Thermosensitive Antibiotic Resistance Plasmids in Enterobacteria

By H. WILLIAMS SMITH, Z. PARSELL AND P. GREEN

*Houghton Poultry Research Station, Houghton, Huntingdon,
Cambridgeshire, PE17 2DA*

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Of 775 conjugative plasmids found in enterobacteria mediating antibiotic resistance, 24 (3.1%) were thermosensitive (ts); they were most common in *Klebsiella pneumoniae*. Ts plasmids were also found in all the samples of sewage and river water examined. Over half of 73 ts plasmids from unrelated sources mediated resistance to chloramphenicol in addition to several other antibiotics. Many of them mediated resistance to mercury (53.4%), arsenite (38.4%) and tellurite (79.5%) but not to copper, cobalt and silver. Fifty-eight belonged to incompatibility group H2 and 12 belonged to the H1 group. Resistance to mercury, arsenite and tellurite was common in strains containing H2 plasmids but not in H1 plasmids. The 73 plasmids transferred at high rates at 22 and 28 °C and at lower rates at 15 °C; they transferred at very low rates or not at all at 37 °C. They could be divided into two sets according to whether they transferred at a high or at a low rate at 33 °C. Unlike the prototype plasmid, Rts 1, they were solely or mainly ts for transfer and not for replication and only one of them brought about a marked reduction in growth rate of its host organism at 42 °C. None of the 73 plasmids mediated colicin or haemolysin production. Three plasmids, all from *K. pneumoniae*, mediated utilization of lactose, two of sucrose and raffinose and three, all belonging to group H1, of citrate. None of the plasmids increased the pathogenicity of *Salmonella typhimurium* for chicks or *Escherichia coli* K12 for mice.

INTRODUCTION

Enterobacteria harbouring chloramphenicol resistance plasmids have been present in the human environment for a considerable time, at least since the antibiotic came into clinical use some 25 years ago. Although many of them can transmit their resistance to *Salmonella typhi* at high rates *in vitro* (Smith, 1973), no outbreaks of chloramphenicol-resistant *S. typhi* infection in human beings were reported until 1972-3 when a large outbreak occurred in Mexico. It was soon followed by others in India, Vietnam and Thailand. The resistance of the strains responsible for these outbreaks, which was plasmid-borne, was principally to tetracycline, streptomycin, spectinomycin and sulphonamide in addition to chloramphenicol. The available evidence revealed that the Mexican outbreak was caused by a single strain of *S. typhi* as was the Indian outbreak, but those in Vietnam and Thailand were caused by at least nine Vi-types of *S. typhi* (Anderson, 1975). Each of these types would have acquired the plasmid determining its antibiotic resistance in a separate event, probably from non-pathogenic enterobacteria. There is no reason to believe that they had acquired the plasmid directly from each other.

Plasmids examined from all four outbreaks were thermosensitive (ts) in that they transferred at high rates at low temperatures, such as 22 and 28 °C, but at very low rates or not at all at 37 °C (Smith, 1974). They belonged to incompatibility group H1. Plasmids of this group, also ts but determining resistance to antibiotics other than chloramphenicol, have also been found in *Salmonella typhimurium* strains isolated in a number of countries

including Britain (Anderson, 1975). Ts plasmids belonging to groups other than H1, or whose compatibility status has not been determined, have been reported by other workers (Terawaki *et al.*, 1967; Coetzee *et al.*, 1972; Yoshida *et al.*, 1974; Grabow *et al.*, 1975; Rodriguez-Lemoine *et al.*, 1975; Sato *et al.*, 1974; Smith & Parsell, 1975). The prototype, Rts 1 (Terawaki *et al.*, 1967), which has been intensively studied, determines kanamycin resistance in a strain of *Proteus vulgaris*. Its transfer frequency at 25 °C is about 10^5 times that at 37 °C. It is spontaneously eliminated from host cells at 42 °C but not at 37 °C, and for that reason its thermosensitivity is ascribed to replication failure. Cultures of Rts 1⁺ *Escherichia coli* also grow poorly at 42 °C, especially in broth containing 0.03% sodium lauryl sulphate (Terawaki *et al.*, 1975). The other ts plasmids have been studied less closely but it is known, for example, that unlike Rts 1 some are thermosensitive for transfer rather than for replication.

Because of the suggestion implicit in the pattern of the chloramphenicol-resistant *S. typhi* outbreaks that ts antibiotic resistance plasmids might have become more prevalent in recent years, it was decided to study the distribution of these plasmids, especially those belonging to group H1, in the environment and their more important characteristics. Special attention was paid to their incidence in sewage and polluted rivers because, as they are thermosensitive, the distinct possibility exists that pathogenic enterobacteria would be more likely to acquire such plasmids in these locations than in the alimentary tract.

METHODS

Plasmids. Only plasmids possessing antibiotic resistance determinants were studied; their origin is given in Table 1. Those that transferred at very low rates, or not at all, at 37 °C and at much higher rates at 28 °C and/or 22 °C were designated thermosensitive (ts) and those that transferred at least as well at 37 °C as at 28 °C or 22 °C were designated thermotolerant (tt). Unless stated otherwise, their bacterial host in all experiments was a *lac*⁺ prototrophic strain of *Escherichia coli* κ 12 called κ 12 Proto.

