***Salmonella* plasmids of the pre-antibiotic era**

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In order to provide a profile of the plasmid gene pool in *Salmonella* prior to the clinical use of antibiotics, a molecular genetic analysis was made of plasmids in strains collected by E. D. G. Murray between 1917 and 1954. These pre-antibiotic era (PAE) salmonellae contain conjugative plasmids of the same incompatibility groups as contemporary enterobacterial plasmids. Upon analysis of total plasmid content, 42 plasmids, sized between 23 and 72 MDa, were found. We defined and investigated six groups of these PAE *Salmonella* plasmids in terms of three groups of genes; those involved in plasmid maintenance and incompatibility, DNA repair and virulence. Of the five groups, three were replicon-typed to groups IncI1, IncX and IncFII; one group exhibited no homology to contemporary Inc/Rep probes, and one group represented virulence plasmids containing a common plasmid-partitioning locus. The results indicated that most of the PAE groups were progenitors of contemporary R-plasmids, except for the virulence plasmids, which have generally not evolved as vectors of antibiotic resistance.

Introduction

Pathogenic enterobacteria from human infections of wide geographical distribution between 1917 and 1954 were collected by E. D. G. Murray and were preserved in sealed glass tubes on agar slants. The majority of this Murray collection of pre-antibiotic era (PAE) bacteria consisted of *Salmonella* (279) and *Shigella* (177 isolates), with small numbers of *Escherichia*, *Klebsiella* and *Proteus* strains. When opened in 1980 about 70% were found to be viable. These unique clinical isolates represent bacterial genera in whose contemporary exemplars plasmid-coded multiple antibiotic resistance is widely prevalent and of major clinical significance.

In a study of the PAE plasmids in the Murray Collection, Hughes & Datta (1983) reported genetic analysis of transferable plasmids among over 400 PAE *Enterobacteriaceae*. Their analysis was made by testing co-transfer of two small indicator plasmids, which are themselves non-conjugative but mobilizable. Of the PAE isolates 24% showed ability to mobilize one or both of the indicator plasmids. Among 84 PAE plasmids transferred to *E. coli* K12, 47 were cryptic (mol. mass estimates 25–70 MDa), 36 determined colicinogeny (mol. mass 55–60 MDa) and one encoded tellurite resistance (mol. mass 100 MDa). None of the transfer-

able PAE plasmids encoded resistance to antibiotics. Datta & Hughes (1983) subsequently labelled these PAE plasmids with the transposons Tn5, Tn7 or Tn9, and tested them for incompatibility with plasmids of the 21 classical incompatibility groups genetically defined (Datta, 1979) since 1971. Their fundamental finding was that conjugative plasmids of the same Inc groups existed in enterobacteria before and after the medical use of antibiotics. Thus the evolution of R-plasmids occurred by insertion of new resistance genes into a pre-existing enterobacterial plasmid gene pool, rather than by the selected dissemination of R-plasmids which existed prior to the introduction of antibiotics.

The aim of this study was a molecular characterization of the plasmid gene pool present in salmonellae prior to the dissemination of transferable antibiotic resistance and also the provision of a point of reference for comparison with current salmonellae. The type of genetic determinants one might expect to find in these progenitors of contemporary R-plasmids would determine such functions as plasmid maintenance and transfer, gene rearrangement and acquisition systems (e.g. the 'integron'; Grinsted *et al.*, 1990), DNA repair systems, colicins (Hughes & Datta, 1983) and virulence. We report the molecular characterization of both conjugative and non-conjugative plasmids present in PAE *Salmonella* strains which were previously shown genetically to contain a conjugative plasmid. This

Abbreviation: PAE, pre-antibiotic era.

complements and completes the original reports (Hughes & Datta, 1983; Datta & Hughes, 1983). These archetypal plasmids were further examined for the presence of partition loci, mutagenic DNA repair genes and mouse-virulence genes. The original identifications of the PAE *Salmonella* strains themselves were also re-examined.

