Persistence of an Antibiotic Resistance Plasmid in Intestinal Escherichia coli of Chickens in the Absence of Selective Pressure

ELISABETH CHASLUS-DANCLA,†* GUY GERBAUD,2 MARYSE LAGORCE,1 JEAN-PIERRE LAFONT,1 AND PATRICE COURVALIN2

Institut National de la Recherche Agronomique, Centre de Tours, 37380 Monnaie,1 and Unité des Agents Antibactériens, Centre National de la Recherche Scientifique, Institut Pasteur, 75724 Paris Cedex 15,2 France

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We studied eight strains of Escherichia coli resistant to high levels of trimethoprim that were isolated during a 6-week period in a commercial breeding flock of broilers. The strains originated from fecal samples and from a carcass immediately after slaughter. Seven of the eight strains belonged to the same inefrequent biotype. They were also resistant to ampicillin and streptomycin, and some were resistant to tetracycline and potassium tellurite. All the strains transferred trimethoprim and ampicillin resistance to E. coli. Analysis of the donors and of the transconjugants by agarose gel electrophoresis after digestion by restriction endonucleases and by nucleic acid hybridization indicated that resistance to trimethoprim (dfrl) and to ampicillin (bla TEM-1) was mediated by a 65-kilobase plasmid, pIP1531. Persistence of resistance to trimethoprim and ampicillin in this flock was therefore due to two cumulative factors, both occurring in the absence of selective pressure, namely the dissemination of a particular plasmid between strains and the ability of an atypical E. coli strain to stably colonize many animals.

Strains of Escherichia coli resistant to multiple antibiotics often predominate in the fecal flora of broiler chickens (2, 15). Resistance is generally due to a high incidence of plasmid carriage which causes concern for both animal and public health. The reasons for this situation have been reviewed elsewhere (14).

It has been demonstrated that in certain flocks, the populations of intestinal E. coli resistant to multiple antibiotics fluctuated to some degree during the rearing period (2, 8, 19). However, these studies have focused mainly on the phenotypic properties of the resistant populations. The nature and evolution of the genetic support for these resistances have not been investigated as much.

During a prospective study on trimethoprim-resistant populations in the intestinal flora of broilers, we observed that a plasmid conferring resistance to ampicillin and trimethoprim persisted in different E. coli strains in the absence of selective pressure during the rearing period. This plasmid was also detected in a strain contaminating a carcass immediately after slaughter.

MATERIALS AND METHODS

Description of flock and origin of samples. The study was carried out with a traditional commercial breeding flock of 5,000 broiler chickens which were slaughtered at 8 weeks of age. The animals were reared on a litter of wood chips on the floor. They had free access to a commercial mash. On days 10 and 11, a preventive treatment of chloramphenicol, dihydrostreptomycin, neomycin, and tetracycline was given. Every week, freshly dropped feces from 10 zones evenly distributed over the floor surface were sampled. A sample of food was also collected weekly before distribution. At slaughter, samples were taken as previously described (3) from 10 fresh carcasses just before packaging and from 4 carcasses after 8 days of storage in polyethylene bags under commercial conditions at 4°C.

Microbiological techniques. A total of 111 samples from these different sources was collected and analyzed. Trimethoprim-resistant E. coli strains were isolated by plating dilutions of feces on citrate-mannitol agar (Difco Laboratories) supplemented with trimethoprim (500 μg/ml). Resistance to other antibiotics was determined by disk agar diffusion on Mueller-Hinton agar (Diagnostics Pasteur). MICs of trimethoprim were determined on the same medium by the agar dilution technique with a multiple inoculator. Galleries (API 20E and API 50CH) were used for identifying and biotyping the strains. The reproducibility of the biotype determination was established by repeated testing, and some unusual characteristics (e.g., urease) were confirmed by classical tests (Diagnostics Pasteur). Bacterial resistance to potassium tellurite (Tel) was assessed as previously described (23).

Conjugation. Conjugation was carried out overnight in brain heart infusion broth (Difco) at 37 or 27°C (26) with two E. coli K-12 strains, BM21 (prototroph, gyrA) and BM14 (pro met azi), as recipients. The antibiotics and concentrations (micrograms per milliliter) used for selection were trimethoprim, 10; nalidixic acid, 40; and sodium azide, 500.

Tests for incompatibility. Because of the unusually high incidence of plasmids belonging to incompatibility group P or I1 in animal strains (4), eight plasmids were studied for incompatibility (1) with plasmids RP4 (Tra+ IncP Ap Km Tc; 50 kilobases [kb]) and pIP112 (Tra+ IncI1 Kmr; 100.5 kb) (11).

