Genetic Instability of R Plasmids in Relation to the Shift of Drug Resistance Patterns in *Salmonella johannesburg*

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Observation of the resistance of *Salmonella johannesburg* to the six drugs ampicillin (A), streptomycin (S), tetracycline (T), chloramphenicol (C), kanamycin (K) and sulphadiazine (Su) was made over the 7 years from 1973 to 1979. Strains with ASTCKSu- and ASCKSu-resistance patterns predominated in the years 1973–1975 and 1976–1979, respectively. These resistances were found to be mediated by autotransferring plasmids belonging to the incompatibility group F_Ime. The ASTCKSu-resistance plasmids were unstable, giving rise to deletion variants at a much higher frequency than ASCKSu-resistance plasmids either of natural origin or derived *in vitro* from the ASTCKSu-resistance plasmids. Thus, the ASCKSu-resistance plasmid might be a deletion variant of the ASTCKSu-resistance plasmid. This is supported by the extensive similarity of their cleavage patterns produced by specific restriction endonucleases.

INTRODUCTION

The F_Ime group of plasmids of *Salmonella* was first described and defined in detail by Threlfall *et al.* (1976) and Anderson *et al.* (1977). These large single-copy plasmids conferred multiple antimicrobial drug resistance, including resistance to ampicillin and chloramphenicol, and were identified in *Salmonella* species isolated in many countries of the Middle East, as well as in North Africa, France and Britain. One characteristic of these plasmids was that the resistance determinants underwent spontaneous segregation. Thus, although the majority of the plasmids coded for resistance to ampicillin (A), streptomycin (S), tetracycline (T), chloramphenicol (C), kanamycin (K) and sulphadiazine (Su) in the host bacterial cells, others showed different combinations of drug resistance, e.g. ASCKSu, ACKSu, etc. Both autotransferring and non-autotransferring members of the F_Ime group have been found; the latter were also of the large single-copy type and were thought to be transfer-defective deletion mutants (Willshaw *et al.*, 1978).

Salmonella johannesburg was prevalent in Hong Kong from 1973 to 1977 following its introduction to this locality in 1972, but the frequency of isolations has subsequently declined. During this period we observed a dramatic change in the patterns of drug resistance, apparently not related to the use of antimicrobial drugs (Chau *et al.*, 1978). This communication describes the spontaneous evolution of drug resistance patterns coded for by F_Ime plasmids that have been identified in *S. johannesburg* during its emergence, prevalence and disappearance in a defined locality, Hong Kong, over a period of 7 years.

METHODS

Bacterial strains and plasmids. Strains of *S. johannesburg* were isolated in the paediatric wards of a general hospital in Hong Kong from 1973 to 1979. All the strains were stored in agar stabs after inoculation from single colonies.

The recipient strain used in transfer experiments (see below) was an *Escherichia coli* K12, plasmid-free, rifampicin-resistant mutant Jp995 (Lac⁺, prototroph, F⁻, λ^- , Rif^R) supplied by Professor A. J. Pittard, Department of Microbiology, University of Melbourne, Australia.

Reference plasmids for incompatibility grouping provided by Professor A. J. Pittard and by Dr R. W. Hedges, Royal Postgraduate Medical School, London, U.K., included representatives of the following groups (the name of the plasmid and its R type are given in parentheses): C (R40a, AKS), FI (R386, T), FII (R1-16, K), FIV (R124, T), H (R27, T), Ia (R144 drd 3, K), I γ (R621a, T), J (R391, K), K (R387, CS), M (R446b, ST), P (RP4, AKT), T (Rts1, K), V (R753, ACSSu), W (S-a, CKSSu) and X (R6K, AS). Plasmids from the collection of the Division of Enteric Pathogens included F-lac and F-T, the FIme plasmid TP243 (K) and the MP10 plasmid K-MP10 (Smith *et al.*, 1973).

Plasmids used for molecular size determination were supplied by Dr E. M. Lederberg, Plasmid Reference Center, Stanford University, Calif., U.S.A.

