Multiple Antimicrobial Resistance Region of a Putative Virulence Plasmid from an Escherichia coli Isolate Incriminated in Avian Colibacillosis

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Abstract
Infections due to *Escherichia coli* have been costly to the poultry industry, but the exact virulence mechanisms used by these organisms to cause disease in birds remain undefined. Several factors have been shown to contribute to the virulence of avian *E. coli*, and many of the genes encoding these factors have been found on large conjugative plasmids. Because of the occurrence of antimicrobial resistance genes on these same plasmids, it is possible that the use of antimicrobial agents may select for persistence of *E. coli* containing such plasmids. In the present study, a subclone of one of these plasmids was identified as likely containing some virulence and antimicrobial resistance genes. In an effort to better understand the relationship between virulence and resistance in these plasmids, this subclone was sequenced and the sequence analyzed. Analysis of this 30-kilobase (kb) region of plasmid pTJ100 revealed a mosaic of virulence genes, insertion sequences, antimicrobial resistance cassettes, and their remnants. Many of the resistance genes found in this region were expressed under laboratory conditions, indicating that certain antimicrobial agents, including disinfectants, antibiotics, and heavy metals, could promote selection of *E. coli* containing such plasmids in the production environment. Also, analysis of the G + C content of this clone indicated that it is the likely consequence of a complex evolution with components derived from various sources. The occurrence of many mobile elements in conjunction with antimicrobial resistance and virulence genes in this 30-kb region may indicate that the genetic constitution of the clone is quite plastic. Although further study will be required to better define this plasmid's role in avian *E. coli* virulence, the sequence described here is, to our knowledge, the longest known contiguous sequence of a ColV plasmid yet presented. Analysis of this sequence indicates that this clone and its parent plasmid may be important to the pathogenesis of avian colibacillosis and the evolution of avian *E. coli* virulence.

Keywords
*Escherichia coli*, avian colibacillosis, antimicrobial resistance, disinfectant resistance, heavy metal resistance, virulence, R plasmid, poultry, Tn21, pTJ100

Disciplines
Veterinary Microbiology and Immunobiology | Veterinary Preventive Medicine, Epidemiology, and Public Health

Comments
This article is from *Avian Diseases* 48, no. 2 (2004): 351–360, doi:10.1637/7121.
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Author(s): Timothy J. Johnson, Jerod Skyberg, Lisa K. Nolan
Published By: American Association of Avian Pathologists
DOI: http://dx.doi.org/10.1637/7121
URL: http://www.bioone.org/doi/full/10.1637/7121

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Multiple Antimicrobial Resistance Region of a Putative Virulence Plasmid from an Escherichia coli Isolate Incriminated in Avian Colibacillosis

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Received 4 October 2003

SUMMARY. Infections due to Escherichia coli have been costly to the poultry industry, but the exact virulence mechanisms used by these organisms to cause disease in birds remain undefined. Several factors have been shown to contribute to the virulence of avian E. coli, and many of the genes encoding these factors have been found on large conjugative plasmids. Because of the occurrence of antimicrobial resistance genes on these same plasmids, it is possible that the use of antimicrobial agents may select for persistence of E. coli containing such plasmids. In the present study, a subclone of one of these plasmids was identified as likely containing some virulence and antimicrobial resistance genes. In an effort to better understand the relationship between virulence and resistance in these plasmids, this subclone was sequenced and the sequence analyzed. Analysis of this 30-kilobase (kb) region of plasmid pTJ100 revealed a mosaic of virulence genes, insertion sequences, antimicrobial resistance cassettes, and their remnants. Many of the resistance genes found in this region were expressed under laboratory conditions, indicating that certain antimicrobial agents, including disinfectants, antibiotics, and heavy metals, could promote selection of E. coli containing such plasmids in the production environment. Also, analysis of the G + C content of this clone indicated that it is the likely consequence of a complex evolution with components derived from various sources. The occurrence of many mobile elements in conjunction with antimicrobial resistance and virulence genes in this 30-kb region may indicate that the genetic constitution of the clone is quite plastic. Although further study will be required to better define this plasmid’s role in avian E. coli virulence, the sequence described here is, to our knowledge, the longest known contiguous sequence of a ColV plasmid yet presented. Analysis of this sequence indicates that this clone and its parent plasmid may be important to the pathogenesis of avian colibacillosis and the evolution of avian E. coli virulence.

