Characteristics of Conjugative R-Plasmids from Pathogenic Avian Escherichia coli

Richard E. Wooley, University of Georgia
Kathy R. Spears, University of Georgia
Lisa K. Nolan, University of Georgia
John Brown, University of Georgia
Mark A. Dekich

Available at: https://works.bepress.com/lisa_nolan/71/
Characteristics of Conjugative R-Plasmids from Pathogenic Avian Escherichia coli

Richard E. Wooley, Kathy R. Spears, John Brown, Lisa K. Nolan, and Mark A. Dekich

Department of Medical Microbiology, College of Veterinary Medicine, University of Georgia, Athens, Georgia 30602

Perdue Farms, Inc., Salisbury, Maryland 21801

Received 16 September 1991

SUMMARY. Three of four virulent avian Escherichia coli isolates transferred a single large molecular-weight R-plasmid to two recipient E. coli strains. Antibiotic resistances transferred included streptomycin (two isolates) and streptomycin-tetracycline-sulfa (one isolate). Production of colicin and siderophores, complement resistance, and embryo lethality present in the virulent isolates were not transferred to recipient organisms.

From the results, it appears that the R-plasmids of these virulent avian E. coli are not associated with virulence.

RESUMEN. Características de plásmidos R conjugados obtenidos de cepas patógenas de Escherichia coli.

Tres de cuatro cepas virulentas de Escherichia coli de origen aviar transfirieron un plásmido R de alto peso molecular, a dos cepas receptores de E. coli. La resistencia a los antibióticos transferida incluyó estreptomicina (dos cepas) y estreptomicina-tetraciclina-sulfa (una cepa). La producción de colicina y sideróforos, resistencia al complemento, sensibilidad a la manosa, hemoaglutinación de eritrocitos, invasión en cultivo celular, movilidad y letalidad para embriones, características estas presentes en las cepas virulentas, no fueron transferidas a los organismos receptores.

De acuerdo con estos resultados, parece que los plásmidos R de estas cepas virulentas de E. coli aviar no están asociados con virulencia.

Microbial characteristics associated with virulent avian Escherichia coli include antibiotic resistance (6), production of colicins and siderophores (9,17,35,37), type 1 pili (1,9,13,27), resistance to host complement (11,35,37), plasmids (17,21,32,35), serotype (7,29), motility (1), and invasion of host cells (35).

Conjugative R-plasmids present in E. coli have been shown to be associated with streptomycin, sulfa, and tetracycline resistance (6,12,19), production of enterotoxins, colonization antigens (12), serum resistance (2), and the production of aerobactin and colicins (37).

The purpose of the present study was to examine the phenotypic characteristics determined by conjugative R-plasmids of virulent avian E. coli and to assess their effects on virulence.

This study was supported by the Veterinary Medical Experiment Station, College of Veterinary Medicine, The University of Georgia.

MATERIALS AND METHODS

Test organisms. Five avian E. coli isolates were studied: three (V-1, V-2, V-3) that were cultured from necropsy samples of broiler chickens with colisepticemia, one (V-4) from a flock with omphalitis, and one (Av) non-pathogenic control. All isolates were inoculated into chickens and determined to be virulent (V-1, V-2, V-3, V-4) and non-pathogenic (Av) by intravenous inoculation of chickens (29).

Antibiotic resistance profiles. Antibiotic resistance profiles of the test organisms were determined by the disk diffusion method (5). Antibiotics tested (and their disc designations) were gentamicin (GM10), streptomycin (S10), kanamycin (K30), ampicillin (AM10), chloramphenicol (C30), polymyxin B (PB300), nalidixic acid (NA30), tetracycline (Te30), and sulfisoxazole (G.25).

Colicin activity. The colicin test organisms consisted of E. coli K-12 (ATCC 23559), a colicin-sensitive control (Col-), and the colicin producer (Col+), E. coli K-12 (ATCC 23558). Colicin production was tested by overlaying chloroform-killed colonies of the...
test organisms with *E. coli* K-12 (ATCC 23559). *E. coli* strain (ATCC 23558) was included on each plate as a positive control (16). Plates were incubated at 37°C for 18 hours and observed for inhibition of growth.