Tests for drug resistance and conjugation experiments. Tests for the susceptibility of bacterial strains to various antimicrobial drugs and for the transfer of drug resistance were performed as described previously (Chau *et al.*, 1978). After overnight matings, transconjugants were selected on MacConkey medium containing ampicillin (25 $\mu\text{g ml}^{-1}$) and chloramphenicol (25 $\mu\text{g ml}^{-1}$) while the donor strains were counterselected with rifampicin (25 $\mu\text{g ml}^{-1}$). Colonies appearing on the selective media were purified on the same medium and their drug susceptibility was tested. The frequency of transfer of resistance was taken as the ratio of transconjugant colony-forming units (c.f.u.) to recipient c.f.u.

Tests for the stability of plasmids. Each of the 12 plasmid-carrying bacterial strains randomly selected in this study (see Table 3) was inoculated into 3 ml nutrient broth and the culture was incubated for 24 h. A sample of 0.1 ml of a 10^{-5} dilution of the first overnight culture (regarded as the first subculture) was inoculated into 3 ml fresh nutrient broth, and this propagation was continued for 5 d. Discrete colonies from each subculture were obtained by plating 0.1 ml of a 10^{-7} or 10^{-8} dilution of the culture on Mueller-Hinton agar (Oxoid). From each subculture, 80–100 colonies were tested for antibiotic susceptibility by replica plating.

Incompatibility grouping. The R plasmid under study was transferred from *S. johannesburg* to *E. coli* Jp995, and the resulting strain was used as the recipient in matings with rifampicin-sensitive *E. coli* donor strains, each harbouring a plasmid of known incompatibility group. In cases when the drug resistance markers of an R plasmid under investigation overlapped with those of the reference plasmid, segregants of the plasmid lacking resistance markers of the reference plasmid were used in mating experiments. To select for transconjugants, the mating mixtures were plated on selective media containing rifampicin and a drug to which the incoming plasmid, but not the resident plasmid, specified resistance. Colonies appearing on selective media were purified by replating on similar media. At least 40 colonies were purified and tested for the presence of both the incoming and resident plasmids.

Phage typing. For phage typing studies, plasmids were transferred from *E. coli* K12 lines into standard strains of *Salmonella typhimurium* phage type 36 or *Salmonella typhi* Vi-phage type A. Colicin E2 was used for counterselection. The *S. typhimurium* transconjugants were phage typed by the method of Callow (1959) as extended by Anderson (1964) and the plasmid-carrying strains of *S. typhi* type A were phage typed by the method of Craigie & Felix (1947).

Gel electrophoresis of plasmid DNA. Plasmid DNA was extracted according to the method of Hansen & Olsen (1978). Electrophoresis was carried out in 0.9% (w/v) agarose slab gels (120 $\times$ 140 $\times$ 3 mm) at 50 mA for 2 h in a Tris/borate/EDTA buffer system (Greene *et al.*, 1974) as described by Willshaw *et al.* (1979).

Restriction enzyme digestion of plasmid DNA. Purified plasmid DNA was prepared according to the method of Humphreys *et al.* (1975). Digestion of purified plasmid DNA by restriction endonuclease *EcoRI* or *HindIII* was carried out at 37 °C for 30 min, with an appropriate amount of DNA and an excess of enzyme. The reaction mixture for *EcoRI* digestion contained 50 mM-NaCl, 100 mM-Tris/HCl pH 7.5, and 100 μg bovine serum albumin (BSA) ml^{-1} . For *HindIII* digestion, the reaction mixture contained 60 mM-NaCl, 70 mM-MgCl₂, 70 mM-Tris/HCl pH 7.4, and 100 μg BSA ml^{-1} . The reaction mixture was heated for 2 min at 65 °C before electrophoresis.

RESULTS AND DISCUSSION

Shift in the antimicrobial drug resistance patterns in S. johannesburg in Hong Kong

The predominant pattern of drug resistance in *S. johannesburg* changed over the period 1973–1979 (Table 1). During the first 3 years after this organism was introduced and became widespread in Hong Kong, 44–53% of the isolates were found to be resistant to six drugs: A, S, T, C, K and Su. In later years, however, most strains were resistant to A, S, C, K and Su, but susceptible to T. In addition, the number of strains with other patterns of drug resistance was dramatically reduced.

Table 1. *Alteration of predominant patterns of drug resistance in S. johannesburg*

Year ...	1973	1974	1975	1976	1977	1978	1979
Total no. of strains examined	63	51	124	118	26	12	3
No. of strains* with resistance pattern:							
ASTCKSu	28 (44)	27 (53)	59 (48)	5 (4)	0	0	0
ASCKSu	0	0	17 (14)	105 (89)	26 (100)	12 (100)	3 (100)
No. of strains* with other resistance patterns	35 (56)	24 (47)	48 (38)	8 (7)	0	0	0

* Figures in parentheses indicate percentages of the total number of strains examined in the year.