RESUMEN. Región de resistencia múltiple a antibióticos de un plásmido putativo de virulencia de una cepa de Escherichia coli incriminada en un brote de colibacilosis.

Las infecciones ocasionadas por cepas de Escherichia coli son sumamente costosas para la industria avícola, pero los mecanismos de virulencia utilizados por estos microorganismos en aves permanecen sin ser clarificados. Se ha demostrado que varios factores contribuyen a la virulencia de las cepas avícolas de E. coli, y muchos de los genes que codifican por estos factores se encuentran alojados en plásmidos de conjugación de gran tamaño. Debido a que estos plásmidos también codifican por genes de resistencia a antibióticos, es posible que el uso de antibióticos seleccione de manera específica las cepas de E. coli que contienen estos plásmidos, aumentando así la persistencia de las mismas en las parvadas. Se identificó un subclon de uno de estos plásmidos, el cual posiblemente contenía algunos factores de virulencia y genes de resistencia a antibióticos. Se analizó la secuencia de este subclon, en un intento de entender mejor la relación entre los factores de virulencia y los genes de resistencia a antibióticos contenidos en este plásmido. El
Colibacillosis has resulted in significant losses for the poultry industry. It is likely that *Escherichia coli*, the causative agent of this disease, employs various virulence mechanisms, most of which may not yet have been elucidated. Several traits have been associated with avian pathogenic *E. coli*, including the ability of a strain to resist the deleterious effects of serum complement (23,24). Some of the factors known to confer complement resistance on bacteria include possession of capsule, a smooth lipopolysaccharide (LPS) layer, and the outer membrane proteins, OmpA, TraT, and Iss (23). Other putative virulence factors include aerobactin production (17,28), the Tsh protein (8), and large plasmids (7,8,14,15).

In a previous study, several of the genes encoding putative virulence factors were localized to a large conjugative plasmid in an avian *E. coli* isolate (15). Thus far, the genes known to occur on this plasmid, pTJ100, include the increased serum survival (*iss*) gene; the temperature-sensitive hemagglutinin (*tsh*) gene; *iucC*, a gene of the aerobactin operon; *intI1*, encoding the class I integrase of the In2 integron; and *cvuC*, the structural gene of the ColV operon (15). pTJ100 also contains genes encoding for several antimicrobial resistances (15). The occurrence of virulence and antimicrobial resistance genes on the same conjugative plasmid indicates that use of certain antimicrobial agents in the production environment could provide pressure for selection of the organisms containing this plasmid or for spread of this plasmid. As initial steps in understanding the relationships of these plasmid-located virulence and antimicrobial resistance genes to one another and the roles of these genes in avian *E. coli* virulence and resistance, regions of pTJ100 were cloned. A 30-kb clone, identified as containing several putative virulence genes and a portion of the antimicrobial resistance–encoding class 1 integron, In2, was selected for sequencing and functional analysis.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** The original source of pTJ100 was a wild-type avian *E. coli* isolate designated A2363 (15) that was isolated from the joint of a chicken clinically diagnosed with colibacillosis. In a prior study, A2363 was mated to *E. coli* DH5α, an avirulent plasmidless strain, and the resulting trans-conjugant, TC4, was used as a source of pTJ100 for the present study (15). pTJ100 is a conjugative R plasmid. It encodes for resistance to tetracycline, ampicillin, and complement and also for production of aerobactin and ColV (15). pTJ100 contains sequences homologous to *iss*, the increased serum survival gene (3,13,23); *tsh*, a gene associated with virulence of avian *E. coli* (8); *intI*, a gene encoding the integrase of class 1 integrons (19); and certain genes of the aerobactin- and ColV-encoding operons. All bacterial isolates and subclones
were stored at −70°C in brain heart infusion broth (Difco Laboratories, Detroit, MI) with 10% glycerol (27) until use.