**Enterobacter (phenolate siderophore) and aerobactin (hydroxamate siderophore) assays.** The siderophore control organisms consisted of *E. coli* AN 193, an enterobactin-non-producing mutant (*Ent*) unable to synthesize dihydroxybenzoic acid but able to utilize enterobactin produced by other organisms (negative control), and *E. coli* AN 194, an Ent+ strain (positive control) (31,33). Enterobacter and aerobactin were bioassayed in petri dishes (60 × 15 mm) containing 10 ml of low-iron agar with 30 µg/ml of deferrated ethylenediamine-N,N'-diacetic acid (EDDA). *Salmonella typhimurium* LT-2 enb-7 and *E. coli* LG1522 (10^6 CFU/ml) were used as the indicator organisms for enterobactin and aerobactin activity, respectively (4,8). *E. coli* strains AN 193 and AN 194 were included in each test. The test organisms were passed five times in low-iron medium, and an overnight culture was concentrated by centrifugation (5000 × g for 10 minutes) and filtration (0.45-µm-pore-size filter). The culture filtrate was concentrated ten times in an evaporator-concentrator (Savant model SVC-100H; Savant Instruments, Inc., Farmingdale, N.Y.). Small wells placed in the agar were filled with 50 µl of the culture filtrate. Plates were incubated for 24 and 48 hours at 37°C and examined for growth around the wells (33).

**Bacterial resistance to complement.** The lytic activity of complement on the test isolates was determined by a quantitative microtiter test method (20,39). Two-hour cultures grown in Luria-Bertani broth were diluted 1:50 in PG broth (1% peptone plus 1% glucose) (25), and 100 µl (10^6 CFU) samples were inoculated into 96-well flat-bottomed microtiter plates (Microtest III, Becton, Dickson & Co., Rutherford, N.J.). Aliquots (100 µl) of guinea pig complement (ICN Biomedicals, Inc., Costa Mesa, Calif.) or heat-inactivated guinea pig complement were added in twofold dilutions from 50% to 0.8%. Media and culture controls were included. Plates were monitored for growth by an enzyme-linked immunosorbent assay reader (Titertek Multiskan, Flow Laboratories, Inc., McLean, Va.) at 492 nm, absorbance mode, with a range of 0.1. Plates were incubated at 35°C and read hourly from 0 to 6 hours (20,39).

**Mannose-sensitive hemagglutination (MSHA) tests.** The ability of the test strains to hemagglutinate erythrocytes was tested in 96-well round-bottomed microtiter plates with a 3% suspension of guinea pig or chicken erythrocytes in saline solution. Brain-heart infusion cultures of the test organisms were incubated statically in air, with subcultures made every 48 to 72 hours for six transfers before testing. Combinations of 25 µl each of culture, erythrocyte suspension, and either saline solution or 2% α-mannose were mixed and incubated at 25°C for 30 minutes before hemagglutination (HA) activity was assessed (18,28).

**Mannose-resistant hemagglutination (MRHA).** The presence of mannose-resistant hemagglutinins was assessed by the method of Old (28) in 96-well round-bottomed microtiter plates using a 3% suspension of guinea pig or chicken erythrocytes in saline solution. Test organisms were grown overnight at 37°C on phosphate-buffered agar (10) and colonization-factor antigen agar (14), and colonies were suspended in saline solution to obtain 10^12 CFU/ml. Combinations of 25 µl of bacterial suspension, erythrocyte suspension, and either saline solution or 2% α-mannose were mixed and incubated at 4°C for 1 hour without agitation before hemagglutination activity was assessed.

**Plasmid isolation and agarose gel electrophoresis.** Plasmid DNA from the test organisms was isolated and purified by the method of Birnboim and Doly (3) from overnight brain-heart infusion broth cultures. *E. coli* strain V-517 was used as a source of reference plasmids (21). 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, visualized on an ultraviolet transilluminator, and photographed (33).

**Tissue-culture invasive test.** The invasion of chicken kidney cells was done by a modification of the method of Miller *et al.* (24). A sample of approximately 10^8 washed bacterial cells from an 18-hour culture was suspended in cell-culture medium, and 3-ml subsamples were inoculated onto primary chicken kidney cell cultures (38) for 6 hours of incubation at 37°C (15). After incubation, the extracellular bacteria were removed by washing, and the cells were treated with kanamycin sulfate solution (20 µg/ml). Intracellular bacteria were released by detergent lysis and counted (24).

