Distribution of Genes for Trimethoprim and Gentamicin Resistance in Bacteria and their Plasmids in a General Hospital

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The incidence of trimethoprim resistance in enterobacteria causing infection in a London hospital increased from 5.6 % in 1970 to 16 % in 1979. The proportion of gentamicin-resistant aerobic Gram-negative bacilli had risen to 6.5 % by 1979. During a 5-month period in 1977, during which no epidemic was recognized, all isolates resistant to either trimethoprim, gentamicin, tobramycin or amikacin were studied. The proportion of enterobacteria resistant to both trimethoprim and gentamicin (3.8 % of the total) was significantly higher than expected assuming no correlation between acquisition of resistance characters. The resistance was transferable in 23 % of trimethoprim-resistant and 76 % of gentamicin-resistant strains. Trimethoprim resistance was carried by plasmids of seven different incompatibility groups and in at least four instances was part of a transposon. Gentamicin resistance was determined by plasmids of three groups – IncC, IncFII and IncW. Transposition of gentamicin resistance was not shown, though this may have been the means of evolution of the gentamicin R plasmids of IncW, which determined aminoglycoside acetyltransferase, AAC(3). Some bacterial strains with their plasmids were endemic. There was evidence for these plasmids (i) acquiring new resistance genes by transposition, (ii) losing resistance genes by deletion and (iii) being transferred between bacterial species in the hospital.

INTRODUCTION

The use of new antimicrobial drugs, to which resistance is initially unusual, provides an opportunity to study the evolution of bacterial resistance. Gentamicin and trimethoprim were introduced in 1963 and 1968, respectively, and have different indications for the treatment of infections. Resistant derivatives of bacterial species previously sensitive to these drugs soon appeared, and the resistance was sometimes plasmid-determined.

In 1972 and 1973 we recognized a strain of *Klebsiella aerogenes* capsular type K9 which was endemic in Hammersmith Hospital and carried a recognizable trimethoprim-sulphonamide R plasmid (Jobanputra & Datta, 1974). By 1976, reported outbreaks of hospital infection with gentamicin-resistant bacteria were not uncommon (Richmond *et al.*, 1975;

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Shafi & Datta, 1975; Shanson *et al.*, 1976; Schaberg *et al.*, 1976), and in March of that year two patients in Hammersmith Hospital (with leukaemia and sickle-cell anaemia, respectively) acquired serious infection with a gentamicin-resistant *Klebsiella* type K10 and were treated with amikacin, then only available by special arrangement with the manufacturers (Bristol-Myers).

In 1977, we began a survey of all aerobic Gram-negative bacilli which infected in-patients in Hammersmith Hospital and which were resistant to either gentamicin, tobramycin, amikacin or trimethoprim. We have monitored changes in the incidence of these resistances, the proportion that was transferable, and attempted to trace the spread of resistant bacteria between patients, plasmids between bacteria, and transposition of resistance genes between plasmids. This paper describes the findings.

METHODS

Bacterial strains and plasmids. The bacteria described here were isolated from clinical specimens, from in-patients, sent to the diagnostic laboratory in the Department of Bacteriology, Royal Postgraduate Medical School between January and June 1977. Sensitivity to antibiotics was tested initially by disc diffusion tests using Stokes's (1975) method. All strains resistant to gentamicin, tobramycin, amikacin or trimethoprim were collected and studied further. They were divided into those causing infections (criteria for infection stated in Table 1) and those considered as commensals, colonizing rather than infecting patients. In our results, only infecting strains are described, one example for each infection. Two multiply-resistant *Klebsiella* strains, isolated in 1976 (see Introduction), are included.

Isolates were identified by the methods of Cowan & Steel (1974); in some cases the API 20E test system was used (Appareils et Procédés d'Information, 38390, Montalieu Vercieu, France). *Klebsiella* species were capsular (K) typed by the method previously described (Casewell, 1972, 1975). This paper describes results with genera included in *Enterobacteriaceae* and 'non-fermenting' Gram-negative bacilli such as *Pseudomonas*. Only clinically relevant acquired resistance was included, e.g. *Pseudomonas* strains were scored for gentamicin resistance but not for trimethoprim resistance.

Laboratory strains used in plasmid studies were *Escherichia coli* K12; J53 *lac⁺ pro met*; J53-1 and J53-2 which are mutants of J53 resistant to nalidixic acid (*nal*) and rifampicin (*rif*, or *rpo*), respectively; J62 *lac pro his trp*; J62-1 and J62-2, *nal* and *rif* mutants of J62 (Bachmann, 1972); PB1150 *his gal recA* (P. L. Bergquist, University of Auckland, New Zealand); and *Pseudomonas aeruginosa* PU21 *ilv leu str rif* (Jacoby, 1974).

Standard plasmids mentioned in the text and used in incompatibility tests are listed by Jacob *et al.* (1977).

Incidence of resistance. Information on number of isolations, numbers of infections and of infected patients and proportions of different bacterial species, from each type of specimen, resistant to each drug, has been stored since 1975 in an ICL 4100 computer. The information on incidence of resistance (Table 2) is taken from print-outs displaying this information, available for 2-month periods. The table shows numbers of infections (numbers of isolations were considerably higher).

Media and antibacterial agents. Nutrient broth was Oxoid no. 2 (CM67). Nutrient agar was Oxoid blood agar base (CM55). All disc sensitivity testing and measurements of inhibitory concentrations were on Oxoid Isosensitest agar (CM471) with 4% (v/v) lysed horse blood. Oxoid Multodisks or individual discs were used. MacConkey agar was Oxoid (CM7b). Minimal salts agar was from the formula given by Clowes & Hayes (1968). Concentrations of antibiotics for selection of resistant strains were as described previously (Coetzee *et al.*, 1972; Jobanputra & Datta, 1974) and gentamicin or tobramycin were used at 4 µg ml⁻¹ and/or 2 µg ml⁻¹ in the medium. [The latter concentration was barely sufficient to prevent growth from the heavy inocula used in mating experiments (see below), but the higher concentration did not always permit the development of transconjugant clones.] All antibacterial drugs used were from commercial sources except for the following gifts: trimethoprim lactate from Wellcome Research Laboratories, nalidixic acid from Winthrop Laboratories, rifampicin from Ciba Laboratories.

