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SUMMARY. An avirulent wild-type avian Escherichia coli strain (Av) was electrotransformed with plasmids coding for complement resistance (pKT107) and Colicin V (ColV) production (pHK11) in order to study the effects of complement resistance and ColV production on virulence. Transformants were also compared with the wild type for embryo lethality, uptake by macrophages, motility, growth rate, plasmid content, and hemolysis. Growth rates and complement resistance patterns of strain Av and transformant Av+pHK11 were similar, but Av+pHK11 caused a significantly greater number of deaths in embryos and acquired motility. Transformant Av+pKT107 had a lower rate of phagocytosis, a slower growth rate, and a greater sensitivity to complement, and it changed from being non-hemolytic to expressing α-hemolytic action. The 35-kb plasmid present in the wild type was not present in the transformants. Although some of the results demonstrate the difficulties encountered in using wild-type organisms as recipients in virulence studies, the results with Av+pHK11 indicate that ColV production plus the acquisition of motility contributes to the virulence of avian E. coli.

RESUMEN. Expresión fenotípica de los plásmidos recombinantes pKT107 y pHK11 de una cepa avirulenta de Escherichia coli aviar.

Con el objeto de estudiar los efectos de la resistencia al complemento y la producción de colicina V sobre la virulencia, una cepa de Escherichia coli aviar fue electrotransformada con plásmidos que codifican para la resistencia al complemento (pKT107) y para la producción de colicina V (pHK11). Las cepas transformadas fueron también comparadas con la cepa original con respecto a la letalidad embrionaria, fagocitosis por macrófagos, motilidad, porcentaje de crecimiento, contenido de plásmidos y hemólisis. Los patrones de crecimiento y resistencia al complemento de las cepas avirulentas y de las cepas avirulentas más pHK11 fueron similares, pero las cepas avirulentas más pHK11 causaron un número significativamente mayor de mortalidad en embriones y adquirieron motilidad. Las cepas transformadas avirulentas más pKT107 tuvieron un porcentaje menor de fagocitosis, un porcentaje de crecimiento más lento y una mayor sensibilidad al complemento y cambiaron de no-hemolíticas a alfa-hemolíticas. El plásmido de 35 kb presente en la cepa original no estaba presente en las cepas transformadas. Aunque algunos de estos resultados demuestran las dificultades encontradas en usar las cepas originales como receptores en estudios de virulencia, los resultados con las cepas avirulentas más pHK11 indican que la producción de colicina V más la adquisición de motilidad contribuyen a la virulencia de E. coli aviar.

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Escberichia coli, a major pathogen of worldwide importance in commercially produced poultry, is responsible for major economic losses to the poultry industry. Microbial characteristics associated with virulent avian E. coli include antibiotic resistance (9), production of colicins and siderophores (10,17,18,36,38), colicinogenicity–complement resistance interaction (3,4,5), type 1 pili (2,10,13,22,30,39), presence of large plasmids (9,12,38,39), motility (2,39), non-hemolytic reactions on blood agar media (36,39), and embryo lethality (23,39). Resistance to the lytic action of host complement has also been implicated as an important virulence-associated factor of pathogenic avian E. coli (23,24,36,39).

Plasmid pKT107 is a hybrid plasmid composed of the cloning vector pACYC184 (3.9 kilobases [kb]) linked to a traT-containing fragment (6 kb) of plasmid R6-5 (20). This 9.9-kb plasmid encodes the outer membrane protein, TraT, which increases the organism's resistance to the lytic action of complement (20). This surface-exposed lipoprotein mediates serum resistance by interfering with the correct assembly or functioning of the complement membrane attack complex (33).

Plasmid pHK11 consists of a 9.4-kb fragment from pColV-K30 cloned into pBR322 (4.4 kb). It contains the structural gene, cvac, which codes for Colicin V (ColV) production; the ColV immunity gene, cvf; and the genes required for export of ColV, cvaA and cvaB (15). In at least four ways, ColV enhances the pathogenicity of E. coli: by providing a competitive advantage in colonization of the intestinal tract; by changing the hydrophobicity of bacterial surface proteins, facilitating attachment to host-cell surfaces; by increasing resistance to host complement; and by contributing antiphagocytic factors (1,32,37).

Molecular studies of bacterial virulence often employ transformation of E. coli K12 derivatives with virulence genes cloned into vector plasmids. Such studies in genetically defined laboratory recipient strains may not result in the true expression of the introduced virulence gene in these recipients. In the present study, cloned virulence genes (pKT107 and pHK11) were evaluated for their effects on the virulence and phenotype within an avirulent, wild-type avian E. coli in order to further clarify the pathogenesis of avian colibacillosis.