Methods

Bacteria, media, culture conditions. PAE strains of salmonellae are listed in Table 1 (a), with their isolation dates and the classically derived incompatibility group of the conjugative plasmids isolated from them. They were serotyped by standard methods (Kauffmann, 1972). Where appropriate they were phage-typed by the methods of Ward *et al.* (1987)

Table 1. *Bacterial strains and DNA probes*

(a) PAE salmonellae					
Murray no.*	Original identification	Correct designation (where appropriate)	Year of collection†	Conjugative Inc group	Plasmid phenotype
M131	<i>S. anatum</i>	<i>S. minneapolis</i>	1938	II	ColI
M149	<i>S. typhimurium</i>		1935	II	ColIa
M476	<i>S. montevideo</i>		1939	II	ColIb
M102	<i>Salmonella</i> sp.	<i>S. typhimurium</i>	1941	II	ColIb
M140	<i>S. typhimurium</i>		1935	II	ColIb
M303	<i>S. typhimurium</i>		1941	II	ColIb
M283	<i>S. typhimurium</i>		1941	II	ColIb
M301	<i>S. typhimurium</i>		1941	II	ColIb
M311	<i>S. typhimurium</i>		1941	II	ColIb
M313	<i>Salmonella</i> sp.	<i>S. typhimurium</i>	1941	II	ColIb
M496	<i>Salmonella</i> sp.	<i>S. typhimurium</i>	1941	II	ColIb
M498	<i>Salmonella</i> sp.	<i>S. typhimurium</i>	1941	II	ColIb
M501	<i>Salmonella</i> sp.	<i>S. typhimurium</i>	1941	II	ColIb
M503	<i>Salmonella</i> sp.	<i>S. typhimurium</i>	1941	II	ColIb
M269	<i>S. oslo</i>		1938	II	Col ⁻
M141	<i>S. virchow</i>		1938	II	Col ⁻
M415	<i>Salmonella</i> sp.	<i>S. paratyphi</i> B	1940	FII	ColB
M443	<i>S. bisebjerg</i>		1938	FII	drd
M444	<i>S. senftenberg</i>		1938	FII	drd
M274	<i>S. potsdam</i>		1938	N	
M204	<i>S. enteritidis</i>		1938	X	
M273	<i>S. moscow</i>		1938	X	
M436	<i>S. typhimurium</i>	<i>S. paratyphi</i> C	1931	X	
M46	<i>S. typhi</i>		1939	X	
M507	<i>S. typhi</i>		1937	X	
M21	<i>S. typhimurium</i>		1917	X	
M25	<i>Salmonella</i> sp.	<i>S. choleraesuis</i>	1926	X	
M581	<i>S. typhi</i>		1935	X	
M155	<i>S. typhimurium</i>		1938	X	
M506	<i>S. eastbourne</i>		1938	Unclassed	
M527	<i>Salmonella</i> sp.	<i>Enterobacter</i> sp.	1940	Unclassed	
M530	<i>Salmonella</i> sp.	<i>Enterobacter</i> sp.	1940	Unclassed	
(b) DNA probes					
Probe	Fragment/size (kb)	Plasmid of origin	Cloned in:	Reference	
FIA	<i>Eco</i> RI/0.9	F	pULB2154	Couturier <i>et al.</i> (1988); NCTC 50547	
FIB	<i>Pst</i> I/1.2	P307	pULB2404	Couturier <i>et al.</i> (1988); NCTC 50549	
FIC	<i>Eco</i> RI- <i>Hind</i> III/0.97	P307	pULB2440	Couturier <i>et al.</i> (1988); NCTC 50550	
FIIA	<i>Pst</i> I/0.5	R1drd-19	pULB2401	Couturier <i>et al.</i> (1988); NCTC 50551	
II	<i>Eco</i> RI- <i>Pst</i> I/1.1	R64drd-11	pULB2428	Couturier <i>et al.</i> (1988); NCTC 50553	
N	<i>Eco</i> RI/1.0	R46	pULB2406	Couturier <i>et al.</i> (1988); NCTC 50555	
X _{R6K}	<i>Hind</i> III/0.9	R6K	pULB2405	Couturier <i>et al.</i> (1988); NCTC 50545	
X _{R485}	<i>Eco</i> RI- <i>Taq</i> I/0.59	R485	pDS49	Stalker & Helinski (1985)	
par	<i>Eco</i> RV/1.9	pStSR100	pYA22027	Tinge & Curtiss (1990a)	
VirA	<i>Eco</i> RI/4.1	pIP1350	pIP1367	Norel <i>et al.</i> (1989a)	
VirB	<i>Cl</i> A/2.9	pIP1350	pIP1366	Norel <i>et al.</i> (1989a)	
impCAB	<i>Eco</i> RV/1.75 and <i>Eco</i> RI- <i>Eco</i> RV/1.0	TP110	pKG10	Glazebrook <i>et al.</i> (1986)	
mucAB	<i>Hinc</i> II/1.1 and 0.9	pKM101	pIC80	Herrera <i>et al.</i> (1988)	

