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Instability of multiple drug resistance plasmids in *Salmonella typhimurium* isolated from poultry

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SUMMARY

Plasmids in five strains of *Salmonella typhimurium* resistant to ampicillin, chloramphenicol, gentamicin, neomycin/kanamycin, streptomycin, sulphonamides, tetracyclines and trimethoprim (ACGKSSuTTm), CGKSSuTTm, ACSSuT or CSSuT which had been isolated from poultry in the first 3 months of 1989 have been characterized and compared with plasmids in two strains of R-types ACGKSSuTTm and ASSuTTm isolated from two patients later in the year. With the exception of the human isolate of R-type ASSuTTm, all strains carried two non-conjugative plasmids, one coding for SSu and belonging to incompatibility group Q, and a second coding for multiple resistance and belonging to the F_I*me* incompatibility group. The human isolate of R-type ASSuTTm did not carry the *Inc*Q SSu plasmid but like the poultry isolates, carried a non-conjugative F_I*me* plasmid.

Restriction endonuclease digestion with the enzymes *Eco*R I, *Pst* I and *Hin*d III demonstrated that the F_I*me* plasmids from strains of different R-types showed a high degree of homology but exhibited numerous fragment size polymorphisms. The restriction digest fingerprint of plasmids in the human isolate of R-type ACGKSSuTTm was indistinguishable from a poultry isolate of the same R-type. Analysis of segregants of one of the poultry isolates of R-type ACGKSSuTTm demonstrated that resistance determinants could be rapidly lost from the F_I*me* plasmid to give rise to a number of R-types and fingerprint patterns. Loss of tetracycline resistance from this plasmid appeared to be correlated with the integration of other plasmid-mediated resistances into the bacterial chromosome. Evidence is presented for the rapid loss of antimicrobial resistance determinants from a multiple resistance plasmid of the F_I*me* incompatibility group in response to withdrawal of antibiotic selective pressure.

INTRODUCTION

In England and Wales multiple antimicrobial drug resistance is common in bovine isolates of *Salmonella typhimurium* but not in poultry isolates [1]. Consequently the appearance of multiply-resistant strains in chickens and turkeys

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on three farms in England early in 1989 was regarded as an unusual event [2]. Although four different patterns of multiple resistance (R-types) were identified – ampicillin, chloramphenicol, gentamicin, kanamycin, streptomycin, sulphonamides, tetracyclines and trimethoprim (ACGKSSuTTm), CGKSSuTTm, ACSSuT and CSSuT, the strains had several features in common: they did not react with the standard *S. typhimurium* typing phages but gave the same reactions with the phages of an ancillary typing scheme developed to differentiate untypable *S. typhimurium*; resistances were plasmid-encoded; and all strains carried two non-conjugative resistance plasmids. One of these plasmids had a molecular weight (MW) of 5.5 megadaltons (MDa) and coded for resistance to streptomycin and sulphonamides; the second had a molecular weight in the range of 60–70 MDa, coded for multiple drug resistance and belonged to the F_I*me* incompatibility group.

F_I*me* resistance plasmids were first identified in 1974 in strains of *S. typhimurium* originating in the Middle East [3]. *S. typhimurium* carrying F_I*me* resistance plasmids have subsequently caused extensive outbreaks in the Middle East, the Indian subcontinent and Africa [4–6] and strains of *S. wien* with F_I*me* plasmids have caused a series of outbreaks in North Africa, Southern Europe and South-east Asia [7–9]. Despite their widespread distribution in salmonellae in developing countries, F_I*me* plasmids have remained relatively uncommon in salmonellae from humans and food animals in Britain (*E. J. Threlfall, unpublished observations*). Thus, although there were considerable differences in their R-types, the finding that all the poultry isolates carried F_I*me* plasmids suggested an epidemiological relationship.

Preliminary studies had demonstrated that with one exception, plasmids in isolates of R-type ACGKSSuTTm were homogeneous in respect of their endonuclease fingerprints, as were plasmids in isolates of R-types CGKSSuTTm and ACSSuT [2]. The purpose of this investigation was to determine whether isolates with the different R-types had evolved within the poultry flocks from a progenitor strain of R-type ACGKSSuTTm or whether a number of strains with different R-types had been introduced, possibly on several occasions. The initial approach was to confirm the relatedness of isolates of different R-types by characterizing representative strains on the basis of the numbers, molecular weights, incompatibility and typing phage restriction properties of carried plasmids, and their ability to produce the aerobactin siderophore. The latter property was included because it has been demonstrated that some F_I*me* plasmids code for the production of the hydroxamate-mediated iron uptake system [10, 11], which is a recognized virulence property of some enteric bacteria. Further studies were directed towards a molecular comparison of carried plasmids on the basis of their restriction endonuclease fingerprints, and an investigation of the stability of resistance determinants in a representative strain of R-type ACGKSSuTTm. Two multiply-resistant strains that gave identical phage typing reactions but which were isolated later in the year from two patients infected in different parts of the country were also characterized in order to compare their molecular identity with those of the poultry isolates.