MATERIALS AND METHODS

Bacterial isolate and plasmids. An avirulent wild-type E. coli strain designated Av (40) was the recipient in the transformation studies. Strain Av had been previously characterized as being sensitive to the action of selected antibiotics (ampicillin, streptomycin, tetracycline, sulfoxazole, and gentamicin), resistant to nalidixic acid, and sensitive to complement. In addition, strain Av did not produce colicins and produced low mortality rates in the intravenous chick assay and the embryo lethality test (23,40). Plasmids used in the present study were pKT107 (20,33), supplied by Dr. F. C. Cabello (Department of Microbiology, New York Medical College, Valhalla, N.Y.), and pHK11 (15), supplied by Dr. R. Kolter (Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Mass.).

Antibiotic resistance profiles. Antibiotic resistance profiles were determined by the disc diffusion method (7). Antibiotics tested were gentamicin (Gm), streptomycin (St), kanamycin (Kn), ampicillin (Ap), chloramphenicol (Cm), nalidixic acid (Na), tetracycline (Tc), and sulfoxazole (Su).

Bacterial resistance to guinea-pig complement. The lytic activity of complement on the test organisms was determined by the viable count method (31). Guinea-pig serum (12% final concentration) (ICN ImmunoBiologicals, Costa Mesa, Calif.) was mixed with phosphate-buffered saline (PBS)-washed cells from an exponentially growing bacterial culture. The bacteria-serum mixtures were incubated in a 37 C water bath and sampled at 0, 1, 2, and 3 hours. Viable organisms were determined by plating on MacConkey agar (31).

Plasmid isolation, purification, and digestion. Plasmid DNA from the E. coli strains containing pKT107 and pHK11 was isolated and purified by the method of Birnboim and Doly (6) from overnight brain-heart infusion (BHI) broth cultures. E. coli strain V517 was used as a source of reference plasmids (19). All restriction enzymes were obtained from Promega Corp., Madison, Wis. For probe construction, traT was removed from pKT107 using BstEII (34). Samples (25 μl) of plasmid DNA were loaded into wells of a 0.7% agarose gel (MC Corp., Rockland, Maine) and run at 40 volts for 12 hours on a horizontal electrophoresis apparatus (Model 452; E-C Apparatus Corp., St. Petersburg, Fla.). Gels were stained with ethidium bromide and visualized on an ultraviolet transilluminator (27). Selected plasmids or their fragments were sliced from the gel and extracted from the agarose using GENECLEAN (Bio101, La Jolla, Calif.).

Probe DNA. Plasmid pKT107 was used as a source of the traT gene. The plasmid was digested with BstEII, and the fragments were separated electrophoretically (34). The 70-base-pair fragment, containing the traT gene (21), was extracted from the agarose with GENE-
CLEAN and labeled according to the Genius 1 Kit protocol (Boehringer Mannheim Corp., Indianapolis, Ind.) for use as a probe. Wild-type and transformant plasmids in agarose were transferred to charge-modified nylon membranes (QIABRANE Nylon plus; Qia-gen, Inc., Chatsworth, Calif.) by the method of Southern (28) and then hybridized with the probe under stringent conditions (25).

**Preparation of genomic DNA.** Chromosomal DNA from the test organisms was isolated and purified by treatment with sodium dodecyl sulfate, proteinase K, and phenol/chloroform/isoamyl alcohol, followed by isopropanol precipitation (2).

**Colicin activity.** Colicin production was tested by overlaying chloroform-killed colonies of the test organisms with a colicin-sensitive *E. coli* K-12 (ATCC 23559; American Type Culture Collection, Rockville, Md.). Plates were incubated at 37°C for 18 hours and then observed for growth inhibition (14).

**Embryo lethality test.** Overnight cultures of the isolates were washed twice in PBS, resuspended and diluted in PBS, and inoculated into the allantoic cavity of thirty 12-day-old chicken embryos. Thirty PBS-inoculated control embryos also were included. Eggs were candied daily, and deaths were recorded for 4 days. Fifteen eggs were used for each of two inoculum dilutions, approximately 10^2 and 10^3 colony-forming units (CFU) in 0.1 ml PBS (23).

**Transformation.** The wild-type avirulent *E. coli* (Av) was made competent for transformation (11,25,29) and transformed with pKT107 and pHK11 using the Gene Pulser Transfection Apparatus and Pulse Controller (Bio-Rad Laboratories, Richmond, Calif.) set at 25 μF, 200 Ω, and 2.5 kV (29). Initial selection of mutants was done on the basis of acquisition of resistance to tetracycline (Tc') (pKT107) or ampicillin (Ap') (pHK11) by incorporating these antibiotics into MacConkey agar plates at concentrations of 10 μg/ml and 50 μg/ml, respectively (15,33). Transforms were then colony-purified three times on selective media before being studied further.