Minimal inhibitory concentration (m.i.c.). Overnight broth cultures, diluted 10⁻² and 10⁻⁴, were plated with a multiple-inoculator on plates incorporating serial twofold dilutions of drugs. The m.i.c. was recorded as the lowest concentration that prevented visible growth from the smaller inoculum (that yielded about 20 colonies after overnight incubation at 37 °C on drug-free medium). The minimal concentration preventing visible growth from the heavier inoculum was usually only twofold higher.

Transfer of R plasmids to *E. coli* K12. The methods were as described by Coetzee *et al.* (1972) and Jobanputra & Datta (1974). When no transconjugants were obtained from broth mating, a surface method was tried as described by Bradley & Chaudhuri (1979). The mixed culture was spread on nutrient agar plates and

Table 1. *Trimethoprim- and gentamicin-resistant Gram-negative infections: sources of organisms*

The following criteria for infection were used. Blood culture: the same organism from all three bottles on one occasion, or from at least one bottle on two or more separate occasions. Wounds: clinical evidence of infection and a moderate growth of one organism. Peritoneal dialysis fluid: at least 10^4 organisms ml^{-1} in fluid. Urine: pus cells plus 10^5 organisms ml^{-1} in urine or 10^5 ml^{-1} of the same organism from more than one specimen. Sputum: presence of pus cells plus at least 10^5 organisms ml^{-1} of pancreatin-digested sputum in patients who had not received antibiotic therapy (the stringency of this criterion led to the exclusion of all sputum isolates from the survey).

Source	No.
Blood cultures	4
Wounds and infected tissues	22
Peritoneal dialysis fluids	13
Urines	48
Total	87

incubated for 2 h, then resuspended and spread on selection plates as described above. With *P. aeruginosa* strains the methods employed were similar, with strain PU21 used in parallel with *E. coli* K12 as a recipient. Where no R transfer to *E. coli* K12 was demonstrated, a known mobilizing plasmid was introduced into the putative donor and the selection was repeated by the same methods.

Transfer of plasmids between lines of E. coli K12. Differences in nutritional requirements were used to separate the recipient strain, with newly acquired antibiotic resistance, from the donor.

Classification of plasmids by incompatibility. R plasmids, after transfer to *E. coli* K12, were tested for compatibility with standard plasmids of different incompatibility groups. All those used are listed by Jacob *et al.* (1977). The method was to introduce a standard plasmid, chosen because it had resistance genes distinguishable from those of the test plasmid, into the strain carrying the plasmid under test, selecting only for a resistance marker of the incoming plasmid. Incompatibility was recognized by elimination of the resident plasmid from all clones tested, or, if 'doubles' carrying both plasmids were obtained, by the instability of the plasmid combination when the clone was grown in drug-free broth. Compatibility was scored when the two plasmids co-existed stably during growth in drug-free broth, their separate identities being shown by separate transmissibility to another K12 strain, or sometimes by their separate identification in agarose gels after electrophoresis.

Assay of gentamicin-modifying enzymes. For assay of gentamicin-modifying enzymes we used the cellulose phosphate paper binding system (Benveniste & Davies, 1973). Suspensions of cells grown at 37°C in nutrient broth were concentrated 10- to 20-fold in 100 mM-Tris/HCl buffer pH 7.6 containing 16 mM-MgCl₂ to approximately 10^9 organisms ml^{-1} and broken ultrasonically (MSE disintegrator). Each tube contained (in a total volume of 55 μl) 200 ng aminoglycoside, 3.5 μmol Tris/HCl buffer pH 7.6, 560 nmol MgCl₂ and 35 μl cell sonicate. For acetylation 2 nmol [1-¹⁴C]acetyl coenzyme A (specific activity 5 Ci mol^{-1} , 185 GBq mol^{-1}) was added and for adenylation 4 nmol [8-¹⁴C]adenosine 5'-triphosphate (specific activity 5 Ci mol^{-1} , 185 GBq mol^{-1}) was added. After incubation at 37°C for 30 min, 50 μl amounts of the mixture were pipetted on to 20×20 mm squares of cellulose phosphate paper. The papers were washed thoroughly in water, dried and the radioactivity bound to the paper was measured. A toluene-based scintillation fluid was used.

Identification of plasmids by agarose gel electrophoresis.

(i) *Single colony lysate gels.* Single colonies were lysed directly on 0.7% (w/v) agarose slab gels ($15 \times 10 \times 0.5$ cm) using the method of Eckhardt (1978). The buffer was 90 mM-Tris/boric acid/EDTA pH 8.3 (Peacock & Dingman, 1967). The gels were run at 170 V, constant voltage, for 4.5 h at room temperature. They were calibrated using standard plasmids of known molecular weights.

(ii) *Restriction enzyme analysis.* Plasmid DNA was isolated using the method of Humphreys *et al.* (1975) and dialysed for 60 h against TNE buffer (50 mM-Tris/50 mM-NaCl/1 mM-EDTA). The plasmid DNAs were digested with the enzyme *Hind*III obtained from Miles Biochemicals; the digestion buffer was that recommended by Miles. Incubations were at 37°C in a total volume of 50 μl . The enzyme reaction was terminated by heating to 60°C for 10 min. Finally 10 μl 1% (w/v) agarose was added, with bromophenol blue tracking dye, to each of the incubation mixtures, which were applied to a horizontal slab gel ($13.3 \times 13.3 \times 0.5$ cm). The gels were run at 130 V, constant voltage, for 4.5 h. They were calibrated with the fragments obtained from *Hind*III digestion of λ DNA (N. Murray, personal communication).

Table 2. *Gram-negative infections in patients in Hammersmith Hospital, 1970-1979: resistance to trimethoprim and/or gentamicin*

The criteria for counting cultures as resistant were unchanged during this time. Before 1977, sensitivity tests were made on Oxoid DST (direct sensitivity test) agar, thereafter on Oxoid Isosensitest agar, both media with added 4% (v/v) lysed horse blood. Comparative tests at the time of change showed that results were the same on the two media.