Table 1. *Plasmids in untypable* S. typhimurium *isolated from poultry and humans in England in 1989*

LAN*	Source	R-type	Molecular weight of plasmid DNA (MDa)				Resistance transfer		
								By mobilization	
							Direct	X (F_{II})	Δ (I_1)
P155251	Chicken	ACGKSSuTTm	70			5·5	—	ACGSSuTTm†	SSu‡
P155261	Turkey	CGKSSuTTm		67		5·5	—	CGSSuTTm	SSu
P158409	Turkey	CSSuT			62	5·5	—	CSSuT	SSu
P156296	Turkey	ACSSuT		68		5·5	—	ACSSuT	SSu
P150201	Turkey	ACGKSSuTTm	72			5·5	—	ACGSSuTTm	SSu
P163245	Human	ASSuTTm		65		—	—	ASSuTTm	—
P164216	Human	ACGKSSuTTm	70			5·5	—	ACGSSuTTm	SSu

* LAN, Laboratory accession number.

Resistance symbols: A, ampicillin; C, chloramphenicol; G, gentamicin; K, kanamycin/neomycin; S, streptomycin; Su, sulphonamides; T, tetracyclines; Tm, trimethoprim.

† Incompatibility Group: $F_I me$.

‡ Incompatibility Group: Q.

MATERIALS AND METHODS

Bacterial strains

Single isolates of R-types ACGKSSuTTm, CGKSSuTTm, CSSuT and ACSSuT were selected from those poultry isolates of *S. typhimurium* described in a previous communication [2]; an additional isolate of R-type ACGKSSuTTm in which restriction fragment polymorphism had been observed was also included. The designations, origins and plasmid profiles of these isolates are shown in Table 1. Two multiply-resistant strains of *S. typhimurium* with identical phage typing reactions to those of the poultry isolates but which were isolated later in 1989 from two patients infected in different areas of the country were also included in this investigation. The R-types and plasmid profiles of these human isolates are also shown in Table 1.

Phage-typing, antimicrobial resistance testing and incompatibility determination

Strains were phage typed by the methods of Callow [12] and Anderson and Williams [13], and tested for antimicrobial drug resistance by the methods of Anderson and Threlfall [14]. Mobilization of non-conjugative plasmids was achieved by using the conjugative plasmids, X (42 MDa, *Inc*F_{II}) and Δ (60 MDa *Inc*I_1). Plasmids were assigned to incompatibility groups on the basis of their incompatibility with selected plasmids of defined groups [15], and also with the plasmids K-MP10 (*Inc*MP10), TP180 (*Inc*F_1*me*), F-*lac* (*Inc*F_I) and NTP3 (*Inc*Q). The origins of K-MP10, TP180 and NTP3 have been described previously [4]. F_1*me* plasmids were displaced from their wild-type host strains by the introduction of F-*lac* and any change in phage type and R-type determined as described above.

Testing for production of aerobactin

Strains were grown for 18 h with aeration in a Tris-succinate medium [16] and the presence of hydroxamate was detected by the ferric perchlorate reaction [17]. An aerobactin-producing strain and a negative control strain were included in all experiments.

Preparation of plasmid DNA, restriction endonuclease digestion and gel electrophoresis

Partially-purified plasmid DNA was isolated by a modification of the method of Kado and Liu [18] as described by Threlfall and colleagues [19] and electrophoresed at 150 V for 3–4 h on a 0·7% (w/v) agarose/trisborate vertical slab gel before staining with ethidium bromide.

For restriction enzyme digest analysis, partially-purified DNA prepared by the method of Olsen [20] was digested with *Hin*d III, *Pst* I and *Eco*R I for 4–5 h under the conditions specified by the manufacturer (Bethesda Research Laboratories). Restriction fragments were separated on horizontal 0·8% (w/v) agarose/Tris-acetate gels (BRL H5 Horizontal Gel Apparatus) at a constant current of 17 mA for 18 h and their molecular weights were determined relative to fragments generated by a *Hind* III, *Pst* I and *Eco*R I digests of bacteriophage lambda.

Molecular relatedness

The molecular relatedness of plasmids was estimated on the basis of the number of fragments in common after digest with *Hind* III and *Pst* I, and was numerically

expressed by using the Dice coefficient of similarity [21] to compare the respective restriction endonuclease fragmentation patterns.

Instability of antimicrobial resistance determinants

In experiments to investigate the stability of resistance determinants, a single colony of P155251 (R-type ACGKSSuTTm) which had been cultured on selective media containing all the antimicrobials to which it was resistant was grown with aeration at 37 °C for 18 h in 3 ml of nutrient broth. After dilution in sterile saline, 100 μl aliquots were spread onto the surface of nutrient agar plates at a dilution estimated to deliver about 100 colonies per plate. After overnight incubation at 37 °C, plates were replicated onto nutrient agar plates containing respectively (mg/l): ampicillin (50), chloramphenicol (20), gentamicin (20), kanamycin (20), streptomycin (20) or tetracycline (10). Colonies that displayed loss of one or more resistance markers were fully R-typed and their phage type, plasmid profile, restriction fragmentation pattern and their ability to produce aerobactin determined as described previously.