* Ordered according to the incompatibility group of the conjugative plasmid identified in the host strain by Hughes & Datta (1983).

† Year of addition into Murray Collection – usually the date of isolation but a few had been isolated earlier.

for *S. enteritidis*, Callow (1959) for *S. typhimurium*, Cragie & Felix (1947) for *S. typhi* and Chambers *et al.* (1987) for *S. virchow*. *Salmonella* and *Escherichia coli* strains were stored on nutrient agar slopes at room temperature and grown on nutrient agar/broth at 37 °C for 18 h. Antibiotics (Sigma) were added to media for growth of DNA probe strains, at the following concentrations ($\mu\text{g ml}^{-1}$): ampicillin (100), chloramphenicol (25), tetracycline (10).

Plasmid analysis and DNA hybridization. PAE *Salmonella* strains were screened for plasmid content by the method of Kado & Liu (1981), and plasmids were visualized by electrophoresis on double-origin, horizontal 0.7% agarose gels. Gels were poured with combs spaced 6 cm apart permitting 36 samples to be run simultaneously (see Fig. 1a). Agarose gels were vacuum-blotted (Pharmacia-LKB Vacu-Gene apparatus) onto Hybond nylon membrane (Amersham). Plasmid DNA for preparation of probe fragments was extracted from 500 ml nutrient broth cultures by cleared lysis and caesium chloride/ethidium bromide density gradient purification (Maniatis *et al.*, 1982). DNA probes were digested with the enzymes reported in Table 1(b) (Gibco-BRL, used according to manufacturer's instructions). Restriction fragments were separated on 1% low melting point agarose (Gibco-BRL). They were labelled with digoxigenin-11-dUTP (Boehringer) by random-primed synthesis in agarose. Membrane filters were hybridized at 68 °C in $5 \times \text{SSC}$, washed (final wash in $0.1 \times \text{SSC}$ at 55 °C), and colour-developed according to Boehringer instructions. DNA probe fragments used in this study are listed in Table 1(b).

Results

Serotyping and phage-typing

Thirty-two strains of PAE *Salmonella* were examined. Following new biotyping and serotyping analysis, two of these strains (M527 and M530) were transferred to the genus *Enterobacter* and two were assigned to different serovars: *S. typhimurium* M436 as *S. paratyphi* C and *S. anatum* M131 as *S. minneapolis*. Another eight strains formerly identified only as *Salmonella* sp. were assigned to the serovars *S. typhimurium*, *S. paratyphi* B and *S. choleraesuis* (see Table 1). The phage types, where known, are given in Table 2.

