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## Evolution and Spread of IncFIV Plasmids Conferring Resistance to Trimethoprim

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Twenty-one IncFIV-group plasmids conferring trimethoprim resistance in *Escherichia coli* isolates from humans and pigs were examined. Three evolutionary lines of plasmids were identified on the basis of restriction enzyme analysis. One was found exclusively in human isolates and another was found in pig isolates, while the third line consisted of plasmids from both sources. All R plasmids readily transferred to laboratory strains, and evidence was found for transfer to other biotypes of *E. coli* in the environment. The  $Tp^r$  genes from representatives of the plasmid lines were cloned and compared by restriction analysis and by hybridization with two characterized  $Tp^r$  dihydrofolate reductase genes. The sequences flanking the  $Tp^r$  genes were different for each line, but all showed homology with the type 2 dihydrofolate reductase gene, irrespective of whether they were of human or animal origin. There was no hybridization to the type 1 gene. The remarkable degree of similarity among plasmids of the third line provided clear evidence of the exchange of plasmid-bearing *E. coli* between humans and pigs.

In a previous study (20) of trimethoprim-resistant isolates from humans and intensively reared pigs, the R plasmids conferring trimethoprim resistance ( $Tp^r$ ) were either 50 to 66 kilobases (kb) in size or greater than 120 kb. The smaller plasmids belonged to the IncN group, while the larger plasmids were incompatible with IncFIV, a relatively rare group (6). The finding of similar proportions of IncFIV and IncN trimethoprim R plasmids in the bacterial flora of both humans and pigs suggested that there was a common pool of plasmids in the two populations. Recently, Wise et al. (23) also found that there was a common pool of R plasmids with overlapping resistance patterns and incompatibility properties in isolates from humans and animals in central England.

The purpose of this investigation was to characterize further the group of IncFIV trimethoprim R plasmids from human and porcine isolates with the view of demonstrating evolutionary trends. Similar research has been conducted by Tschäpe and Tietze (22) on R plasmids incompatible with the IncK plasmid R387, by Jørgensen et al. (14) on chloramphenicol-resistant IncFII plasmids, and by Konarska-Kozłowska and Iyer (15) on a group of IncN plasmids. Results of these studies found that many of the restriction enzyme fragments generated were common and that plasmid functions were retained on restriction fragments of the same size. Our results suggest that there has been considerable evolution among the IncFIV plasmids and that a  $Tp^r$  *Escherichia coli* has transferred from humans to pigs (or vice versa) and successfully colonized both ecosystems.

### MATERIALS AND METHODS

**Bacterial strains and plasmids.** Standard bacterial strains and plasmids used in this study are described in Table 1. The human isolates were provided by the Combined Clinical Microbiology Service, Sir Charles Gairdner Hospital, Perth, Western Australia. The porcine isolates were obtained as

normal flora from the feces of animals held at the Government Pig Research Station, located 50 km from the hospital. Strain identification, initial transfer, and incompatibility testing of trimethoprim R plasmids have been reported previously (20). Tables 2 and 3 show data on the human and pig IncFIV trimethoprim R plasmids and the strains from which they were isolated.

**Resistance to antimicrobial agents.** Resistance to the following antimicrobial agents was determined by replica plating onto MacConkey agar incorporating the agent at the following concentrations (in micrograms per milliliter): ampicillin, 25; tetracycline, 25, 12.5, 6.25, and 3.12; chloramphenicol, 25; kanamycin, 25; streptomycin, 25 and 12.5; nalidixic acid, 50. Resistance to sulfafurazole at 250 and 125  $\mu\text{g/ml}$  and trimethoprim at 20  $\mu\text{g/ml}$  was determined on Sensitest agar (CM261; Oxoid Ltd., London, England). Resistance to mercuric ions was determined on CLED agar (CM301; Oxoid) containing  $10^{-4}$  M  $\text{HgCl}_2$ .

**Plasmid DNA preparation.** Plasmid DNA was prepared by the method of Birnboim and Doly (2) and purified by ultracentrifugation in a cesium chloride gradient in the presence of ethidium bromide.

**Restriction enzyme analysis.** Restriction enzymes were used as recommended by the manufacturers (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Agarose gel electrophoresis (AGE) and DNA restriction fragment detection were carried out by standard procedures (18). The sizes of lambda restriction fragments were obtained from Daniels et al. (5).

**Cloning of the  $Tp^r$  gene.** Plasmid DNA from representatives of the various sets of trimethoprim R plasmids was digested to completion with *EcoRI* and ligated to pACYC184 digested with *EcoRI*. Ligated DNA mixtures were used to transform *E. coli* JP3438 to  $Tp^r$ . Transformation of plasmid DNA was done by the method described by Kushner (16).

**Preparation of DNA probes.** The type 1 (pFE506) and type 2 (pFE420) dihydrofolate reductase (DHFR) probes used in this study have been described previously in detail (9). Plasmid pFE506 is a ColE1::Tn7 derivative containing the replication origin of ColE1 and 4.1 kb of Tn7 DNA which

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TABLE 1. Standard *E. coli* strains and plasmids

Strain or plasmid	Characteristics	Reference
<i>E. coli</i>		
JP990	<i>argE3 his-4 ilvC7 proA2 thi-1 supE44 galK2 λ<sup>-</sup>nalA368</i>	10
JP3438	<i>thr-1 leu-1 lacY1 gal351 supE44 tonA2 hsdR4 rpoB364 recA</i>	7 ( <i>recA</i> derivative of JP777)
Plasmid		
pFE506	Tp <sup>r</sup> type 1 DHFR gene (ColE1::Tn7)	9
pFE420	Tp <sup>r</sup> type 2 DHFR gene (R67, <i>EcoRI-BamHI</i> fragment in pSC101)	9
pACYC184	Cm <sup>r</sup> Tc <sup>r</sup>	3

includes the trimethoprim resistance determinant. pFE420 consists of a 2.4-kb *EcoRI-BamHI* fragment of R67, which contains the type 2 DHFR gene, cloned into pSC101. In each case the entire plasmid was labeled with biotinylated dUTP with the Enzo Bio-Probe nick translation kit (Enzo Biochem, Inc.).

