (23) and loaded into the wells of 12% polyacrylamide gels in a Hoefer SE600 Vertical Slab Electrophoresis unit. The electrophoresis was run at 20 mA using a constant-current power source (Model PS 500X; Hoefer) until the bromophenol dye had migrated 10 cm (23). Commercially obtained rough and smooth LPS samples (Sigma Chemical Co., St. Louis, Missouri) were run on each gel for comparative purposes. Each gel also contained a protein sample as described earlier. Gels were stained initially with Coomassie blue to detect gross protein contamination followed by silver staining (Bio-Rad Silver Stain Kit) to detect the LPS. Gels were illumi-

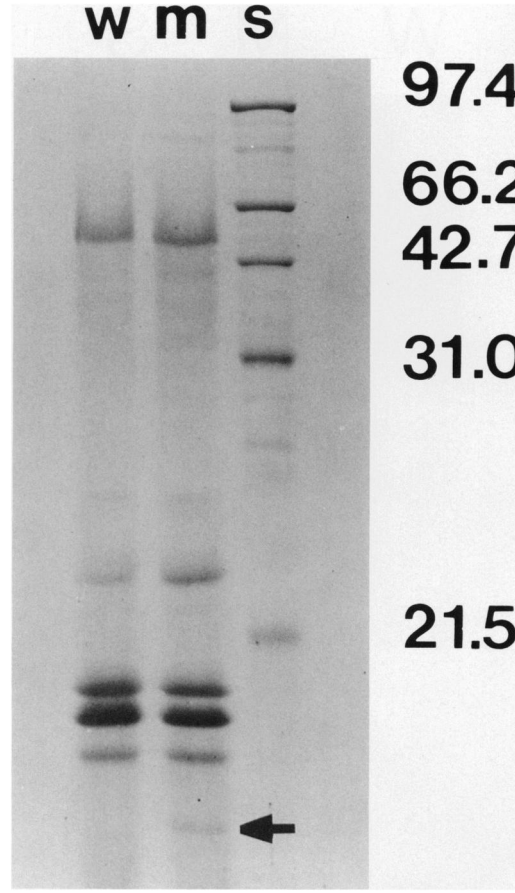

Fig. 2. SDS-PAGE of wild-type and mutant *E. coli* outer-membrane proteins. Lane W = wild-type's outer membrane proteins; Lane M = mutant's outer-membrane proteins, including a 16.2-kilodalton band not seen in the wild-type (arrow); Lane S = molecular-weight marker (×1000).

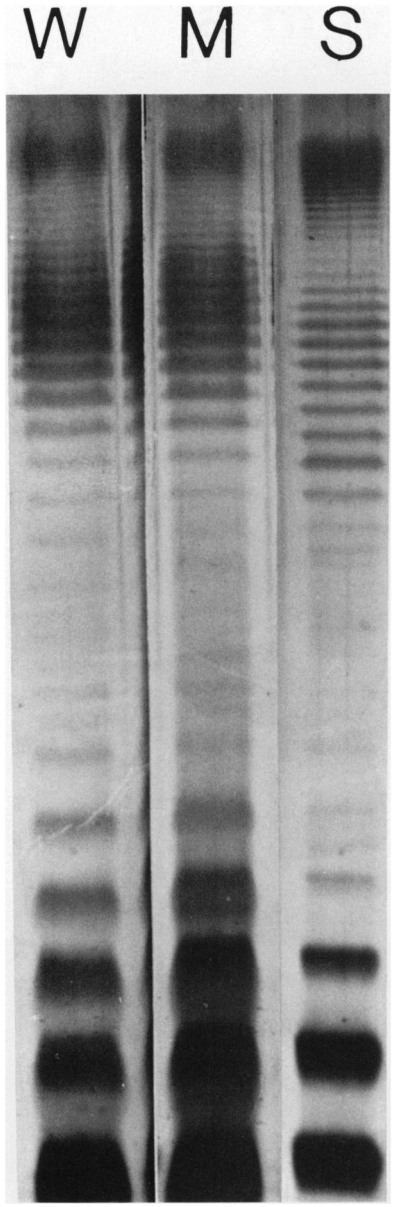

Fig. 3. SDS-PAGE of wild-type and mutant *E. coli* LPS. Lane W = wild-type's LPS; Lane M = mutant's LPS; Lane S = commercially obtained LPS derived from the smooth strain, *E. coli* O111:B4.

nated with white light and photographed with an Olympus OM-1 camera with a 50-mm, F3.5 macro lens (Olympus, Tokyo, Japan) using Kodak Technical Pan Film (Eastman Kodak Co., Rochester, N.Y.).

Bacteriophage susceptibility. Dr. A. S. Cross of the Department of Bacterial Diseases, Walter Reed Army Institute of Research in Washington, D.C., tested the mutant and wild-type *E. coli* for susceptibility to a set of rough-LPS-specific bacteriophages. This testing was deemed necessary, since the LPS patterns of partially rough and smooth *E. coli* isolates may be indistinguishable on SDS-PAGE (2).