Assay for beta-lactamase. Bacterial extracts were obtained after sonication (5), and the presence of a beta-lactamase was detected by an adaptation of the iodometric method in agar (12). The enzymes were characterized by a microacidimetric method (A. Philippom, R. Labia, G. Paul, and P. Nevot, Program Abstr. 1st Mediterr. Congr. Chemother., p. 211–216, 1978). The following plasmid-mediated
beta-lactamases were used as references: R111 (TEM-1), RP4 (TEM-2), RGN238 (OXA-1), R55 (OXA-3), and RPL11 (PSE-1) (11, 25). Isoelectric focusing of S100 preparations in polyacrylamide gels was as described elsewhere (18).

**Preparation of plasmid DNA and agarose gel electrophoresis.** Plasmid DNA was prepared, and agarose gel electrophoresis was performed as previously described (24). Plasmids RP4 and pIP112 and strain V517 (16) were used for molecular size standards.

**Hybridization.** Plasmid pFE872 (7) and a derivative of plasmid pFE364 (6; L. Elwell, personal communication) were used to construct probes specific for dihydrofolate reductase types I and II. Plasmid pFE872 has a 500-base-pair (bp) HpaI insert containing the gene for type I enzyme. The pFE364 derivative contains the dihydrofolate reductase type II gene on an 800-bp EcoRI fragment. Purification of the DNA fragments by agarose gel electrophoresis in 0.8% low melting agarose (type VII; Sigma Chemical Co.) was as described elsewhere (17). 32P labeling of the purified restriction DNA fragments was performed with a nick translation reagent kit (Bethesda Research Laboratories, according to the recommendations of the manufacturer. Nitrocellulose filters (BA-85; Schleicher & Schuell, Inc.) were incubated with a multiple inoculator. After 3 h of incubation on Mueller-Hinton agar, colonies were lysed, and hybridization was carried out in 50% formamide at 42°C as previously described (17). Positive controls [strains C600(pFE872) and C600(pPE364)] and negative controls [strains HB101 and HB101(pBR322)] were included.

**Enzymes.** Restriction endonucleases EcoRI and HindIII (Boehringer Mannheim Biochemicals) were used according to the recommendations of the manufacturer. Digestion of DNA was carried out after ethanol precipitation as previously described (24).

**Chemicals.** 5'[^α-32P]ATP (triethylammonium salt) was obtained from the Radiochemical Centre, Amersham, England. The antibiotics were provided by the following laboratories: ampicillin, carbenicillin, and cephalothin by Beecham Laboratories; cephaloridine by Eli Lilly & Co.; nalidixic acid by Winthrop Laboratories; oxacillin by Bristol Laboratories; penicillin by Specia; and trimethoprim by Sigma. Potassium tellurite and sodium azide were provided by Prolabo.

**RESULTS**

Resistance to trimethoprim was recorded with all fecal samples studied, with an incidence of 1 to 5% of the total E. coli population. Strains of E. coli resistant to trimethoprim

<table>
<thead>
<tr>
<th>TABLE 1. Origin and properties of the E. coli strains studied</th>
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<td><strong>Designation</strong></td>
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<td><strong>Wild strains</strong></td>
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<td><strong>Transconjugants</strong></td>
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* Hybridization number in Fig. 4.
* Tel, Resistance to potassium tellurite; the nomenclature of other phenotypic characters is according to Novick et al. (20).

[FIG. 1. Agarose gel electrophoresis of plasmid DNA from strains V517 (I) and BM21pIP112 (II) and of transconjugants from strains BN976 (A), BN1342 (B), BN1333 (C), BN1143 (D), BN1171 (E), BN781 (F), BN1290 (G), and BN1340 (H).]

[FIG. 2. Agarose gel electrophoresis of plasmid DNA after digestion with EcoRI. The DNA of transconjugants from strains BN976 (A), BN1342 (B), BN1333 (C), BN1143 (D), BN1171 (E), BN781 (F), BN1290 (G), and BN1340 (H) was used. The sizes of the restriction fragments were determined by comparison with fragments obtained by digestion of λ DNA with EcoRI. △, Undigested plasmid DNA.]
were also isolated from a food sample during week 7 and from fresh and chilled carcasses. We studied 209 trimethoprim-resistant isolates from all sources by the disk agar diffusion test. All were also resistant to one or several other inhibitory agents, including sulfonamide in 93% and potassium tellurite in 67% of cases tested. To avoid redundancy among the strains studied, a single isolate representative of each resistance pattern was selected for further studies. A total of 78 strains were thus conjugated with *E. coli*, and 70 strains transferred resistance to trimethoprim. Cotransfer of many other resistances was observed. Eight transconjugants acquired only resistance to ampicillin and to trimethoprim. Since this was the most frequently transferred short pattern of resistance, these transconjugants were retained for further study. They were the only transconjugants obtained from the corresponding natural isolates by selection on trimethoprim.