**DNA-DNA hybridizations.** DNA homology between biotin-labeled probes containing the type 1 and type 2 DHFR genes and the cloned Tp<sup>r</sup> genes from the IncFIV plasmids was detected by a modification of the technique of Southern (21). Restriction fragments of cloned Tp<sup>r</sup> genes were separated by AGE and transferred electrophoretically to the hybridization membrane (Gene Screen Plus; New England Nuclear Corp., Boston, Mass.). Hybridization was performed for 16 to 24 h at 65°C in 1% sodium dodecyl sulfate-1 M sodium chloride-10% dextran sulfate-100 µg of salmon sperm DNA per ml with a probe concentration of 5 ng/ml. The membrane was washed twice, for 5 min with 0.3 M sodium chloride-0.03 M sodium citrate (2× SSC) at room temperature, for 30 min with 2× SSC-1% sodium dodecyl sulfate at 65°C, and for 30 min with 0.1× SSC at room temperature. Hybridization was detected by using the Enzo Bio-Probe

streptavidin and biotinylated acid phosphatase system (Detek 1-ACP; Enzo Biochem).

## RESULTS

**Plasmid collection from humans and pigs.** The plasmids listed in Tables 2 and 3 are a selection of those recovered from human and porcine isolates during a 15-month survey and are considered to be representative of those circulating at that time. Based on antibiotic resistance patterns and transfer frequencies, the R plasmids were categorized into seven sets. The human plasmids comprised sets A, B, C, and D; and the porcine plasmids comprised sets D', E, and F. Plasmids with the simplest antibiotic resistance pattern were allocated to set A which included the sulfafurazole and trimethoprim resistance plasmids pHTB1 and pHTB3. The plasmids pHTB19, pHTB20, and pHTB53 were included in set B. Some members of this set conferred very low levels of resistance to tetracycline. For example the MICs for tetracycline conferred by plasmids pHTB19 and pHTB20 were 3.12 and 6.25 µg/ml, respectively. Both of these plasmids were recovered from the same isolate. The host to which these plasmids were transferred had an MIC to tetracycline of less than 1 µg/ml. The set C plasmids pHTB14, pHTB21, and pHTB23 all conferred resistance to streptomycin, sulfafurazole, tetracycline, and trimethoprim, although pHTB21 exhibited lower levels of resistance to streptomycin and sulfafurazole. Set D comprised the chloramphenicol, sulfafurazole, tetracycline, trimethoprim, and mercuric ion resistance plasmids pHTB8, pHTB9, and pHTB31 and were the only human plasmids which conferred resistance to mercuric ions. All the pig plasmids conferred resistance to mercuric ions. The pig plasmids pPTB34, pPTB151, and pPTB197 were included in set D' because in both size and resistance pattern they resembled the set D plasmids. In addition, the biotypes of the *E. coli* isolates from which both the set D and set D' plasmids were recovered were identical. The porcine plasmids conferring resistance to sulfafurazole, trimethoprim, and mercuric ions were included in set E; and the sulfafurazole, tetracycline, trimethoprim, and mercuric ion resistance plasmids were included in set F.

TABLE 2. Properties of the human isolates and IncFIV trimethoprim R plasmids

Date of isolation (mo/day/yr)	Wild-type <i>E. coli</i> isolates		Properties of plasmids <sup>a</sup>				
	Resistance pattern <sup>b</sup>	Biotype <sup>c</sup>	Plasmid designation	Transfer frequency <sup>d</sup>	Resistance pattern	Size (kb) <sup>e</sup>	Set <sup>f</sup>
5/17/80	Ap Cm Km Sm Su Tc Tp	51445722	pHTB1	10 <sup>-5</sup>	Su Tp	155	A
10/17/80	Su Tp	51445722	pHTB3	10 <sup>-6</sup>	Su Tp	155	A
11/10/80	Ap Cm Km Sm Su Tc Tp	51445723	pHTB19	10 <sup>-4</sup>	Su Tc <sub>c</sub> Tp	164	B
			pHTB20	10 <sup>-4</sup>	Su Tc <sub>b</sub> Tp	164	B
3/2/81	Ap Cm Km Sm Su Tc Tp	51445722	pHTB53	10 <sup>-4</sup>	Su Tc <sub>a</sub> Tp	164	B
8/1/80	Sm Su Tc Tp	71445722	pHTB14	10 <sup>-5</sup>	Sm Su Tc Tp	171	C
8/3/80	Sm Su Tc Tp	50445520	pHTB21	10 <sup>-4</sup>	Sm <sub>a</sub> Su <sub>a</sub> Tc Tp	170	C
			pHTB23	10 <sup>-7</sup>	Sm Su TcTp	170	C
4/3/80	Ap Cm Sm Su Tc Tp	50445522	pHTB31	10 <sup>-5</sup>	Cm Su Tc Tp Hg	158	D
6/11/80	Ap Cm Nx Sm Su Tc Tp	50445522	pHTB8	10 <sup>-5</sup>	Cm Su Tc Tp Hg	142	D
			pHTB9	10 <sup>-5</sup>	Cm Su Tc Tp Hg	158	D

<sup>a</sup> The host for all transconjugants was JP990.