Bacterial strains. Those examined for ts plasmids were believed to be epidemiologically unrelated. They had been maintained in this laboratory for at least 1 year on Dorset egg medium at 5 °C. A rough plasmid-free *Salmonella typhimurium* strain of phage type 36 was used as plasmid recipient during one stage of the isolation of plasmids from sewage and river water but in all other plasmid transfer studies a *lac pro his trp phe* strain of *E. coli* κ 12 called κ 12 Auxo was used. Both of these strains were spontaneous chromosomal mutants resistant to sodium nalidixate (Nal^r) and rifampicin (Rif^r).

Cultural conditions. Unless stated otherwise, bacteria were grown in 10 ml nutrient broth (Oxoid, no. 2) for 24 h in a stationary water bath. Cultures on solid media were incubated for the same period in an air incubator.

Identification of strains possessing ts plasmids: the determination of plasmid transfer rate. Nutrient broth, pre-heated to 37 °C, was inoculated with 2 ml of a broth culture of an antibiotic-resistant strain and 2 ml of the κ 12 Auxo strain, both of which had been incubated at 37 °C. After incubation at this temperature, 0.1 ml of the mating mixture was inoculated in a uniformly reproducible manner on to a plate of MacConkey agar containing sodium nalidixate and one of the antibiotics to which the donor strain was resistant (each at 20 μ g ml⁻¹). The plate was incubated at 37 °C and the degree of bacterial growth upon it was taken as an indicator of the amount of plasmid transfer that had occurred. The transfer that occurred at 28 °C and at 22 °C was assessed in the same manner except that all the broth cultures were incubated at these temperatures instead of at 37 °C. On the basis of these results, plasmids were provisionally classified as ts or tt.

The rate of plasmid transfer at different temperatures was assessed using a similar procedure. The total numbers of viable κ 12 Auxo organisms in the different mating mixtures were determined by a modification of the method of Miles & Misra (1938) using MacConkey medium containing sodium nalidixate, and the numbers that had acquired the plasmid of the donor strain were determined by duplicate counts on similar medium with an additional antibiotic to which the plasmid provided resistance. The dilution procedures were performed rapidly in phosphate buffer at 5 °C and the culture plates were pre-heated to 37 °C.

Sewage and river water. Specimens of untreated sewage were obtained on different occasions from sewage works in Essex and Cambridgeshire. River water was obtained from rivers in Essex, The Midlands and South Wales that were known to be polluted. Apart from those from the Welsh rivers, all specimens were examined within a few hours of collection.

Isolation of ts plasmids from sewage and river water. (1) *Direct method.* Specimens were filtered through

butter muslin, centrifuged at 2500 g for 30 min and the deposit was inoculated in a manner likely to yield well-separated colonies on to five plates of MacConkey agar, each plate containing a different antibiotic (ampicillin, chloramphenicol, neomycin, streptomycin or tetracycline) at 20 µg ml⁻¹. After incubation at 37 °C, up to 20 colonies from each plate were purified by replating on plain MacConkey agar and broth cultures were then examined for ts plasmids.

(2) *Enrichment method.* The centrifuged deposit from 1 l sewage or river water that had been filtered through butter muslin was divided into five aliquots and each aliquot was incubated at 37 °C in 20 ml MacConkey broth (Oxoid, CM5) containing novobiocin (10 µg ml⁻¹) and one of each of the five antibiotics (30 µg ml⁻¹) used in the direct method. The centrifuged deposit of each of these cultures was added to 10 ml nutrient broth which had been inoculated with 2 ml of a broth culture of the *S. typhimurium* strain. After incubation at 22 °C, each of the five cultures was inoculated on to plates of MacConkey agar containing sodium nalidixate and the antibiotic with which it had previously been grown (each at 20 µg ml⁻¹). Ten *S. typhimurium* colonies were picked from each plate, purified by replating and examined for ts plasmids. The *S. typhimurium* strain had been chosen for use in this method because it was much less susceptible than *E. coli* κ12, for example, to the activity of phages and bacteriocins that were commonly present in the specimens of sewage and river water.

Whether obtained by the direct or the enrichment method, only one plasmid determining any particular antibiotic resistance pattern was retained from any one specimen of sewage or river water for further study; it was implanted in κ12 Proto for this purpose.

Stability of plasmids at different temperatures. Approximately 10⁸ viable organisms of a plasmid-containing (R⁺) κ12 Proto strain were inoculated into nutrient broth and each culture was incubated at a different temperature. Every 24 h a similar number of organisms was transferred from each culture to fresh broth at the same temperature and incubation was continued. At intervals, the cultures were replica-plated (Lederberg & Lederberg, 1952) on to MacConkey agar with or without one of the antibiotics (20 µg ml⁻¹) against which the plasmid provided resistance and the proportion of R⁻/R⁺ colonies was assessed.

