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R-plasmid transfer *in vivo*: a prospective study

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Summary. Two hundred and eighty-seven coliform bacteria were isolated from 116 rectal swabs or faecal specimens obtained from 113 patients. By means of plasmid analysis and resistance transfer (R-transfer) *Escherichia coli* was found to differ from other enteric genera in plasmid distribution. Criteria were proposed that enabled in-vivo "R-transfer potential" and in-vivo "R-transfer rate" to be calculated. From each of 22 of the 113 patients numerous coliforms were isolated, of which at least one per patient contained one or more self-transmissible R-plasmids potentially transferable to 43 other coliforms. Evidence indicated that R-plasmid transfer had occurred *in vivo* on two of the 43 potential occasions. These results are discussed in the context of plasmid ecology in the human host.

Introduction

The clinical problems associated with infections caused by resistant coliform bacteria and the role of plasmids in resistance are well known (Casewell, 1982; Stuart-Harris and Harris, 1982). The importance of resistance transfer (R-transfer) *in vivo* is commonly inferred from epidemiological evidence (Knight and Casewell, 1981; Platt and Sommerville, 1981*a*). However, detailed studies of in-vivo transfer are few and have often been limited by the experimental system used. Jarolmen and Kemp (1969) and Smith (1971) concluded that in-vivo transfer in animals was a rare event influenced by the characteristics of the donor and recipient organisms, the plasmid, the host environment and antimicrobial therapy. Studies in man have also suggested the infrequency of in-vivo R-transfer (Hartley and Richmond, 1975; Petrocheilou *et al.*, 1976; Richmond and Petrocheilou, 1978). More recently, reports have described the acquisition of several R-plasmids by *Salmonella* species in individual patients (Datta *et al.*, 1981; Platt *et al.*, 1984*a*). The observation that most strains of *E. coli*, collected irrespective of drug resistance, harbour plasmids (Platt *et al.*, 1984*b*) prompted us to examine the distribution of plasmids among enteric organisms and to reconsider in-vivo R-transfer on a prospective basis.

Materials and methods

Specimen collection and processing

Sixty-two faecal specimens were obtained from the routine diagnostic laboratory and 54 rectal swabs from in-patients in the peripheral vascular disease unit, Glasgow Royal Infirmary. None of the latter were known to have any gastro-intestinal infection at the time of specimen collection, but patients with suspected mesenteric ischaemia were not excluded.

Faecal suspensions (*c.* 0·5 g/10 ml of sterile distilled water) and rectal swabs were plated on CLED (Mast DM110) and MacConkey (Oxoid, CM7B) Agars for isolation of single colonies. A direct sensitivity test was performed on Isosensitest Agar (Oxoid CM471) with antibiotic disks (Oxoid) containing carbenicillin 100 μg, cefazolin 30 μg, tetracycline 10 μg, kanamycin 30 μg, streptomycin 10 μg, chloramphenicol 10 μg, sulphamethoxazole 25 μg and trimethoprim 1·25 μg to select any resistant coliforms that were present in small numbers. The plates were examined after overnight incubation aerobically at 37°C. From the CLED and MacConkey plates six colonies of each distinct colony type were suspended in 2·5 ml of Isosensitest Broth (Oxoid, CM473) and plated separately for purity on CLED. They were tested for homogeneity in disk-diffusion sensitivity tests with disks containing: nalidixic acid 30 μg, rifampicin 50 μg, ampicillin 10 μg, carbenicillin 100 μg, cefazolin 30 μg, cefamandole 30 μg, colistin sulphate 25 μg, tetracycline 10 μg, kanamycin 30 μg, streptomycin 10 μg, gentamicin 10 μg, tobramycin 10 μg, amikacin 10 μg, sulphamethoxazole 25 μg, trimethoprim 1·25 μg, and chloramphenicol 10 μg.

Any coliform colonies growing within the inhibition zones on the direct sensitivity plates were similarly purified and their sensitivities were tested. Pure cultures were identified by means of the API 20E system and stored for further investigation.

Plasmid detection

Cell lysis, electrophoresis and plasmid visualisation were performed as described by Platt and Sommerville (1981*b*).