<sup>b</sup> Resistance abbreviations and concentrations (in micrograms per milliliter): ampicillin (Ap); tetracycline (Tc), 25; Tc<sub>a</sub>, 12.5; Tc<sub>b</sub>, 6.25; Tc<sub>c</sub>, 3.12; chloramphenicol (Cm); kanamycin (Km); streptomycin (Sm), 25; Sm<sub>a</sub>, 12.5; sulfafurazole (Su), 250; Su<sub>a</sub>, 125; trimethoprim (Tp); nalidixic acid (Nx); and mercuric ions (Hg).

<sup>c</sup> The biotype for the wild-type isolate comprises the seven-digit API profile number according to the manufacturers (Analytab Products, Plainview, N.Y.). The final digit was derived from the sum of the values for dulcitol and raffinose fermentation, which were given as 1 and 2, respectively; a value of zero was allocated when neither was fermented. The precautions taken to ensure reproducibility have been described previously (20).

<sup>d</sup> Transfer frequency is expressed as the ratio of the number of transconjugants to the number of donors after a 4-h broth mating at 37°C.

<sup>e</sup> Plasmid sizes were determined by the summation of *EcoRI* restriction fragments larger than 0.85 kb.

<sup>f</sup> See text for explanation of sets.

TABLE 3. Properties of the porcine isolates and IncFIV R plasmids<sup>a</sup>

Date of isolation (mo/day/yr)	Wild-type <i>E. coli</i> isolates		Properties of plasmids				
	Resistance pattern	Biotype	Plasmid designation	Transfer frequency	Resistance pattern	Size (kb)	Set
2/2/80	Ap Cm Sm Su Tc Tp	50445522	pPTB34	10 <sup>-4</sup>	Cm Su Tc Tp Hg	148	D'
3/30/81	Ap Cm Sm Su Tc Tp	50445522	pPTB151	10 <sup>-4</sup>	Cm Su Tc Tp Hg	162	D'
5/4/81	Ap Cm Sm Su Tc Tp	50445522	pPTB197 <sup>b</sup>	10 <sup>-4</sup>	Cm Su Tc Tp Hg	147	D'
2/2/80	Cm Sm Su Tp	50445521	pPTB41	10 <sup>-3</sup>	Su Tp Hg	124	E
2/19/80	Cm Sm Su Tc Tp	10445521	pPTB44	10 <sup>-3</sup>	Su Tp Hg	124	E
3/3/80	Sm Su Tp	50445521	pPTB64	10 <sup>-3</sup>	Su Tp Hg	124	E
6/9/80	Sm Su Tp	51445531	pPTB92	10 <sup>-1</sup>	Su Tp Hg	124	E
2/23/81	Su Tp	50445520	pPTB128	10 <sup>-3</sup>	Su Tp Hg	124	E
3/17/80	Ap Cm Sm Su Tc Tp	71445520	pPTB1	10 <sup>-5</sup>	Su Tc Tp Hg	130	F
2/16/81	Ap Cm Sm Su Tc Tp	51445723	pPTB104	10 <sup>-3</sup>	Su Tc Tp Hg	130	F

<sup>a</sup> See footnotes a through f in Table 2 for explanation of abbreviations and experimental details.

<sup>b</sup> This transconjugant contained a 3-kb cryptic plasmid which was mobilized at a high frequency by the Tp<sup>r</sup> plasmid.

**Restriction enzyme analysis of the plasmid sets.** The *EcoRI* restriction fragment patterns of representatives of the seven plasmid sets are presented in Fig. 1. The sizes of restriction fragments greater than 0.85 kb are presented in Table 4 and are numbered on the basis of relative size. The fragment patterns were consistent with the sets established on the basis of resistance patterns and size. The set A plasmids differed only in the size of their second largest *EcoRI* fragments (3 and 4), while all the set B plasmids had identical restriction profiles (only pHTB20 shown). The set C plasmids pHTB21 and pHTB23 had identical restriction fragment profiles, even though pHTB21 exhibited lower levels of resistance to streptomycin and sulfafurazole. Differences found in the other set C plasmid, pHTB14, could be accounted for by the insertion of a 1.37-kb segment of DNA into fragment 34 of pHTB21 or pHTB23 to give one new fragment, 29. Plasmids in sets A, B, and C shared 13 *EcoRI* fragments among all representatives, with three other fragments (fragments 2, 4, and 34) being common to all but one of the representatives. Plasmids in these three sets were transferred from *E. coli* of four different biotypes.