Growth rate of R⁺ κ12 Proto strains at 42 °C. This was studied by the method of Terawaki *et al.* (1975) employing Penassay broth (PAB, Difco) with or without 0.03 % (w/v) sodium lauryl sulphate. Bacterial growth rates were estimated by viable counts and by photometric methods.

Identification of H1 and H2 plasmids. Plasmids were designated H1 or H2 solely on their compatibility properties. They were first screened by the rapid test of Anderson (1975) and were designated H1 if they were incompatible with F and with prototype H1 plasmids, and H2 if they were compatible with F and incompatible with the H1 prototypes. No measurements of molecular weight or percentage homology were made (Anderson, 1975). Compatibility was tested as described by Datta & Olarte (1974).

Antibiotic sensitivity tests. These were done on Sensitest agar (Oxoid) by the disc method (Smith, 1977) using discs containing 25 µg ampicillin (Ap), 50 µg chloramphenicol (Cm), 30 µg neomycin (Nm), 300 units polymyxin, 25 µg rifampicin, 25 µg spectinomycin (Spc), 25 µg streptomycin (Sm), 50 µg tetracycline (Tc), 15 µg furazolidone, 30 µg sodium nalidixate, 300 µg sulphonamides (Su) and 1.25 µg trimethoprim.

Identification of plasmids providing resistance to inorganic compounds. The plasmids were tested in the usual host organism, κ12 Proto, except that they were tested against sodium arsenite in the *S. typhimurium* strain, as strain κ12 Proto and all the other κ12 strains available in this laboratory were moderately resistant to sodium arsenite. Broth cultures of the R⁺ organisms and of the R⁻ host organism, diluted 100-fold, were streaked on plates of culture medium at right angles to strips of filter paper that had been soaked in aqueous solutions of copper sulphate (5 %, w/v), cobalt sulphate (5 %, w/v), sodium arsenite (1 %, w/v), mercuric chloride (0.1 %, w/v), silver nitrate (0.1 %, w/v) and potassium tellurite (0.01 %, w/v). The plates were incubated at 37 °C and the relative susceptibility of the cultures was assessed by measuring the zones of inhibition of bacterial growth. The culture medium used for the silver nitrate tests was TYE agar (McHugh *et al.*, 1975) and for the other tests was Sensitest agar.

Minimum inhibitory concentrations were determined by spot-inoculating a series of plates of Sensitest agar containing twofold differences in concentration of the substance under test with broth cultures of different R⁺ organisms suitably diluted so that each spot contained approximately 200 viable organisms. The plates were incubated at 37 °C and the lowest concentration that prevented visible growth was recorded as the minimum inhibitory concentration.

Colicinogenicity and lysogenicity tests. To identify colicinogenic strains, broth cultures were spot-inoculated on to plates of tryptose agar (Difco) that had been inoculated with 'lawns' of κ12 Auxo and of the *S. typhimurium* strain. The plates were observed after incubation at 37 °C. The technique of identifying lysogenic strains was similar except that the culture medium was supplemented with sodium nalidixate (20 µg ml⁻¹) and the strains were tested as supernates of centrifuged broth cultures.

Biochemical tests. These were done by the methods described by Cruickshank (1970); Elek's medium was used to detect urease production and Koser's liquid citrate medium (5 ml) to detect citrate utilization, the

Table 1. *Presence of ts plasmids in R⁺ enterobacteria from different sources and in sewage and river water samples*

Material examined	No. of strains or samples examined	ts plasmids	
		No. found	Resistance*
R ⁺ <i>Escherichia coli</i> strains from man and animals	355	9	SmSuCmTcSpcNm (1) SmSuCmTcAp (1) SmSuCmSpc (1) SmCmTc (1) Tc (4) Ap (1) SmSuCmTcSpc (3)
R ⁺ <i>Klebsiella pneumoniae</i> strains from animals	8	3	
R ⁺ <i>Salmonella</i> strains from man and animals	18	1	Ap (1)
R ⁺ <i>Shigella flexneri</i> strains from man	2	0	
R ⁺ enterobacterial strains† from rivers	145	9	SmSuCmTcSpc (2) SmSuCmSpc (5) SmSuCm (1) ApSm (1)
R ⁺ enterobacterial strains† from sewage plants	247	2	SmSuCmTcSpc (1) SmSuCmSpc (1)
Samples of water‡ from 23 rivers	28	32	SmSuCmTcNmSpcAp (1) SmSuCmTcSpcNm (1) SmSuCmTcSpc (4) SmSuCmSpc (9) SmSuTcSpc (2) SmTcNmAp (2) SuCmAp (1) SuCm (1) Tc (11)
Samples of water‡ from 5 sewage plants	11	17	SmSuCmTcSpc (2) SmSuCmSpc (3) SmSuTcSpc (2) TcNm (2) Tc (8)

* Sm, Streptomycin; Su, sulphonamide; Cm, chloramphenicol; Tc, tetracycline; Spc, spectinomycin; Nm, neomycin; Ap, ampicillin. The numbers of plasmids that mediated the resistance quoted are given in parentheses.

† Classified as enterobacteria by their appearance on MacConkey agar.