Plasmid characterization: general considerations

Plasmid detection and yield by the procedure of Kado & Liu (1981) was not noticeably different for the PAE strains from results with contemporary salmonellae (our unpublished observations). Forty-two plasmids in all were detected; among these only two plasmids showed consistently low yield. A 25 MDa IncX plasmid of *S. typhi* gave an undetectable low yield in many agarose gel experiments (faint lower plasmid band in track 30 of Fig. 1a) but nonetheless hybridized with DNA probes. A 24 MDa IncFII plasmid of *S. seftenberg* was visible in agarose gels (track 21, Fig. 1a). Other *Salmonella* plasmids, detected reproducibly, varied in size between 23 MDa and 72 MDa ('cryptic' plasmids of *S. potsdam*

M274). With respect to size classes, the largest was the 65 MDa class, all but four of which were present in *S. typhimurium*. The next largest was the 25 MDa class, spread between serovars *S. choleraesuis*, *S. enteritidis*, *S. moscow*, *S. typhimurium* and *S. typhi*. All other size classes (Table 2) had only one or two representatives. Twenty PAE *Salmonella* strains contained only a single plasmid. It is noteworthy that of the twenty such strains, only one was a (mouse) virulence plasmid (*S. paratyphi* C M436), whilst that of the others belonged to other plasmid types (twelve IncII, four IncX, two IncFII and one unclassified exemplar).

The other ten strains contained two plasmids. In four serovars (*S. enteritidis*, *S. moscow*, *S. typhi*, *S. typhimurium*) IncX and virulence plasmids co-existed. In *S. typhimurium* IncII plasmid(s) co-existed with either virulence plasmids (two strains) or IncFII plasmids (two strains).

In order to correlate the hybridization data and the classical Inc-typing results of Datta & Hughes (1983), the primary *E. coli* K12 transconjugants containing conjugative *Salmonella* plasmids were also analysed (data not shown). The sizes of the transferred plasmids were compared to plasmids in the PAE salmonellae (Table 1a) by running samples side-by-side in agarose gel tracks. All strains, M131 to M506, were shown to contain a plasmid of equivalent molecular mass. In cases where more than one plasmid co-existed, the original conjugative plasmid was thereby identified. These are indicated by asterisks in Table 2.

The overall approach was to order the strains as in Table 1(a), viz. according to the previously reported presence of a conjugative plasmid of known Inc group. This order was preserved in all gel analyses (see Fig. 1a) and their related hybridization experiments (Figs 1b, 2a, b). The thirteen DNA probes defined in Table 1(b) were hybridized with individual Southern blots and washed under stringent conditions as described in Methods.

IncII PAE plasmids carrying mutagenic DNA repair genes

Five serovars contained large (62–65 MDa) plasmids which hybridized with two of the eight replicon probes, II and FIC. This pattern of hybridization identified them as belonging to the Inc group II (Couturier *et al.*, 1988). It should be noted that the FIC probe also hybridizes with plasmids of Inc group FII. However, these are distinguished from Inc group II plasmids by their failure to hybridize with the II probe and their hybridization with the FIIA probe. (Compare *S. paratyphi* B M415 and *S. minneapolis* M131 in Table 2.)

In two *S. typhimurium* strains, M149 and M140, the II-hybridizing plasmid co-migrated with a similarly sized

Fig. 1. The plasmid content of the strains is shown, and represents the standard gel format blotted for all experiments (cf. Figs 1*b* and 2*a*, *b* for example). Plasmid molecular mass standards (strain 39R861; NCTC 50192) are shown in tracks 1 and 18, labelled in MDa on the left. Chromosomal fragments (Chr) are indicated. The tracks contained: 1, M131; 2, M149; 3, M476; 4, M102; 5, M140; 6, M303; 7, M283; 8, M301; 9, M311; 10, M313; 11, M496; 12, M498; 13, M501; 14, M503; 15, M269; 16, M141; 17, M415; 18, 39R861; 19, M443; 20, M415; 21, M444; 22, M274; 23, M204; 24, M273; 25, M436; 26, M46; 27, M507; 28, M21; 29, M25; 30, M581; 31, M155; 32, M506; 33, M527; 34, M530. (b) Southern blot of gel as shown in (a) hybridized with the FIC replicon probe.

virulence plasmid. (Fig. 1*a*, tracks 3 and 8, appear as 'single' strong bands.) The existence of two plasmids in these cases were resolved by examining *Pst*I digests of

total plasmids DNA from the strains (data not shown). In two *S. typhimurium* strains of phage type 100, the IncII plasmid co-existed with a smaller plasmid (Fig. 1*a*,

Table 2. *Molecular characterization of the plasmids*

+ or – indicates homology or no homology detected under the conditions described in Methods. DNA probes FIA, FIB, X_{R6K} and *muc* were also tested against these plasmids but no homologies were found.