DNA isolation and purification. Plasmid DNA of the mutant and wild-type isolates was isolated, purified, and resuspended in Tris-acetate-EDTA buffer (18). Plasmids were electrophoretically separated in 1% agarose gels run at 50 volts for 6 hours on a horizontal electrophoresis unit (Model MPH; IBI, New Haven, Conn.) with a constant voltage power source (Model 452; E-C Apparatus Corp.) and stained with ethidium bromide (18). Plasmid DNA derived from *E. coli* V517 (11) was included on each gel for molecular size comparisons. The DNA in gels was visualized with an ultraviolet transilluminator (Model TM 36; Ultra-Violet Products, Inc., San Gabriel, Calif.). Photographs were taken with Polaroid type 55 film with a no. 23A Wratten gelatin filter (Eastman Kodak Co.) on a Polaroid MP4 Land camera.

Hybridization study with a *traT* probe. Plasmid pKT107 was used as a source of the *traT* gene (14), which encodes TraT, an OMP that contributes to the complement resistance of some *E. coli* strains (14,21,22). This plasmid was digested with *Bst*EII (Boehringer Mannheim, Indianapolis, Inc.), and the fragments were separated electrophoretically (15,21). The 700-bp fragment, containing most of the *traT* gene (15), was extracted from the agarose with GENECLEAN (Bio101, La Jolla, Calif.) and labeled with the Genius 1 Kit (Boehringer Mannheim) for use as a probe. Mutant and wild-type plasmids in agarose were transferred to charge-modified nylon membranes (QIABRANE Nylon plus; Qiagen, Inc., Chatsworth, Calif.) by the method of Southern (20) and hybridized with the probe under stringent conditions (18). That is, the filter was prehybridized in aqueous solution for 12 hours at 68 C and hybridized in the same solution with the denatured probe for 12 hours at 68 C. The filter was washed successively in 2× SSC (SSC: 0.15 M NaCl + 0.015 M sodium citrate) (5 Prime → 3 Prime, Inc., Boulder, Colo.) with 0.1% SDS (Sigma); in 1× SSC with 0.1% SDS; in 0.5× SSC with 0.1% SDS; and in 0.1× SSC with 0.1% SDS. Each wash was carried out with mild agitation for 15 minutes at 68 C. The hybridized probe was detected using the protocol in the Genius 1 Kit.

RESULTS AND DISCUSSION

The growth curves of the wild-type and mutant *E. coli* isolates were similar (Fig. 1), and both cultures contained similar concentrations of bacteria following overnight incubation. No capsule was detected around either organism with Maneval staining, and neither the wild-type nor mutant *E. coli* possessed K1 antigen.

The virulent isolate and its mutant did differ in outer-membrane-protein (OMP) profiles, with the mutant *E. coli* possessing a 16.2-kilodalton protein not present in the wild-type organism (arrow, Fig. 2). No differences were noted between the two organisms in LPS: both the mutant and wild-type produced a smooth LPS pattern on SDS-PAGE (Fig. 3), and both were resistant to rough-LPS-specific bacteriophages (2). Both organisms contained the *traT* gene within their plasmid DNA.

The only structural characteristic that clearly distinguished the two organisms in this study was the presence of the 16.2-kilodalton OMP in the mutant, not found in the wild-type isolate. TraT, a 25-kilodalton OMP encoded by the plasmid-located *traT* gene, is known to contribute to the complement resistance of some *E. coli* strains (14). Since the 16.2-kilodalton band in the mutant might represent a truncated TraT protein produced from an interrupted *traT* gene, the plasmid DNA from the mutant and wild-type were probed with a labeled *traT* gene. Although this gene was present in both organisms, it was found on larger plasmids than the mutant plasmid previously shown to be affected by transposon insertion (16). Thus, it is unlikely that the deviations from the wild-type traits seen in the mutant are associated with a mutation of *traT*. Therefore, the 16.2-kilodalton band may represent a protein that in its native form contributes to the complement resistance and virulence of the wild-type isolate.

Differences in the growth of the two organisms might also produce differences in complement resistance and virulence. Since the complement-resistance test used to classify the mutant and wild-type corrects for growth differences (10), only the reduction in virulence noted in the mutant might be explained by growth differences. Since the growth curves of the wild-type and its mutant were similar, it is unlikely that growth differences between the organisms can account for the mutant's reduced virulence.

In summary, the only detectable, clearcut difference between the two organisms that might account for the mutant's reduced complement resistance and virulence was in OMP profiles. This OMP change did not appear to be due to a mutation, caused by transposon insertion, of the *traT* gene. If an alteration in this OMP is responsible for the loss in the mutant's complement resistance and virulence, then this OMP, not previously known for its importance in complement resistance and virulence, may be contributing to these two traits in this wild-type avian *E. coli* isolate.

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