**Plasmid isolation and visualization.** pTJ100 DNA was initially obtained from an overnight culture of TC4 in Luria-Bertani broth (Difco Laboratories) containing ampicillin (100 µg/ml) and naladixic acid (30 µg/ml), according to the method of Wang and Rossman (29). In some cases, this DNA was digested with restriction enzymes (New England Biolabs, Beverly, MA). The plasmid DNA or its fragments were then separated by horizontal gel electrophoresis unit as previously described (15). Appropriate molecular size standards were run on each gel for comparative purposes (Life Technologies, Gaithersburg, MD).

**Cloning of pTJ100.** A library of pTJ100 was generated using the restriction endonuclease NarI (New England Biolabs). Fragments were cloned into the ClaI site of the cloning vector, pBluescript (Stratagene, La Jolla, CA), using an overnight ligation reaction at 16°C and a insert:vector molar ratio of 5:1. *Escherichia coli* DH5α (26) was transformed with the ligation products via electroporation using a Gene Pulser (BioRad, Hercules, CA) set at 2.5 kV, 200 ohms, and 960 uFD with an 0.2-cm gap. Immediately following electroporation, transformation mixtures were cultured in 1 ml of SOC media at 37°C for 1 hr (26). Samples of this culture were then plated on LB agar containing ampicillin (100 µg/ml), X-gal (0.004%), and IPTG (0.5 mM). White colonies were picked and screened for insert size. Also, to identify particular clones of interest, clones were surveyed for possession of various virulence genes using polymerase chain reaction (PCR) (see below). One clone, containing insert DNA of approximately 30 kb in size, was designated p92. Using the amplification methods described below, it was found to contain traT, iucC, and intI1, but not iss or tsh. Since it contained several of the genes of interest, it was selected for further study. In order to subclone p92 for further study, plasmid DNA was isolated using the method of Kado and Liu (16) and digested with EcoRI (New England Biolabs). The fragments were subjected to horizontal gel electrophoresis, and the fragments were gel purified from remaining salts and enzymes using the Freeze N Squeeze Kit (BioRad). Fragments were ligated into the multiple cloning site of pBluescript, and the recombinant plasmids were transformed into *E. coli* DH5α using methods described above. The transformants, containing the subclones of interest, were selected on media containing X-gal (0.004%), IPTG (0.5 mM), and ampicillin (100 µg/ml).

**PCR for virulence genes.** Recombinant plasmids generated in this study were examined for the presence of iss using a protocol previously described (24). All primers, including those for iss, were obtained from Sigma-Genosys (The Woodlands, TX) (Table 1). Amplification for iss was performed according to the following parameters: 5 min at 95°C; 9 cycles of 1 min at 95°C, 30 sec at 51.6°C, and 30 sec at 72°C; 25 cycles of 30 sec at 94°C, 30 sec at 51.6°C, and 30 sec at 72°C; 7 min at 72°C; and held at 4°C. Amplicons were subjected to horizontal gel electrophoresis. iss⁺ and iss⁻ control strains were examined with each amplification procedure. An isolate was considered to contain iss if it produced an amplicon of 760 base pairs (bp) (24).

Using similar techniques, recombinant plasmids were surveyed for the presence of several other genes linked to pTJ100. These included tsh, a gene associated with virulence in APEC strains (8,21); traT, which encodes an outer membrane protein (18,24); intI1, the gene encoding the integrase of class I integrons (1,19); and iucC (iuc, iron uptake chelate gene) of the aerobactin operon (20). Primers specific for each gene used in this study are listed in Table 1. Amplification protocols for all genes were the same as that described for iss, except annealing temperatures were modified appropriately for each primer pair used. Annealing temperatures used were 51°C for truT, 61.1°C for iucC, 51.6°C for iss, 55°C for tsh, and 55°C for intI1.