The set D plasmids pHTB9 and pHTB31 had identical restriction profiles. Plasmid pHTB8 differed from pHTB9, even though it was recovered from the same isolate. It was missing fragments 11, 17, and 22a or b, while additional fragments 19 and 26 were present. Both plasmids pHTB8 and pHTB9, following retransfer to other recipients, retained their restriction fragment patterns. Set D plasmids differed extensively from those of sets A, B, and C, with only three *EcoRI* fragments being common among all representatives (fragments 12a or b, 22a or b, and 38). The set D' plasmids pPTB34, pPTB151, and pPTB197 all had similar restriction profiles, with 15 *EcoRI* fragments being common. Differences between these plasmids was confined mainly to the larger *EcoRI* fragments. These plasmids showed remarkable similarity to the set D plasmids from humans. In particular pPTB34 shared 19 of its 20 *EcoRI* fragments with pHTB9 and pHTB31. Overall sets D and D' had 14 common fragments, with another four fragments being common to all but one of the representatives (fragments 16, 22a or b, 24 a or b, and 43). All set E plasmids had identical restriction profiles (pPTB64; Fig. 1). The set F plasmids were also identical and differed from set E plasmids by the addition of only one fragment (fragment 30) which was presumably associated with the acquisition of tetracycline resistance by this set. In the collection of pig plasmids there was little similarity, with only two *EcoRI* fragments being common to all representatives (fragments 12a or b and 38). Fragments of

this size were found in all 21 Tp<sup>r</sup> plasmids that were analyzed. All seven E and F set plasmids were transferred from *E. coli* with different biotypes or antibiograms or both.

**Cloning of the Tp<sup>r</sup> gene.** The Tp<sup>r</sup> genes from representatives of each of the plasmid sets were cloned into the *EcoRI* site of pACYC184. The *EcoRI* fragment containing the Tp<sup>r</sup> gene from each of the sets is indicated in Table 4. In each

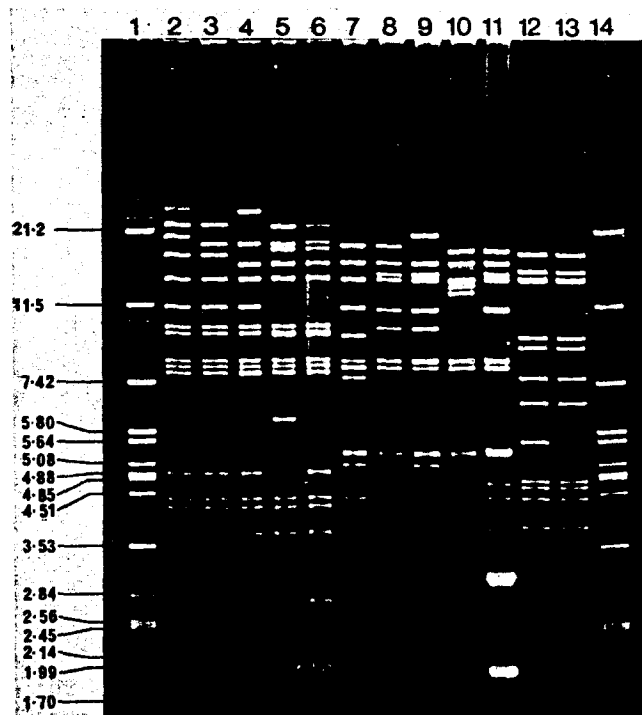


FIG. 1. AGE (1.0%) of *EcoRI* restriction digestion fragments of representatives of the human and pig trimethoprim R plasmids. The less intense bands of highest molecular weight in lanes 2, 4, 5, 10, and 11 are partial digestion fragments and were not included in Table 4. Lanes 1 and 14, a mixture of lambda DNA digested with *EcoRI* and lambda DNA digested with *PstI*; lanes 2 and 3, set A plasmids pHTB1 and pHTB3; lane 4, set B plasmid pHTB20; lanes 5 and 6, set C plasmids pHTB14 and pHTB21; lanes 7 and 8, set D plasmids pHTB8 and pHTB9; lanes 9, 10, and 11, set D' plasmids pPTB34, pPTB151, and pPTB197; lane 12, set F plasmid pPTB1; lane 13, set E plasmid pPTB64. Numbers on the left are sizes (in kilobases) of *EcoRI* and *PstI* fragments of lambda DNA.

TABLE 4. *EcoRI* restriction fragments of IncFIV trimethoprim R plasmids<sup>a</sup>