Murray no.	Designation	Phage type	Mol. mass (MDa)	DNA probe homologies								
				FIC	FIIA	I1	<i>par</i>	N	X _{R485}	VirA	VirB	<i>imp</i>
M131	<i>S. minneapolis</i>		65*	+	–	+	–	–	–	–	–	+
M149	<i>S. typhimurium</i>	4	65*†	+	–	+	–	–	–	–	–	+
			65†	–	–	–	+	–	–	+	+	–
M476	<i>S. montevideo</i>		65*	+	–	+	–	–	–	–	–	+
M102	<i>S. typhimurium</i>	100	65*	+	–	+	–	–	–	–	–	+
			34	+	+	–	–	–	–	–	–	–
M140	<i>S. typhimurium</i>	RDNC‡	65*†	+	–	+	–	–	–	–	–	+
			65†	–	–	–	+	–	–	+	+	–
M303	<i>S. typhimurium</i>	12	65*	+	–	+	–	–	–	–	–	+
M283	<i>S. typhimurium</i>	12	65*	+	–	+	–	–	–	–	–	+
M301	<i>S. typhimurium</i>	100	65*	+	–	+	–	–	–	–	–	+
			34	+	+	–	–	–	–	–	–	–
M311	<i>S. typhimurium</i>	12	65*	+	–	+	–	–	–	–	–	+
M313	<i>S. typhimurium</i>	132	65*	+	–	+	–	–	–	–	–	+
M496	<i>S. typhimurium</i>	12	65*	+	–	+	–	–	–	–	–	+
M498	<i>S. typhimurium</i>	100	65*	+	–	+	–	–	–	–	–	+
M501	<i>S. typhimurium</i>	20	65*	+	–	+	–	–	–	–	–	+
M503	<i>S. typhimurium</i>	100	65*	+	–	+	–	–	–	–	–	+
M269	<i>S. oslo</i>		62*	+	–	+	–	–	–	–	–	+
M141	<i>S. virchow</i>	34	62*	+	–	+	–	–	–	–	–	+
M415	<i>S. paratyphi B</i>		48*	+	+	–	–	–	–	–	–	–
M443	<i>S. bisebjerg</i>		70	–	–	–	+	–	–	–	+	–
			48*	+	+	–	–	–	–	–	–	–
M444	<i>S. senftenberg</i>		24*	–	–	–	–	–	–	–	–	–
M274	<i>S. potsdam</i>		72	–	–	–	–	–	–	–	–	–
			23*	–	–	–	–	–	–	–	–	–
M204	<i>S. enteritidis</i>	6a	38	–	–	–	+	–	–	+	+	–
			26*	–	–	–	–	–	+	–	–	–
M273	<i>S. moscow</i>		38	–	–	–	+	–	–	+	+	–
			26*	–	–	–	–	–	+	–	–	–
M436	<i>S. paratyphi C</i>		36*	–	–	–	+	–	–	+	+	–
M46	<i>S. typhi</i>	46	25*	–	–	–	–	–	+	–	–	–
M507	<i>S. typhi</i>		25*	–	–	–	–	–	+	–	–	–
M21	<i>S. typhimurium</i>	RDNC‡	25*	–	–	–	–	–	+	–	–	–
M25	<i>S. choleraesuis</i>		30	–	–	–	+	–	–	+	+	–
			25*	–	–	–	–	–	+	–	–	–
M581	<i>S. typhi</i>		25*	–	–	–	–	–	+	–	–	–
M155	<i>S. typhimurium</i>		60	–	–	–	+	–	–	+	+	–
			25*	–	–	–	–	–	+	–	–	–
M506	<i>S. eastbourne</i>		32*	–	–	–	–	–	–	–	–	–

* Conjugative plasmid as originally identified by Datta & Hughes (1983).