**Capsule production.** Capsule production was identified by the acid fuchsin staining technique of Maneval (22).

**Bacterial matings and selection of transconjugants.** Conjugative recipients consisted of *E. coli* strain 1932, which has the following characteristics: it is a lactose non-fermenter (Lac-); it is nalidixic acid-resistant (Nal'); it is an enterobactin-producer (Ent+); it is complement-sensitive, non-encapsulated, coli- cin-negative (Col-), mannose-sensitive hemagglutination-positive (MSHA+), non-hemolytic, and motile. *E. coli* strain 1932 is also invasive in chick kidney cell cultures and lethal to 0% and 5% of 12-day-old embryos when inoculated with 10^5 and 10^6 colony-forming units (CFU) of bacteria, respectively, into the allantoic cavity (30,33). A second recipient consisted of a mutant strain of *Av*, designated *Av*av (avirulent
Table 1. Characteristics of avian Escherichia coli isolates.

<table>
<thead>
<tr>
<th>E. coli isolate</th>
<th>Antibiotic resistance</th>
<th>Colicin</th>
<th>Sensitivity to complement</th>
<th>MSHA</th>
<th>Plasmids (megadaltons)</th>
<th>% Dead in IV chick assay</th>
<th>% Dead in embryo lethality assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>V-1</td>
<td>Am, St, Te, Su</td>
<td>+</td>
<td>R</td>
<td>+</td>
<td>42.4, 37.4, 3.7, 3.6</td>
<td>75</td>
<td>90</td>
</tr>
<tr>
<td>V-2</td>
<td>St, Te, Su</td>
<td>+</td>
<td>R</td>
<td>−</td>
<td>50.2, 38.4, 3.5, 2.2, 1.2</td>
<td>60</td>
<td>75</td>
</tr>
<tr>
<td>V-3</td>
<td>Gm, St, Te, Su</td>
<td>+</td>
<td>R</td>
<td>+</td>
<td>43.3, 36.9, 3.7, 2.9, 1.2</td>
<td>40</td>
<td>85</td>
</tr>
<tr>
<td>V-4</td>
<td>St, Te, Su</td>
<td>+</td>
<td>I</td>
<td>+</td>
<td>42.2</td>
<td>45</td>
<td>10</td>
</tr>
<tr>
<td>Av</td>
<td>None</td>
<td>−</td>
<td>S</td>
<td>+</td>
<td>None</td>
<td>0</td>
<td>5</td>
</tr>
</tbody>
</table>

*Isolates V-1, V-2, V-3, V-4, and Av are: Ent*, MRHA*, aerobactin-negative, non-hemolytic, motile, non-encapsulated, and invasive in chick kidney cell cultures.

**Antibiotic resistance: Am = ampicillin, St = streptomycin, Te = tetracycline, Su = sulfa, Gm = gentamicin, Na = nalidixic acid.

<table>
<thead>
<tr>
<th>E. coli</th>
<th>R-plasmid</th>
<th>R-plasmid</th>
<th>Colicin</th>
<th>Sensitivity to complement</th>
<th>MSHA</th>
<th>Plasmids (megadaltons)</th>
<th>% Dead in IV chick assay</th>
<th>% Dead in embryo lethality assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>V-2 × 1932</td>
<td>10&lt;sup&gt;-6.2&lt;/sup&gt;</td>
<td>St</td>
<td>38.4</td>
<td>−</td>
<td>S</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>V-2 × Av&lt;sup&gt;α&lt;/sup&gt;</td>
<td>10&lt;sup&gt;-3.2&lt;/sup&gt;</td>
<td>St</td>
<td>38.4</td>
<td>−</td>
<td>S</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>V-3 × 1932</td>
<td>10&lt;sup&gt;-6.2&lt;/sup&gt;</td>
<td>St</td>
<td>36.9</td>
<td>−</td>
<td>S</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>V-3 × Av&lt;sup&gt;α&lt;/sup&gt;</td>
<td>10&lt;sup&gt;-4.3&lt;/sup&gt;</td>
<td>St</td>
<td>36.9</td>
<td>−</td>
<td>S</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>V-4 × 1932</td>
<td>10&lt;sup&gt;-5.7&lt;/sup&gt;</td>
<td>St, Te, Su</td>
<td>42.2</td>
<td>−</td>
<td>S</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>V-4 × Av&lt;sup&gt;α&lt;/sup&gt;</td>
<td>10&lt;sup&gt;-5.7&lt;/sup&gt;</td>
<td>St, Te, Su</td>
<td>42.2</td>
<td>−</td>
<td>S</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
</tbody>
</table>

<sup>α</sup>Mating frequencies incubated at 37 C.