Fragment no. <sup>b</sup>	Sizes (kb) of <i>EcoRI</i> fragments in the following plasmid sets:											
	pHTB1, A	pHTB3, A	pHTB20, B <sup>c</sup>	pHTB14, C	pHTB21, C <sup>d</sup>	pHTB8, D	pHTB9, D <sup>e</sup>	pPTB34, D'	pPTB151, D'	pPTB197, D'	pPTB1, F <sup>f</sup>	PTB64, E <sup>g</sup>
1			24.0 <sup>h</sup>									
2	21.6 <sup>h</sup>	21.6 <sup>h</sup>		21.6 <sup>h</sup>	21.6 <sup>h</sup>							
3	19.9							19.9				
4		19.2	19.2	19.2	19.2	19.2	19.2					
5				18.4	18.4				18.4	18.4		
6											18.3	18.3
7	17.4	17.4										
8						16.7	16.7	16.7	16.7	16.7		
9			16.5	16.5	16.5							
10											15.8	15.8
11							15.3	15.3		15.3		
12a and b	14.6	14.6	14.6	14.6	14.6	14.6	14.6	14.6	<u>14.6</u>	14.6	<u>14.6</u>	<u>14.6</u>
13									14.1			
14									13.4			
15	11.9	11.9	11.9									
16						11.8	11.8	11.8		11.8		
17	10.5	10.5	10.5	10.5	10.5		10.5	10.5				
18a and b	9.87	9.87	9.87	<u>9.87</u>	<u>9.87</u>							
19						9.85						
20											9.79	9.79
21											8.92 <sup>h</sup>	8.92 <sup>h</sup>
22a and b	8.36	8.36	8.36	8.36	8.36	8.36	8.36	8.36	<u>8.36</u>	<u>8.36</u>		
23	8.16	8.16	8.16	8.16	8.16							
24a and b						<u>8.06</u>	<u>8.06</u>	8.06	<u>8.06</u>	<u>8.06</u>		
25a and b	7.80	7.80	<u>7.80</u>	7.80	7.80							
26						7.65						
27											7.58	7.58
28											6.66	6.66
29				6.21								
30											5.55	
31						5.30	5.30	5.30	5.30	5.30		
32						5.28 <sup>h</sup>	5.28 <sup>h</sup>	5.28 <sup>h</sup>	5.28 <sup>h</sup>	5.28 <sup>h</sup>		
33						5.03	5.03	5.03				
34	4.88	4.88	4.88		4.88						4.84	4.84
35												
36									4.71	4.71		
37											4.62	4.62
38	4.44	4.44	4.44	4.44	4.44	4.44	4.44	4.44	4.44	4.44	4.44	4.44
39	4.25	4.25	4.25	4.25	4.25							
40											3.90	3.90
41						3.88	3.88	3.88	3.88	3.88		
42	3.78	3.78	3.78	3.78	3.78							
43						3.46	3.46		3.46	3.46		
44											3.20	3.20
45						2.88	2.88	2.88	2.88	2.88		
46	2.83	2.83	2.83	2.83	2.83							
47											2.45	2.45
48											2.06	2.06
49	2.03	2.03	2.03	2.03	2.03							
50						1.85	1.85	1.85	1.85	1.85		
51						1.69	1.69	1.69	1.69	1.69		
52	1.65	1.65	1.65	1.65	1.65							
53											1.38	1.38
54						1.35	1.35	1.35	1.35	1.35		
55	1.32	1.32	1.32	1.32	1.32							
56											1.26	1.26
57						1.25	1.25	1.25	1.25	1.25		
58						1.00	1.00	1.00	1.00	1.00		

<sup>a</sup> Values are the average of at least three independent determinations. The sizes of fragments greater than 3 kb were determined by using 0.7% agarose with *EcoRI* fragments of lambda as molecular weight standards. Sizes of fragments less than 3 kb were determined by using 1.5% agarose with *PstI* fragments of lambda as molecular weight standards. Fragment sizes that are underlined indicate the presence of two fragments of the same size.

<sup>b</sup> The relative size of a fragment is indicated by its position on the table and the fragment number (largest to smallest).

<sup>c</sup> Set B plasmids pHTB19 and pHTB53 had identical *EcoRI* restriction profiles to that of pHTB20.

<sup>d</sup> Set C plasmid pHTB23 had an identical *EcoRI* restriction profile to that of pHTB21.

<sup>e</sup> Set D plasmid pHTB31 had an identical *EcoRI* restriction profile to that of pHTB9.

<sup>f</sup> Set F plasmid pPTB104 had an identical *EcoRI* restriction profile to that of pPTB1.

<sup>g</sup> Set E plasmids pPTB41, pPTB44, pPTB92, and pPT128 had identical *EcoRI* restriction profiles to that of pPTB64.

<sup>h</sup> The restriction fragment carries the gene for T<sub>p</sub><sup>r</sup>.