The origin and properties of the wild strains which transferred ampicillin and trimethoprim resistance only are given in Table 1. They originated from fecal samples collected during weeks 2 (one strain), 6 (one strain), and 7 (five strains) and from the internal swab of a fresh carcass (one strain). The eight strains were resistant to high levels of trimethoprim (MIC, >1,024 μg/ml). They were also resistant to streptomycin but susceptible to spectinomycin, and six strains were also resistant to tetracycline; however, none of these other resistances were acquired by ampicillin- and trimethoprim-resistant transconjugants.

Except for BN781, all strains belonged to the unusual biotype of *E. coli* with urease- and cellbiose-positive characteristics. Resistance to potassium tellurite was observed in all strains, except for strain BN781. This resistance was never present in ampicillin and trimethoprim-resistant transconjugants.

In all strains, beta-lactamase activity was detected by the iodometric test. Results (not shown) from the microcildimetric method and isoelectric focusing indicated that the enzyme responsible was TEM-1.

Analysis of crude bacterial lysates by agarose gel electrophoresis revealed a striking similarity between the total plasmid contents of all the natural isolates except BN781 (data not shown). Plasmids of approximately 65 kb were present in all donor strains and were recovered in every Ap Tp transconjugant (Fig. 1). These 65-kb plasmids were indistinguishable by restriction endonuclease digestion followed by agarose gel electrophoresis. After *EcoRI* digestion, five fragments were observed (Fig. 2). Digestion with *HindIII* generated nine DNA bands common to the different preparations (Fig. 3). The added sizes of the *EcoRI* and *HindIII* DNA fragments, 66 and 61.2 kb, respectively, are in agreement with those estimated for the undigested plasmids. All the plasmids could stably coexist with pLp112 and RP4 and therefore do not belong to incompatibility group I1 or P.

The eight *E. coli* strains and the corresponding transconjugants were hybridized with the 32P-labeled 500-bp *HpaI* fragment of pFE872 and the 800-bp *EcoRI* fragment of the pFE364 plasmid derivative (Fig. 4). There was hybridization of the pFE872 insert with all strains studied. This DNA fragment contains the *dfrI* gene of Tn7 (7), so this observation indicates that the plasmids studied also encode a dihydrofolate reductase of type I.

**DISCUSSION**

From samples taken at the beginning of the present study, strains resistant to trimethoprim were isolated on unsupplemented citrate-mannitol agar plates. This resistance is, in
our experience, infrequent in the *E. coli* intestinal populations of chickens, and trimethoprim had not been used for therapy in the flock studied. The present work therefore focused on this particular resistance. The majority of the transconjugants obtained from the trimethoprim-resistant isolates acquired resistance to various antibiotics in addition to trimethoprim-resistance. The shorter and most frequently transferred pattern was resistance to ampicillin and trimethoprim, and eight transconjugants of this type were studied further.

The plasmids present in the transconjugants showed identical resistance phenotypes, mechanisms of resistance, and fragment patterns generated by two restriction endonucleases (Fig. 2 and 3). A single plasmid, designated pIP1531, encoding a TEM-1 beta-lactamase and a dihydrofolate reductase type I, was therefore present at least from week 2 to 7 in different intestinal *E. coli* strains of this chicken flock (Table 1). Plasmid pIP1531 was widespread in the animal population, as judged by its recovery from samples taken during week 7 from five areas of the floor surface. Furthermore, an isolate harboring plasmid pIP1531 was also obtained from one fresh carcass immediately after slaughter. However, pIP1531 was not detected in *E. coli* in food samples or in chilled carcasses. Most interesting, the persistence of this ubiquitous plasmid was apparently not favored by selective pressure since neither ampicillin nor trimethoprim was distributed in this flock. Plasmid pIP1531 was present in strains belonging to different biotypes, which suggests its dissemination in the *E. coli* population of the animals. It was initially recovered from a typical *E. coli* isolated during week 2. In subsequent samples, it was harbored by an unusual urease- and cellulase-positive *E. coli* (10; API 20E Analytical Profile Index). The isolation of this strain during week 7 from five sites on the litter demonstrates a large colonization of the flock. The detection of the same strain during weeks 6 and 7 and in a fresh carcass at slaughter indicates its stability in this particular ecosystem. Urease-positive *E. coli* has already been reported among strains of animal origin (21). This metabolic ability was not transferred with plasmid pIP1531. It could have conferred an ecological advantage on this particular strain, which was able to persist and spread without selective pressure by ampicillin or trimethoprim.

The intestinal flora of farm animals seems to be characterized by a turnover of antibiotic-resistant strains (9, 19). In experimental conditions, it has been observed that a single plasmid could persist and spread in the *E. coli* population of chickens (13) and pigs (22). The present study extends this notion to field conditions and suggests that such persistence may occur in the absence of selective pressure.

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**LITERATURE CITED**