**DNA sequencing.** The four subclones created by digesting p92 with EcoRI were sequenced using the EZ:TN Transposon Insertion Kit (Epigence Technologies, Madison, WI), according to manufacturer’s directions. Briefly, a transposon containing a kanamycin resistance cassette and bidirectional sequencing primer sites was randomly inserted at a 1:1 ratio into target DNA isolated from each subclone. The clones containing transposon insertions were transformed into *E. coli* DH5α via electroporation (2.5 kV, 960 uFD, 0.2-cm gap), and transformants were selected on LB agar containing kanamycin (25 µg/ml). Plasmid DNA for each clone was isolated and purified using the method of Kado and Liu (16). Clones containing single transposon insertions were visualized on a 1.0% TAE agarose gel, and IR800-labeled primers (Epigence Technologies) were used to sequence away from the site of transposon insertion in each clone using the Thermosequenase cycle sequencing kit from Epigence and a LICOR 4000L automated sequencer (Lincoln, NE), following manufacturers’ instructions. Cycling reactions were performed on an Eppendorf Mastercycler (Brinkmann-Eppendorf, Westbury, NY). Sequences were collected using Baselmager software (LICOR).

**DNA sequence analysis and annotation.** For each of four p92 subclones, random, overlapping DNA sequences were aligned using the BioEdit contig assembly program version 5.0.9 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Gaps in the sequence alignment were closed with primer walking using primers designed based on a previously obtained sequence, a USB terminator dye sequencing kit (Cleveland, OH), and the LICOR 4000L sequencer. Open reading frames (ORFs) were identified using software available through the National Center for
Biotechnology Information (NCBI). ORFs were compared to the current NCBI protein database using the BlastP program, and homologous protein sequences were identified. Similarly, homologous DNA sequences were identified using a standard nucleotide blast (BlastN) program. ORFs were further analyzed for G+C content. The contiguous sequence thus identified was submitted to GenBank (AY214164).

**Antimicrobial susceptibility testing.** The original donor isolate from which pTJ100 was derived, E. coli A2363, the recipient strain, E. coli DH5α, their transconjugant, TC4, and the E. coli DH5α transformant containing p92 were compared for susceptibility to nine antimicrobial agents. These agents were selected for use since genes purported to encode resistance to them were identified in the p92 sequence obtained in this study. The disc diffusion method was used to test susceptibility to ampicillin, gentamicin, streptomycin, spectinomycin, tetracycline, sulfisoxazole, and chloramphenicol. The assay was performed using BBL Sensi-Disc Antimicrobial Susceptibility Tests (Becton Dickinson, Le Pont de Claix, France) in accordance with the methods described by Carter and Cole (6). Zones of inhibition were measured and interpreted according to manufacturer’s guidelines.

In order to compare the susceptibility of the donor, recipient, transconjugant, and transformant to a quaternary ammonium compound, minimum inhibitory concentrations (MICs) for each strain to benzalkonium chloride were determined (22). A single colony of an organism to be tested was used to inoculate a dilution series of benzalkonium chloride, ranging from 0 to 18 μg/ml in LB broth. Following overnight incubation at 37°C with shaking, the most dilute concentration of benzalkonium chloride inhibiting all visible growth for each organism was recorded as its MIC for that agent. In similar fashion, the susceptibility of these organisms to a heavy metal-containing compound, silver nitrate, was compared (11). In this case, the MICs were determined using a range of silver nitrate from 0 to 100 μg/ml.