‡ By the indirect method, which did not require the isolation of the R⁺ donor organisms.

inoculum for this test consisting of approximately 10^4 viable organisms ml⁻¹ and the temperature and time of incubation being 37 °C and 6 d. A minimal salts agar medium, supplemented when necessary with growth factors, was used to detect utilization of lactose, sucrose and raffinose (Smith & Parsell, 1975).

Pathogenicity tests. Each R⁺ *S. typhimurium* strain was given orally to a group of six 1 d-old chicks in a dose of approximately 10^9 viable organisms; the R⁻ strain was given to three groups. The chicks were observed daily for 16 d. In an attempt to identify plasmids that synthesized lethal toxin, 0.5 ml of a shaken Tryptic Soy Broth (Difco) culture containing 10^9 viable organisms ml⁻¹ was inoculated subcutaneously into two young adult white Swiss mice and two 4 d-old chicks. The animals were observed daily for 5 d.

RESULTS

Incidence of thermosensitivity in enterobacterial antibiotic resistance plasmids; the isolation of thermosensitive plasmids from sewage and river water

Of 775 antibiotic resistance plasmids in enterobacterial strains that had been isolated from healthy and diseased human beings and domestic animals or, by the direct method, from sewage and rivers, 24 (3.1 %) were thermosensitive (Table 1). There was no association

Table 2. Transfer rate of *ts* and *tt* plasmids at different temperatures

Plasmids F, I and *Sh. dysenteriae* are *tt*; the remainder are *ts*. Plasmids of groups H1 and H2 are marked as such. The plasmids were transferred from the κ 12 Proto strain to the κ 12 Auxo strain, and the *ts* plasmids were divided into two sets according to their degree of transfer at 33 °C.

No.	Plasmid		Proportion of recipient organisms that acquired the plasmid at (°C):				
	Set	Determinants	15	22	28	33	37
47 (H2)	1	SmSuCmSpc	5×10^{-3}	1×10^{-1}	2×10^{-2}	1×10^{-7}	1×10^{-7}
49 (H2)	1	SmSuCmSpc	—	2×10^{-2}	2×10^{-3}	$< 1 \times 10^{-7}$	$< 1 \times 10^{-7}$
71 (H2)	1	SmSuCmSpc	3×10^{-4}	6×10^{-2}	9×10^{-4}	1×10^{-7}	$< 1 \times 10^{-7}$
12 (H2)	1	SmSuCmSpc	9×10^{-3}	7×10^{-2}	3×10^{-2}	$< 1 \times 10^{-7}$	$< 1 \times 10^{-7}$
9 (H2)	1	SmSuCmTcSpc	2×10^{-3}	1×10^{-1}	3×10^{-3}	1×10^{-7}	$< 1 \times 10^{-7}$
59 (H2)	1	SmSuCmTcSpc	5×10^{-4}	5×10^{-3}	9×10^{-5}	$< 1 \times 10^{-7}$	$< 1 \times 10^{-7}$
66 (H2)	2	SmSuCmTcSpc	1×10^{-4}	4×10^{-2}	6×10^{-3}	3×10^{-3}	$< 1 \times 10^{-7}$
58 (H2)	2	SmSuCmSpc	1×10^{-2}	2×10^{-1}	3×10^{-2}	2×10^{-2}	1×10^{-5}
68 (H2)	2	SmSuCmTcSpc	2×10^{-4}	1×10^{-2}	1×10^{-2}	2×10^{-3}	$< 1 \times 10^{-7}$
55 (H2)	2	SmCmTc	4×10^{-5}	8×10^{-3}	2×10^{-2}	5×10^{-3}	1×10^{-7}
87	2	Ap	—	3×10^{-4}	1×10^{-3}	1×10^{-3}	$< 1 \times 10^{-6}$
32 (H2)	2	Tc	1×10^{-3}	9×10^{-2}	6×10^{-1}	5×10^{-1}	2×10^{-4}
30 (H1)	2	Tc	—	4×10^{-3}	3×10^{-2}	9×10^{-3}	$< 1 \times 10^{-7}$
44 (H1)	2	SuCm	1×10^{-4}	2×10^{-1}	1×10^{-1}	9×10^{-2}	$< 1 \times 10^{-7}$
Rts 1	2	Nm	2×10^{-4}	5×10^{-2}	3×10^{-1}	3×10^{-1}	$< 1 \times 10^{-7}$
<i>S. typhi</i> 14 (H1)	2	SmSuCmTcSpc	5×10^{-5}	4×10^{-2}	7×10^{-2}	6×10^{-2}	$< 1 \times 10^{-7}$
<i>S. thompson</i>	2	Tc	1×10^{-1}	8×10^{-1}	7×10^{-1}	2×10^{-3}	$< 1 \times 10^{-7}$
F		Tc	1×10^{-7}	1×10^{-6}	6×10^{-3}	3×10^{-1}	7×10^{-1}
I		Tc	6×10^{-6}	1×10^{-3}	1×10^0	1×10^0	9×10^{-1}
<i>Sh. dysenteriae</i>		SmSuCmTcSpc	1×10^{-6}	1×10^{-6}	3×10^{-4}	9×10^{-2}	2×10^{-2}