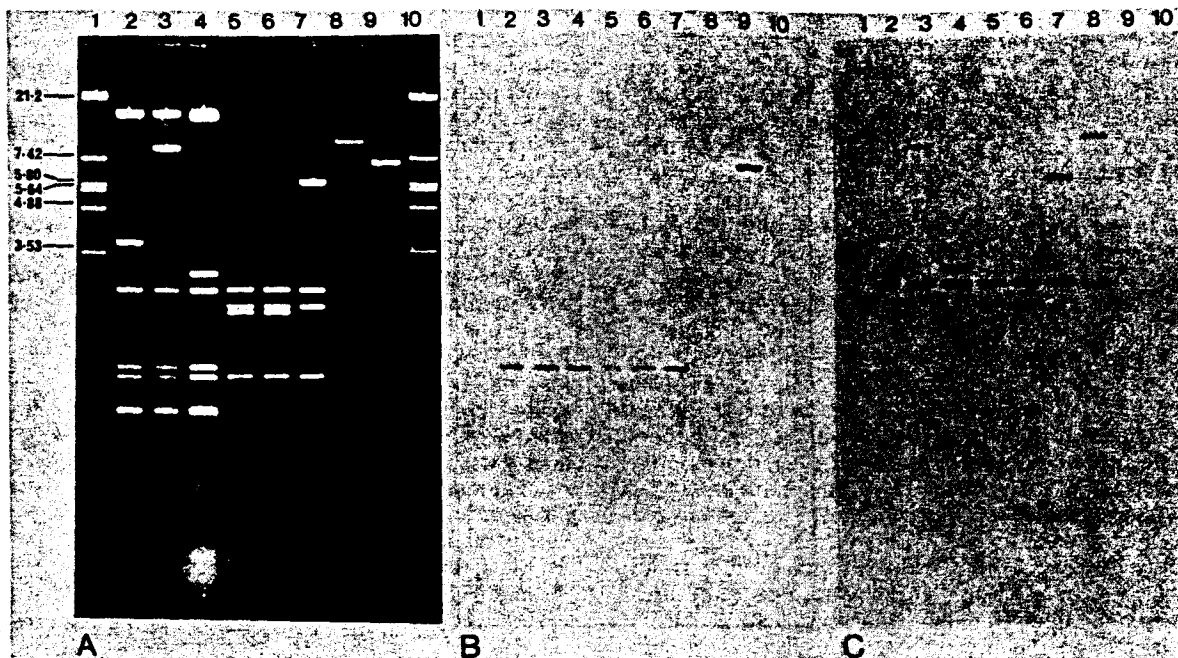


FIG. 2. Hybridization of biotin-labeled type 1 and type 2 probes to cloned Tp<sup>f</sup> genes from the various plasmid sets which were digested with *EcoRI* and *HindIII* (lanes 2 through 7). Hybridization was detected with a streptavidin and biotinylated acid phosphatase system. (A) A 0.7% agarose gel stained with ethidium bromide and photographed under UV light. The 2.6- and 1.4-kb fragments common in lanes 2 through 7 are *EcoRI-HindIII* fragments associated with the vector pACYC184. Cleavage of pFE420 with *EcoRI* and *BamHI* (lane 8) produces an 8.5-kb fragment of pSC101 and a 2.4-kb fragment of Tn7. The smaller fragment is not visible in panel A of this photograph. Numbers on the left are sizes (in kilobases) of *EcoRI* restriction fragments of lambda DNA. (B and C) Photographs of enzymatically stained membranes showing hybridization to the type 1 and type 2 probes, respectively. Lanes 1 and 10, lambda DNA digested with *EcoRI*; lanes 2, set A Tp<sup>f</sup> gene (from pHTB1); lanes 3, set B Tp<sup>f</sup> gene (from pHTB20); lanes 4, set C Tp<sup>f</sup> gene (from pHTB23); lanes 5, set D Tp<sup>f</sup> gene (from pHTB31); lanes 6, set D' Tp<sup>f</sup> gene (from pPTB151); lanes 7, set E (and set F) Tp<sup>f</sup> gene (from pPTB64); lanes 8, pFE420 (type 2 DHFR) digested with *EcoRI*; lanes 9, pFE506 (type 1 DHFR) digested with *EcoRI* and *BamHI*.

plasmid of the sets A, B, and C, the Tp<sup>f</sup> gene was located on the largest *EcoRI* fragment, which varied in size from 24.0 to 21.6 kb. The Tp<sup>f</sup> gene on the sets D and D' plasmids was located on the 5.28-kb fragment (fragment 32). Sets E and F had the Tp<sup>f</sup> gene contained within the 8.92-kb fragment (fragment 21).

**Comparison of Tp<sup>f</sup> clones by double digestion.** The Tp<sup>f</sup> hybrids were doubly digested with *EcoRI* and *HindIII*, and the fragments generated were analyzed by AGE (Fig. 2A). Set A, B, and C plasmids showed extensive similarities, as indicated by the number of common fragments, although each set did produce one unique fragment which, in each case, contained the DHFR gene. Set A contained a unique 3.87-kb *HindIII* fragment, set B contained an 8.72-kb *EcoRI-HindIII* fragment, and set C contained a 3.12-kb *HindIII* fragment. The largest *HindIII* fragment of set C also appeared to be slightly smaller than the corresponding fragments from sets A and B. Set D produced two *EcoRI-HindIII* fragments (excluding the vector) which were identical to those produced by set D', confirming the close relationship between these sets. Sets E and F were identical with each producing two *EcoRI-HindIII* fragments (excluding the vector), the smaller of which was identical in size to the largest fragment from sets D and D'. By examination of these clones with restriction enzymes *BamHI* and *PstI* (data not shown), it was shown that the regions flanking the Tp<sup>f</sup> genes in the set D, D', E, and F plasmids were identical and that these regions were different from those regions in sets A, B, and C.

**Hybridization of Tp<sup>f</sup> clones with the type 1 and type 2 DHFR probes.** Biotin-labeled probes containing the type 1 and type 2 DHFR genes were hybridized to Southern blots of the double-digested fragments. The results of hybridization with the type 1 probe are presented in Fig. 2B. Hybridization occurred with the control (Fig. 2B, lane 9) and with the smaller of the two vector fragments in lanes 2 through 7 (Fig. 2B) which were produced as a result of *EcoRI-HindIII* cleavage of pACYC184. This smaller fragment contained the ColE1-related origin of replication of pACYC184. No hybridization occurred with any of the fragments associated with the inserted DNA. The results of hybridization with the type 2 probe are presented in Fig. 2C. Hybridization was observed with the 8.5-kb pSC101 fragment and the 2.4-kb Tn7 fragment (not visible in Fig. 2A) of the control (Fig. 2C, lane 8). Hybridization with the larger of the two vector fragments in lanes 2 through 7 (Fig. 2C) is associated with the tetracycline resistance gene common to pACYC184 and pSC101. No hybridization occurred with the smaller vector fragment or with pFE506 (Fig. 2C, lane 9), as the pSC101 replicon is distinct from the ColE1 replicon. In addition to the vector, the type 2 probe hybridized to a single *HindIII* or *EcoRI-HindIII* fragment containing the Tp<sup>f</sup> gene in each of the lanes 2 through 7 (Fig. 2C). Thus, all the IncFIV plasmids contained the type 2 DHFR gene which was associated with unique *HindIII* or *EcoRI-HindIII* fragments of sets A, B, and C; a common 2.37-kb *EcoRI-HindIII* fragment in sets D and D'; and a common 5.90-kb *EcoRI-HindIII* fragment in sets E and F.