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DNA purification and digestion by restriction endonucleases

DNA was prepared from 5 ml of an overnight culture in Brain Heart Infusion Broth (Oxoid, CM225) by the alkaline SDS method of Birnboim and Doly (1979) as modified by Ish-Horowitz and Burke (1981). After extraction with a mixture of phenol and chloroform, DNA was precipitated with an equal volume of isopropanol at room temperature. The precipitate was dissolved in 100 μl of TE buffer (10 mM Tris, 1 mM EDTA, *p*H 8·0); 50 μl of 7·5 M ammonium acetate were added with mixing followed by 300 μl of ice-cold ethanol. After at least 30 min at −18°C the precipitate was deposited by centrifugation and redissolved in 80 μl of TE buffer, treated with 9 μl of RNAase 1 mg/ml and incubated for 30 min at 37°C. A 10-μl volume of 2·5 M sodium chloride was added and DNA was obtained by isopropanol precipitation, ammonium acetate treatment, and ethanol reprecipitation as above. The final pellet was resuspended in 30 μl of TE buffer. The restriction enzymes *Hind* III, *Pst* 1, *Sma* 1, *Alu* 1, and *Hae* III were obtained from BRL (Bethesda Research Laboratories) and 4 μl of each enzyme were used in a 50-μl reaction mixture in eppendorf tubes containing 10 μl of DNA with buffers, as recommended by the manufacturer. The tubes were incubated for 4 h at 37°C after which 5 μl of bromophenol blue tracker dye were added to each and the contents loaded on to 0·7–1·0% horizontal agarose gels. Electrophoresis was performed at 18 mA overnight. The running buffer was tris-borate, *p*H 8·2, containing ethidium bromide 0·3 μg/ml. The mol. wts of restriction fragments were calculated by comparison with *Hind* III, *Pst* 1 and *Hae* III digests of phage lambda DNA.

Antibiotic resistance transfer

Organisms resistant to antimicrobial agents other than those to which the species was intrinsically resistant were tested for R-transfer after overnight broth mating by standard methods (Kraft *et al.*, 1983). Recipient organisms used were *E. coli* K12 J53-2 (lac^+, rif^r) and K12 J62-2 (lac^-, rif^r) (Bachmann, 1972) and *Enterobacter aerogenes* GRI 9880 (Platt and Sommerville, 1981*b*). Methods used for the confirmation of R-transfer were as described in detail by Kraft *et al.* (1983).

Antimicrobial therapy

The antimicrobial therapy prescribed during the 30 days preceding the collection of specimens was recorded.

Calculation of in-vivo "R-transfer potential" and in-vivo "R-transfer rate"

Two indices were calculated to describe the likelihood of R-transfer *in vivo*. The in-vivo R-transfer potential was the number of combinations of (i) a transferable plasmid of mol. wt $>20\times10^6$ in a donor with (ii) a potential recipient in the same specimen. The in-vivo R-transfer rate was the number of times transfer of a plasmid of mol. wt $>20\times10^6$ was detected/the R-transfer potential.

The following criteria were applied separately to each specimen for the purposes of the calculation. (a) A given specimen must have yielded at least two coliforms distinguishable biochemically or by resistance pattern. (b) At least one coliform must have harboured one or more R-plasmids transferrable to *E. coli* K12. (c) Each coliform that fulfilled criterion (b) was considered as a separate donor. (d) In the presence of a plasmid donor every other distinguishable coliform isolated from a given specimen was considered as a separate potential recipient. (e) When a single donor transferred more than one plasmid of mol. wt $>20\times10^6$ independently, the R-transfer potential was calculated separately for each plasmid; and conversely when co-transfer of two or more plasmids occurred without demonstrable segregation the R-transfer potential was calculated as for a single plasmid.

Statistical analysis

The chi-squared test was used to compare the plasmid distributions (Siegel, 1956).

Results

One hundred and sixteen specimens were obtained from 113 patients. Two hundred and eighty-seven coliforms were isolated, of which 161 were *E. coli*. The other 126 belonged to diverse enteric genera and are listed in table I, which also shows the numbers of plasmids harboured and the resistance of strains to antimicrobial agents. Drug resistance was more common among *E. coli* isolates than among those of other enteric genera. Although the numbers of isolates of enteric genera other than *E. coli* were too small for individual analyses, they appeared similar with respect to a low incidence of resistance and small numbers of plasmids. The latter is most clearly seen in fig. 1, which shows the distribution of plasmids among *E. coli* and other enteric genera. The distribution of plasmids was significantly different ($\chi^2_{5df}=54{\cdot}104$; $p<0{\cdot}001$) and, with the possible exception of *Enterobacter* spp. for which the sample size was very small, strains of other enteric genera contained fewer plasmids than *E. coli*. However, when the distribution of plasmids among strains that contained one or more plasmids was compared there was no significant difference between *E. coli* and other enteric genera ($\chi^2_{3df}=0{\cdot}696$; $p>0{\cdot}8$) (fig. 2). This indicates that the difference between *E. coli* and other enteric genera was due to differences in the number of plasmid-free strains rather than to an overall carriage of fewer plasmids by the other enteric genera.