<sup>β</sup>R-plasmid passed: St = streptomycin, Te = tetracycline, Su = sulfa.

<sup>γ</sup>Sensitivity to complement; S = sensitive.

<sup>δ</sup>Embryo lethality: Colony-forming units (CFU) injected into allantoic cavity of twenty 12-day-old chickens.

R. E. Wooley et al.

Table 2. Characteristics of transconjugants of matings of virulent avian Escherichia coli isolates to recipient E. coli strains 1932 and Av<sup>α</sup>.

**Chick lethality.** To assay the virulence of the isolates in chickens, 10<sup>6</sup> E. coli organisms were inoculated intravenously into twenty 21-day-old birds (29). These birds were observed for a 10-day period; mortality was recorded daily, and dead birds were necropsied.

**Embryo lethality.** To assay embryo lethality, overnight cultures of the isolates were washed twice in phosphate-buffered saline (PBS), resuspended and

mutant), which was Lac<sup>−</sup> and Na<sup>+</sup> (23). R-plasmids were transferred by mixing 0.2 ml of exponentially grown donor cells with 1.8 ml of an overnight culture of recipient cells in PennAssay broth. Mixtures were incubated at 25 C, 37 C, and 42 C for 18 hours. Transconjugants were selected on MacConkey agar plates containing a donor-inhibiting concentration of nalidixic acid (30 μg/ml) and a recipient-inhibiting concentration of the counter-selector antibiotics of streptomycin (30 μg/ml) or tetracycline (15 μg/ml) (36). Samples from the selector plates were picked and re-identified, and their antibiotic-resistance patterns were determined. Frequencies of transconjugants were expressed relative to the number of donor cells in the mating mixture (36).
diluted in PBS, and inoculated into the allantoic cavity of twenty 12-day-old chicken embryos. Twenty PBS-inoculated control embryos also were included. Eggs were candled daily, and deaths were recorded for 4 days. Twenty eggs were used for each of two inoculum dilutions, approximately 10^2 and 10^5 CFU in 0.1 ml of PBS (25).

RESULTS AND DISCUSSION

Table 1 shows the phenotypic characteristics of isolates V-1, V-2, V-3, V-4, and Av. The virulent isolates (V-1, V-2, V-3, and V-4) were resistant to three or more antibiotics, with streptomycin-tetracycline-sulfa being a common resistance profile. Isolate Av lacked resistance to any of the antibiotics tested. The virulent isolates were colicin producers, whereas the Av isolate was colicin-negative. Isolates V-1, V-2, and V-3 were resistant to the action of complement; V-4 was classified as intermediate; and Av was sensitive. In the chick and embryo lethality assays, the virulent isolates had greater mortality rates than the Av isolate and recipient E. coli strain 1932. Plasmid content also differed; the virulent isolates had a greater number of plasmids. Isolate V-1 was non-conjugative with the recipient organisms used in the present study. Isolates V-2, V-3, and V-4 passed R-plasmids to both recipients, with the highest conjugative frequencies occurring at 37 C. In each mating, only one large plasmid was passed. In two of the matings, streptomycin was passed; in one transfer, streptomycin-tetracycline-sulfa was passed. Transfer frequencies were greater in Av™ than in E. coli strain 1932. No other phenotypic characteristics associated with virulence were passed. No differences in embryo lethality were observed between the transconjugants and the recipients (Table 2).

Virulent avian E. coli possess virulence-associated characteristics such as colicin production, which enables the organism to survive in the alimentary tract of birds, resistance to host complement, and the presence of large molecular-weight plasmids (55). Large conjugative plasmids and large conjugative R-plasmids have been shown to encode for colicin production (ColV) (2,12,37) and complement resistance (R100 and R6-5) (12,34). Genetic studies have identified two determinants of complement resistance, namely traT, which is encoded by plasmids R100, R6-5, and ColV, and iss which is encoded by ColV. The traT and iss genes encode for outer-membrane proteins that modify sites on the cell surface that are sensitive to the membrane attack complex of complement resulting in surface exclusion (12,34). Although the virulent E. coli isolates used in the present study possess these virulence-associated characteristics, they were not transferred when mated with an E. coli laboratory recipient (strain 1932) and a wild-type recipient (E. coli Av™).

In conclusion, the conjugative R-plasmids of the virulent E. coli studied do not appear to be associated with virulence.

REFERENCES


