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Factors influencing the establishment and spread of R-plasmids in an experimental model of urinary tract infection

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SUMMARY

An experimental model simulating infection of the urinary tract was used to investigate factors influencing the transfer of plasmid-mediated drug resistance in the human bladder in the absence of antibiotic therapy. When a small number of cells carrying plasmid-mediated drug resistance was added to an established population of sensitive cells, it was found that varying the physical conditions of the bladder model had no significant effect on the spread of the plasmid and, under all conditions used, exponential spread of the plasmid through the originally sensitive population occurred. When the initial donor cells were killed shortly after addition to the model, exponential transfer of resistance amongst the established sensitive population still occurred. Thus, even in the absence of antibiotic therapy, only a small number of initial transfer events was required for a fully resistant population to be developed. This was true of both actively multiplying and early stationary phase cultures. An initially greater proportion of resistant cells, or the use of an antimicrobial agent to which some of the cells are resistant, would ensure that this process occurred more rapidly.

INTRODUCTION

Previous workers (e.g. Willetts, 1974; Broda, Cullum & Collins, 1977; Cullum, Collins & Broda, 1978*a*, *b*) have attempted quantitative analysis of the spread of plasmids conferring transferable antibiotic resistance under non-selective conditions through populations of *Escherichia coli*. The process of spread is complex, including both transfer (retransfer from original recipient cells as well as from primary donors) and the growth of donor, progeny and recipient cells. Cullum *et al.* (1978*b*) concluded that the three most important factors in determining the proportion of a recipient population to which a plasmid spreads were (i) the amount of initial transfer by the original donors (ii) the rate of spread of the plasmid between recipient cells, and (iii) the time during which retransfer can occur before the conditions in the mating mixture become unsuitable for plasmid transfer. With the plasmids studied by these workers it seemed that there were two main constraints upon 'epidemic spread'; firstly, that after the initial transfer from donor to recipient cells, subsequent rounds of transfer could only occur at about 30 min intervals during active exponential growth and less frequently in slowly

Table 1. *Bacterial strains and plasmids*

	Characteristics	Reference
E. coli K12		
CSH1	Thiamine and tryptophan requiring; streptomycin-resistant	Miller (1972)
J53.2	Proline and methionine-requiring; rifampicin-resistant	Coetzee, Datta & Hedges (1972)
Plasmids		
R388	Confers resistance to trimethoprim and sulphonamides	Datta & Hedges (1972)
R386	Confers resistance to tetracycline; derepressed for transfer	Dennison (1972)

growing cultures; and secondly, it took about 60–90 min for the descendants of newly infected cells to become proficient donors. Therefore, in mixed cultures the number of progeny did not increase dramatically more rapidly than did the total cell count (Cullum *et al.* 1978*a*, *b*).

The purpose of the work described in this paper was to examine a number of the possible factors which might influence the transfer of R-plasmids in an experimental model of the human bladder. Acute infection of the normal urinary tract is nearly always caused by a single bacterial strain, usually of *E. coli*, and plasmid-mediated resistance to a particular drug is therefore likely to be present or absent throughout an infection rather than emerging during it. Hospital-associated infections, however, are caused by a wider range of organisms and are often mixed, especially in patients who have been repeatedly catheterised (Gillespie, 1976). Simultaneous infection with a drug-sensitive and a drug-resistant strain can occur. We decided, therefore, to determine the effect on plasmid spread, and hence the proportion of a population which might be expected to carry a plasmid under non-selective conditions, of varying a number of parameters in a model which mimics at least some of the *in vivo* features of human urinary tract infection.

MATERIALS AND METHODS

Bacterial strains and plasmids are listed in Table 1.

The Bladder Model. The model used was originally designed for the purpose of studying the treatment of bacterial cystitis and has been described in full elsewhere (Greenwood & O'Grady, 1978). Like the human bladder, the model is a semi-continuous culture system in which the contents are continuously diluted by fresh 'urine' and are discharged (micturated) at intervals. The system is fully automatic, so that dilution and 'micturition' can be controlled precisely for prolonged periods. Bacterial growth in the bladder model was continuously monitored by measuring the turbidity of the culture using a photometer and a potentiometric pen-recorder.