**RESULTS**

A library of pTJ100 was created using the restriction endonuclease NarI. PCR was performed on the subclones to identify genes of interest. One 30-kilobase (kb) subclone, designated p92, produced amplicons for intI1, a gene of class 1 integrons, which are genetic units that are associated with multiple antimicrobial resistance (19), and the virulence genes, iucC (17) and traT (18). Neither tsh nor iss appeared to be present in p92. This subclone was sequenced. A total of 320 sequencing reactions were performed, resulting in approximately 240,000 bp of data, which correspond to approximately an eightfold coverage of the region described. Initial sequencing data were combined with primer walking to create one contiguous sequence of 30,054 bp (Fig. 1). Open reading frames were identified (Table 2) using software available from NCBI. Individual ORFs were compared to known
proteins using the BlastN program from NCBI. This 30-kb region contained certain plasmid transfer genes (traIDC) (18); silP, a gene encoding heavy metal resistance (11); tetA and tetR, which are involved in tetracycline resistance (12); two transposons, Tn21 and Tn3 (19); and bla, a gene encoding resistance to beta-lactam antibiotics (5). The Tn21-like region includes tmrARM, genes involved in the transposition of this transposon (19), and a class I integron (In2) (Fig. 1). In2 contains intI1, the gene encoding the integrase of this integron (19); several genes encoding antimicrobial resistance (aadB, catB3, qacEΔ1, and sulI) (19); orf5, a gene of unknown function; and istA and istB, which compose the insertion sequence IS1326 (19). Altogether, this 30-kb region contained genes suspected to encode resistance to 8 multiple antimicrobial agents (Table 2). Analysis of the region for G + C content was also undertaken. The overall G + C content of this region was 56% (Fig. 2).

The donor, recipient, transconjugant, and transformant were tested for susceptibility to nine antimicrobial agents for which corresponding resistance genes were identified in the p92 clone. The results are shown in Fig. 3. Briefly, the donor, transconjugant, and transformant gave similar results for tetracycline, ampicillin, streptomycin, sulfisoxazole, and benzalkonium chloride. All were more resistant to these agents than the recipient organism. However, all four organisms were susceptible to the actions of spectinomycin, gentamicin, and chloramphenicol. The MICs of silver nitrate for the donor and transconjugant were identical, as were the MICs for the recipient and transformant. The donor and transconjugant were more resistant to silver nitrate (100 μg/ml) than were the recipient and transformant (60 μg/ml).

**DISCUSSION**

Many factors have been associated with virulence of avian *E. coli*, but a complete picture of avian colibacillosis pathogenesis has yet to evolve. Of particular interest in recent years has been the role that plasmid-located genes may play in the virulence of the *E. coli* causing disease in poultry, and several accounts linking large aerobactin- or ColV-encoding plasmids to avian *E. coli* virulence are found in the literature (8,9,10,14,15). Interestingly, it has been
shown that while ColV production itself is not essential for virulence in *E. coli*, the presence of a ColV plasmid in a strain increases the likelihood that the strain will be virulent (25). It has also been suggested that if individual virulence genes are genetically linked to each other on large plasmids, then the strains containing these plasmids will have an increased adaptive advantage because of the coordination of these genes (30). Recently, our lab described a ColV-producing, aerobactin-encoding conjugative R plasmid containing many of the genes commonly associated with virulent avian *E. coli* (15). Included within this set of genes are *iss, tsh, cvaC, intI1,* and *traT.* In order to better understand the relationship of virulence and antimicrobial resistance genes on this plasmid, a 30-kb clone of pTJ100, identified as containing several virulence genes and *intI1* of class 1 integrons, was analyzed.

Sequencing of this large clone revealed that it did contain the genes previously identified by PCR, including *intI1,* a class 1 integron with several antimicrobial resistance cassettes, other portions of *Tn21,* several mobile elements, and other genes or their remnants (Fig. 1). Bass and colleagues (1) had previously reported *Tn21*’s presence in avian *E. coli.* *Tn21* or transposon 21 has been called the “flagship of the floating genome” because of its ability to facilitate the movement of genes within and between genomes (19). *Tn21* typically contains *Tn10,* a transposon encoding tetracycline resistance; a class 1 integron known as In2; the mercury resistance (*mer*) operon; and a large transposase gene, *tnpA,* used in *Tn21*’s transposition (19). The region of pTJ100 described here contains the tetracycline resistance genes, but *Tn10*’s other components are lacking. This *Tn21*-like region also contains an intact class I integron, In2, with three gene cassettes, but the portion of *Tn21* found in pTJ100 lacks the *mer* operon. Such deviations from the prototypical structure of *Tn21* have been noted previously (19) and may be due to failure of *Tn21* to acquire them or *Tn21*’s loss of them during its evolution.