between *ts* plasmids and any disease condition or any animal species. Three of the eight laboratory strains of *Klebsiella pneumoniae* were *ts*. Three of the eleven enterobacterial strains from sewage or rivers found to possess *ts* plasmids also belonged to this species; two others were *E. coli*, two *Citrobacter* spp. and four were taxonomically anomalous. When examined by the enrichment technique, *ts* plasmids were found in all the samples of sewage and river water that were examined. Of the 73 *ts* plasmids identified in this survey, 41 determined resistance to at least four antibiotics and 39 determined resistance to chloramphenicol, in addition to other antibiotics. Genes mediating chloramphenicol resistance were common amongst *ts* and *tt* (thermotolerant) plasmids in enterobacterial strains in sewage and river water. For example, although in the direct method of examining river water, organisms were picked as being potentially R^+ because they were resistant to ampicillin, streptomycin, neomycin, tetracycline or chloramphenicol, 95 out of 133 that were ultimately proved to be R^+ possessed resistance determinants that included those mediating chloramphenicol resistance.

Twelve of the 73 *ts* plasmids belonged to compatibility group H1. One was $SuCmAp^+$, one $SuCm^+$, two $TcNm^+$ and eight Tc^+ . Ten had been obtained by the enrichment method (four from sewage and six from river water) and two, both Tc^+ , had been found in *E. coli* isolated from the faeces of healthy pigs. Of the remaining 61 plasmids, 58 belonged to group H2 and three belonged neither to this group nor to H1. The three that belonged to neither group comprised an Ap^+ plasmid found in an *E. coli* strain from the faeces of a healthy pig, another Ap^+ plasmid found in a *Salmonella cholerae-suis* strain and an $ApSm^+$ plasmid that had been found in an *E. coli* strain from river water.

Table 3. *Rate of loss of ts plasmids during daily passage in broth at different temperatures of the κ 12 Proto strain in which they had been implanted*

Between 30 and 100 colonies from each culture were examined for plasmid loss.

Plasmid no.	Percentage loss after the number of passages indicated at:						
	37 °C	40 °C		42 °C		44 °C	
	three	one	three	one	three	one	three
87	0	2	8	7	9	52	100
17	7	3	66	3	55	6	88
19	3	0	14	4	25	0	50
12	0	0	0	1	1	12	45
6	0	0	0	0	6	0	2
Rts 1	0	2	63	82	90	100	100
<i>S. typhi</i> 14 (H1)	0	—	—	—	—	0	3
<i>S. thompson</i>	0	—	—	—	—	0	2

Transfer rate of ts plasmids at different temperatures

The transfer rate of all 73 ts plasmids was estimated at 22, 28, 33 and 37 °C; that of 19 of them was also estimated at 15 °C. The results obtained for a selection of them are summarized in Table 2. Those for three 'stock' ts plasmids [Rts 1 of Terawaki *et al.* (1967), a plasmid from the Vietnamese *S. typhi* 14 strain of Butler *et al.* (1973) and a plasmid from the *Salmonella thompson* strain of Smith & Parsell (1975)] and three tt plasmids [F, *Idrd*16 of Hardy *et al.* (1973) and a group O plasmid from a *Shigella dysenteriae* strain isolated by Datta & Olarte (1974)] are included for comparison. According to their pattern of transfer at the different temperatures, the 73 ts plasmids were divided into two sets. Set 1 comprised 16 plasmids that transferred best at 22 °C and poorly or not at all at 33 °C and above, and set 2 comprised 57 plasmids that transferred best at 22, 28 and 33 °C and poorly or not at all at 37 °C and above; all 19 plasmids studied at 15 °C transferred less efficiently at that temperature than at 22 °C. Differences were found between the transfer rate patterns of some of the plasmids in set 2 but they did not appear sufficient to merit further sub-division. The plasmids in set 1 had all been obtained from sewage or river water. All 16 mediated chloramphenicol resistance (six were SmSuCmTcSp⁺ and ten SmSuCmSp⁺) whereas only 23 of the 57 set 2 plasmids did so; the 23 Tc⁺ plasmids all belonged to set 2. The 12 H1 plasmids, the three plasmids whose incompatibility group was not known, and the H1 *S. typhi* 14 plasmid were in set 2 as were Rts 1 and the *S. thompson* plasmid; H2 plasmids were in both sets, and comprised all those in set 1.

In general, the 12 H1 plasmids transferred as efficiently as the *S. typhi* 14 plasmid and the H2 plasmids. At 22 °C, two of the H1 plasmids and three H2 plasmids of set 1, the only plasmids tested, transferred from the κ 12 Proto strain to a Vi⁺ strain of *S. typhi* at a rate of 10⁻¹, the same rate at which the *S. typhi* 14 H1 plasmid transferred.

In contrast to the ts plasmids, the three tt plasmids transferred very efficiently at the higher temperatures and much less efficiently at the lower ones, the temperature effect being especially noticeable with F and the *Sh. dysenteriae* plasmid.