The number of different coliforms isolated from each specimen varied between zero and seven and

Table I. The distribution of plasmids and antibiotic resistance among the genera studied

Organism	Total number of isolates	Number of isolates containing the stated number of plasmids per isolate							Total number of antibiotic-resistant isolates
		0	1	2	3	4	5	6	
E. coli	161	21(3)*	55(22)	47(20)	23(9)	7(4)	6(4)	2(1)	63
Klebsiella spp.	51	27(0)	12(3)	10(2)	2(1)	0	0	0	6
Proteus spp.	34	24(1)	6(0)	4(2)	0	0	0	0	3
Enterobacter spp.	22	5	3	4	5	3	1	1	0
Citrobacter sp.	16	9	4(1)	2	0	1	0	0	1
Salmonella sp.	1	1	0	0	0	0	0	0	0
Providencia sp.	1	1	0	0	0	0	0	0	0
Yersinia sp.	1	1	0	0	0	0	0	0	0
Total	287	89	80(26)	67(24)	30(10)	11(4)	7(4)	3(1)	73

* Number of isolates resistant to at least one antibiotic shown in parentheses.

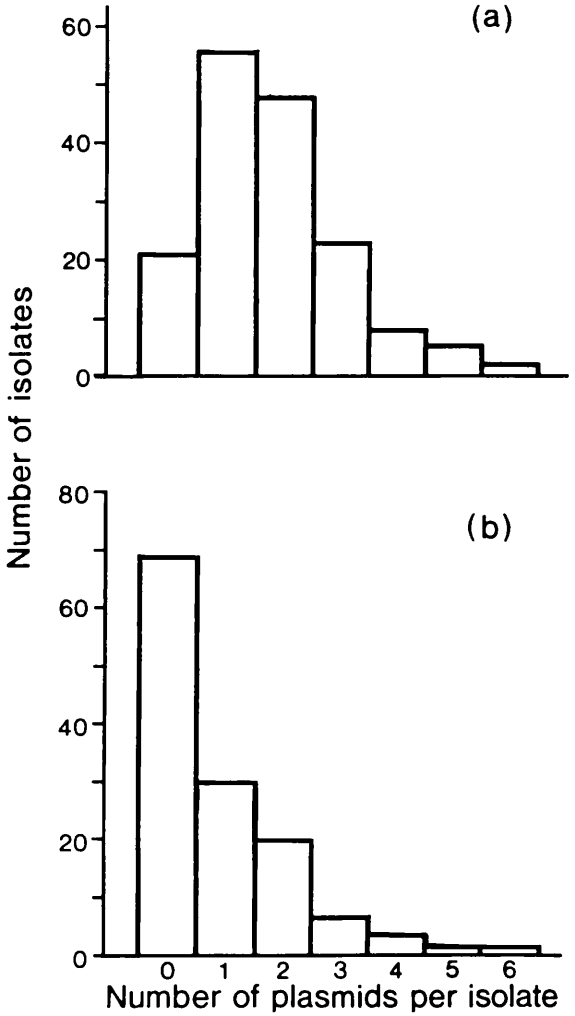

Fig. 1. The distribution of plasmids among isolates of (a) *E. coli*, (b) other enteric genera.

their distribution is shown in table II. The seven specimens that yielded no coliforms were all obtained from patients receiving at least two antimicrobial agents and included three patients undergoing gut "sterilisation' with Fracon (framycetin, colistin and neomycin). The modal value was one coliform per specimen.