† Strain carries two co-migrating plasmids. On separation the *imp*/I1/FIC homologies were located on a I1 plasmid, while the VirA,B/*par* homologies on the other plasmid (data not shown).

‡ Reacted with *S. typhimurium* typing phages but did not conform to any designated pattern.

tracks 5 and 9) which was identified as IncFII. By the above criteria, sixteen strains contained IncI1 plasmids. By comparison with the primary *E. coli* transconjugants of Datta & Hughes (1983) it was established that all these plasmids were conjugative. The entire plasmid set was next hybridized with cloned genes encoding mutagenic DNA repair functions. These were the *impCAB* locus of the IncI1 plasmid TP110 (Glazebrook *et al.*, 1986), and the *mucAB* locus of the IncN plasmid pKM101 (Herrera *et al.*, 1988). In our experiments all sixteen PAE IncI1 plasmids hybridized positively with the *impCAB* probe, whilst none of the other 26 plasmids did so.

PAE IncX plasmids

Two types of contemporary IncX plasmids have been distinguished on the basis of their non-homologous replicons. The corresponding replication loci have been cloned from the plasmid R6K (Couturier *et al.*, 1988), which was isolated from *E. coli*, and from R485 (Stalker & Helinski, 1985), which was isolated from *Proteus morganii*. Both these probes were employed to screen for IncX PAE plasmids. The R6K probe detected no homologous replicons. The R485 probe detected eight IncX PAE replicons, all around the same size (25 MDa;

Fig. 2. (a) Southern blot (for format cf. Fig. 1) hybridized with the IncX replicon probe. (b) Southern blot (cf. Fig. 1) hybridized with the IncII replicon probe.

Table 2) and distributed in five serovars, *S. choleraesuis*, *S. enteritidis*, *S. moscow*, *S. typhi* and *S. typhimurium*. Numerically, IncX plasmids represented the second largest plasmid class among these PAE salmonellae.

PAE virulence plasmids

The plasmid blots were screened for homology with probes containing the VirA, VirB (Norel *et al.*, 1989a)

Table 3. Occurrence of PAE plasmid classes in the *Salmonella* serovars

Plasmid class	No. of examples	Serovar and (no. of plasmids found)
I1	16	<i>S. minneapolis</i> (1) <i>S. montevideo</i> (1) <i>S. oslo</i> (1) <i>S. typhimurium</i> (12) <i>S. virchow</i> (1)
X	8	<i>S. choleraesuis</i> (1) <i>S. enteritidis</i> (1) <i>S. moscow</i> (1) <i>S. typhimurium</i> (2) <i>S. typhi</i> (3)
VirA ⁺	7	<i>S. choleraesuis</i> (1) <i>S. enteritidis</i> (1) <i>S. moscow</i> (1) <i>S. paratyphi</i> C (1) <i>S. typhimurium</i> (3)
FIIA	4	<i>S. bisebjerg</i> (1) <i>S. paratyphi</i> B (1) <i>S. typhimurium</i> (2)

and *par* (Tinge & Curtiss, 1990a) loci previously cloned from the *S. typhimurium* virulence plasmid. An identical pattern of positive hybridization was found with each of these probes, with a single plasmid of seven strains belonging to the five serovars *S. typhimurium*, *S. enteritidis*, *S. moscow*, *S. paratyphi* C and *S. choleraesuis* (see also Table 3). Under the stringent conditions used in this study no homologies were found for the FIB or FIIA probes. The sizes of these serotype-specific virulence plasmids were, respectively, 60, 38, 38, 36 and 30 MDa. Interestingly, *S. enteritidis* M204 and *S. moscow* M273, which are closely related serologically, each contained co-existing IncX and virulence plasmids. The two plasmids were of the same molecular masses in each serovar (compare Fig. 1a, tracks 23 and 24, where the upper band is the plasmid hybridizing with the *par* probes).

