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SUMMARY. The role of complement resistance in the virulence of an avian Escherichia coli isolate was examined with transposon mutagenesis. A suicide plasmid containing a kanamycin-encoding mini-transposon was used to transform a virulent complement-resistant avian E. coli isolate. A less resistant mutant was identified that contained a transposon insertion in a plasmid and in the chromosome. This loss of complement resistance was associated with a drop in virulence in an embryo assay. No other phenotypic changes were detected in the mutant. These results suggest that complement resistance is associated with the virulence of this organism.

RESUMEN. Mutagenesis de transposones usada para el estudio del papel de la resistencia al complemento en la virulencia de una cepa de Escherichia coli aviar.

Se examinó el papel de la resistencia al complemento en la virulencia de una cepa de Escherichia coli usando mutagenesis por transposones. Se usó un plásmido suicida que contenía un minitransposón que codifica la kanamicina, para transformar una cepa virulenta de E. coli aviar resistente al complemento. Se identificó una mutante menos resistente que contenía un transposón insertado en un plásmido y en el cromosoma. Esta pérdida de resistencia al complemento se asoció con una disminución en la prueba de virulencia para el embrión. No se detectaron otros cambios fenotípicos en la mutante. Estos resultados sugieren que la resistencia al complemento está asociada con la virulencia de este organismo.

The virulence of Escherichia coli in avian hosts has been attributed to a plethora of virulence factors (5,13,19). This statement has been inferred from comparisons of the phenotypes possessed by many virulent and avirulent E. coli isolates. The creation of mutant E. coli differing from the wild-type E. coli only in the single factor of interest offers a more definitive means of examining the role of single phenotypic characteristics in virulence.

The purpose of the present study was to examine the single trait of complement resistance for its contribution to the virulence of an avian E. coli isolate.

MATERIALS AND METHODS

Test organisms. A virulent E. coli isolate cultured from a chicken with systemic colibacillosis and an avirulent E. coli isolate were used.

Media. Bacterial isolates were maintained in Luria-Bertani (LB) broth (15). For the tests of complement resistance, isolates were grown in PG broth (1% peptone plus 1% glucose) with or without bromothymol blue (0.5%) (11). LB agar (1.1%) containing kanamycin (45 μg/ml) or ampicillin (100 μg/ml) was used for screening (15). Guinea pig complement (ICN ImmunoBiologics, Costa Mesa, Calif.) was used in the complement-resistance tests.

Transposon and vector. A mini-transposon constructed from the kanamycin resistance (Knr) gene of TN903 and two IS10 units (7,9) was obtained from Dr. Nancy Kleckner (Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Mass.). This transposon, designated as derivative 103, was provided on a suicide plasmid, pNK2794, which
codes for ampicillin resistance (Ap') as well as for a transposase.

**Transposon mutagenesis and screening.** The wild-type virulent *E. coli* was made competent for transformation (14) and was transformed with pNK2794 using the Gene Pulser Transfection Apparatus and Pulse Controller (BioRad Laboratories, Richmond, Calif.) set at 25 μF, 200 Ω, and 2.5 kV (17). Initial selection of mutants was done on the basis of acquisition of Kn'. To confirm loss of the suicide plasmid, mutants were further selected for ampicillin sensitivity (Ap'). The Kn'Ap' mutants were retained for further study.

Complement resistance of the mutants was initially examined using a qualitative microtiter test (11) with guinea pig complement (20). The wild-type isolate, which is virulent and complement-resistant, and the avirulent, complement-sensitive *E. coli* were included on each plate as controls. Sensitive mutants identified by this method were further tested using a quantitative microtiter test (10) with guinea pig complement and the same sources for comparison. Sensitive mutants were stored at −70°C in LB broth for further testing.

**Phenotypic characterization.** The wild-type and mutant organisms were characterized by their ability to use the 95 different carbon sources included in Biolog GN microplates (Biolog, Hayward, Calif.). They were tested for motility by stab-inoculation of motility test agar (Bacto Motility Test Medium; Difco Laboratories, Detroit, Mich.) (1) and for colicin production by the method of Fredericq (6) using a colicin-sensitive *E. coli* (ATCC 23559) and a colicin-producing *E. coli* (ATCC 23558) as controls. Their antibiograms were determined by the disc diffusion method using ampicillin, chloramphenicol, gentamicin, kanamycin, nalidixic acid, polymyxin B, streptomycin, sulfadiazine, and tetracycline (3). Hemolysis production was tested by streaking an isolate on 5% bovine blood agar (Bacto Blood Agar Base; Difco) and incubating for 18 hours at 37°C.

**DNA isolation, purification, and digestion.** Chromosomal and plasmid DNA was isolated, purified, and resuspended in Tris-acetate-EDTA buffer (14). All restriction enzymes were obtained from Boehringer Mannheim (Indianapolis, Ind.). Chromosomal DNA was digested with *Eco*RI and *Cla*I, and pNK2794 was digested with *Bam*HI to remove the transposon for probe construction (9). All DNA fragments or plasmids were electrophoretically separated in 0.8% agarose gels run at 50 volts for 6 hours on a horizontal electrophoresis apparatus (Model MPH; IBI, New Haven, Conn.) with a constant voltage power source (Model 452; E-C Apparatus Corp., St. Petersburg, Fla.) and stained with ethidium bromide (14). The DNA in gels was visualized with an ultra violet transilluminator (Model TM36; Ultra-Violet Products, Inc., San Gabriel, Calif.) Photographs were taken with Polaroid type 55 film (Polaroid Corp., Cambridge, Mass.) with a no. 23A Wratten gelatin filter (Eastman Kodak Co., Rochester, N.Y.) on a Polaroid MP4 Land camera. When necessary, selected fragments of plasmids were cut out of the gel and extracted from the agarose using GENECLEAN (Bio 101, La Jolla, Calif.).