### Table 2. Characteristics of ORFs in the sequenced fragment of pTJ100.

| ORF | Gene | ORF location | Description of homologous protein | % Identity | Accession no.
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>traI</em></td>
<td>3324-1</td>
<td>TraI; plasmid helicase</td>
<td>98</td>
<td>AAK62053</td>
</tr>
<tr>
<td>2</td>
<td><em>traD</em></td>
<td>4908-3231</td>
<td>TraD, partial sequence</td>
<td>90</td>
<td>X55815</td>
</tr>
<tr>
<td>3</td>
<td><em>silF</em></td>
<td>5647-5964</td>
<td>Hypothetical protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td><em>tetA</em></td>
<td>6062-8542</td>
<td>SIlP; silver &amp; heavy metal resistance</td>
<td>98</td>
<td>Q9ZHC7</td>
</tr>
<tr>
<td>5</td>
<td><em>tetR</em></td>
<td>9164-8814</td>
<td>Similar to lysostaphin</td>
<td>99</td>
<td>AAD11751</td>
</tr>
<tr>
<td>6</td>
<td><em>tnpA</em></td>
<td>10093-9419</td>
<td>Putative transposase</td>
<td>99</td>
<td>AAM08042</td>
</tr>
<tr>
<td>7</td>
<td><em>tnpR</em></td>
<td>11986-10712</td>
<td>TetA; tetracycline resistance</td>
<td>94</td>
<td>AAM22221</td>
</tr>
<tr>
<td>8</td>
<td><em>intI1</em></td>
<td>11990-12667</td>
<td>TetR; repressor</td>
<td>100</td>
<td>AAM22220</td>
</tr>
<tr>
<td>9</td>
<td><em>aadA4</em></td>
<td>15964-12998</td>
<td>Tn1721 transposase</td>
<td>100</td>
<td>X61337</td>
</tr>
<tr>
<td>10</td>
<td><em>aadB</em></td>
<td>16342-16968</td>
<td>Tn1721 resolvase</td>
<td>90</td>
<td>CAA08841</td>
</tr>
<tr>
<td>11</td>
<td><em>catB</em></td>
<td>17156-16704</td>
<td>Tn21 modulator</td>
<td>100</td>
<td>BAA78803</td>
</tr>
<tr>
<td>12</td>
<td><em>intI1</em></td>
<td>17837-17256</td>
<td>IntI1; integrase for class I integron</td>
<td>99</td>
<td>NP_052898</td>
</tr>
<tr>
<td>13</td>
<td><em>tetA</em></td>
<td>18198-18947</td>
<td>Aminoglycoside adenyltransferase; aminoglycoside resistance</td>
<td>100</td>
<td>CAA28209</td>
</tr>
<tr>
<td>14</td>
<td><em>aadA4</em></td>
<td>19030-19773</td>
<td>Chloramphenicol acetyltransferase; chloramphenicol resistance</td>
<td>99</td>
<td>AAD50826</td>
</tr>
<tr>
<td>15</td>
<td><em>aacA4</em></td>
<td>19717-20505</td>
<td>Aminoglycoside adenyltransferase; streptomycin/spectinomycin resistance</td>
<td>100</td>
<td>NP_052895</td>
</tr>
<tr>
<td>16</td>
<td><em>qacEA1</em></td>
<td>27011-21058</td>
<td>Quaternary ammonium compound resistance</td>
<td>100</td>
<td>NP_052895</td>
</tr>
<tr>
<td>17</td>
<td><em>sul</em></td>
<td>21052-21891</td>
<td>Sulf; dihydropteroate synthetase type I; sulfonamide resistance</td>
<td>100</td>
<td>NP_052895</td>
</tr>
<tr>
<td>18</td>
<td><em>isb</em></td>
<td>23475-22690</td>
<td>IstB; IS1326 transposase</td>
<td>100</td>
<td>AAC33916</td>
</tr>
<tr>
<td>19</td>
<td><em>isa</em></td>
<td>23462-24280</td>
<td>IstA; IS1326 transposase</td>
<td>100</td>
<td>AAC33917</td>
</tr>
<tr>
<td>20</td>
<td><em>bla</em></td>
<td>24417-25208</td>
<td>Hypothetical protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td><em>tnpA</em></td>
<td>25826-25272</td>
<td>Tn3 transposase</td>
<td>100</td>
<td>P03008</td>
</tr>
<tr>
<td>22</td>
<td><em>tnpR</em></td>
<td>28448-29005</td>
<td>Tn3 resolvase</td>
<td>100</td>
<td>P03011</td>
</tr>
<tr>
<td>23</td>
<td><em>bla</em></td>
<td>29194-30054</td>
<td>Beta-lactamase; beta lactam resistance</td>
<td>100</td>
<td>CAD24670</td>
</tr>
</tbody>
</table>