Table 2. *In vivo change of resistance patterns in S. johannesburg in individual cases*

The data in this table are from cases from which two or more isolates of *S. johannesburg* were made.

Resistance patterns of subsequent isolates when initial isolates were:			
ASTCKSu-resistant (38 cases)		ASCKSu-resistant (25 cases)	
Resistance pattern	No. of strains*	Resistance pattern	No. of strains*
ASTCKSu	64 (60.5)	ASTCKSu	2 (3.9)
ASCKSu	7 (6.6)	ASCKSu	48 (94.1)
ASTCSu	6 (5.7)		
ATCK	2 (1.9)		
TCSu	2 (1.9)		
ASTKSu	1 (0.9)		
TSu	11 (10.4)		
T	2 (1.9)		
ATSu	1 (0.9)		
ASSu	1 (0.9)	ASSu	1 (2.0)
ATSu	1 (0.9)		
ST	3 (2.8)		
ASKSu	5 (4.7)		
Total no. of subsequent isolates	106 (100)		51 (100)

* Figures in parentheses indicate percentages of the total number of subsequent isolates.

Alteration of resistance patterns in vivo

Since *S. johannesburg* was isolated repeatedly from a number of patients, changes in the resistance patterns of strains could be observed from individual cases (Table 2). When the first isolates were ASTCKSu-resistant, 40% of the subsequent strains showed up to 13 different patterns of resistance. However, from patients initially infected with an ASCKSu-resistant strain, only 6% of the subsequent strains showed different resistance patterns. Thus the resistance pattern ASCKSu appeared to be more stable *in vivo* than the pattern ASTCKSu.

Alteration of resistance patterns in vitro

The ASTCKSu-resistance plasmids could be transferred *in toto* from *S. johannesburg* to recipient *E. coli* strains at frequencies ranging from 2×10^{-3} to 1×10^{-5} in overnight matings at 37 °C. Of the transconjugants, 41% showed a variety of resistance patterns different from that of the donor. The plasmids carrying ASCKSu resistances could also be transferred *in*

Table 3. *Frequency of progeny colonies with different resistance patterns obtained during daily subculture of resistant S. johannesburg parent strains*

Strains were subcultured daily for 5 d and the resistance patterns of the progeny colonies were determined for each subculture (see Methods).

Resistance pattern of parent strain . . .	ASTCKSu (natural isolates)	ASCKSu (natural isolates)	ASCKSu (derived from ASTCKSu- resistant parent)
No. of strains tested	4	4	4
Progeny colonies with parental resistance pattern (%)	79.2	98.7	99.0
Progeny colonies with other resistance patterns (%)	20.8	1.3	1.0

toto to recipient *E. coli* strains. However, plasmid segregants showing various resistance patterns other than ASCKSu were obtained at a lower frequency of 17%. This suggested that during transfer plasmids carrying ASCKSu resistances were more stable than those carrying ASTCKSu resistances.

The stability of drug resistance markers was therefore compared during daily subculture of the ASTCKSu-resistant and ASCKSu-resistant strains of *S. johannesburg* (Table 3). Colonies becoming sensitive to one or more drugs were obtained at frequencies of 21% and 1.3%, respectively. Similar studies were performed on the ASCKSu-resistant progenies of the ASTCKSu-resistant parents. Progeny colonies showing different resistance patterns were obtained at a frequency of 1% (Table 3). This supported the hypothesis that plasmids carrying ASCKSu resistances were much more stable than those determining ASTCKSu resistances.

Incompatibility grouping of the R plasmids

Each of the eight plasmids in this study which coded for ASCKSu resistance and ASTCKSu plasmids that had spontaneously lost the T-resistance determinant were incompatible with F-lac and F-T and with the group FI plasmid R386, being completely displaced by the incoming standard plasmid. They were compatible with the other groups of plasmids mentioned in Methods. Thus it was concluded that these *S. johannesburg* plasmids were members of the FI complex.