Plasmid loss during incubation at high temperatures

After three daily passages in broth at 44 °C, no plasmid loss was detected from forms of the κ 12 Proto strain in which 34 of the 73 ts plasmids had been implanted. The rate of loss from 12 forms was 1 to 3 %, of 9 forms was 4 to 10 %, of 11 forms was 11 to 30 % and of 7 forms was 31 to 100 %. In general, set 1 plasmids were lost faster than set 2 plasmids. The rate of loss of H1 plasmids was always small. Four of the R⁺ forms that had suffered

Fig. 1. The growth rate of different R⁺ forms of *E. coli* $\kappa 12$ Proto in 0.03% sodium lauryl sulphate broth at 42 °C: ○, *E. coli* $\kappa 12$ Proto; ●, *E. coli* $\kappa 12$ Proto R8⁺; ■, *E. coli* $\kappa 12$ Proto Rts 1⁺; □, *E. coli* $\kappa 12$ Proto R37⁺.

the greatest rate of plasmid loss and one that had suffered only a slight loss were re-examined after one or three passages at 37, 40, 42 and 44 °C; their rate of plasmid loss was never as great as that of Rts 1 (Table 3).

Because some of the plasmids could have lost conjugative ability but not their resistance determinants in the passage experiments, 48 of the R⁺ $\kappa 12$ Proto forms which had suffered no loss or a very low rate of loss were re-passaged three times at 44 °C and 10 isolates from each were tested for ability to transfer their determinants. None of the 10 isolates from each of 45 strains had lost conjugative ability, but seven of one and one each of another two strains had done so.

Thirteen tt plasmids found in sewage or river water were also examined for thermostability by submitting cultures of forms of the $\kappa 12$ Proto strain in which they had been implanted to three daily passages at 44 °C. As a group, they were lost at a rather higher rate than the ts plasmids.

Effect of the ts plasmids on the growth of the $\kappa 12$ Proto strain in sodium lauryl sulphate broth

All the forms of the $\kappa 12$ Proto strain in which one of 72 of the ts plasmids had been implanted grew at the same rate as the R⁻ Proto strain in 0.03% sodium lauryl sulphate broth at 42 °C. The growth of the remaining form (R37⁺) which was harbouring an H2 plasmid of set 2 was inhibited as markedly as that of a form harbouring the Rts 1 plasmid, but both forms suffered a sharp initial decrease in colony-forming organisms (Fig. 1).

Resistance to inorganic compounds mediated by the ts plasmids

Sodium arsenite. By strip diffusion tests, 28 (38.4%) of the 73 R⁺ forms of the *S. typhimurium* strain were classified as resistant and 45 as sensitive. The minimum inhibitory concentration (m.i.c.) of sodium arsenite for the resistant forms was 250 to 2000 $\mu\text{g ml}^{-1}$ (median, 2000 $\mu\text{g ml}^{-1}$) and was <30 $\mu\text{g ml}^{-1}$ for the sensitive forms and for the R⁻ parent strain.

Mercuric chloride. The m.i.c. of mercuric chloride for 39 (53.4%) of the R⁺ forms of strain

$\kappa 12$ Proto classed as resistant by strip diffusion tests was 32 to 128 $\mu\text{g ml}^{-1}$ (median 32 $\mu\text{g ml}^{-1}$) and was 8 $\mu\text{g ml}^{-1}$ for the 43 classed as sensitive and the R^- parent strain.

Potassium tellurite. The m.i.c. of potassium tellurite for 58 (79.5%) of the R^+ forms of strain $\kappa 12$ Proto classed as resistant by strip diffusion tests was 32 to 128 $\mu\text{g ml}^{-1}$ (median 64 $\mu\text{g ml}^{-1}$) and for the 15 classed as sensitive and the R^- parent strain was 4 $\mu\text{g ml}^{-1}$.

Other compounds. Apart from five plasmids which mediated a slightly increased resistance to cobalt nitrate, all 73 plasmids were shown in strip diffusion tests not to mediate resistance to this compound, silver nitrate or copper sulphate.

A high proportion of the plasmids that mediated resistance to chloramphenicol in addition to other antibiotics, including all 16 in set 1, also mediated resistance to mercury, arsenite and tellurite whereas none of the Cm^- ones did. Plasmids that were Tc^+ only either mediated resistance to tellurite only or to none of the three compounds. Apart from one that mediated mercury resistance, none of the 12 H1 plasmids, the *S. typhi* H1 plasmid or the Rts 1 plasmid mediated resistance to any of the compounds. Of the six plasmids found in *K. pneumoniae* and which mediated resistance to chloramphenicol in addition to other antibiotics, all were resistant to mercury, arsenite and tellurite.

Because of the close association between chloramphenicol and mercury resistance in the ts plasmids, 357 wild-type *E. coli* strains known to possess tt plasmids mediating resistance to chloramphenicol in addition to other antibiotics were examined and 316 (88%) were found to be mercury resistant. All forms of the *S. typhimurium* recipient strain to which the plasmids of 50 of the mercury-resistant strains were transferred had also acquired mercury resistance; none of the forms were arsenite- or tellurite-resistant.