From each of two patients, two coliforms were isolated (patient A, *E. coli* and *Klebsiella pneumoniae*; patient B, *E. coli* and *Citrobacter freundi*). The pairs of isolates each harboured transferrable R-plasmids that were indistinguishable on the basis of resistance markers transferred, mol. wt and restriction-enzyme fingerprints (table III). We interpret this finding as evidence of in-vivo R-transfer. The 36×10^6-mol. wt conjugative plasmid harboured by the strains of *E. coli* and *K. pneumoniae* isolated from patient A gave rise to transconjugants with two resistance patterns that differed with respect to sulphonamide resistance. Both Tc^r Tp^r pSm^r Su^r and Tc^r Tp^r pSm^r phenotypes were isolated with *E. coli* as the plasmid donor. However, only Tc^r Tp^r pSm^r transconjugants were obtained when *K. pneumoniae* was the donor and *E. coli* K12 the recipient. Transconjugants of both phenotypes were isolated from the *K. pneumoniae* strain when *Ent. aerogenes* replaced *E. coli* K12 as the recipient. Furthermore, when plasmids with the Su^s phenotype were retransferred to *E. coli* K12 from either of the above transconjugants both Su^s and Su^r phenotypes were isolated. This indicates that the difference in phenotype was the result of altered plasmid expression. Comparison of both *Hae* III and *Alu* I fingerprints of the Su^s and Su^r transconjugant plasmids showed that loss of the Su phenotype was associated with an increase in the size of the largest

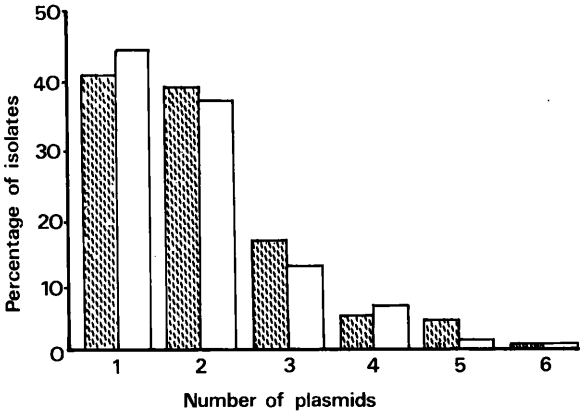

Fig. 2. The proportional distribution of plasmids among *E. coli* ▨ and other enteric genera □ after the exclusion of plasmid-free isolates.

Table II. The distribution of specimens from which distinguishable coliforms were isolated

Number of coliforms isolated	Number of specimens
0	7
1	30
2	26
3	22
4	22
5	5
6	2
7	2

restriction fragment (table III). Low level streptomycin resistance (pSm^r) was also plasmid-mediated and expressed in the transconjugants and in the *K. pneumoniae* donor but not in the *E. coli* donor from patient A.

Patient A had not received antibiotics within the previous 30 days. Patient B had been treated with amoxycillin for 5 days at the time the organisms were isolated and the plasmids described in the strains conferred resistance to amoxycillin.

R-plasmid transfer to *E. coli* K12 was demonstrated from 32 of the isolates studied and from at least one of the coliforms isolated from each of 30 patients. However, from eight of these patients no other coliform was isolated and therefore the in-vivo "R-transfer potential" was calculated on the basis of 22 patients and found to be 43. Combining these data with the two in-vivo transfer events described above gives an in-vivo R-transfer rate of 1/21·5.

Discussion

The development of rapid, simple methods for the physical detection, separation and fingerprinting of plasmid DNA from diverse genera (Platt, 1983) has provided the means to investigate plasmid ecology by enabling a large number of strains to be studied in detail. Thus, resistant organisms can be investigated with sensitive strains isolated in parallel, their plasmids compared and a better interpretation of the role of plasmids obtained. Hughes and Datta (1983) and Datta and Hughes (1983) studied

Table III. Summary of the characters of R-plasmids transferred *in vivo*

	Details of clinical isolate			Details of transconjugants			
Patient	Species	Resistance	Mol. wt (10^6) of plasmid	Resistance	Mol. wt (10^6) of plasmid	Restriction fragment sizes (kilobase pairs) with the given endonuclease	
A	*E. coli*	Tc Tp Su	62, 36	Tc Tp pSm Su*	36	*Hae* III 1·35, 1·30, 1·05, 0·74, 0·69 *Alu* 1 1·35, 1·25, 1·10, 0·98, 0·86	
				Tc Tp pSm*	36	*Hae* III 1·7, 1·30, 1·05, 0·74, 0·69 *Alu* 1 1·7, 1·25, 1·10, 0·98, 0·86	*Pst* 1 2·05, 1·85
A	*K. pneumoniae*	Tc Tp Su pSm	62, 36	Tc Tp pSm*	36	*Hae* III 1·7, 1·30, 1·05, 0·74, 0·69 *Alu* 1 1·7, 1·25, 1·10, 0·98, 0·86	
				Tc Tp pSm Su†	36	*Hae* III 1·35, 1·30, 1·05, 0·74, 0·69 *Alu* 1 1·35, 1·25, 1·10, 0·98, 0·86	
B	*E. coli*	Ap	62, 30, 10	Ap*	62	*Pst* 1 4·0, 3·3, 3·1, 2·8, 2·5, 2·3 1·8, 1·6, 1·3	
B	*C. freundi*	Ap	62	Ap*	62	*Sma* 1 10·0, 7·6, 6·9, 6·0, 5·1, 3·5, 2·6, 2·5, 2·25, 2·15, 2·0, 1·9	