Media. Oxoid Isosensitest Broth was used as 'urine' in the bladder model. This has been found to be a good substitute for human urine and alleviates problems caused by precipitates, storage, sterility and stability (Greenwood & O'Grady, 1978).

Oxoid DST Agar was chosen as the basis for the selective media used. Antibiotics

Table 2. *Variable parameters of the model*

1. Bladder conditions:
   (*a*) rate of delivery of urine.
   (*b*) interval between micturitions.
   (*c*) residual volume after a micturition.
2. The point in the dilution/micturition cycle at which the donor cells were added.
3. The time(s) at which the bladder contents were sampled.
4. The length of time for which the donor and recipient cells were grown together.
5. The transfer characteristics of the plasmid used.

(obtained from standard commercial sources) were added to DST, at the concentrations listed below, in appropriate combinations to distinguish between different bacterial strains with or without a plasmid: streptomycin sulphate, 200 μg/ml; trimethoprim lactate, 50 μg/ml; tetracycline hydrochloride, 10 μg/ml; rifampicin, 100 μg/ml.

When trimethoprim lactate was included in a selective medium, 4% lysed horse blood was also added.

Basic experimental design. The basic design of the experiment involved growing CSH1 (Table 1) as the recipient strain in the bladder model until a steady state was reached. This usually took about 16 h. 0·1 ml of the plasmid-carrying donor J53.2 (Table 1) was then added and the dilution/micturition cycle continued. The bladder contents were sampled at intervals to determine the types of cell present (i.e. donor or recipient, with or without a plasmid). This was done by spreading dilutions onto plates of the appropriate selective media. After 24 h incubation at 37 °C, the number of colonies on the plates was counted using a Chiltern CC50 colony counter. Counts were done in duplicate and mean values calculated. These values were then used to calculate the numbers of each type of cell in the original culture.

The parameters which it was possible to vary in this basic experiment are listed in Table 2; however, for the experiments described in this paper the 'urine' flow rate was maintained at the normal day-time value of 1 ml/min.

RESULTS

Maintenance of a plasmid in the bladder under non-selective conditions

To check that carriage of a plasmid was not disadvantageous to a bacterial cell when growing in the bladder model, a culture of J53.2 carrying the plasmid R388 (Table 1) was grown in the bladder overnight. During this initial period broth containing 10 μg/ml trimethoprim lactate was used as the 'urine' to ensure that only plasmid-carrying cells could grow. When a steady state had been reached, the 'urine' was changed to normal trimethoprim-free broth and the dilution/micturition cycle continued for a further 24 h. At the end of this time the bladder contents were sampled and appropriate dilutions plated out onto agar containing rifampicin only (to select for J53.2) and incubated overnight. One hundred randomly-selected colonies were subcultured onto agar containing trimethoprim. Two of the 100 colonies failed to grow (i.e. 2% of the colonies sampled had lost R388).

Table 3. *Effect on plasmid transfer of varying the physical conditions in the bladder model*

Micturition interval (h)	Residual volume (ml)	Transfer* after 6 h	Transfer* after 24 h
1	20	$2{\cdot}0 \times 10^{-5}$	$5{\cdot}0 \times 10^{-3}$
1	1	$6{\cdot}2 \times 10^{-5}$	$1{\cdot}7 \times 10^{-2}$
2	20	$5{\cdot}7 \times 10^{-7}$	$2{\cdot}7 \times 10^{-3}$
2	1	$5{\cdot}1 \times 10^{-6}$	$8{\cdot}0 \times 10^{-3}$

* Transfer is expressed in terms of progeny/ml divided by total recipients/ml.

Effect on plasmid transfer of varying the physical conditions in the bladder model

For these experiments the micturition interval and residual volume in the bladder model were varied. The normal interval between micturitions is four hours or longer, but this decreases during urinary tract infection. As the 'bladder' was too small to hold four hours' 'urine', intervals of one and two hours were chosen.

The volume of urine remaining in a normal healthy bladder after micturition has been reported to be about 1 ml (Shand *et al.* 1968), but this may increase a great deal with disease (Shand *et al.* 1970). Therefore, for these experiments, residual volumes of 1 ml and 20 ml were used.

