Spontaneous deletions of drug-resistance determinants from *Salmonella typhimurium* in *Escherichia coli*

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Summary. Plasmids isolated from two different clinical isolates of *Salmonella typhimurium*, both resistant to the antibiotics ampicillin, tetracycline, streptomycin and chloramphenicol, were used to transform *Escherichia coli*. Segregation of antibiotic-resistance determinants occurred in both cases. Analysis of plasmids from one set of segregants by DNA-DNA hybridisation indicated that the segregation was due to precise deletions in the transforming plasmid.

Introduction

One aspect of R-plasmid-mediated spread of antibiotic resistance amongst enterobacteria which has remained enigmatic is the mechanism or mechanisms by which R factors evolve during their dissemination. Although the emergence of multi-resistant *Salmonella typhimurium* in Britain correlated with the prophylactic use of antibiotics in cattle feed, no such obvious reason can be adduced to explain the appearance of plasmid-borne multi-resistance in *S. typhimurium* strains that have caused several outbreaks of human salmonellosis in India in recent years (Sood and Basu, 1983). One attractive hypothesis is that R plasmids are acquired from a repository in commensal bacteria in response to antibiotic therapy, as has been suggested to have caused the appearance of antibiotic resistant *S. typhi* (Datta *et al.*, 1981; Threlfall *et al.*, 1982). Threlfall *et al.* (1982), however, found that the spectrum of resistances encoded in the plasmid acquired by *S. typhi* during antibiotic therapy was different from that of a multi-resistant *S. typhimurium* strain which was co-isolated. They suggested that intestinal enterobacteria may have been the sources of R factors, a not unreasonable suggestion in view of the frequently reported opportunist infections by antibiotic-resistant *Escherichia coli* (Grüneberg and Bendall, 1979; Christie, 1980; Gross *et al.*, 1983) and the well documented spread of R plasmids across generic barriers (Datta *et al.*, 1980; Tompkins *et al.*, 1980). In the present report we describe experiments to show whether transfer

of an R plasmid from a multi-resistant isolate of *S. typhimurium* into *E. coli* K12 may result in plasmid instability leading to an altered plasmid genotype.

Materials and methods

Bacterial strains

S. typhimurium strains 993 and 16408 were clinical isolates obtained from Professor Srinivasan, Department of Microbiology, All India Institute of Medical Sciences, New Delhi. *E. coli* K12 strain ED 8654 (*met⁻ rk⁻ mk⁺ supE trpR⁻*) was obtained from Dr D. Botstein, Massachusetts Institute of Technology, Cambridge, MA, USA and *E. coli* K12 strain DH1 (*endoI⁻ recA⁻ rk⁻ mk⁻ Nal^R*) from Dr B. Hohn, Friedrich Miescher Institute, Basel, Switzerland.

Plasmid DNA preparation

Cleared lysates were prepared from bacteria as described by Clewell and Helinski (1970). Protein was removed from the lysates by two extractions with phenol saturated with Tris-EDTA (TE) (0.01 M Tris-HCl, pH 7.6; 0.001 M EDTA) followed by two extractions with chloroform. The DNAs present in the lysate were precipitated with ethanol and dissolved in TE before electrophoresis or transformation.

Transformation

E. coli strain ED 8654 was transformed with *S. typhimurium* plasmid DNA, as described by Maniatis *et al.* (1982). Transformants were selected on tryptone-agar plates containing ampicillin 50 µg/ml, streptomycin 50

$\mu\text{g/ml}$, tetracycline 20 $\mu\text{g/ml}$ or chloramphenicol 20 $\mu\text{g/ml}$.

Probe DNAs

Plasmids pMS6 and pMS7 were radiolabelled with [$\alpha^{32}\text{P}$]dCTP by nick-translation (Maniatis *et al.*, 1982).

