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Characterization of plasmids conferring resistance to gentamicin and apramycin in strains of *Salmonella typhimurium* phage type 204c isolated in Britain

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SUMMARY

In *Salmonella typhimurium* phage type 204c isolated in Britain, gentamicin resistance is specified by plasmids of the I_1 compatibility group which also confer resistance to apramycin. These plasmids have been subdivided into three types within the I_1 group on the basis of their antibiotic resistance specificity, their ability to produce colicin Ib and their restriction enzyme digest fragmentation patterns. All three have been identified in strains from cattle, but as yet only two types have been found in strains from humans.

It is suggested that the use of apramycin in animal husbandry is responsible for the appearance of gentamicin resistance in multiresistant strains of phage type 204c, a phage type already epidemic in bovine animals and with an increasing incidence in humans.

INTRODUCTION

Salmonella typhimurium phage type 204c (DT204c) was identified in 1979 in calves in Somerset, England, and in 1984 was responsible for 18·6% of incidents in adult cattle and for 67·8% of incidents in calves in England, Wales and Scotland (Anonymous, 1985). In humans, 265 infections caused by DT204c were recognized in 1984 (Threlfall *et al.* 1985) and this phage type ranked in the ten most common phage types amongst isolates referred to the Division of Enteric Pathogens.

All strains of DT204c are multiply antibiotic-resistant. In 1979 and 1980 the most common resistance pattern was chloramphenicol (C), streptomycin (S), sulphonamides (Su), tetracyclines (T) and trimethoprim (Tm) (R-type CSSuTTm) but in subsequent years strains of R-type ACKSSuTTm (A, ampicillin; K, neomycin-kanamycin) have predominated. Gentamicin resistance first appeared in DT204c in 1983 in strains from cattle (Threlfall *et al.* 1983) and during 1985 24·9% of bovine and 11·6% of human isolates of this phage type were gentamicin-resistant.

Previous studies have demonstrated that gentamicin resistance is plasmid-encoded in DT204c, and that the plasmids which code for resistance to gentamicin also confer resistance to the aminoglycoside antibiotic apramycin. Three types of gentamicin–apramycin resistance plasmid have been identified (Threlfall *et al.*

1985). The genetic characterization of these three plasmids is presented and their epidemiological distribution is reviewed.

MATERIALS AND METHODS

Bacterial strains and plasmids

The origins, year of isolation and R-types of representative strains of DT204c carrying these plasmids are listed in Table 1.

Resistance testing

Resistance to antimicrobial drugs was determined by the methods of Anderson & Threlfall (1974). The levels of resistance to aminoglycoside antibiotics were determined by observing the ability of inoculums of approximately 10^3 exponentially grown cells/ml to grow for 18 h in doubling dilutions of 1 ml of the respective antibiotics at concentrations ranging from 0·5 to 2000 μg/ml, in nutrient broth for gentamicin and streptomycin, and in Mueller–Hinton broth for apramycin, netilmicin and tobramycin.

Plasmid characterization

Plasmids were transferred either directly or by mobilization with conjugative plasmids to a plasmid-free laboratory strain of *Escherichia coli* K12F$^-$ *lac*$^+$, nalidixic acid-resistant (= K12 *nal*^{r}). Plasmids were then assigned to compatibility groups on the basis of incompatibility with standard plasmids of the groups listed by Jacob *et al.* (1977) and with the cryptic *S. typhimurium* plasmid MP10 (Smith *et al.* 1973). Intra-group classification was achieved by determining the colicinogeny and antimicrobial resistances specified by different plasmids, and by their restriction endonuclease fragmentation patterns.

Identification of aminoglycoside-modifying enzymes

The aminoglycoside-modifying enzymes specified by the gentamicin–apramycin resistance plasmids were identified by the cellulose phosphate paper binding method of Shannon & Phillips (1983). Radiolabelled ATP and acetyl-coenzyme A were obtained from Amersham International plc. Radioactivity was measured in 5 ml of scintillation fluid, using a Packard Tri-Carb 4530 scintillation counter.

Preparation of partially purified DNA and agarose gel electrophoresis

Partially purified plasmid DNA from clinical isolates of DT204c and from K12 *nal*^{r} into which plasmids had been transferred or mobilized was isolated by the method of Birnboim & Doly (1979). Samples of plasmid DNA dissolved in TE buffer were subjected to electrophoresis on vertical 0·6% agarose slab gels, of approximate dimensions 16 × 18 cm. Electrophoresis was performed at 140 V for 3–4 h at room temperature, after which gels were stained for 30 min in distilled water containing 5 μg/ml of ethidium bromide (Sigma Chemical Co.). Molecular weights (MW's) were determined in relation to the mobility of four reference plasmids with MW's of 98, 42·0, 23·9 and 4·6 megadaltons (MDa). These were carried in a strain of K12 *nal*^{r}, designated 39R861.