Substrate utilization by the ts plasmids

None of the forms of the $\kappa 12$ Proto strain in which one of the 73 ts plasmids had been implanted utilized raffinose as sole energy source. Three of the six forms harbouring the *K. pneumoniae* ts plasmids utilized lactose (when implanted in the *lac* $\kappa 12$ Auxo strain) and two, one from sewage and one from river water, utilized sucrose; the plasmids for sucrose utilization also mediated use of raffinose when present in the *S. typhimurium* strain but not when they were in the $\kappa 12$ Proto strain.

Forms of strain $\kappa 12$ Proto harbouring three of the twelve H1 plasmids and the H1 plasmid from the *S. typhi* 14 strain, but none of the other plasmids, utilized citrate as sole energy source. The three Cit^+ plasmids had been obtained by the enrichment method from sewage; two were TcNm^+ and one was Tc^+ .

The properties of the 73 thermosensitive plasmids are summarized in Table 4.

Mediation of pathogenicity by the ts plasmids

Either none or one death occurred in each of the groups of six chicks inoculated orally with one of the 73 R^+ forms of the *S. typhimurium* strain; two of 18 died in the group given the R^- form. No signs of ill-health were noted in groups of two mice and four chicks inoculated intramuscularly with 0.5 ml of Tryptic Soy Broth cultures of each of the 73 R^+ forms of the $\kappa 12$ Proto strain.

Mediation of other characteristics by the ts plasmids

None of the 73 forms of the $\kappa 12$ Proto strain that were harbouring different ts plasmids produced colicin, haemolysin or urease; neither did they liquefy gelatin or produce hydrogen sulphide. The appearance of cultures of these forms and of the corresponding forms of the *S. typhimurium* strain in broth and on nutrient and MacConkey agar resembled those of cultures of the R^- $\kappa 12$ Proto or *S. typhimurium* parent strains. None of the R^+ forms were lysogenic for the R^- parent.

Table 4. *Properties of thermosensitive plasmids*

No. of plasmids	Incom- patibility group	Antibiotic resistances mediated	Resistance mediated to			Utilized				High trans- mission rate at 33 °C	Unstable in host at 44 °C	Inhibited multipli- cation of host
			asi	mer	tel	lac	suc	raf	cit			
1	H1	SuCmAp	—	—	—	—	—	—	—	+	+	—
1	H1	SuCm	—	+	—	—	—	—	—	+	+	—
1	H1	TcNm	—	—	—	—	—	—	+	+	—	—
1	H1	TcNm	—	—	—	—	—	—	+	+	+	—
1	H1	Tc	—	—	—	—	—	—	+	+	+	—
1	H1	Tc	—	—	—	—	—	—	—	+	—	—
6	H1	Tc	—	—	—	—	—	—	—	+	+	—
1	H2	SmSuCmTcSpcNmAp	—	—	+	—	—	—	—	+	—	—
2	H2	SmSuCmTcSpcNm	—	+	+	—	—	—	—	+	—	—
2	H2	SmSuCmTcSpc	+	+	+	—	+	+	—	—	—	—
1	H2	SmSuCmTcSpc	+	+	+	—	—	—	—	—	—	—
3	H2	SmSuCmTcSpc	+	+	+	—	—	—	—	—	+	—
2	H2	SmSuCmTcSpc	+	+	+	+	—	—	—	+	—	—
1	H2	SmSuCmTcSpc	+	+	+	+	—	—	—	+	+	—
2	H2	SmSuCmTcSpc	+	+	+	—	—	—	—	+	—	—
1	H2	SmSuCmTcSpc	+	+	+	—	—	—	—	+	+	—
10	H2	SmSuCmSpc	+	+	+	—	—	—	—	—	+	—
5	H2	SmSuCmSpc	+	+	+	—	—	—	—	+	—	—
1	H2	SmSuCmSpc	—	+	+	—	—	—	—	+	—	—
3	H2	SmSuCmSpc	—	+	+	—	—	—	—	+	+	—
1	H2	SmSuCmTcAp	—	+	+	—	—	—	—	+	—	—
1	H2	SmSuCm	+	+	+	—	—	—	—	+	—	—
1	H2	SmCmTc	—	—	+	—	—	—	—	+	—	—
2	H2	SmSuTcSpc	—	+	+	—	—	—	—	+	+	—
1	H2	SmSuTcSpc	—	—	+	—	—	—	—	+	—	—
1	H2	SmSuTcSpc	—	+	—	—	—	—	—	+	+	—
2	H2	SmTcNmAp	—	—	+	—	—	—	—	+	+	—
12	H2	Tc	—	—	+	—	—	—	—	+	—	—
2	H2	Tc	—	—	+	—	—	—	—	+	+	—
1	H2	Tc	—	—	+	—	—	—	—	+	+	+
1	Not H1 or H2	SmAp	—	—	—	—	—	—	—	+	+	—
1	Not H1 or H2	Ap	—	—	+	—	—	—	—	+	+	—
1	Not H1 or H2	Ap	—	—	—	—	—	—	—	+	+	—

asi, arsenite; mer, mercury; tel, tellurite; lac, lactose; suc, sucrose; raf, raffinose; cit, citrate.