Year	Enterobacteria			<i>P. aeruginosa</i>	
	Total	Tp ^r (%)	Gm ^r (%)	Total	Gm ^r (%)
1970*	429	24 (5.6)	0 (0)	95	0 (0)
1975†	638	70 (11)	15 (2.4)	187	18 (9.6)
1976	1705	226 (13)	38 (2.2)	424	43 (10)
1977	1618	215 (13)	63 (3.9)	404	45 (11)
1978	1758	272 (16)	88 (5.0)	451	56 (12)
1979‡	598	92 (15)	35 (5.9)	138	10 (7.2)

* Results from Anderson *et al.* (1972).

† Last 6 months.

‡ First 4 months.

Tests for transposition of resistance genes.

(i) *Genetic analysis.* The presence of a transposon carrying resistance genes on a plasmid can often be identified by the introduction of a second, incompatible, plasmid into the R⁺ strain. The resident plasmid is lost but the transposon may be transposed to the incoming plasmid or retained in the chromosome. Where such evidence of transposition was seen, the test was repeated in a *recA* strain.

(ii) *Restriction enzyme analysis.* When a plasmid carrying Tn7 is digested with *Hind*III there are two characteristic internal fragments of Tn7 which can be recognized by their migration in gel electrophoresis (Richards & Nugent, 1979). The trimethoprim R plasmids from this survey were analysed by restriction enzyme digestion with *Hind*III and compared with known Tn7-carrying plasmids.

RESULTS

Incidence and distribution of resistance

The frequency of resistance to trimethoprim rose rapidly and to gentamicin more slowly between 1970 and 1979 in Hammersmith Hospital (Table 2). Table 3 shows the distribution of species amongst the 87 resistant bacteria collected during the survey that covered part of 1977, each considered to have caused an infection. The relatively high proportion of strains resistant to both drugs represents the presence in the hospital of multiply-resistant strains, many of them shown (see below) to carry R plasmids. The origins of the strains are shown in Table 1.

R transfer from resistant strains

Of the strains collected during the 1977 survey, those from 34 infections demonstrated transfer of trimethoprim and/or gentamicin resistance to recipient bacteria in conjugation experiments. From them we identified 31 conjugative plasmids and 5 non-conjugative plasmids (Table 4). The two multiply-resistant *Klebsiella* strains isolated in 1976 both transferred gentamicin resistance. Only one strain was resistant to tobramycin and not gentamicin; transfer of this resistance could not be demonstrated. None of the isolates of *P. aeruginosa*, *Acinetobacter* or *Alcaligenes* spp. transferred resistance to *E. coli* K12 or *P. aeruginosa* PU21. Among the enterobacteria from which no transfer of trimethoprim or gentamicin resistance occurred there were 21 resistant to trimethoprim with lower levels of resistance than have been reported as plasmid-determined. Minimal inhibitory concentrations of trimethoprim for these 21 strains were between 10 and 1000 µg ml⁻¹, whereas all those that transferred trimethoprim resistance grew well with 1000 µg ml⁻¹; the mechanism of low-level resistance to trimethoprim in these strains is unknown. There were two strains that showed a generalized and low-level resistance to aminoglycosides, including amikacin,

Table 3. *Gram-negative infections in Hammersmith Hospital, 1 January–1 June 1977: incidence of trimethoprim resistance and gentamicin resistance*

Genus	Total	Tp ^s Gm ^s (%)	Tp ^r Gm ^s (%)	Tp ^s Gm ^r (%)	Tp ^r Gm ^r (%)
<i>Escherichia</i>	343	310 (90)	23 (6.7)	3 (0.9)	7 (2.0)
<i>Klebsiella</i>	143	120 (84)	14 (10)	2 (1.4)	7 (4.9)
<i>Enterobacter</i>	14	6 (43)	5 (36)	1 (7.1)	2 (14)
<i>Citrobacter</i>	8	2 (25)	3 (38)	0	3 (38)
<i>Proteus/Providencia</i>	142	133 (94)	3 (2.1)	3 (2.1)	3 (2.1)
Others*	9	6 (67)	0	0	3 (33)
All enterobacteria	659	577 (88)	48 (7.3)	9 (1.4)	25 (3.8)†
<i>Pseudomonas</i>	169			5 (3.0)	

* *Acinetobacter* 2, *Alcaligenes* 1, unclassified 6 (no *Serratia*).

† The proportion of enterobacteria resistant to both trimethoprim and gentamicin is significantly higher ($P \leq 0.001$) than would be the case assuming no correlation between the acquisition of the two resistances (see text).

that could not be transferred to other bacterial strains; presumably this resistance is chromosomal (Mawer & Greenwood, 1977; Seal & Strangeways, 1977): these were the only amikacin-resistant isolates. Four strains died or were lost before being tested for transfer: this leaves 17 whose resistance patterns suggested the presence of R plasmids but where the results of transfer experiments were negative. Some of these transferred R plasmids carrying resistance to drugs other than trimethoprim or gentamicin.

Plasmids from the resistant strains

The strains from which R plasmids were derived and the characters of the plasmids themselves are shown in Table 4. Nearly all the donor strains were resistant to many antibacterial agents, usually including both trimethoprim and gentamicin. From many of these strains, resistance to both drugs was transferred to *E. coli* K12. The very high frequency of resistance to HgCl₂ seen here is similar to that described elsewhere in hospital bacteria (Nakahara *et al.*, 1977).

Trimethoprim R plasmids. Included in Table 4 is one example of *Klebsiella* serotype K9, with its trimethoprim-sulphonamide R plasmid, pHH720, that was identified as endemic in the hospital in 1972–73. That plasmid belonged to incompatibility group W (IncW) and was the only kind of trimethoprim R plasmid found in bacteria isolated from in-patients in Hammersmith Hospital (Jobanputra & Datta, 1974). It was indistinguishable from plasmid R388 (Datta & Hedges, 1972; Jacob *et al.*, 1977). In 1977 (Table 4), trimethoprim R plasmids in the hospital were more frequently members of group IncW than of any other incompatibility group, but there were important differences from the earlier findings. The 1977 IncW plasmids were not found in a *Klebsiella* host. They determined resistance to ampicillin, HgCl₂ and gentamicin as well as to sulphonamides and trimethoprim, and were often non-conjugative. The non-conjugative IncW plasmids (Table 4) were mobilized to *E. coli* K12 either by separate plasmids present in the wild strains or by the introduction of known plasmids. The molecular mass of pHH720 and of R388 is 21 megadaltons (Md) similar to that of other IncW plasmids (Jacob *et al.*, 1977). The IncW plasmids described here have molecular masses of 27 Md.