Southern blot hybridisation

Plasmid DNAs were separated by electrophoresis in 0.7% agarose gels at 3 V/cm for 5 h and, after denaturation, transferred on to nitrocellulose filters and hybridised with ^{32}P -labelled probe for 18 h (Maniatis *et al.*, 1982). The filters were washed at room temperature with double strength SSC (SSC contained 0.15M NaCl, 0.01M sodium citrate), containing sodium dodecyl sulphate 0.1% ($2 \times \text{SSC}$, 0.1% SDS) followed by washing with $2 \times \text{SSC}$, 0.1% SDS at 68°C for 2 h to increase the stringency of hybridisation. The filters were dried and autoradiographs prepared by exposing the dried filters to X-ray film for 24 h at -20°C before the films were developed.

Results

S. typhimurium strains 993 and 16408, both resistant to ampicillin, tetracycline, streptomycin and chloramphenicol, harboured plasmids (fig. 1). *S. typhimurium* strain 16408 (lane 2) harboured a single plasmid of mol. wt 12×10^6 . *S. typhimurium* strain 993 (lane 1) harboured two plasmids—a larger one similar in size to the plasmid of strain 16408 and a smaller one of mol. wt 5.8×10^6 . The two leading bands in lane 1 represent the covalently closed circular and open circular forms of the smaller plasmid.

Fig. 1. Agarose gel electrophoresis of plasmids isolated from *S. typhimurium* strains 993 (lane 1) and 16408 (lane 2), (a) large plasmids (mol. wt 12×10^6), (b) small plasmid (mol. wt 5.8×10^6), covalently closed circular form, (c) small plasmid open circular form.

Plasmid preparations from *S. typhimurium* strains 993 and 16408 were used separately to transform *E. coli* K12 ED 8654. The transformants were selected on tryptone-agar plates containing ampicillin, tetracycline, streptomycin or chloramphenicol. The transformants obtained with the plasmid from *S. typhimurium* strain 16408, which harboured a single plasmid, were expected to be resistant to all four antibiotics irrespective of the selecting antibiotic, whereas the segregation of resistance determinants in transformants with *S. typhimurium* strain 993 plasmid DNA preparations was expected to indicate the distribution of resistance determinants between the two plasmids present in this strain. Most transformants with plasmids from both *S. typhimurium* strains were resistant to all four antibiotics, indicating that, in both cases, all four resistance determinants were present on the same replicon. However, a considerable proportion of the transformants (20.4% and 32.2% respectively) showed segregation of resistance determinants (tables I and II). Although many of the possible combinations of resistance phenotypes were observed, there was a marked predominance of those that retained both ampicillin and tetracycline resistance. With transformation by *S. typhimurium* strain 993 plasmid DNA, resistance to all four antibiotics may have been the result of co-transformation with two plasmids carrying different resistance determinants, but no clear-cut segregation pattern consistent with the resistance determinants being present on different replicons was observed (table II). Thus, as well as the predominant phenotype, ApTcSmCp (resistance to ampicillin, tetracycline, streptomycin and chloramphenicol), various phenotypes (Ap, ApTc, ApTcCp, ApSmCp, SmCp and Sm) were observed amongst the segregants.

Agarose gel electrophoresis of plasmids isolated from different transformants revealed heterogeneity in their sizes (fig. 2). MS5 (Sm; lane 2), MS6 (SmCp; lane 3), MS10 (Tc; lane 4) and MS7 (Ap; lane 5), harboured plasmids of mol. wts (10^6) 1.7, 2.4, 0.8 and 2.8 respectively. It appeared that part of the original plasmid had been deleted in the segregants, the heterogeneity in sizes resulting from the deletions being of different sizes. To confirm that the loss of resistance determinants also resulted from these deletions in the transforming plasmid, plasmids from transformants *E. coli* MS6 (SmCp; fig. 2, lane 3) and *E. coli* MS7 (Ap; lane 5) obtained by transformation of *E. coli* ED 8654 with *S. typhimurium* strain 993 plasmid DNA, were radio-labelled by nick-translation and plasmid preparations from several segregants obtained after transformation with *S. typhimurium* strain 993

Table 1. Phenotypes of *E. coli* transformants obtained by transforming *E. coli* ED 8654 with the plasmid DNA from *S. typhimurium* strain 16408.