## DISCUSSION

In our previous study (20) we established that IncFIV and IncN trimethoprim R plasmids are present in similar proportions in isolates from humans and pigs. However, the limited physical analysis of the plasmids reported in that study precluded the formation of any firm conclusions concerning the relationships between them. The aim of this study was to characterize the IncFIV trimethoprim R plasmids to gain insight into their evolution and spread within and between isolates from humans and pigs.

Trimethoprim was introduced into clinical use in Australia in 1973, and Tp<sup>r</sup> isolates began to appear at low frequencies approximately 6 years later. The relative simplicity of the resistance patterns of the plasmids conferring Tp<sup>r</sup> and their incompatibility reaction with R124, the IncFIV reference plasmid (11), was fortunate. This provided a unique opportunity to study the development and spread of trimethoprim R plasmids from an early stage in their evolution in Perth.

Studies designed to follow the evolution of R plasmids and to assess their spread, such as that described by Jørgensen (13), are few. The major difficulty in this type of study is the generally high level of plasmid-borne resistance to the antibiotic under study that is already circulating. Such problems were not encountered in our study. As a result we were able to follow the evolution of trimethoprim R plasmids within the two ecosystems and provide conclusive evidence of the interchange of R plasmid-bearing *E. coli* between humans and animals.

The plasmids reported here represent the largest single collection of IncFIV plasmids analyzed by restriction enzymes and hybridization, with there having been only four other reports presented previously (4, 8, 12, 17). The results indicate that considerable plasmid evolution has occurred over a relatively short time span, especially when compared with similar analyses of N- (15), FII- (14), and K- (22) group plasmids in which fewer evolutionary changes were observed, despite greater geographic and temporal spread. However the recovery of all 21 trimethoprim R plasmids from *E. coli* and their association with the rare IncFIV group and with the less commonly encountered type 2 DHFR gene (1, 19) suggest a common ancestry because it is very unlikely that such properties could have arisen independently within two ecosystems.

Detailed analysis indicates that three lines of IncFIV plasmids have evolved up to the time of the survey. Sets A, B, and C represent one evolutionary line of plasmids exclusive to human isolates. Most representatives of these sets had identical restriction fragment profiles among the smaller fragments. Differences were confined mainly to the five largest *EcoRI* fragments of each set and could be explained by relatively minor rearrangements of the DNA sequence. Cloning of the Tp<sup>r</sup> gene demonstrated that it was located on the largest *EcoRI* fragment, and while the size of the subfragment containing the Tp<sup>r</sup> gene varied for each set, the sequences flanking the gene were essentially identical. Another line of plasmid evolution, represented by sets E and F, was exclusive to the pigs. These sets were virtually identical, and restriction profiles remained unchanged throughout the survey.

Members of these two lines probably represent distantly related plasmids which evolved from an IncFIV progenitor carrying the type 2 DHFR gene present either within the hospital or piggery, but not both. The progenitor IncFIV plasmid probably spread between different *E. coli* biotypes and from one ecosystem to the other. The acquisition of

additional resistance markers may then have occurred independently. The evolution of these two lines of plasmids proceeded to the extent that they now share only two *EcoRI* restriction fragments. Furthermore, the Tp<sup>r</sup> gene common to all was flanked by DNA regions which also underwent considerable evolution. Because the two lines have evolved to such an extent, the data do not provide conclusive evidence as to the direction in which plasmid exchange has occurred.

Sets D and D' represent a third line of plasmid evolution and were transferred from isolates recovered from both humans and pigs. These plasmids were similar in many respects and were all obtained from strains of *E. coli* with identical biotypes. The restriction profiles of some of the plasmids were almost identical, even though they were isolated from different ecosystems. While there were variations between the plasmids of sets D and D', these differences were no greater than those between members of the line of human plasmids of sets A, B, and C. The relative ease with which variations in restriction profiles can occur was demonstrated by the fact that quite different plasmids, pHTB8, and pHTB9, were transferred from the same isolate. Plasmid pHTB8 probably represents a deletion derivative of pHTB9 which was selected perchance from that cross. The plasmids in this evolutionary line contained Tp<sup>r</sup> genes which were part of an *EcoRI* restriction fragment which appeared to be identical, irrespective of whether they were from human or porcine strains. The remarkable similarity between the plasmids in this line provides clear evidence of recent interchange of R plasmid-bearing *E. coli* between humans and pigs but conflicting evidence for the direction of plasmid transfer. First, cloning of the Tp<sup>r</sup> genes from these plasmids revealed that the sequences flanking the DHFR genes were homologous with flanking sequences in the porcine plasmid line. Second, they resembled the porcine plasmids in that they possessed a gene for mercury resistance, a marker not present on any of the human plasmid lines. This indicates that they evolved from the porcine plasmid line and that a clone carrying this plasmid recently entered the hospital environment. However, plasmids of sets D and D' shared more *EcoRI* restriction fragments with the human plasmids than they did with porcine plasmids. The possession of characteristics of both the human and porcine plasmids cannot be explained easily by a single plasmid exchange event in either direction. On the basis of the shared *EcoRI* restriction fragments, the D and D' plasmids are more likely to be of human origin. If this is true, then at some stage a human IncFIV plasmid must have transferred to the porcine ecosystem and there acquired resistance to mercury and the porcine-type Tp<sup>r</sup> gene. This plasmid spread throughout the porcine ecosystem and transferred back into the human ecosystem.