In 1977, trimethoprim R plasmids belonging to seven different incompatibility groups were found (Table 4). In some cases Tn7-like transposons were identified (see below). All but two of the trimethoprim R plasmids also determined sulphonamide resistance.

Gentamicin R plasmids. The plasmids identified as carrying gentamicin resistance all belonged to one of three incompatibility groups – IncFII, IncC and IncW. The IncW

Table 4. *Gentamicin and/or trimethoprim R plasmids and their naturally occurring host bacteria*

Strain of origin			Plasmid			
No.	Species	Resistances	No.	Resistances	Tra	Inc
D1463*	<i>Klebsiella</i> K10	<i>Tp</i> GmTmKmSmSuCmApHg	pHH1140	GmTmKmSuCmHg	+	FII
D1414*	<i>Klebsiella</i> K10	<i>Tp</i> GmTmKmSmSuCmApHg	pHH1141	GmTmKmSuCmHg	+	FII
D1647	<i>Klebsiella</i> K10	<i>Tp</i> GmTmKmSmSuTcCmApHg	pHH1325	GmTmSuCmHg	+	FII
D1648	<i>Klebsiella</i> K10	<i>Tp</i> GmTmKmSmSuTcCmApHg	pHH1326	GmTmSuCmApHg	+	FII
D1649	<i>Klebsiella</i> K10	<i>Tp</i> GmTmKmSmSuTcCmApHg	pHH1327	GmTmSuCmHg	+	FII
D1651	<i>Klebsiella</i> K10	<i>Tp</i> GmTmKmSmSuTcCmApHg	pHH1329	GmTmSuCmHg	+	FII
D1623	<i>Klebsiella</i> K10	<i>Tp</i> GmTmKmSmSuTcCmApHg	pHH1301	GmTmKmSuCmHg	+	FII
D1618	<i>Klebsiella</i> K10	<i>Tp</i> GmTmKmSmSuTcCmApHg	pHH1296a	GmTmKmSuTcCmAp	+	C
			pHH1296b	GmTmSmSuCmApHg	+	FII
D1652	<i>Klebsiella</i> NT	GmTmKmSuCmApHg	pHH1330	GmTmSuCmHg	+	FII
D1654	<i>Klebsiella</i> NC	<i>Tp</i> GmTmKmSmSuTcCmApHg	pHH1332	GmTmSuCmHg	+	FII
D1650	<i>Klebsiella</i> K21	<i>Tp</i> GmTmKmSmSuTcCmApHg	pHH1328	GmTmSuCmApHg	+	FII
D1653	<i>Klebsiella</i> K21	<i>Tp</i> GmTmKmSmSuTcCmApHg	pHH1331	GmTmKmSuApHg	+	FII
D1617	<i>E. coli</i>	<i>Tp</i> GmTmKmSmSuTcCmApHg	pHH1295	GmTmKmSuCmApHg	+	FII
D1510	<i>Enterobacter</i>	<i>Tp</i> GmTmKmSmSuCmApHg	pHH1186	GmTmKmSuCmHg	+	FII
D1626	<i>Enterobacter</i>	<i>Tp</i> GmTmKmSmSuTcCmApHg	pHH1304	GmTmKmSuCmApHg	+	FII
D1509	<i>Citrobacter</i>	<i>Tp</i> GmTmKmSmSuTcCmApHg	pHH1185a	GmTmKmSuTcCm	+	C
			pHH1185b	GmTmCmHg	+	FII
D1593	<i>E. coli</i>	<i>Tp</i> SmSuTcCmApHg	pHH1169	<i>Tp</i> SmSuCmHg	+	FII
D1496	<i>E. coli</i>	<i>Tp</i> SmSuTcCmApHg	pHH1172	<i>Tp</i> SmSuCmHg	+	FII
D1508	<i>E. coli</i>	<i>Tp</i> GmTmKmSmSuTcCmAp	pHH1184	GmTmKmSuTcCm	+	C
D1621	<i>P. mirabilis</i>	<i>Tp</i> GmTmKmSuTcCmPx	pHH1299	GmTmKmSuTcCm	+	C
D1622	<i>Providencia</i>	<i>Tp</i> GmTmKmSmSuTcCmApNal PxHg	pHH1300	GmTmKmSmSuApHg	—	C
D1514	<i>E. coli</i>	<i>Tp</i> GmTmKmSmSuCmApHg	pHH1190	<i>Tp</i> GmTmKmSmSuCm ApHg	+	C
D1056†	<i>Klebsiella</i> K9	<i>Tp</i> SmSuTcCmAp	pHH720‡	<i>Tp</i> Su	+	W
D1515	<i>Enterobacter</i>	<i>Tp</i> KmSmSuTcApHg	pHH1191	<i>Tp</i> KmSu	+	W
D1517	<i>Enterobacter</i>	<i>Tp</i> GmKmSmSuTcApHg	pHH1193	<i>Tp</i> GmSuApHg	+	W
D1625	<i>Enterobacter</i>	<i>Tp</i> GmKmSmSuTcApHg	pHH1303	<i>Tp</i> GmKmSuApHg	—	W
D1629	<i>Enterobacter</i>	<i>Tp</i> GmSmSuTcApHg	pHH1307	<i>Tp</i> GmSuApHg	—	W
D1512	<i>E. coli</i>	<i>Tp</i> GmKmSmSuTcApHg	pHH1188	<i>Tp</i> GmKmSuApHg	+	W
D1516	<i>E. coli</i>	<i>Tp</i> GmKmSmSuCmApHg	pHH1302	<i>Tp</i> GmSuApHg	—	W
D1628	<i>E. coli</i>	<i>Tp</i> GmSuApHg	pHH1306	<i>Tp</i> GmSuApHg	—	W
D1511	<i>E. coli</i>	<i>Tp</i> KmSmSuTcCmApHg	pHH1187	<i>Tp</i> SmSu	+	X
D1494	<i>E. coli</i>	<i>Tp</i> Su	pHH1170	<i>Tp</i> Su	+	N
D1497	<i>Enterobacter</i>	<i>Tp</i> SmSuTcApHg	pHH1173	<i>Tp</i> SuHg	+	P
D1513	<i>E. coli</i>	<i>Tp</i> SmSuTc	pHH1189	<i>Tp</i> Sm	+	Iδ
D1627	<i>E. coli</i>	<i>Tp</i> GmTmKmSmSuTcCmApHg	pHH1305	<i>Tp</i> Sm	+	Iδ
D1630	<i>Enterobacter</i>	<i>Tp</i> SmSuTcCmApHg	pHH1308	<i>Tp</i> SmSuTcCmApHg	+	C
D1507	<i>Klebsiella</i>	<i>Tp</i> SmSuTcCmApHg	pHH1183	<i>Tp</i> SmSuTcCmApHg	+	C