Other PAE plasmids: IncFII and unclassified

The next largest class of PAE plasmids was made up of those hybridizing with both the FIIA and FIC probes. Fig. 1(b) shows the FIC probe data and four strains (tracks 5, 9, 19 and 20) subsequently also hybridized with the FIIA probe. In tracks 5 and 9, the plasmids concerned are the smaller of the two FIC-positives. This result identified two 34 MDa plasmids of *S. typhimurium*, and two 48 MDa plasmids in *S. paratyphi* and *S. bisebjerg* as belonging to IncFII (criteria of Couturier *et al.*, 1988).

A single exemplar previously published as plasmid incompatibility group N was found in *S. potsdam* M274. It did not hybridize with the probe for IncN (Table 1b) or

with *mucAB*, which are plasmid-borne genes encoding DNA mutagenic repair in some contemporary IncN plasmids like R46.

The 70 MDa plasmid of *S. bisebjerg* cannot be considered a virulence plasmid as it hybridized only with *par* and *virB* and not with the *virA* probe. Both these regions are concerned with plasmid stability and not directly with virulence functions.

Two other plasmids were observed which failed to hybridize with any tested probe. One of these had transferred an indicator plasmid in the genetic system of Hughes & Datta (1983); this was the 32 MDa plasmid of *S. eastbourne* M506. The other, 72 MDa, was detected uniquely in these experiments, in *S. potsdam* M274, where it co-existed with the 'IncN' plasmid discussed above.

Discussion

Couturier *et al.* (1988) have developed a molecular classification of plasmids on the basis of their 'basic replicon' content, i.e. the genes required for autonomous plasmid replication and its control. The 'basic replicon' of a plasmid may be cloned as a locus expressing incompatibility functions associated with its replication. These clones were then used as DNA probes, enabling identification of plasmids belonging to different incompatibility groups by molecular replicon typing. Many multi-replicon plasmids which gave ambivalent results by classical phenotypic Inc typing have been thus unequivocally characterized. This Inc/Rep method characterized five Inc groups among the PAE *Salmonella* plasmids, whilst a sixth group, representing virulence plasmids, did not react with the Inc/Rep probes listed in Table 1. This contrasts with the findings of Pohl *et al.* (1987) who found that the virulence plasmid of *S. typhimurium* hybridized with both the FIA and FII probes. Therefore, we deduce that *Salmonella* virulence plasmids have replicons which are related but not identical to IncFI and IncFII plasmids. Tinge & Curtiss (1990b) reached a similar conclusion on the basis of weak homology of the replication regions of the *S. typhimurium* virulence plasmid R100 (IncFII) of F (IncFI). Sixteen IncI1 plasmids were detected (Table 2), as determined by reaction with FIC and I1 probes. We discuss these below in terms of their carriage of DNA repair determinants.

Eight IncX PAE plasmids of about 25 MDa were observed to hybridize with the R485, but not the R6K replicon probe. R6K is, in fact, the only example of this IncX replicon type reported. R485 is a contemporary plasmid carrying only sulphonamide resistance. Other contemporary R485-type replicons identified carried multiple resistances encoded by identifiable transposons

Tn3, Tn7 and Tn21 (D. Osborne and C. Jones, unpublished). In this case the data clearly show the evolution of R-plasmids from progenitor PAE plasmids by acquisition of transposable resistance genes. Four IncFII PAE plasmids were identified. The IncFII group of contemporary enterobacteria contains numerous examples of multiple antibiotic resistance plasmids such as R100 (Watanabe *et al.*, 1964). The transfer system of R100 has been particularly well-studied at a molecular genetic level (reviewed by Willetts & Skurray, 1980) and this plasmid was also the source of the multi-resistance transposon Tn21 which carries a highly recombinogenic gene-acquisition system termed an 'integron' (Grinsted *et al.*, 1990).