Selecting antibiotic	Number of transformant colonies of each phenotype obtained					
	ApTcCpSm	ApTcSm	ApTcCp	ApCpSm	ApTc	Sm
Ampicillin	29	1	0	1	5	0
Tetracycline	23	0	0	0	14	0
Chloramphenicol	24	0	1	0	0	0
Streptomycin	14	0	0	0	0	1

Ap, Tc, Cp, Sm = resistance to ampicillin, tetracycline, chloramphenicol and streptomycin respectively.

Table II. Phenotype of *E. coli* transformants obtained by transforming *E. coli* ED 8654 with plasmid DNA from *S. typhimurium* strain 993

Selecting antibiotic	Number of transformant colonies of each phenotype obtained							
	ApTcCpSm	ApTcSm	ApTcCp	ApCpSm	ApTc	CpSm	Ap	Sm
Ampicillin	20	0	1	1	4	0	24	0
Tetracycline	21	1	2	0	2	0	0	0
Chloramphenicol	29	0	3	0	0	0	0	0
Streptomycin	28	0	0	0	0	1	0	9

Abbreviations: see footnote to table I.

Fig. 2. Agarose gel electrophoresis of plasmids isolated from *S. typhimurium* strain 993 (lane 1) and from *E. coli* transformants MS5 (lane 2), MS6 (lane 3), MS10 (lane 4) and MS7 (lane 5). Lanes 6 and 7 contain plasmids pBR322 and ColE1 respectively. **Fig. 3.** Hybridisation of labelled DNA pMS6 (probe) with a Southern blot of the gel shown in fig. 2.

plasmid DNA were probed with the labelled DNAs.

Plasmid preparations from *E. coli* transformants MS5 (Sm; fig. 2, lane 2), MS6 (SmCp; lane 3), MS10 (Tc; lane 4) and MS7 (Ap; lane 5) were electrophoresed on two identical 0.7% agarose gels with a plasmid preparation from the parent *S. typhimurium* strain 993 (fig. 2, lane 1). Plasmids pBR322 (Bolivar and Backman, 1979), bearing ampicillin

and tetracycline resistance determinants, and ColE1, were also run on the same gel to serve as positive and negative controls respectively (fig. 2, lanes 6 and 7). The DNAs in the two gels were transferred to nitrocellulose (Southern blot) and the blots were hybridised separately with radiolabelled pMS6 (the plasmid from *E. coli* MS6) and pMS7 (the plasmid from *E. coli* MS7) used at a dose of $c. 10^6$ cpm/blot in stringent conditions, and autoradiographed (figs 3 and 4). As expected, pMS6

Fig. 4. Hybridisation of labelled DNA pMS7 (probe) with a Southern blot of the gel shown in fig. 2.

carrying streptomycin and chloramphenicol resistance determinants hybridised with its parent plasmid (the two bands in fig. 3, lane 1, corresponding to the two leading bands in fig. 2, lane 1), with the plasmid from *E. coli* MS5, resistant to streptomycin only (fig. 3, lane 2) and with itself (fig. 3, lane 3). Similarly pMS7, which conferred resistance to ampicillin, hybridised with the parent plasmid (fig. 4, lane 1), with itself (fig. 4, lane 5) and with pBR322 which harbours a β -lactamase determinant. Neither pMS6 (conferring streptomycin and chloramphenicol resistance) nor pMS7 (conferring ampicillin resistance only) hybridised with the plasmid preparation from *E. coli* MS10, a tetracycline-resistant segregant obtained after subculturing an ApTc segregant in the presence of tetracycline. The original ApTc segregant retained both resistance markers when grown in the presence of ampicillin but tended to become osmotically fragile; growth in the presence of tetracycline readily resulted in the loss of the ampicillin-resistance marker. Spontaneous mini-plasmid formation occurred in these strains (fig. 2, lane 4) and these tetracycline-resistant segregants were osmotically stable. There was thus a very good concordance between the resistance phenotypes and the hybridisation profile, with little or no cross-hybridisation. This indicated that the mechanism of segregation of the antibiotic-resistance determinants was the deletion of various discrete lengths of plasmid DNA. Furthermore pMS7, which is in the same size range as pBR322, appeared to harbour a β -lactamase determinant homologous with the TEM β -lactamase gene of pBR322.