Although other workers in the field of plasmid transfer have expressed their results in terms of numbers of progeny per ml, this seemed unsuitable for experiments using the bladder model in which the actual concentration of organisms per ml varied during the dilution/micturition cycle. It was therefore felt that the clearest way to present the results in this and subsequent experiments was to express the amount of plasmid transfer as the number of progeny divided by the total number of recipients. Implicit in this method was the assumption that the total number of recipients at the end of each cycle remained constant. Viable counts showed that this was the case. It was also of interest that the small number of donor cells added at the beginning of each experiment also remained constant and hence no change in the relative proportion of original donor and recipient cells occurred during the course of the experiments.

Results obtained using the different combinations of conditions are summarized in Table 3. Although there was some variation between the 6 h results obtained, it seemed that after 24 h the plasmid used (R388) was successfully established in the bladder in all four experiments.

Rate of plasmid transfer with respect to time

The experiment was carried out using R388, the shorter micturition interval of one hour and the smaller residual volume of 1 ml. The original donor cells were introduced into the 'bladder' immediately after a micturition. Results obtained are depicted in Fig. 1. The progeny obtained at zero time represent the result of matings on the surface of the agar plate. Within two hours of adding the donor culture, the rate of increase in the number of resistant recipient cells became exponential, only beginning to slow down 10 h after the addition of the donor cells.

The experiment was repeated, adding the donor cells immediately before a

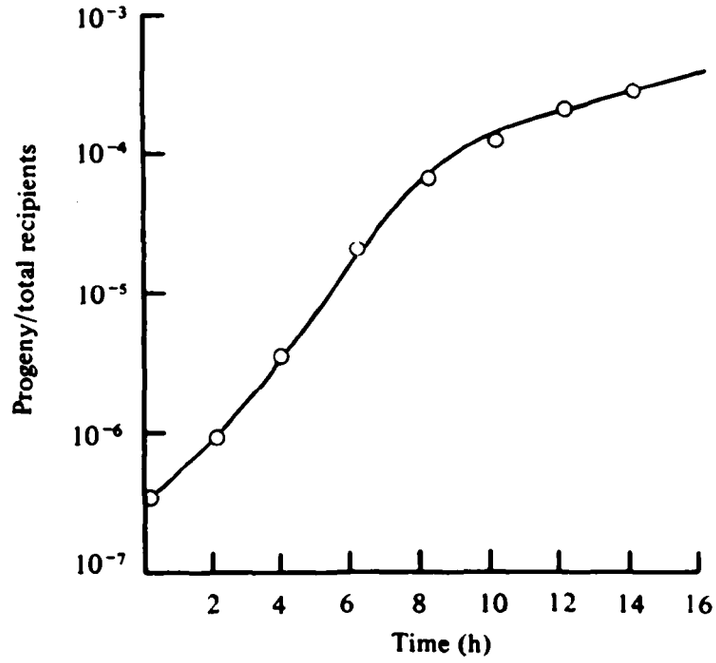

Fig. 1. Rate of transfer of R388 in the bladder model. The dilution rate was 1 ml/min, the micturition interval 1 h and the residual volume 1 ml.

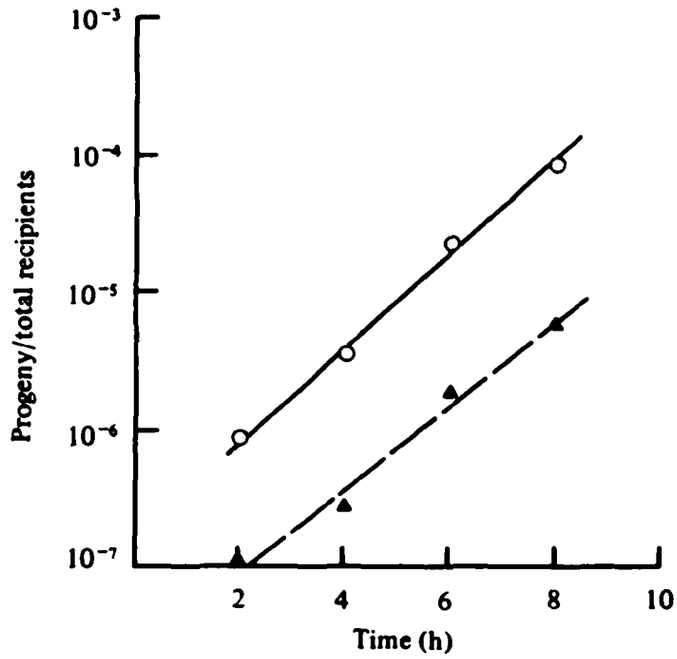

Fig. 2. Effect of stopping donor-to-recipient transfer by the addition of streptomycin. The experiment was carried out using plasmid R388, a dilution rate of 1 ml/min, a micturition interval of 1 h and a residual volume of 1 ml. ○——○, no streptomycin or streptomycin added after 1 or 2 h; ▲——▲, streptomycin added immediately after donor cells.

micturition. No significant difference in the amount of plasmid transfer was observed.