*a Denotes nucleotide positions in sequenced fragment from start to stop codon.
*Identity presented as percentage of amino acid identity to referenced protein via BlastN.
Each gene marked with an asterisk encodes resistance to one or more antimicrobial agents.
In2 in pTJ100 contains three gene cassettes, encoding resistance to spectinomycin/streptomycin (aadA4) (31), chloramphenicol (catB3) (19), and gentamicin (aadB) (19). These cassettes are inserted directly downstream of the attI site; each was associated with a 59-bp element capable of acting as a recombination site. Additionally, In2 contains qacE_D1, which is known to encode a low-level resistance to quaternary ammonium compounds (19), and sulI, which encodes sulfonamide resistance (19). It may be that many APEC strains contain In2 (1), which acts as a “docking bay” for up to seven antimicrobial resistance genes (2).

Also found within this 30-kb region are genes involved in encoding resistance to ampicillin (bla) (5) and silver and other heavy metals (silP) (11). All the resistance genes within this 30-kb clone either are contained within or flank In2 of Tn21, indicating that Tn21 may play an important role in the transmission and acquisition of antimicrobial resistance genes in this E. coli isolate.

Several mobile elements were found in the region sequenced in this study. In2 itself is a mobile element, and the tetracycline resistance genes are flanked upstream by the insertion sequence IS26. Similarly, silP and bla, genes encoding for resistances to heavy metal and beta-lactam antimicrobials, respectively, and In2, the class I Integron of Tn21, are all surrounded by mobile elements. Two diverse transposases, directly upstream of Tn21 and bla, are capable of recognizing and interacting with other inverted repeats to facilitate transposition (19). These transposases can also direct one-ended transposition. Therefore, the genetic plasticity of this region may be or may have been at one time enhanced by its fragmented and intact insertion sequences. The recurring pattern of mobile elements flanking resistance genes might indicate that these genes were acquired at different times during the evolution of this strain. Support for this supposition was found in the G + C analysis of the region’s individual ORFs (Fig. 2), which revealed a much lower G + C content for the regions encompassing silP, qacE_D1, and istB than in the rest of this 30-kb region of pTJ100. Furthermore, the overall content of this 30-kb region (56%) shows an intriguing deviation from that of the genome of E. coli strain MG1655 (51%) (4), which also indicates that many
of the genes of this clone and, perhaps, of pTJ100, may have originated from a non–E. coli source. The relationship between this resistance-encoding region with several putative virulence genes and mobile elements indicates that this plasmid may be a more important player in virulence and in the evolution of virulence among APEC than previously thought.

DNA sequencing also revealed that the mercury resistance operon, which is typically a component of Tn\textsuperscript{21}, appears to be absent in the portion of Tn\textsuperscript{21} found in pTJ100. That the sequencing data revealed the absence of \textit{merA} in the presence of some of Tn\textsuperscript{21}'s other components was foreshadowed by previously reported results obtained by PCR. That is, PCR results had indicated that pTJ100 contained the \textit{intI1} gene but did not contain the \textit{merA} gene (15). Elsewhere, it was suggested that the mercury resistance operon evolved separately from Tn\textsuperscript{21} (19); perhaps this result lends support to this statement. The absence of this operon in pTJ100 indicates that pTJ100 either acquired Tn\textsuperscript{21} without the mercury resistance operon or that this operon was initially present in pTJ100 and was later lost. This Tn\textsuperscript{21} region is flanked by two transposases that may have been involved in the original mobilization of Tn\textsuperscript{21} into pTJ100.