TC = Tetracycline, Tp = trimethoprim, Su = sulphonamide, pSm = low level streptomycin, Ap = ampicillin.
* *E. coli* K12 transconjugants.
† *Enterobacter* transconjugant.

the transferability of various markers from Murray's collection of enteric bacteria isolated in the pre-antibiotic era and concluded that conjugative plasmids were as common then as they are in drug-sensitive strains today.

In a previous study of defined populations of *E. coli* we compared the frequency distributions of strains that harboured 0, 1, 2, 3 ... 7 plasmids per isolate (Platt *et al.*, 1984*b*). The population collected on the basis of antibiotic resistance possessed 26% more plasmids than the sensitive population. In this study the distribution of plasmids among *E. coli* isolates was similar. However, other enteric genera collected in parallel with *E. coli* harboured fewer plasmids (mean 0·89 plasmids/isolate compared with 1·78 plasmids/isolate among *E. coli*) (fig. 1). This suggests that *E. coli* is either a better plasmid recipient than other enteric genera or better able to maintain plasmids once acquired. Several possible explanations could account for this. The principal habitat of *E. coli* is the human and animal gut, where it is the predominant coliform; the other enteric genera tend to be minority residents and are also adapted to a free-living existence. Plasmid carriage constitutes a biosynthetic burden (Zund and Lebek, 1980; Sherratt, 1982). It seems likely that a well-adapted organism is readily able to accumulate plasmids. In the absence of selection pressure this may confer an energetic disadvantage on the host. Alternatively, the high spontaneous mutation rate in genera such as *Serratia* (Platt *et al.*, 1983) and *Klebsiella* (Smith, 1976) may confer sufficient genetic flexibility to reduce the benefits of plasmid carriage. It could also be argued that the differences in the distribution of plasmids might be attributable to the greater incidence of antibiotic resistance among *E. coli* isolates as compared with other enteric genera (table I). However, the effect of antibiotic resistance on plasmid distribution was minimal in our previous study (Platt *et al.* 1984*b*) and it seems unlikely that it would cause the striking differences observed here.

Earlier workers who found a low absolute incidence of R-transfer *in vivo* have emphasised such factors as donor ability, recipient ability (Jarolman and Kemp, 1969; Smith 1971) and possible inhibitory effects of other organisms (Anderson, 1975). These arguments reflect the systems studied and neglect the plasmid's contribution to its own transfer. In particular, we consider that specimens from which either single coliform strains or coliform strains lacking conjugative R-plasmids are isolated should be excluded, and therefore we have proposed the concept of in-vivo "R-transfer potential" and "R-transfer rate". Although the restrictions imposed by the criteria used to define them minimise the actual values obtained we refrained from the use of the prefix 'minimal" because other biological characters of the plasmids, e.g., surface exclusion, incompatibility, DNA restriction, could further decrease the real transfer potential. Despite these minimising influences the observations that of 116 specimens examined 43 potential transfer events were possible within 22 specimens, and that two such events were realised *in vivo*, suggests that R-transfer is very much more common *in vivo* than was hitherto suggested. This is consistent with recent reports of transfer in individual patients (Datta *et al.*, 1981; Platt *et al.*, 1984*b*).

Further refinements in methodology such as the use of alternative recipients to *E. coli* K12 for plasmids from other genera (Platt and Sommerville, 1981*b*; Sommerville, unpublished data) and the more extensive use of restriction-enzyme fingerprints to compare plasmids that acquire transposons (Kraft *et al.*, 1984) will no doubt necessitate the modification of our proposals. Nevertheless, the cautious use of the suggested terms should lead to a better appreciation of plasmid ecology.

REFERENCES

Anderson J D 1975 Factors that may prevent transfer of antibiotic resistance between Gram-negative bacteria in the gut. *Journal of Medical Microbiology* **8**:83–88.