DISCUSSION

As a group, the 73 ts plasmids, which were obtained from a wide variety of sources, differed markedly from Rts 1, the ts plasmid that was first discovered and that has been most extensively studied (Terawaki *et al.*, 1967). For example, following their implantation in the κ 12 Proto strain, only one of them, an H2 plasmid, markedly reduced the growth rate of this strain at 42 °C. However, much of the reduction in the case of this and the Rts 1⁺ form was really due to an initial sharp reduction in the numbers of colony-forming organisms. This would not be apparent when using photometric methods for measuring bacterial growth (Terawaki *et al.*, 1967). Although instability in the κ 12 Proto strain at high temperatures was a feature of some of the plasmids, the incidence of this instability was no higher than in the group of tt plasmids that we examined. Neither was its degree sufficient to warrant their classification with Rts 1 as being thermosensitive for replication; they were solely or mainly thermosensitive for transfer. The incompatibility grouping results also

indicate that plasmids of group H2 may be far more common than plasmids of group T, the group to which Rts 1 belongs.

Although our ts plasmids always transferred at very low rates at 37 °C, or not at all, differences in transfer rate were sometimes noted when some of them were tested at one or other of the lower temperatures on more than one occasion. Their division into two sets according to degree of transfer at 33 °C, however, appeared to be a reliable one. It was supported by the finding that all the plasmids in set 1 provided resistance against chloramphenicol and several other antibiotics and against mercury, arsenite and tellurite whereas only some of those in set 2 did so. Also set 2 included all the H1 plasmids. Practically all the plasmids transferred at high rates at 22 °C, environmental temperatures that prevail in many countries, thus making their transfer in voided excreta, sewage and polluted rivers a definite possibility. Transfer in the alimentary tract of homothermic animals, even with the selection pressure provided by antibiotics, seems less likely. In the conjugation experiments between *E. coli* κ 12 strains and between *E. coli* κ 12 and *S. typhi*, H2 plasmids transferred as efficiently as H1 plasmids at all temperatures. However, although we found that more of our ts plasmids, and especially those that mediated chloramphenicol resistance, belonged to group H2 than to group H1, all the strains examined from outbreaks of chloramphenicol-resistant *S. typhi* in Mexico, Vietnam, Thailand and India harboured H1, not H2, plasmids (Anderson, 1975). *Salmonella typhimurium* strains mediating resistance to antibiotics other than chloramphenicol and causing disease in the Far East and the United Kingdom also harboured H1 plasmids (Anderson, 1975). If the relative distribution of H1 and H2 plasmids in countries in which outbreaks of disease caused by organisms harbouring ts plasmids is similar to that found in our survey, then H1 plasmids may well possess properties not shared with H2 plasmids that facilitate their conjugation with pathogenic enterobacteria in extra-intestinal situations, properties not brought to light in the present study. On the other hand, it may be that the relative distribution of H1 and H2 plasmids differs in different countries. It is noteworthy in this respect that Anderson (1975) found H2 plasmids in a chloramphenicol-resistant strain of *S. typhi* from a patient infected in Spain and in *S. typhimurium* strains isolated in Portugal, Belgium, Canada and Israel. Regarding the source of our H1 plasmids, it is of interest that two that mediated tetracycline resistance had been found in *E. coli* isolated from the faeces of pigs, a species which in the United Kingdom constitutes an enormous reservoir of tetracycline-resistant *E. coli*, the consequence of the past policy of continuously feeding them on diets containing tetracyclines for growth promotional purposes.

Our collection of H1 plasmids showed certain differences from the H2 collection. Only one of them mediated mercury resistance and none of them arsenite or tellurite resistance. More interestingly, they included three plasmids with citrate-utilizing ability which the present study has revealed to be yet another property that can be plasmid-mediated.

A high proportion of our ts plasmids mediated resistance to mercury, arsenite and tellurite; the mercury and arsenite resistance, but not the tellurite resistance, was largely confined to plasmids that provided resistance to chloramphenicol in addition to several other antibiotics. Mercury resistance is commonly associated with plasmids providing this kind of antibiotic resistance and was found in 316 of the 357 R⁺ wild-type *E. coli* strains we examined, in clinical isolates of *E. coli* and *K. pneumoniae* examined by Nakahara *et al.* (1977a, b) and in different kinds of enterobacteria and related bacteria examined by Joly & Cluzel (1975). Plasmids mediating resistance to tellurite and arsenite may be less common than our survey of ts plasmids suggest. For example, none were found amongst the 50 tt plasmids we examined. A lower incidence of tellurite and arsenate resistance was also found by Summers & Jacoby (1977) during an examination of 'stock' plasmids of *Pseudomonas aeruginosa* and *E. coli* while Joly & Cluzel (1975) did not find arsenate resistance plasmids in their study. In these two surveys and those of Nakahara *et al.* (1977a, b) the temperature-sensitive status of the plasmids was not determined.

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