Abbreviations: NT, non-typable; NC, non-capsulated; Tra, conjugative property. Resistances: *Tp*, trimethoprim (m.i.c. 1000 µg ml⁻¹); *Tp*, trimethoprim (lower m.i.c.); Gm, gentamicin; Tm, tobramycin; Km, kanamycin; Sm, streptomycin; Su, sulphonamide; Tc, tetracycline; Cm, chloramphenicol; Ap, ampicillin; Px, polymyxin; Nal, nalidixic acid; Hg, mercuric chloride. Resistances are listed only if the m.i.c. was at least four times that of *E. coli* K12 R⁻. All Gm R plasmids determined some Km resistance as seen with disc diffusion tests but it is not entered here except on its m.i.c. level. This table does not distinguish between higher levels of resistance except in the case of trimethoprim.

* 1976 isolate. † 1972 isolate. All other strains isolated 1 January–1 June 1977.

‡ pHH720 is indistinguishable from R388, identified in *E. coli* from another London hospital (Datta & Hedges, 1972; Jacob *et al.*, 1977).

plasmids were those that also carried trimethoprim-sulphonamide resistance. Only one IncW plasmid (pHH1191) carried trimethoprim resistance without gentamicin resistance.

Endemic strains and plasmids

Some recognizable plasmid-bearing strains were isolated from several patients. One was a *Klebsiella* (e.g. D1463, Table 4). The host strain was recognized by its biotype and serotype (K10), the plasmid by its incompatibility (IncFII) and its resistance pattern (GmTmSuCmHg). This strain was first seen in 1976 (see Introduction) and appeared repeatedly during the 1977 survey (Table 4). It seems certain that cross-infection of patients with *Klebsiella* K10 was occurring, although an epidemic was not recognized clinically. These K10 strains with IncFII plasmids were isolated from patients in wards in three well-separated hospital blocks and no clustering of cases was seen [clustering being defined as four or more patients infected or colonized in a ward in a 4-week period (Casewell & Phillips, 1978)].

Another endemic R⁺ strain was the *Enterobacter* sp. (e.g. D1517) that carried the trimethoprim-gentamicin R plasmid of IncW. The different isolates were all of the same somewhat unusual biotype. Like the R⁺ *Klebsiella* K10, they were found sporadically in widely separated ward blocks.

Evidence for plasmid transfer in the hospital environment

What appeared to be the same IncFII gentamicin R plasmid, as judged by incompatibility properties and resistance pattern, was found in several bacterial species in each of three wards with *Klebsiella* K10, strongly suggesting plasmid transfer. The IncW plasmid, too, was found in *E. coli* strains in the same wards as its characteristic *Enterobacter* host. Thus the transfer probably occurred between bacteria colonizing or infecting patients. Three patients yielded related plasmids from more than one species of bacterium, e.g. *Klebsiella* K10 (D1618) and *Citrobacter freundii* (D1509) were isolated from one patient and each strain carried two gentamicin R plasmids (IncFII and IncC, Table 4). From another patient came *Klebsiella* K21 (D1650) and *E. coli* (D1617) each with an IncFII plasmid. This patient remained in hospital for over 6 months, almost continuously colonized or infected by the resistant strains. Trimethoprim R plasmids pHH1183 and pHH1308 (Table 4) were also from different bacterial genera from a single patient.

Levels of resistance and their clinical significance

All the trimethoprim R plasmids determined high levels of resistance, allowing uninhibited growth of their host bacteria on medium containing 1000 µg trimethoprim ml⁻¹. Wild strains with lower levels of trimethoprim resistance were many (Table 4), but these strains never transferred their resistance. There were four cultures highly resistant to trimethoprim (2 *E. coli*, 1 *Klebsiella*, 1 *Citrobacter*) that failed to transfer this character.

The levels of resistance to gentamicin determined by plasmids of IncFII and IncW were low and the IncW plasmids did not confer significant levels of tobramycin resistance (Table 5). *Klebsiella* isolates that carried gentamicin R plasmids of IncFII were more resistant to gentamicin and tobramycin than were the *E. coli* K12 transconjugants with the same plasmids (Table 5). In some cases the m.i.c. of gentamicin was lower than the concentration that may be achieved in patients' serum. Nevertheless, the clinical significance of these levels in rendering therapy ineffective is implied by the fact that in at least four cases the organisms were isolated from clinical specimens while the patient was receiving gentamicin or tobramycin therapy. These isolates included examples of bacteria each carrying one of the three kinds of gentamicin R plasmid. Some patients were colonized or infected by plasmid-bearing strains for prolonged periods despite antibiotic therapy. One patient carried *Klebsiella* and *E. coli* each with an IncFII gentamicin R plasmid for over

Table 5. *Minimal inhibitory concentrations (m.i.c.) of gentamicin and tobramycin*

Results given are the mean values of six tests for the plasmid-free controls and the mean values for all the relevant cultures (see Table 4) for the R⁺ strains; the range of values from which the mean was found is shown in parentheses. No culture was counted as resistant unless its m.i.c. was at least four times that of *E. coli* K12 R⁻.