Effect of stopping donor-to-recipient transfer

For these experiments the donor cells (J53.2) were killed by the addition of streptomycin, to which the recipient cells (CSH1) were resistant. This stopped further donor-to-recipient transfer, although any transfer event that was in

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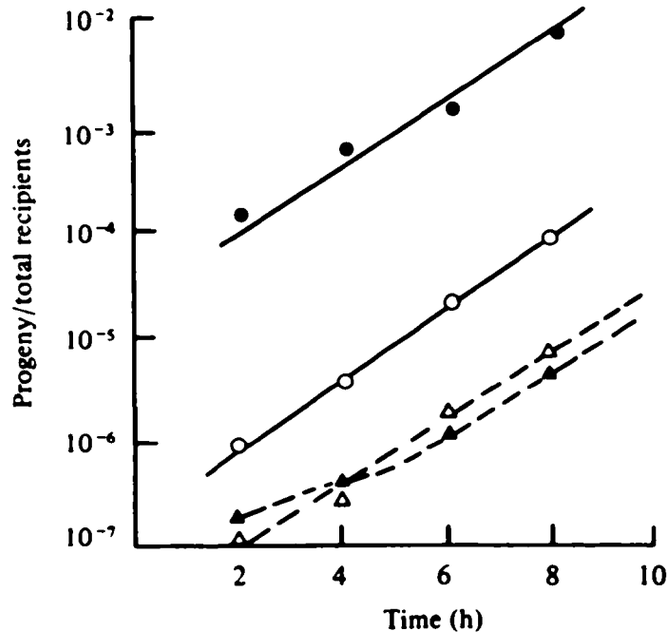

Fig. 3. Comparison between plasmids R388 and R386. The dilution rate was 1 ml/min, the micturition interval 1 h and the residual volume 1 ml. ○——○, R388, no streptomycin; △- - -△, R388, streptomycin added at zero time; ●——●, R386, no streptomycin; ▲---▲, R386, streptomycin added at zero time.

progress at the time of addition of the antibiotic could still continue, and re-transfer from initial progeny to further recipients was unaffected. By changing the 'urine' to Isosensitest broth containing 200 μg/ml streptomycin sulphate at different times and comparing the different patterns of transfer, it was possible to assess the relative importance of donor-to-recipient and recipient-to-recipient transfer at different times. Results obtained are depicted in Fig. 2. The amount of plasmid transfer appeared to be unaffected by adding streptomycin either one or two hours after introduction of the donor cells, but adding the antibiotic immediately after the donors significantly reduced the amount of transfer.

Effect of using a derepressed plasmid

In all of the previous experiments a repressed plasmid, R388, was used. In order to examine whether any of the characteristics of the transfer pattern were due to the transfer characteristics of the particular plasmid used, the experiments with and without the addition of streptomycin were repeated using the derepressed plasmid R386. Results obtained are depicted in Fig. 3. When streptomycin was added immediately following the addition of the donor cells, similar results were obtained for both plasmids. However, in the absence of streptomycin, R386 gave a higher overall amount of transfer than R388, but the rate of transfer (as measured by the slope of the graph) was the same.

Comparison with a static system

It was decided to repeat some of the 'bladder' experiments using a static system to discover whether the bladder model gave fundamentally different results. The experiments repeated were those investigating plasmid transfer with and without

the addition of streptomycin immediately after introducing the donor cells. For each of these experiments a culture of the recipient was grown in Isosensitest broth until close to the end of the exponential stage of growth (to mimic a recipient culture which had reached stability in the bladder just after a micturition). An overnight culture of donor cells was then added to give the same proportions of donors and recipients as in the bladder model. The actual amounts used were, of course, larger to allow for sampling as, unlike the bladder, no more broth was being added.