**Rate of phagocytosis.** The bioassay for uptake of the test bacteria was done using avian macrophages. The *E. coli* strains were radiolabeled by adding 100 μl of an overnight culture in BHI broth into 4 ml of fresh BHI broth with 80 μCi [3H]thymidine. The *E. coli* were then grown to log phase, washed three times, and diluted in PBS to 10^6 CFU as determined from a standard curve of optical density (OD) at 595 nm vs. viable CFU (16). Avian macrophages were collected from 45-week-old Hy-Line W36 laying hens. Chickens were injected intraperitoneally with dextran beads (Sephadex G-50; Sigma Chemical Co., St. Louis, Missouri), and peritoneal macrophages were collected 18 hours later at necropsy. The cells were washed and resuspended in RPMI-1640 (Gibco Laboratories, Grand Island, N.Y.) containing 10% heat-inactivated fetal bovine serum to a concentration of 10^7 cells/ml (8,16,35).

The rate of phagocytosis was measured by the method of Harmon et al. (16). Aliquots (200 μl) of the macrophage suspension were placed into 7 ml sterile borosilicate glass scintillation vials (Kimble, Toledo, Ohio), centrifuged for 30 seconds at 200 g and incubated for 1 hour at 40°C. The media were then decanted, and aliquots consisting of 10^6 radiolabeled *E. coli* in 200 μl PBS with 10% complete chicken serum were added to the vials for a final bacteria: cells ratio of 50:1. Test vials containing macrophages and radiolabeled *E. coli* were incubated at 40°C for 45 minutes; the cells were washed three times in PBS; and the phagocytosed, radioactive bacteria were counted. The phagocytosis index for each strain was then calculated (16).

**Growth curve.** Bacterial growth curves were done in Luria-Bertani (LB) broth (26) using a 1% vol/vol inoculum from an overnight culture. Samples were taken and plated on MacConkey agar plates every hour from 0 to 7 hours.

**Hemolytic reactions.** The type of hemolysis was determined by the stab inoculation of bovine blood agar plates with the test organisms, followed by incubation at 37°C overnight.

**Motility reactions.** Motility was determined by stab inoculation of Motility Test Media (Difco Laboratories, Detroit, Mich.), followed by overnight incubation at 37°C.

**Statistical analysis.** The percentages of embryo deaths (embryo lethality) in the three groups were compared by a chi-square/analysis of variance with a follow-up Tukey multiple comparison test. The phagocytic percentages of the three groups were compared using a Kruskal-Wallis test with a Tukey-type multiple comparison test (41).

**RESULTS**

Electrotransformation of pHK11 and pKT107 into an avirulent avian *E. coli* (Av) was partially successful, based on acquisition of antibiotic resistances coded for the recombinant plasmids, pKT107 and pHK11. As a result of electrotransformation, transformant Av+pKT107 acquired both Tc' and Cm'. This antibiotic resistance pattern verifies transformation of Av with the pACYC184 portion of pKT107, but it also indicates that the *traT*-containing portion of pKT107 was lost in the process, since the presence of the *traT* fragment in pKT107 inactivates the Cm' gene of pACYC184. Transformant Av+pHK11 acquired Ap' and the ability to produce ColV, indicating that this transformation was complete. Table 1 shows antibiotic resistance profiles of the parent and its transformants.
Table 1. Antibiotic resistance profiles* of plasmids pKT107 and pHK11, recipient wild-type avian E. coli (Av), and their transformants.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Resistance profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKT107</td>
<td>Tc</td>
</tr>
<tr>
<td>pHK11</td>
<td>Ap</td>
</tr>
<tr>
<td>Av</td>
<td>Na</td>
</tr>
<tr>
<td>Av+pKT107</td>
<td>Na&gt;Tc&gt;Cm</td>
</tr>
<tr>
<td>Av+pHK11</td>
<td>Na-Ap</td>
</tr>
</tbody>
</table>

*Antibiotics tested: ampicillin (Ap), chloramphenicol (Cm), gentamicin (Gm), kanamycin (Kn), nalidixic acid (Na), streptomycin (St), sulfisoxazole (Su), and tetracycline (Tc).

Table 2 shows changes in embryo lethality, mean percent phagocytic rate, and motility of Av and its progeny, Av+pHK11 and Av+pKT107. Eggs inoculated with Av+pHK11 had significantly (P < 0.05) more deaths than those inoculated with either Av or Av+pKT107. Av+pHK11 also acquired motility. In the phagocytic uptake studies, Av+pKT107 was significantly less likely to be phagocytized than its parent (Av).

Fig. 1 shows the effect of 12% guinea pig serum on the three test strains sampled over 3 hours. Av and Av+pHK11 had similar curves and were moderately sensitive to the lytic effect of complement; Av+pKT107 was significantly more sensitive.

Fig. 2 shows the growth curves of Av, Av+pHK11, and Av+pKT107. The growth curves of Av and Av+pHK11 were similar, whereas Av+pKT107 had a slower growth rate than its parent.