The DNA of plasmids conferring resistance to apramycin–gentamicin was

Table 1. *Plasmids in gentamicin-resistant* Salmonella typhimurium *DT204c*

Strain	Source	Year of isolation	Resistance pattern*	Molecular weights of plasmids† present (MDa) 1	2	3	4	5	6	7
23M3424	Bovine	1983	ACGKSSuTTm	120	·	·	70	62	36	4·2
23M5519	Bovine	1983	ACGKSSuTTm	120	·	74	·	62	36	4·2
P66477	Bovine	1985	ACGKSSuTTm	120	77	·	·	62	36	4·2

* A, ampicillin; C, chloramphenicol; G, gentamicin-apramycin; K, neomycin-kanamycin; S, streptomycin; Su, sulphonamides; T, tetracyclines; Tm, trimethoprim.

† 1, CSSuTTm, Inc H_2; 2–4, Inc I_1, see Table 2; 5, T, Inc MP10; 6, AK, Inc X; 7, Su, unclassified.

Table 2. *Gentamicin–apramycin resistance plasmids in DT204c*

Designation	MW (MDa)	Resistances specified	Colicinogeny	Original host strain
TP305	70	G	ColIb	23M3424
TP306	74	GSTm	ColIb	23M5519
TP307	77	GS	—	P66477

Resistance symbols as Table 1.

digested with the restriction endonuclease *Eco*RI for 5 h at 37 °C under conditions specified by the enzyme manufacturer (Boehringer Mannheim). Restriction fragments were separated and their molecular weights calculated as described by Willshaw *et al.* (1980).

RESULTS

Plasmid profile analysis and plasmid characterization

The molecular weights (MW's) of plasmids in representative strains of DT204c of R-type ACGKSSuTTm (G, gentamicin-apramycin) are shown in Table 1.

All strains carried five plasmids with MWs of approximately 120, 70–77, 62, 36 and 4·2 MDa. The plasmids of 120 MDa coded for CSSuTTm, those of 70, 74 and 77 MDa conferred resistance to G, GSTm and GS respectively, and the 62 MDa plasmid coded for tetracycline resistance (T). The 36 MDa plasmid coded for AK and the 4·2 MDa plasmid for sulphonamide resistance (Su). The 120 MDa, 70, 74 and 77 MDa, and 36 MDa plasmids were conjugative and of the H_2, I_1 and X compatibility groups respectively. The 62 MDa tetracycline-resistance plasmid was non-conjugative but mobilizable, and was incompatible with the cryptic *S. typhimurium* plasmid MP10. The 4·2 MDa plasmid was also non-conjugative and was compatible with standard non-conjugative plasmids.

The plasmids conferring resistance to gentamicin–apramycin were designated TP305, TP306 and TP307 (Table 2). TP305 and TP306 both coded for the production of colicin Ib (ColIb), but differed in that TP305 coded for gentamicin–apramycin resistance but not for resistance to other antibiotics (GColIb), whereas TP306 conferred resistance to gentamicin–apramycin, streptomycin and trimethoprim (GSTmColIb). In contrast, TP307 coded for resistance to gentamicin–apramycin and streptomycin but did not code for ColIb production (GS). These plasmids also coded for resistance to the aminoglycosides netilmicin and tobramycin but not to amikacin. The MIC's to gentamicin and apramycin of strains of DT204c and *E. coli* K12 carrying TP305 and TP306 were 64 and 125 μg/ml respectively (Table 3). Strains carrying TP307 had MIC's of 500 μg/ml to apramycin and 256 μg/ml to gentamicin.

Enzymic mechanism of aminoglycoside inactivation

In cellulose phosphate paper binding assays, strains carrying TP305, TP306 and TP307 were unable to adenylylate gentamicin but all produced aminoglycoside 3-*N*-acetyltransferase IV. This enzyme has been designated AAC(3)IV (Davies & O'Connor, 1978).

Molecular studies of gentamicin–apramycin-resistance plasmids

TP305, TP306 and TP307 were digested with the restriction endonuclease *Eco*R1 and the DNA fragments were separated by gel electrophoresis. The results are shown in Fig. 1 and the MW's of the *Eco*R1-generated fragments are summarized in Table 4.

TP305 and TP306 had similar fragmentation patterns and had 9 of 11 DNA fragments in common. However *Eco*R1 digest of TP306 produced a fragment of 5·0 MDa whereas in the digest of TP305, the MW of the fragment was 4·0 MDa.