In spite of the fact that, apart from the initial stages of the experiment, the cells were in the early stationary phase of growth, it was found that both with and without the addition of streptomycin, no significant difference in the amount of plasmid transfer was observed between the bladder model and the static system. Thus, even in conditions where cells are not actively growing, it seems that exponential spread of a transferable R-plasmid through a population will still occur.

DISCUSSION

Although it has often been stated (e.g. Chabbert, Baudens & Bouanchaud, 1969; Anderson, 1974; Lacey, 1975) that plasmid carriage can adversely affect bacterial growth under non-selective conditions, this has been shown to only occur significantly with limiting environmental conditions. Melling, Ellwood & Robinson (1977) and Wouters, Rops & Van Andel (1978) showed that plasmid-carrying strains were at a disadvantage under conditions of phosphorus limitation, while Dale & Smith (1979) obtained similar results for oxygen limitation. Isosensitest broth is sufficiently rich in phosphate and growth conditions were probably sufficiently aerobic for neither of these limiting factors to be involved in the experiments described in this paper. Thus, only 2 % of the cells of a plasmid-carrying culture had lost their plasmid after 24 h growth in the bladder model. As the majority of the experiments were carried out over a shorter period of time, this small amount of plasmid loss was unlikely to have significantly affected the results.

Successful establishment of a plasmid in the recipient culture occurred when only a relatively small number of donor cells had been introduced, even in the absence of any antibiotic therapy. Thus, in the model, only a small number of cells carrying this type of antibiotic resistance were necessary for the development of an antibiotic-resistant bacterial cystitis. The proportion of recipient cells which acquired a plasmid was surprisingly constant, regardless of the micturition interval or the residual volume. Thus the physical conditions prevailing in the bladder affected the end result of plasmid transfer very little.

Plasmid spread amongst recipient cells became exponential within two hours of the introduction of the donor culture. This implies that within this time, transfer from initial progeny (i.e. original recipients) to further recipients had become more important than continued transfer from original donors to recipients (which would give a linear increase). When streptomycin was used to stop donor-to-recipient transfer after one or two hours, no significant difference was observed. However, when streptomycin was added immediately after the introduction of the donor cells, transfer was reduced. Thus it seemed that donor-to-recipient transfer was only important within the first hour, after which exponential transfer amongst the

recipient population was far more important in the spread of resistance. Thus only a short period of superinfection with a resistant culture is necessary for spread of resistance to occur. This was true of both actively multiplying and early stationary phase cultures.

Using the derepressed plasmid R386, it was shown that when streptomycin was added immediately after the introduction of the donor cells, the pattern of transfer was similar to that obtained for the repressed plasmid R388. When no streptomycin was added, the transfer *rate* was the same as for the repressed plasmid, but the actual *amount* of transfer in a given time was much greater (by a factor of about 10^2). This was consistent with previous work, in that repressed plasmids are known to be derepressed when newly introduced into recipient cells, while for established repressed plasmids, only a small proportion of the population can engage in plasmid transfer (Stocker, Smith & Ozeki, 1963; Falkow, 1975). From the results obtained, it appeared that about 10^{-2} of the original donor cells infected with R388 could transfer it at any given time.

Experiments with a static system gave results very similar to those obtained with the bladder model. Thus, unlike plasmid spread in the gastrointestinal tract (Smith, 1969), it appears that the spread of a plasmid through a bacterial population in urinary tract infection (at least in so far as urinary infection is mimicked by this model), is quite similar to the situation observed with *in vitro* static systems.

The clinical importance of these results is that it should be expected that if a mixed urinary tract infection occurs with a drug-sensitive strain and a strain carrying a transferable R-plasmid, it is likely that the R-plasmid will become established in the originally sensitive population, even when the originally resistant cells form a minority of the infecting population and when no antibiotics are being used. An initially greater proportion of resistant cells, or the use of an antimicrobial agent to which the cells are resistant, will only ensure that this process occurs more rapidly, leading to the development of a fully resistant population before natural clearance mechanisms, helped by antibiotic therapy, have any opportunity to take effect.

We are greatly indebted to Dr D. Greenwood for his advice on the setting-up and use of the bladder model.

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