Discussion

The absence of hybridisation between segregant

plasmids conferring different phenotypes seems to indicate a precise excision of resistance determinants rather than random deletions because in random deletions, rigid correspondence between hybridisation profile and phenotype would not be expected. That the deletions were indeed post-transformation events was indicated by the observation that the *E. coli* transformants, segregants as well as those which harboured all four resistance determinants, lost resistance determinants within a few generations unless an appropriate antibiotic pressure was applied, in contrast to wild-type *S. typhimurium* strain 993 which inherited all four resistance markers stably even in the absence of antibiotic pressure. This instability was also observed when the antibiotic-resistance-bearing plasmid from *S. typhimurium* strain 993 was transferred to *E. coli* DH1 by conjugation.

The instability of certain plasmids when transferred from *Salmonella* strains to *E. coli* has been observed previously (Anderson and Lewis, 1965). However the reverse observation, the instability of *E. coli* Inc FII plasmids such as R1, R6 and R100 in *S. typhimurium* (Watanabe *et al.*, 1964) or *Proteus* species (Morris and Rownd, 1974) is better documented. In this case, recombination between the insertion sequences IS 1 flanking the resistance determinants was shown to cause its separation from the resistance-transfer factor (Chandler *et al.*, 1977). A similar mechanism would account for the precise deletions taking place in *E. coli* transformed with *S. typhimurium* strain 993 R factor. Watanabe *et al.* (1980 and 1982) isolated a mutant with a function *dor* of *S. typhimurium* LT2 which caused deletions of plasmids resulting from legitimate recombination between the homologous sequences flanking resistance determinant-bearing transposons, but had no effect on illegitimate deletions taking place as a result of the transposition phenomenon. This mutation left Rec functions intact as judged by resistance to UV radiation. If the observed segregations of plasmids from *S. typhimurium* strains 993 and 16408 were due to legitimate recombination events in *E. coli* similar to that directed by *dor* in *S. typhimurium*, either a plasmid-encoded repressor for such recombination events effective in *S. typhimurium* but not in *E. coli*, or a difference in sensitivity of the plasmid to this function in the two species, may be postulated. There are reports which suggest that the plasmids R1-19 and R100 code for a repressor-like molecule that may interact with the *E. coli* gene product involved in recombination (Chernin and Ovadis, 1980). Furthermore, these plasmids are stable in *E. coli* and not in *S. typhimurium*, which has been

attributed to the *dor* gene function of *S. typhimurium* (Watanabe *et al.*, 1982).

Furthermore, because pMS6 (SmCp) and pMS7 (Ap) share no homology, and neither hybridises with pMS10 (Tc), multiple non-homologous replication origins may be present in the parent *S. typhimurium* plasmid; otherwise the shared replicator regions would have been expected to hybridise, assuming that these regions are not too small to be detected by hybridisation done under stringent conditions. Comparison of the mol. wts of the segregant plasmids conferring mutually exclusive phenotypes, pMS6 (1.7×10^6), pMS7 (2.8×10^6)

and pMS10 (0.8×10^6), with that of the parent *S. typhimurium* R factor plasmid (5.8×10^6) appears to admit this possibility.

Our transformation experiments have simulated conditions which may prevail in the gut during the course of antibiotic therapy in that a selective pressure must select resistant bacteria resulting from the interspecies transfer of plasmids, but the stability of plasmids may vary in different host environments. Transposability of genes from one replicon to another may have provided one method by which this problem may be avoided.

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