Table 3. *Resistance to aminoglycosides in DT204c*

Strain	Aminoglycoside antibiotic					
	Amikacin	Apramycin	Gentamicin	Netilmicin	Streptomycin	Tobramycin
23M3424	−	+ (125)	+ (64)	+ (64)	+ (64)	+ (64)
K12 (TP305)	−	+ (125)	+ (64)	+ (64)	− (0·5)	+ (64)
23M5519	−	+ (125)	+ (64)	+ (64)	+ (125)	+ (64)
K12 (TP306)	−	+ (125)	+ (64)	+ (64)	+ (32)	+ (64)
P66477	−	+ (500)	+ (256)	+ (125)	+ (250)	+ (125)
K12 (TP307)	−	+ (500)	+ (256)	+ (125)	+ (64)	+ (125)

+, Resistant; −, sensitive. MICs in parentheses (μg/ml).

Table 4. *Digest of gentamicin–apramycin resistance plasmids with EcoR1*

Plasmid	Fragment sizes (MDa)														
TP305	17·2	13·5	8·0	·	·	4·8	4·0	3·8	·	·	2·8	2·65	2·45	·	1·85	·
TP306	17·2	13·5	8·0	·	5·0	4·8	·	3·8	·	·	2·8	2·65	2·45	·	1·85	·
TP307	17·2	13·5	8·0	5·9	·	·	·	·	3·3	3·0	·	2·65	·	2·05	·	1·38

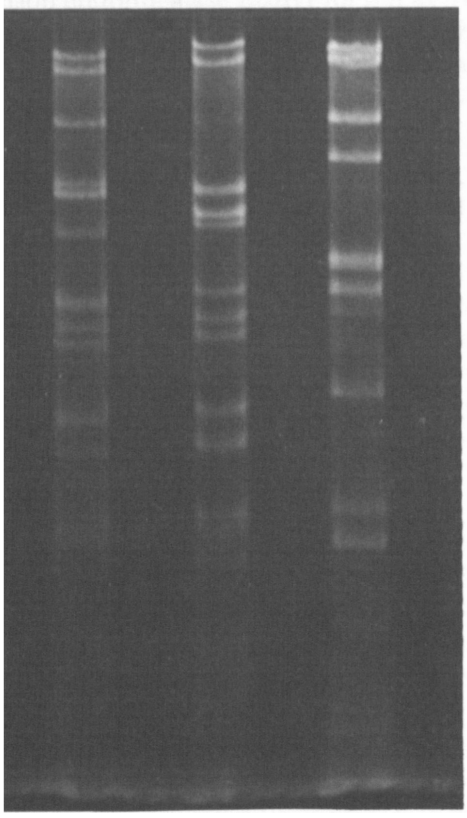

Fig. 1. EcoR1 digest of gentamicin–apramycin resistance plasmids in DT204c. Track 1, TP306 (GSTmColIb); track 2, TP305 (GColIb); track 3, TP307 (GS).

In contrast, TP307 had only 4 of 9 fragments in common with TP305 and v TP306.

Distribution of gentamicin–apramycin resistance plasmids in DT204c

From 1983 to 1985, 346 isolates of DT204c from cattle and 33 isolates fi humans were gentamicin-resistant. The occurrence of TP305, TP306 and TP30 DT204c was investigated in 79 of the 346 isolates from cattle and in all of 33 isol from humans (Table 5).

Of the bovine isolates 33 (41·8 %) carried TP305, 18 (22·8 %) TP306 and (35·4 %) TP307. Of the human isolates, 31 (93 %) carried TP307 and 2 (6 %) TP The two isolates carrying the TP305 plasmid were isolated in the last 9 mor of 1985. As yet, plasmids of the TP306 type have not been identified in DT2 from humans.

DISCUSSION

These studies show that strains of *S. typhimurium* DT204c of R-type ACG SuTTm carry five unrelated drug-resistance plasmids, as shown in Table 1. Tl plasmids of compatibility groups H_2, I_1 and X are conjugative and two plasn

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Table 5. *Distribution of TP305, TP306 and TP307 in DT204c ACGKSSuSTTm*

	Isolates from cattle				Isolates from humans			
		Plasmid types				Plasmid types		
	No. examined	TP305	TP306	TP307	No. examined	TP305	TP306	TP307
1983	21 (21)	13	6	2	1 (1)	0	0	1
1984	22 (73)	9	6	7	8 (8)	0	0	8
1985	36 (252)	11	6	19	24 (24)	2	0	22
Totals	79 (346)	33	18	28	33 (33)	2	0	31
Per cent		41·8	22·8	35·4		6·1	0	93·9

Figures in parentheses indicate the total isolates of DT204c ACGKSSuTTm from the respective sources, from 1983–85.

are non-conjugative. Of the two non-conjugative plasmids, one of 4·2 MDa codes for sulphonamide resistance and the second, of 62 MDa, codes for tetracyline resistance. The 62 MDa plasmid has been identified in all strains of DT204c studied, and is incompatible with the cryptic *S. typhimurium* plasmid MP10 (Smith *et al.* 1973). Thus, this plasmid may represent a tetracycline-resistant derivative of the MP10-like plasmids which have been identified in many phage types of *S. typhimurium* (Anderson & Smith, 1972).