**Detection of transposon by hybridization.** The DNA to be used as the probe was obtained by digesting pNK2794 with *Bam*HI, separating the fragments electrophoretically, and extracting the transposon portion with GENECLEAN. This 1.6-kb fragment was radioactively labeled with [32P]dCTP (Amersham, Arlington Heights, Ill.) by random oligonucleotide-primer synthesis (18). Mutant and wild-type chromosomal fragments and plasmids in agarose were transferred to charge-modified nylon membranes (Sigma Chemical Co., St. Louis, Missouri) by the method of Southern (16) and hybridized with the probe under stringent conditions (14). 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 then washed successively in 2× SSC (5 Prime − 3 Prime, Inc., Boulder, Colo.) with 0.1% sodium dodecyl sulfate (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. Hybridized filters were used to expose Kodak X-Omat-Ar film (X-Ray of Georgia, Norcross, Ga.) for 12 hours at −70°C.

**Virulence tests.** A laboratory-based chicken embryo lethality test was used to assess virulence (12). Overnight cultures of the isolates were washed twice in phosphate-buffered saline (PBS), resuspended, diluted in PBS, and inoculated into the allantoic cavity of 12-day-old chicken embryos. PBS-inoculated embryos were included as controls. Twenty eggs were used for each of two inoculum dilutions of approximately 10⁹ and 10¹ colony-forming units (CFU) in 0.1 ml. Eggs were candled daily, and deaths were recorded for 96 hours.

**RESULTS**

**Identification of mutants.** More than 1200 Kn' transformants were selected on LB-kanamycin plates. These transformants were further screened for acquisition of Ap' as an indication of suicide plasmid retention. The 700 Ap'Kn' mutants identified were examined for loss of complement resistance in a qualitative test. The eight mutants thus identified were further studied with a quantitative complement resistance test. All eight were more sensitive than the wild-type organism but more resistant than the avirulent control organism.
Virulence of mutants. Of the eight identified complement-sensitive mutants, the four most sensitive were selected for virulence testing. The most complement-sensitive and least virulent mutant killed 10% of the embryos in which it was tested, in contrast to the wild-type, which killed 75% of the embryos. This mutant was selected for phenotypic characterization and hybridization studies.

Phenotypic analysis of the complement-resistant avirulent mutant. This mutant appeared identical to the wild-type isolate in colicin production, lack of hemolysin production, colony morphology, motility, antibiotic profiles (except for Kn'), and fermentative abilities.

Estimation of insertion sites in the complement-sensitive avirulent mutant. The hybridization studies revealed a single radioactive band in the plasmid DNA (lane B, Fig. 1). Comparing the location of this band to its location in the agarose gel that was probed (lane B, Fig. 2), it was possible to determine the likely location of the insert. Because this radioactive band migrated around 2.7 kb, the 1.6-kb transposon probably inserted into the 1.6-kb plasmid. A single radioactive band was also identified in the chromosomal digest that resulted from EcoRI digestion (lane D, Fig. 1). EcoRI does not cut within the transposon. Three radioactive bands were identified within the Clal chromosomal digest of the mutant (lane E, Fig. 1). The radioactive 2.7-kb band in lane E may be due to plasmid contamination of the chromosomal DNA. The remaining two radioactive bands were the result of a single cut by Clal.
within the inserted transposon (9). Such information suggested that there had been a single insertion into plasmid and chromosomal DNA.

DISCUSSION

Complement resistance appears to play a role in the pathogenesis of many bacterial diseases, including colibacillosis (4,8,19). This resistance may be mediated by one or more bacterial factors, such as elaboration of a sialic acid-containing capsule, long-chain lipopolysaccharide, or certain outer-membrane proteins (4,8,19). These factors may be chromosomal or plasmid-encoded (19). We have attempted to ascertain the importance of complement resistance in the virulence of an avian E. coli isolate and the location of the virulence-associated complement-resistance-encoding region. With one of four mutants showing increased complement sensitivity, there was a concomitant decline in virulence in chicken embryos. The lack of effect on virulence with decreased complement resistance in the three other mutants may reflect insertions into different complement-resistance genes. The disrupted genes may yield similar resistance profiles in vitro but produce quite different results in vivo owing to their different mechanisms of action. Unknown virulence-encoding regions linked to the disrupted complement-resistance segments in the less virulent mutant also may have been affected by the transposon insertion (2). Furthermore, the insertion of the transposon into two sites within the less virulent mutant creates the possibility that some virulence-associated characteristic other than complement resistance has been affected in this organism. Phenotypic characterization of this mutant, however, has revealed no deviation from the wild-type patterns except in complement resistance and virulence profiles.

REFERENCES