Table 2. Characteristics of avirulent E. coli Av and its transformants containing pKT107 and pHK11.

<table>
<thead>
<tr>
<th>E. coli</th>
<th>% Embryo deathsa</th>
<th>% Mean phagocytic rateb</th>
<th>Motilityc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Av</td>
<td>3.33</td>
<td>1.94</td>
<td>-</td>
</tr>
<tr>
<td>Av+pKT107</td>
<td>6.67</td>
<td>1.33</td>
<td>-</td>
</tr>
<tr>
<td>Av+pHK11</td>
<td>13.33</td>
<td>2.09</td>
<td>+</td>
</tr>
</tbody>
</table>

*aEmbryo lethality test. Percent dead of 50 eggs. Values followed by the same lower case superscript are not significantly different (P > 0.05).

bPhagocytic uptake of organisms. Mean of three replicate tests. Values followed by the same lower case superscript are not significantly different (P > 0.05).

c- = negative reaction, + = positive reaction.

Other phenotypes that differed between Av and its progeny were type of hemolysis and ColV production. Both Av and Av+pHK11 were non-hemolytic, whereas Av+pKT107 expressed a slight α-hemolytic reaction. Av and Av+pKT107 remained non-producers of colicin, but Av+pHK11 produced ColV.

Fig. 2. Growth curves of Av, Av+pHK11, and Av+pKT107 in Luria-Bertani broth.
Expression of pKT107 and pHK11 in E. coli

Fig. 3. Plasmid screen of Av, pKT107, pHK11, and their progeny, Av+pKT107 and Av+pHK11. Arrow indicates 35-kb plasmid present in wild-type avian E. coli (Av).

Plasmid isolation and visualization of transformants showed that when Av was transformed with pHK11 and with pKT107, it lost a 35-kb plasmid (Fig. 3). A plasmid screen of Av+pHK11 confirmed its acquisition of the total recombinant plasmid. A plasmid screen of Av+pKT107 was unable to confirm the acquisition of pKT107.

Hybridization studies using labeled traT as a probe showed that traT was present in the 35-kb cryptic plasmid of Av and in pKT107 but was not observed in Av+pKT107 (Fig. 4). The traT gene also was not observed in genomic DNA preparations of Av or Av+pKT107.

DISCUSSION

There are inherent difficulties in transforming wild-type organisms with plasmids in order to assess those plasmids' contributions to virulence. It is for this reason that the recipient organisms employed in such studies are usually avirulent E. coli K-12 strains. Unfortunately, there are also inherent difficulties in assessing the virulence contributions of DNA put into such genetically refined backgrounds as those of these E. coli K-12 derivatives. For this reason, we attempted to transform an avirulent wild-type avi-
an *E. coli* with two recombinant plasmids (pKT107 and pHK11) known to produce putative virulence proteins (14,33). The results demonstrate both the benefits and drawbacks to this approach.

First, the transformation of Av with pHK11 was successful, as revealed by tests confirming the acquisition of Ap', a plasmid of proper size, and ColV production. It appears that ColV production made Av more virulent, but since the transformants also became motile, this increase in virulence may not be totally attributed to ColV production. The acquisition of motility may be the result of some complementation event occurring within the transformant. Changes in complement resistance and uptake by macrophages were not observed in Av+pHK11, although increases in complement resistance and anti-phagocytic properties have been attributed to the ColV plasmid in mammalian *E. coli* isolates (31,36).

The transformation of Av with pKT107 was fraught with ambiguous results. For example, the transformation appeared to be successful based on the acquisition of Tc', but the acquisition of Cm' also indicated that the *traT* gene of pKT107 was lost. Also, the acquisition of the plasmid pKT107 by Av could not be confirmed by plasmid and genomic isolation and DNA probe analysis. Changes in the transformant included an increased sensitivity to complement, a slower growth rate, and a decrease in the rate of phagocytosis. Such changes may reflect a recombination event within this transformant that was not related to the virulence gene, *traT*. Therefore, it is not possible to assess the effect
of traT on the virulence or complement resistance in this system.

Furthermore, both transformants appeared to lose a 35-kb cryptic plasmid present in Av. Plasmid-group incompatibilities between the 35-kb plasmid and the introduced plasmids may account for the loss of the 35-kb plasmid in the Av-transformants.

In summary, these results suggest that CoV production, motility, or the combination of CoV production and motility contributes to the virulence of avian E. coli. The loss of the 35-kb plasmid and the unexpected acquisition of motility do not invalidate this suggestion but certainly must be considered while investigating its plausibility. These results do not, however, provide evidence that traT contributes to virulence. These results also indicate that there are many pitfalls in the use of wild-type recipients for virulence studies.

REFERENCES


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