Characterization of gentamicin–apramycin resistance plasmids in DT204c has demonstrated the existence of three plasmid types, the prototypes of which are TP305 (GColIb), TP306 (GSTmColIb) and TP307 (GS). Thus, three genetic lines of DT204c with the same phenotypic resistance pattern (ACGKSSuTTm) have been identified. Up to the end of 1985, DT204c ACGKSSuTTm with the TP306 plasmid had been found only in cattle but strains with the TP305 and TP307 plasmids had been identified in cattle and humans. However, the nature of spread of DT204c from cattle to humans is such that the appearance in humans of strains with the TP306 plasmid type is to be expected in the future.

In gentamicin-resistant DT204c, resistance to gentamicin is mediated by production of the aminoglycoside-modifying enzyme AAC(3)IV. This enzyme also inactivates the aminoglycoside antibiotic apramycin, which is used in veterinary medicine. We understand that apramycin has been used extensively in calves over the last 3 years in attempts to combat salmonellosis, whereas gentamicin is rarely used in bovine husbandry. It is reasonable to conclude that this useage is responsible for the appearance of gentamicin resistance in a phage type already epidemic in cattle in Britain and with an increasing incidence in humans.

In 1969 the Joint Committee on the use of Antibiotics in Animal Husbandry and Veterinary Medicine (Swann Committee) stated that they considered it sensible to reduce as far as possible the actual and potential dangers to man which may result from the giving of antibiotics to animals (Anonymous, 1969). From the evidence presented above it is apparent that attempts to control bovine salmonellosis with apramycin have resulted in the appearance of *S. typhimurium* resistant to gentamicin, a drug of vital importance in the treatment of Gram-negative septicaemia in humans.

REFERENCES

ANDERSON, E. S. & SMITH, H. R. (1972). Fertility inhibition in strains of *Salmonella typhimurium*. *Molecular and General Genetics* **118**, 79–84.

ANDERSON, E. S. & THRELFALL, E. J. (1974). The characterization of plasmids in the enterobacteria. *Journal of Hygiene* **72**, 471–487.

ANONYMOUS (1969). *Report of the Joint Committee on the use of Antibiotics in Animal Husbandry and Veterinary Medicine*. London: Her Majesty's Stationery Office.

ANONYMOUS (1985). *Animal Salmonellosis*. 1984 *Annual Summaries*. Ministry of Agriculture, Fisheries and Food; Welsh Office Agriculture Department; Department of Agriculture and Fisheries for Scotland.

BIRNBOIM, H. C. & DOLY, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* **7**, 1513–1523.

DAVIES, J. & O'CONNOR, S. (1978). Enzymatic modifications of aminoglycoside antibiotics: 3-*N*-acetyltransferase with broad specificity that determines resistance to the novel aminoglycoside apramycin. *Antimicrobial Agents and Chemotherapy* **14**, 69–72.

JACOB, A. E., SHAPIRO, J. A., YAMAMOTO, L., SMITH, D. I., COHEN, S. N. & BERG, D. (1977). Appendix B: Bacterial plasmids. In *DNA Insertion Elements, Plasmids and Episomes* (ed. A. I. Bukhari, J. A. Shapiro and S. L. Adhya), pp. 607–670. Cold Spring Harbor Laboratory.

SHANNON, K. P. & PHILLIPS, I. (1983). Detection of aminoglycoside-modifying strain of bacteria. In *Antibiotics: Methods of Assessing Antimicrobial Activity and the Occurrence of Resistance* (ed. A. D. Russell, and L. B. Quesnel), pp. 183–198. SAB Technical Series, no. 18. London: Academic Press.

SMITH, H. R., HUMPHREYS, G. O., GRINDLEY, N. D. F., GRINDLEY, J. N. & ANDERSON, E. S. (1973). Molecular studies of an fi^+ plasmid from strains of *Salmonella typhimurium*. *Molecular and General Genetics* **126**, 143–145.

THRELFALL, E. J., ROWE, B., FERGUSON, J. L. & WARD, L. R. (1983). Gentamicin-resistant *Salmonella typhimurium*. *Veterinary Record* **113**, 627.

THRELFALL, E. J., WARD, L. R., FERGUSON, J. L. & ROWE, B. (1985). The increasing incidence of resistance to gentamicin and related aminoglycosides in *Salmonella typhimurium* phage type 204c in England, Wales and Scotland. *Veterinary Record* **117**, 355–357.

WILLSHAW, G. A., BARCLAY, E. A., SMITH, H. R., MCCONNELL, M. M. & ROWE, B. (1980). Molecular comparison of plasmids encoding heat-labile enterotoxin isolated from *Escherichia coli* strains of human origin. *Journal of Bacteriology* **143**, 168–175.