p92 contained several genes suspected to encode for antimicrobial resistance. In an effort to determine whether the presence of these genes corresponded to phenotypic differences in resistance among the donor strain, recipient strain, their transconjugant, and the p92-containing transformant, their susceptibilities to tetracycline, ampicillin, streptomycin, gentamicin, sulfisoxazole, chloramphenicol, benzalkonium chloride, and silver nitrate were assessed. The donor, transconjugant, and transformant showed similar resistance profiles to tetracycline, ampicillin, streptomycin, sulfisoxazole, and benzalkonium chloride; in these cases, the donor, transconjugant, and transformant were all more resistant than the recipient strain. Therefore, the genes present in p92 could account for the resistances to these agents as seen in the transformant, transconjugant, and donor.

<table>
<thead>
<tr>
<th>Donor (2363) with pTJ100</th>
<th>Tc</th>
<th>Ap</th>
<th>Sp</th>
<th>St</th>
<th>Gn</th>
<th>Su</th>
<th>Cm</th>
<th>Be</th>
<th>An</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R\textsuperscript{4}</td>
<td>R</td>
<td>S\textsuperscript{2}</td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>LS\textsuperscript{4}</td>
<td>LS</td>
</tr>
<tr>
<td>Recipient (DH5\textalpha)</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>MS\textsuperscript{5}</td>
<td>MS</td>
</tr>
<tr>
<td>Transconjugant (DH5\textalpha + pTJ100)</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>LS</td>
<td>LS</td>
</tr>
<tr>
<td>Transformant (DH5\textalpha + p92)</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>LS</td>
<td>MS</td>
</tr>
</tbody>
</table>

Fig. 3. Susceptibilities of donor, recipient, transconjugant, and transformant for antimicrobial agents corresponding to resistance genes found in the p92 sequence. Susceptibilities to the antimicrobial agents in the nonshaded portion of the figure were determined by the disk diffusion method. Relative susceptibilities of the organisms to silver nitrate and benzalkonium chloride were assigned to the organisms based on their MICs for each agent; these results are provided in the shaded areas of the figure. \( ^{31} \text{R} = \) resistant to the action of the drug; \( ^{2} \text{S} = \) susceptible to the action of the drug; \( ^{1} \text{I} = \) intermediate susceptibility to the drug; \( ^{4,5} \text{LS} = \) less susceptible to this agent than the MS = more susceptible organism. Tc = tetracycline; Ap = ampicillin; Sp = spectinomycin; St = streptomycin; Gn = gentamicin; Su = sulfisoxazole; Cm = chloramphenicol; Be = benzalkonium chloride; and An = silver nitrate. Changes in susceptibility showing acquisition of donor traits by the transconjugant or transformant are highlighted in bold italic.
all the genes required to produce the heavy metal resistance of the silver operon, but p92 does not.

In summary, analysis of a 30-kb region of pTJ100 is a mosaic of putative virulence genes, insertion sequences, antimicrobial resistance cassettes, or their remnants. It appears that the genetic constitution of this region of the plasmid may be quite plastic and the consequence of a complex evolution. Although further study will be required to better define this plasmid’s role in avian E. coli virulence, the sequence described here indicates that this plasmid may comprise a unique virulence unit important to the pathogenesis of avian colibacillosis. Further, the occurrence of multiple antimicrobial resistance genes on this conjugative plasmid indicates that there are many selectors that may promote the maintenance of E. coli containing such plasmids in the production environment. In other words, APEC containing these putative virulence plasmids may flourish under selective pressure of various antimicrobials, including antibiotics, disinfectants, and heavy metals. Therefore, producers may wish to exercise caution in their choice of antimicrobial agents to be used in poultry operations.

REFERENCES


ACKNOWLEDGMENTS

We gratefully acknowledge the assistance and advice of Cathy Giddings and Dr. Shelley Horne in the completion of this project.