Bacteria carrying Gm R plasmid of group:	Wild strains		<i>E. coli</i> K12	
	Gentamicin ($\mu\text{g ml}^{-1}$)	Tobramycin ($\mu\text{g ml}^{-1}$)	Gentamicin ($\mu\text{g ml}^{-1}$)	Tobramycin ($\mu\text{g ml}^{-1}$)
None			0.19 (0.125-0.25)	0.25 (0.25)
IncFII	9 (4-16)	9 (4-16)	2.6 (1-8)	2.9 (1-8)
IncC	24 (8-32)	24 (16-32)	16 (8-32)	23 (8-32)
IncFII & IncC*	64	64		
IncW	7.3 (4-16)	0.5 (0.25-1)	5.3 (2-8)	0.8 (0.25-1)

* D1618 (see Table 4).

15 weeks. Another retained an *E. coli* strain with an IncC plasmid for 6 months. Both of these patients had had kidney transplants and the bacteria were isolated from urine, wound drainage and blood culture. In other instances the effect of gentamicin or tobramycin therapy in selection and establishment of plasmid-bearing strains was indicated by the isolation of the resistant organism within a few days of a course of treatment with these drugs.

However, not all of the patients infected with gentamicin-resistant strains had received gentamicin. For example, strains D1509 and D1618, highly resistant to gentamicin and tobramycin and each carrying two gentamicin R plasmids, were isolated from a patient who had received cotrimoxazole but no aminoglycoside.

Gentamicin-modifying enzymes

Escherichia coli K12 strains with gentamicin R plasmids of IncC determined synthesis of an enzyme that adenylylated gentamicin, tobramycin and kanamycin, but not neomycin or amikacin. The same was true of the *Klebsiella* isolates carrying the IncFII plasmid (Table 6a). Extracts of *E. coli* K12 transconjugants carrying the IncFII plasmid exhibited very low adenylyltransferase activity, reflecting the low levels of resistance found in *E. coli* K12 carrying these plasmids (Table 5). Considering the adenylylating activity determined by each plasmid against each substrate separately, the differences from the drug-free control were small and usually not statistically significant. However, for each R⁺ culture, the average counts were always higher when gentamicin, tobramycin or kanamycin (Table 6a: columns 1, 2 and 3) were in the reaction mixture than when neomycin, amikacin or no drug (Table 6a: columns 4, 5 and 6) was present. Differences between counts of radioactivity (on a log₁₀ scale) were investigated using a two-way analysis of variance. This showed highly significant differences between extracts ($P < 0.001$) and between substrates ($P < 0.001$). Tukey's test for non-additivity did not give a significant value, implying that there was no statistical interaction between extracts and substrates, i.e. that each extract showed the same ratio of results with the range of substrates. The studentized range test was then applied, and showed that differences between substrates were almost entirely due to a difference between results shown in columns 1, 2 and 3 from those in columns 4, 5 and 6 ($P < 0.01$). There were no significant differences between the results shown in columns 1, 2 and 3 or columns 4, 5 and 6. Thus the IncFII plasmids determined the adenylylation of gentamicin, tobramycin and kanamycin, but not neomycin or amikacin, enzyme reactions typical of aminoglycoside 2''-O-adenylyltransferase, AAD(2'') (Davies & Smith, 1978).

The gentamicin R plasmids of IncW determined the synthesis of an enzyme that acetylated gentamicin, including its C₁ component. The enzyme from pH1302, but not that

Table 6. *Substrate profiles of aminoglycoside-modifying enzymes*
 (a) *Assays for adenylyltransferase (uptake of [8-¹⁴C]adenosine 5'-triphosphate)*

Strain and plasmid	Plasmid Inc group	Radioactivity (c.p.m.) bound with substrate					
		(1) Genta- micin	(2) Tobra- mycin	(3) Kana- mycin	(4) Neo- mycin	(5) Ami- kacin	(6) No anti- biotic
<i>E. coli</i> K12 (pHH1184)	IncC	440	585	889	91	90	88
<i>E. coli</i> K12 (pHH1185a)	IncC	288	388	653	67	84	75
<i>E. coli</i> K12 (pHH1190)	IncC	265	340	464	80	74	57
<i>E. coli</i> K12 (pHH1296a)	IncC	562	625	786	229	218	234
<i>E. coli</i> K12 (pHH1299)	IncC	437	501	462	194	200	196
<i>E. coli</i> K12 (pHH1300)	IncC	460	532	578	218	200	200
<i>Klebsiella</i> K10 (pHH1326)	IncFII	340	313	354	171	198	209
<i>Klebsiella</i> K10 (pHH1327)	IncFII	281	288	260	154	166	153
<i>Klebsiella</i> K21 (pHH1328)	IncFII	236	241	240	121	129	128
<i>Klebsiella</i> K10 (pHH1329)	IncFII	331	365	328	187	182	184
<i>E. coli</i> K12 (pHH1186)	IncFII	124	112	133	75	92	72
<i>E. coli</i> K12 (pHH1325)	IncFII	289	293	256	212	197	230
<i>E. coli</i> K12 (pHH1326)	IncFII	158	149	138	113	135	131
<i>E. coli</i> K12 (pHH1327)	IncFII	439	511	440	380	423	367
<i>E. coli</i> K12 (pHH1328)	IncFII	293	309	260	240	241	216
<i>E. coli</i> K12 (pHH1329)	IncFII	389	399	338	315	290	339
<i>E. coli</i> K12 (pHH1330)	IncFII	397	383	390	308	301	330
<i>E. coli</i> K12 (pHH1331)	IncFII	233	203	204	172	152	156
<i>E. coli</i> K12 (pHH1332)	IncFII	318	320	301	214	231	274

(b) *Assays for acetyltransferase (uptake of [1-¹⁴C]acetyl coenzyme A)*

		Radioactivity (c.p.m.) bound with substrate*						
		Genta- micin	Genta- micin C ₁	Tobra- mycin	Kana- mycin	Neo- mycin	Ami- kacin	No anti- biotic
<i>E. coli</i> K12 (pHH1188)	IncW	229	176	117	122	136	128	121
<i>E. coli</i> K12 (pHH1302)	IncW	185	153	94	77	67	73	54

There was no evidence that any culture produced both adenylyl- and acetyltransferase.