It can be deduced from results obtained with the first two plasmid lines that there was exchange of *E. coli* between the two ecosystems and conjugal transfer of the R plasmids within each. Plasmids of the third line probably arose as a result of bidirectional exchange of an R plasmid-bearing *E. coli* between the two ecosystems. It is reasonable to assume that these plasmids are widespread throughout the community because it is unlikely that, from the large range of environments which could have been included in the initial investigation, we selected the only two with nearly identical plasmids. With this in mind, the problems associated with deciding from which environment the resistance arose become less important, considering the ease and frequency with which the R plasmids are exchanged. Policies on

antibiotic use therefore should consider not only the proposed use in either clinical or veterinary practice but also the capacity of the agent to select for R plasmid-mediated resistance.

## LITERATURE CITED

1. Acar, J. F., and F. W. Goldstein. 1983. Resistance. Genetics and medical implications, p. 243-258. In G. W. Hitchings (ed.), Handbook of experimental pharmacology, vol. 64 Springer-Verlag, Berlin.
2. Birnboim, H. C., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* 7:1513-1523.
3. Chang, A. C. Y., and S. N. Cohen. 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from P15A cryptic miniplasmid. *J. Bacteriol.* 134:1141-1156.
4. Chun, D., D. T. Cho, S. Y. Seol, M. H. Suh, and Y. C. Lee. 1984. R plasmids conferring multiple drug resistance from shigella isolated in Korea. *J. Hyg. Camb.* 92:153-160.
5. Daniels, D., J. Schroeder, W. Szybalski, F. Sanger, A. Coulson, G. Hong, D. Hill, G. Petersen, and F. Blattner. 1983. Complete annotated Lambda sequence, p. 519-676. In R. W. Hendrix, J. W. Roberts, F. W. Stahl, and R. A. Weisberg (ed.), Lambda II. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
6. Datta, N. 1979. Plasmid classifications: incompatibility grouping, p. 3-12. In K. N. Timmis and A. Puhler (ed.), Plasmids of medical, environmental and commercial importance, Elsevier/North-Holland Publishing Co., Amsterdam.
7. Davey, R. B., P. I. Bird, S. M. Nikolett, J. Praszki, and J. Pittard. 1984. The use of mini-gal plasmids for the rapid incompatibility grouping of conjugative R plasmids. *Plasmid* 11:234-242.
8. De La Cruz, F., J. C. Zabala, and J. M. Ortiz. 1979. Incompatibility among  $\alpha$ -hemolytic plasmids studied after inactivation of the  $\alpha$ -hemolysin gene by transposition of Tn802. *Plasmid* 2:507-519.
9. Fling, M. E., and L. P. Elwell. 1980. Protein expression in *Escherichia coli* minicells containing recombinant plasmids specifying trimethoprim-resistant dihydrofolate reductases. *J. Bacteriol.* 141:779-785.
10. Grant, A. J., P. I. Bird, and J. Pittard. 1980. Naturally occurring plasmids exhibiting incompatibility with members of incompatibility groups I and P. *J. Bacteriol.* 144:758-765.
11. Hedges, R. W., and N. Datta. 1972. R124, an  $fi^+$  R factor of a new compatibility class. *J. Gen. Microbiol.* 71:403-405.
12. Hedges, R. W., and K. P. Shannon. 1984. Resistance to apramycin in *Escherichia coli* isolated from animals: detection of a novel aminoglycoside-modifying enzyme. *J. Gen. Microbiol.* 130:473-482.
13. Jørgensen, S. T. 1983. Relatedness of chloramphenicol resistance plasmids in epidemiologically unrelated strains of pathogenic *Escherichia coli* from man and animals. *J. Med. Microbiol.* 16:165-173.
14. Jørgensen, S. T., J. Grinsted, P. Bennett, and M. H. Richmond. 1980. Persistence and spread of a chloramphenicol resistance-mediating plasmid in antigenic types of *Escherichia coli* pathogenic for piglets. *Plasmid* 4:123-129.
15. Konarska-Kozłowska, M., and V. N. Iyer. 1983. Sequence homology between the IncN group plasmids. *Plasmid* 10:211-223.
16. Kushner, S. R. 1978. An improved method for transformation of *Escherichia coli* with ColE1 derived plasmids, p. 17-23. In H. W. Boyer and S. Nicosia (ed.), Genetic Engineering, Elsevier/North-Holland Publishing Co., Amsterdam.
17. Lebek, G., and L. Petri. 1980. Incompatibility testing of  $fi^+$  R-factors from the area of Berne, p. 209-211. In S. Mitsuhashi, L. Rosival, and V. Krcmery (ed.), Antibiotic resistance, transposition and other mechanisms. Springer Verlag, Berlin.
18. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. Mayer, K. H., M. E. Fling, J. D. Hopkins, and T. F. O'Brien. 1985. Trimethoprim resistance in multiple genera of Enterobacteriaceae at a U.S. hospital: spread of the type II dihydrofolate reductase gene by a single plasmid. *J. Infect. Dis.* 151:783-789.
20. Mee, B. J., and S. M. Nikolett. 1983. Plasmids encoding trimethoprim resistance in bacterial isolates from man and pigs. *J. Appl. Bacteriol.* 54:225-235.
21. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503-517.
22. Tschäpe, H., and E. Tietze. 1980. Genetic and molecular characterization of R plasmids incompatible with R387 (IncK). *J. Gen. Microbiol.* 118:515-521.
23. Wise, P. J., K. J. Towner, C. A. Webster, R. C. B. Slack, and T. O. Jones. 1985. Trimethoprim resistance plasmids in *Escherichia coli* isolated from cases of diarrhoea in cattle, pigs and sheep. *J. Appl. Bacteriol.* 58:555-561.