Two classes of plasmid-borne mutagenic DNA repair genes have been characterized. These are *mucAB*, restricted to IncN plasmids, and *impCAB*, found in IncI1 and IncB plasmids (reviewed by Strike & Lodwick, 1987). Functional homologues of the *impCAB* locus of contemporary plasmids were shown to exist in two Murray PAE plasmids of Inc groups I1 and B by Sedgewick *et al.* (1989). This 100% conserved linkage of *imp* genes to IncI1 replicons may be contrasted with the variability reported (Hughes & Datta, 1983) for the colicin phenotype of these plasmids: the PAE IncI1 plasmids either did not encode colicins or encoded one of three types, ColIb, ColIa or ColI. Of nine contemporary IncI1 plasmids tested seven carried the *impCAB* locus (reviewed by Strike & Lodwick, 1987), along with single (e.g. TP110) or multiple antibiotic resistances (e.g. R62).

Five serovars contained plasmids hybridizing with the VirA locus which was cloned from a large plasmid of *S. typhimurium* LT2 and which suppresses the fertility of plasmid F and also confers a virulence phenotype in mouse infection studies. This virulence plasmid has been variously termed pSLT (Jones *et al.*, 1982), pLT2 (Brown *et al.*, 1986), pIP1350 (Norel *et al.*, 1989a), pEX102 (Rhen *et al.*, 1989) and pStSR100 (Tinge & Curtiss, 1990a). Two linked loci of this plasmid have been characterized in some detail – they are termed VirA and VirB. Physical-genetic maps of both regions have been presented (e.g. Norel *et al.*, 1989a). Four genes in the VirA region of *S. typhimurium* have been characterized to the nucleotide sequence level and are termed *mkf* (mouse killing factor)A, *mkfB*, ORF2 (Norel *et al.*, 1989b, c) and *vagA* (Pullinger *et al.*, 1989). The VirA region is conserved in plasmids of 11 contemporary serovars of salmonellae (Williamson *et al.*, 1988). The virulence genes of one of these serovars, *S. choleraesuis*, have been shown to be conserved at the sequence level (Matsui *et al.*, 1990a,b). Virulence plasmids have characteristic incompatibility behaviour with respect to homologous plasmids of other *Salmonella* serovars (Ou *et al.*, 1990).

Brown *et al.* (1986) and Woodward *et al.* (1989) have provided evidence that possession of these large plasmids is a serotype-specific property. A plasmid-partitioning (*par*) locus which is also responsible for incompatibility has been cloned from the virulence plasmid of *S. typhimurium* (Tinge & Curtiss, 1990a) and is invariably linked to VirA-carrying plasmids (Tinge & Curtiss, 1990b). The fact that PAE virulence plasmids of *S. typhimurium*, *S. enteritidis* and *S. choleraesuis* hybridized positively with the partition locus (*par*) probe is consistent with the report that these virulence plasmids are incompatible with each other in contemporary serovars (Ou *et al.*, 1990). The molecular masses of PAE virulence plasmids observed were not different from those reported for contemporary representatives of the five serovars where they were found (Ou *et al.*, 1990, Woodward *et al.*, 1989). Of the six PAE serovars which contained plasmids hybridizing with *par*, three (*S. typhimurium*, *S. choleraesuis* and *S. enteritidis*) contemporary serovars have been reported to harbour plasmids which do so (Tinge & Curtiss, 1990b). Our results indicate that *Salmonella* virulence plasmids have not undergone detectable molecular evolution under the influence of antibiotic selection pressure. This is not unexpected, since they are serotype-specific, poorly transferable and are not vectors of antibiotic resistance.

The data presented in this report provide a molecular picture of *Salmonella* plasmids prior to the imposition of selective pressure due to the medical and agricultural use of antibiotics. As such the data provide a 'baseline' for the study of molecular evolution in the plasmid groups analysed and it could be, for instance, usefully compared with results from present-day salmonellae at the levels of individual plasmid loci, of plasmid classes, and of population genetics.

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