These results indicate that the IncC and IncFII plasmids determine aminoglycoside 2''-O-adenylyl-(or nucleotidyl)-transferase, AAD(2'') or ANT(2''), and that the IncW plasmids determine aminoglycoside 3-N-acetyltransferase, AAC(3); for details, see text.

* Means of five determinations for each culture.

from pHH1188, also seemed to acetylate tobramycin, albeit very poorly (Table 6b). No other aminoglycosides were acetylated. The substrate profiles were incompatible with 2'- or 6'-N-acetyltransferases but were compatible with a 3-N-acetyltransferase (Davies & Smith, 1978). Extracts of pHH1188 and pHH1302 exhibited much lower enzymic activity than that shown by extracts of isolates from St Thomas' Hospital that produced aminoglycoside 3-N-acetyltransferase I [AAC(3)I] or AAC(3)II (unpublished observations). Taking into account the sensitivity to tobramycin of the strains carrying IncW plasmids, it seems that the enzyme is most likely to be AAC(3)I. Without more detailed studies we cannot accurately classify the acetyltransferase determined by the IncW plasmids.

Insertions into and deletions from R plasmids

Specific insertions. Six IncFII plasmids determined ampicillin resistance in addition to their characteristic (GmTmSuCmHg) pattern, as shown by co-elimination in incompatibility tests. Those that included ampicillin resistance had slightly higher molecular masses

Fig. 1. Agarose gel electrophoresis showing the characteristic internal fragments of *Hind*III-digested Tn7. Purified, *Hind*III-digested DNA: 1, λ , molecular masses (Md) of fragments are shown on the left; 2, RP4:Tn7; 3, pHH1187. The molecular masses (Md) of the internal fragments of Tn7 are shown on the right.

than those without, e.g. the migration of pHH1140, pHH1186 and pHH1325 in agarose gel electrophoresis showed molecular mass values of 57 Md while pHH1326 and pHH1328 were each 59 Md, and we therefore assume that the IncFII plasmid had, in these instances, acquired an ampicillin resistance transposon.

Three IncC plasmids carried ampicillin resistance in addition to their GmTmKmSuTcCm pattern and one (pHH1190) also carried trimethoprim, streptomycin and HgCl₂ resistance. The trimethoprim and streptomycin resistances were transposable: when RA1, a plasmid incompatible with pHH1190, was introduced into a *recA* strain carrying pHH1190, pHH1190 was eliminated, but in some clones trimethoprim-streptomycin resistance was retained and was now incorporated into RA1. The transposon was not identified by restriction analysis (RA1 and pHH1190 each have a molecular mass of 86 Md, too large for easy isolation) but resembles Tn7 in the resistance genes transposed. Of the other trimethoprim R plasmids, pHH1189 and pHH1305, (both of IncI δ) and pHH1187 (IncX) were shown by genetic analysis to carry transposable trimethoprim-streptomycin resistances. On digestion of pHH1187 plasmid DNA with the enzyme *Hind*III, fragments were generated with molecular masses equal to those generated when Tn7 is cleaved with *Hind*III, evidence that the plasmid carries a Tn7-like sequence (Fig. 1).

The characteristics of two trimethoprim R plasmids, pHH1169 and pHH1172, (IncFII, TpSmSuCmHg) suggested that Tn7 might be inserted into a gentamicin R plasmid of the kind exemplified by pHH1140 (IncFII, GmTmSuCmHg) and at a locus to prevent expression of the aminoglycoside resistance gene. However, the molecular masses of the plasmids (pHH1169 and pHH1172, each 58 Md; pHH1140, 57 Md) did not confirm this suggestion nor was transposition of TpSm demonstrated.

Because the IncW plasmids determined TpGmSuApHg resistance and their molecular

mass was 27 Md, whereas the IncW plasmids identified previously in the same hospital (Jobanputra & Datta, 1974) carried only TpSu resistance and had molecular masses of 21 Md, we supposed that the extra genes might have been inserted as transposons into the earlier plasmid species, sometimes in loci to inactivate the transfer genes (see above). Both ampicillin and HgCl₂ resistance genes have been shown in other situations to be transposable (Hedges & Jacob, 1974; Stanisich *et al.*, 1977) and we were interested to see whether gentamicin resistance might also be transposable. However, genetic tests for transposition of the Ap, Hg or Gm resistances of the IncW plasmids were all negative.

Specific deletions. Strains D1516 and D1628 (Table 4), each carrying an IncW plasmid (R1302 and R1306, respectively) conferring the resistance pattern TpGmSuApHg, gave rise to clones in which the IncW plasmid was retained but trimethoprim resistance was lost. *Escherichia coli* K12 Rec⁺ strains, but not a *recA* strain, carrying R1302 or R1306, also lost trimethoprim resistance at variable frequencies. Loss of trimethoprim resistance from cultures with other IncW plasmids was not observed. Experiments are in progress to show whether independently isolated trimethoprim-sensitive clones all show the same alterations in plasmid DNA sequences.

DISCUSSION

An unexpected finding in this study was that, among bacteria collected because they were resistant either to trimethoprim or to gentamicin, so many (Table 3) were resistant to both these drugs. Similar multiple-resistance patterns appeared in bacteria of different genera (Table 4). Much, but not all, of the resistance was plasmid-determined. Multiply-resistant Gram-negative bacteria of the kind identified here resemble those that currently cause outbreaks of hospital infection (e.g. Schaberg *et al.*, 1976; Casewell *et al.*, 1977; Rennie & Duncan, 1977; Curie *et al.*, 1978; Gerding *et al.*, 1979), but during the period of the survey and up to the present no epidemic has occurred.