Plasmids that originally coded for ASCKSu or ASTCKSu resistance but which had lost the K resistance marker were tested for compatibility with the non-autotransferring plasmid K-MP10 and with the FIme resistance factor TP243. Transconjugants were screened for the presence of the incoming and resident plasmids as described in Methods and both *S. johannesburg* plasmids were found to be incompatible with K-MP10 and with TP243. These plasmids were therefore assigned to the FIme subgroup on the basis of their incompatibility with the F factor and R386, and with K-MP10.

Phage typing studies

When ASCKSu or ASTCKSu plasmids were introduced into *S. typhimurium* type 36, lysis of the plasmid-carrying *S. typhimurium* transconjugants by 24 of the typing phages was reduced or abolished and the resultant pattern of phage lysis corresponded to that designated NC10 (Anderson *et al.*, 1977). Similarly, acquisition of these plasmids converted *S. typhi* type A, sensitive to all the Vi-typing phages, into type 44, which was sensitive only to phage 44. These restriction patterns in the respective hosts are typical of those produced by FIme plasmids and have previously been observed with FIme plasmids from *S. typhimurium* and *S. typhi* (Anderson *et al.*, 1977).

Fig. 1. Agarose gel electrophoresis of plasmid DNA in *E. coli* transconjugants. Tracks: 1, strain A (ASTCKSu-resistant); 2, strain B (ASCKSu-resistant); 3, strain C (ASTCKSu-resistant); 4, strain D (ASTCKSu-resistant); 5, strain D' (derived from strain D, ASCKSu-resistant); 6, strain E (ASTCKSu-resistant); 7, strain E' (derived from strain E, ASCKSu-resistant); 8, reference plasmid TP124 (120 Mdal); 9, reference plasmid TP181 (98.2 Mdal); 10, reference plasmid RA1 (86 Mdal).

Characterization of the R plasmids by agarose gel electrophoresis

Gel electrophoresis of plasmid DNA demonstrated the presence of a large plasmid in strains of *S. johannesburg* (results not shown) and in *E. coli* transconjugants which had acquired the drug resistance pattern of the donor. These R plasmids coding for ASCKSu or ASTCKSu resistance varied in size but were around 110 megadaltons (Mdal). Figure 1 shows the size estimation by agarose gel electrophoresis of plasmid DNA from *E. coli* transconjugants, with reference to plasmids of known molecular size.

In order to examine the molecular similarity of these R plasmids, they were subjected to enzymic cleavage by the restriction endonucleases *Eco*RI or *Hind*III, and the DNA fragments generated were separated in agarose gels. The two plasmids chosen for this study were prepared from an ASCKSu- and an ASTCKSu-resistant strain. The two plasmids produced very similar restriction patterns (Fig. 2), indicating their molecular relatedness.

The FIme plasmids described in this paper were similar to those found in *S. typhimurium* (Anderson *et al.*, 1977) and *S. wien* (McConnell *et al.*, 1979) in their resistance pattern, molecular size and genetic instability. The wide distribution of the FIme plasmid-bearing *S. typhimurium* strains of apparently clonal origin in the Middle East, North Africa and Great Britain has prompted Anderson and his colleagues to suggest that these plasmids might contribute to the communicability and virulence of the host bacterial cells, in addition to their drug resistance. Our finding of the prevalence of these plasmids in *S. johannesburg* and the dramatic increase in the incidence of *S. johannesburg* in Hong Kong might add further weight to their argument.

The primary purpose of this communication is to illustrate the change of resistance patterns in a given *Salmonella* serotype, which was evidently due to the instability of the

Fig. 2. Agarose gel electrophoresis of DNA fragments. Tracks 1 and 2 show *Eco*RI digests of a plasmid coding for ASTCKSu-resistance and ASCKSu-resistance, respectively. Tracks 3 and 4 show *Hind*III digests of the same plasmids as in Tracks 1 and 2.

ASTCKSu-resistance plasmid. This instability appeared to be associated with the presence of the T-resistance determinant, since its deletion from the four ASTCKSu resistance plasmids rendered it genetically more stable (Table 3). The reason for the initial predominance of the ASTCKSu-resistance plasmid was unclear since it was apparently not very stable. We suspect it was a newly evolved plasmid, formed, for example, by integration of two plasmids into one. The ASCKSu-resistance plasmids apparently evolved from the ASTCKSu-resistance plasmids. Their molecular similarity indicated by restriction enzyme digestion and their belonging to the same F1me group support this view.

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