Bachmann B J 1972 Pedigrees of some mutant strains of *Escherichia coli* K-12. *Bacteriological Reviews* **36**:525–557.

Birnboim H C, Doly J 1979 A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* **7**:1513–1523.

Casewell M 1982 The role of multiply resistant coliforms in hospital-acquired infection. In: Reeves D S, Geddes A M (eds) Recent advances in infection 2. Churchill Livingstone, Edinburgh, pp 31–50.

Datta N, Hughes V M 1983 Plasmids of the same Inc groups in Enterobacteria before and after the medical use of antibiotics. *Nature* **306**:616–617.

Datta N, Richards H, Datta C 1981 *Salmonella typhi in vivo* acquires resistance to both chloramphenicol and co-trimoxazole. *Lancet* **1**:1181–1183.

Hartley C L, Richmond M H 1975 Antibiotic resistance and the survival of *E. coli* in the alimentary tract. *British Medical Journal* **4**:71–74.

Hughes V M, Datta N 1983 Conjugative plasmids in bacteria of the 'pre-antibiotic' era. *Nature* **302**:725–726.

Ish-Horowitz D, Burke J F 1981 Rapid and efficient cosmid cloning. *Nucleic Acids Research* **9**:2989–2998.

Jarolmen H, Kemp G 1969 R-factor transmission *in vivo*. *Journal of Bacteriology* **99**:487–490.

Knight S, Casewell M 1981 Dissemination of resistance plasmids

among gentamicin-resistant enterobacteria from hospital patients. *British Medical Journal* **283:**755–756.

Kraft C A, Platt D J, Timbury M C 1983 Distribution and transferability of plasmids in trimethoprim resistant urinary *Escherichia coli*. *Journal of Medical Microbiology* **16:**433–441.

Kraft C A, Platt D J, Timbury M C 1984 Distribution and transferability of plasmids in trimethoprim-resistant urinary strains of *Escherichia coli*: a comparative study of hospital isolates. *Journal of Medical Microbiology* **18:**95–105.

Petrocheilou V, Grinsted J. Richmond M H 1976 R-plasmid transfer *in vivo* in the absence of antibiotic selection pressure. *Antimicrobial Agents and Chemotherapy* **10:**753–761.

Platt D J 1983 Bacterial plasmids and their fingerprints. *Disease Markers* **1:**107–115.

Platt D J, Sommerville J S 1981*a* *Serratia* species isolated from patients in a general hospital. *Journal of Hospital Infection* **2:**341–348.

Platt D J, Sommerville J S 1981*b* A simple method for the detection of resistance plasmids in *Serratia* species by transfer to member of the genus *Enterobacter*. *Journal of Antimicrobial Chemotherapy* **8:**145–152.

Platt D J, Sommerville J S, Gribben J 1984*a* Sequential acquisition of R-plasmids *in vivo* by *Salmonella typhimurium*. *Journal of Antimicrobial Chemotherapy* **13:**65–69.

Platt D J, Sommerville J S, McGroarty J A 1983 Characterization of spontaneous resistant variants of *Serratia marcescens* selected in the presence of carbenicillin. *Journal of Antimicrobial Chemotherapy* **12:**329–335.

Platt D J, Sommerville J S, Kraft C A, Timbury M C 1984*b* Antimicrobial resistance and the ecology of *Escherichia coli* plasmids. *Journal of Hygiene* **93:**181–188.

Richmond M H, Petrocheilou V 1978 R-factor transfer *in vivo* in humans. In: Schlessinger D (ed) Microbiology. American Society of Microbiology, Washington DC, pp 273–276.

Sherratt D J 1982 The maintenance and propagation of plasmid genes in bacterial populations. *Journal of General Microbiology* **128:**655–661.

Siegel S 1956 Non-parametric statistics for the behavioral sciences. McGraw Hill Book Company Inc., New York.

Smith H W 1971 Observations on the *in vivo* transfer of R factors. *Annals of the New York Academy of Sciences* **182:**80–90.

Smith H W 1976 Mutants of *Klebsiella pneumoniae* resistant to several antibiotics. *Nature* **259:**307–308.

Stuart-Harris C H, Harris D M 1982 The control of antibiotic-resistant bacteria. Academic Press, New York.

Zünd P, Lebek G 1980 Generation time-prolonging R-plasmids: correlation between increases in the generation time of *Escherichia coli* caused by R-plasmids and their molecular size. *Plasmid* **3:**65–69.