The incidence of trimethoprim resistance in Gram-negative infections in the hospital during this decade has risen from 5.6 to 16 % (Table 2), frequencies that fall within the local variations reported in other surveys of in-patient infections (Grüneberg, 1976; Marks *et al.*, 1977; Amyes *et al.*, 1978; McGill, 1978; Grey *et al.*, 1979). In a majority of trimethoprim-resistant strains isolated during the 1977 survey (53/73, i.e. 77 %; Tables 3 and 4), the trimethoprim resistance was non-transmissible and expressed at lower levels than has been reported as plasmid-determined. We infer that this resistance is chromosomally-determined. Trimethoprim resistance was shown to be plasmid-determined in 17 strains (23 %), a proportion similar to that found in 1972-73 (Jobanputra & Datta, 1974), but the distribution of trimethoprim resistance genes among plasmids was very different. In 1972-73 we concluded that the only trimethoprim R plasmids identified in this hospital constituted a single molecular clone of incompatibility group W that carried resistance to trimethoprim and sulphonamides (TpSu) and was found most often in a host strain of *Klebsiella* that was endemic in the hospital. In the 1977 collection of trimethoprim R plasmids, members of seven different incompatibility groups were identified (Table 4), showing the acquisition of genes for trimethoprim resistance by a range of plasmids in the enterobacteria (see also Acar *et al.*, 1977). Some of this spread of trimethoprim resistance genes has resulted from the distribution in hospitals and in farm animals of the trimethoprim-streptomycin resistance transposon Tn7 (Barth & Datta, 1977; Richards *et al.*, 1978; West & White, 1979; Datta *et al.*, 1979) and in the present study we identified Tn7-like sequences in plasmids of three different groups - IncC, IncI δ and IncX. In one instance, the IncC plasmid pHH1190, it seems likely that acquisition of resistance determinants by transposition occurred in the hospital, since plasmids resembling pHH1190 in incompatibility properties and resistance patterns, but without trimethoprim, streptomycin or ampicillin resistance, were found (e.g. pHH1184, Table 4).

Trimethoprim R plasmids, possibly related to the IncW (TpSu) plasmids of the early

1970s, were found in the present survey but carried, in addition, resistances to gentamicin, ampicillin and HgCl_2 (i.e. the pattern was now TpSuGmApHg, e.g. pHH1193, Table 4). Possibly these represent the original TpSu plasmids with DNA insertions of about 6 Md, but we have no direct evidence for this. The gentamicin resistance of the IncW plasmids was associated with an aminoglycoside acetylating enzyme. We found no other plasmids in the hospital that determined the same enzyme, and certainly have no evidence for on-the-spot transposition of the gene.

Of the 34 gentamicin-resistant enterobacteria in the survey, 26 (76 %) carried gentamicin R plasmids. The plasmid-determined gentamicin resistance genes showed less evidence than those for trimethoprim resistance of dissemination between replicons. The only evidence for Gm transposition was the circumstantial evidence, discussed above, for the acquisition of gentamicin resistance by IncW plasmids. The only other gentamicin R plasmids we found belonged to groups IncC and IncFII. Plasmids of both groups determined aminoglycoside 2''-O-adenylyltransferase. In the naturally occurring hosts of these plasmids the levels of gentamicin resistance were such as to render gentamicin therapy ineffective.

The constancy of their respective resistance patterns suggests that the same IncC or IncFII plasmids were present in different genera. Ampicillin resistance was often included in the resistance pattern of IncC and IncFII plasmids and in one case (pHH1190, see above), TpSm was also included. The inserts were probable or proved transposons. In other instances (pHH1169 and pHH1172) a Tn7-like sequence might have inserted into an IncFII gentamicin resistance plasmid such as pHH1140 (GmTmSuCmHg) and a subsequent deletion led to loss of both transposability and the aminoglycoside resistance.

Some of the first gentamicin R plasmids described in enterobacteria belonged to IncC and have been found in many bacterial species, including *P. aeruginosa*, in Paris hospitals (Witchitz & Chabbert, 1971, 1972). Like the IncW plasmids referred to above, they represent an 'epidemic plasmid' with occasional insertions or deletions of inessential sequences (Chabbert *et al.*, 1979). The IncC plasmids identified in our survey, e.g. pHH1184, pHH1190 (Table 4), are probably related to this epidemic plasmid; they are not only of the same incompatibility group but also determine the same aminoglycoside-modifying enzyme, though some of their other resistance genes are different.

Gentamicin R plasmids of IncFII determining AAD(2'') have also been reported before. An early example was JR66b from a *Klebsiella* strain that caused an outbreak of hospital infection (Martin *et al.*, 1971; Benveniste & Davies, 1971; Datta & Hedges, 1973). Gentamicin R plasmids from *Klebsiella* responsible for an outbreak of hospital infection in Toronto also belonged to group IncFII and determined AAD(2'') (Rennie & Duncan, 1977, and unpublished observations). Identical R plasmids have been found in different bacterial genera isolated in different continents (Anderson *et al.*, 1977; Barth & Grinter, 1974; Cohen, 1977; Datta *et al.*, 1979) and it is not improbable that a gene for gentamicin resistance, firmly linked to the replication genes of an IncFII plasmid, has been carried between continents.

As in other studies of plasmids in hospital bacteria (e.g. Witchitz & Chabbert, 1972; Richmond *et al.*, 1975; Sadowski *et al.*, 1979; Datta *et al.*, 1979) we found evidence for transfer of plasmids between bacteria of different genera during colonization or infection of individual patients. In addition, and more commonly, we found that identifiable strains of bacteria, carrying their identifiable plasmids, colonized or infected patients in different parts of the hospital on different, unrelated occasions.

Patients in hospital may be infected or colonized with *Klebsiella* strains derived from their own intestinal flora (Seldon *et al.*, 1971; Cooke *et al.*, 1979) but, in addition, some strains may give rise to small outbreaks (Casewell & Phillips, 1978). It would be of interest to know to what extent multiple resistance is a factor in allowing strains such as our *Klebsiella* K10 with its IncFII plasmid or *Enterobacter* sp. with its IncW plasmid to become established in a hospital. It is of even greater importance to clarify the factors which lead to major out-

breaks of infection, such as that described by Curie *et al.* (1978), rather than the low-level endemicity found here.

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