RESULTS

Plasmid characterization

The MWs of plasmids in the five untypable isolates from poultry and in the two human isolates of R-types ASSuTTm and ACGKSSuTTm are shown in Table 1.

All poultry isolates carried two non-conjugative plasmids, one with a MW of 5·5 MDa which coded for SSu and a second with a MW ranging from 62–75 MDa which coded for ACGSSuTTm in P155251 and P155201, CGSSuTTm in P155261, ACSSuT in P156296 and CSSuT in P158409. Kanamycin resistance in P155251, P155201 (ACGKSSuTTm) and P155261 (CGKSSuTTm) was neither transferable nor mobilizable. The SSu plasmids were readily mobilizable with the *Inc*I_1 plasmid Δ and were incompatible with NTP3, a standard plasmid of incompatibility group Q; in contrast the large molecular mass plasmids could not be mobilized with Δ but were readily mobilizable with the *Inc*F_{II} plasmid, X. These plasmids were incompatible with K-MP10, with F-*lac* and with TP180 and on the basis of these incompatibility results were assigned to the F_I*me* incompatibility group.

The human isolate of R-type ASSuTTm carried a single non-conjugative plasmid of 70 MDa which coded for the complete resistance spectrum of its host strain. This plasmid was mobilizable with X and belonged to the F_I*me* incompatibility group. The isolate of R-type ACGKSSuTTm carried two non-conjugative plasmids, a 5·5 MDa SSu plasmid which belonged to incompatibility group Q and a 70 MDa F_I*me* plasmid which coded for ACGSSuTTm. As in the poultry isolates of R-types ACGKSSuTTm and CGKSSuTTm, kanamycin resistance was neither transferable nor mobilizable.

Influence of plasmids on phage type

When the F_I*me* plasmids from both the poultry and human isolates were introduced into *S. typhimurium* phage type 36, which is lysed by all the *S. typhimurium* typing phages, the resultant phage restriction patterns matched that previously described as non-conforming, type 10 (NC10) [4]. This restriction pattern is typical of that produced by plasmids of the F_I*me* incompatibility group.

F_I*me* plasmids have been shown to be phage type determining in wild-type

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strains of *S. typhimurium* [4]. In order to investigate the influence of the F_I*me* plasmids from the poultry and human *S. typhimurium* isolates on the phage types of their hosts, the F_I*me* plasmids were displaced from their wild-type host strains by the introduction of the *Inc* F_I plasmid F-*lac*. In all cases the resultant *lac*$^+$ *S. typhimurium* from which the F_I*me* plasmids had been displaced reacted with one of the standard *S. typhimurium* typing phages and their lysis pattern corresponded to that of phage type 32.

Plasmid analysis of strains from which the F_I*me* plasmid had been displaced by F-*lac* confirmed that the 5·5 MDa plasmid which coded for SSu had been retained by the six isolates which originally carried this plasmid. Kanamycin resistance had been retained by the three poultry isolates and the single human isolate which were resistant to this antibiotic but since no plasmid DNA other than that of F-*lac* was detected in the strains, it was concluded that kanamycin resistance could be chromosomal.

Aerobactin production

When tested for the production of the aerobactin, all isolates from poultry and humans, and transconjugants into which the F_I*me* plasmids from these isolates had been transferred produced the brownish red colour indicative of aerobactin production. In contrast plasmid-free control strains of *S. typhimurium* and *E. coli* did not alter the colouration of the growth medium.

Restriction endonuclease fingerprinting of plasmid DNA

Previous studies had demonstrated that with the exception of P155201, all poultry isolates of R-type ACGKSSuTTm had identical plasmid DNA fragmentation patterns when cleaved with the restriction enzymes *Eco*R I, *Hind* III and *Pst* I [2]. For this reason P155251 was chosen as the representative isolate of strains of R-type ACGKSSuTTm; P155201 was included because of the previously-observed fragment length polymorphism in the F_I*me* plasmid in this isolate.

The *Hind* III and *Pst* I restriction endonuclease digest profiles of plasmids in the five representative poultry isolates and of the human isolate P163245 (ASSuT) are shown in Fig. 1; their respective Dice coefficients of similarity are provided in Table 2. The digest profiles of the second human isolate P164216 (ACGKSSuTTm) were identical to those of the poultry isolate P155251 and have therefore not been included in Fig. 1 and Table 2.

The results demonstrate a high degree of relatedness among the plasmids in the isolates. Coefficients of similarity ranged from 63% between P150201 (ACGSSuTTm) and P158409 (CSSuT), to 94% between P158409 (CSSuT) and P155261 (CGSSuTTm). P156296 (ACSSuT) showed about 80% similarity with isolates of other R-types. The human isolate P163245 (ASSuTTm) had between 71 and 80% similarity with poultry isolates of different R-types.

Segregation of antimicrobial resistance determinants

When a single colony of P155251 was cultured for 18 h in non-selective media, spontaneous segregation of antimicrobial resistance determinants was observed at the following frequencies (%): A, 18; C, 11; G, 9; K, 3; S, $< 0{\cdot}1$; T, 12. Eighty-

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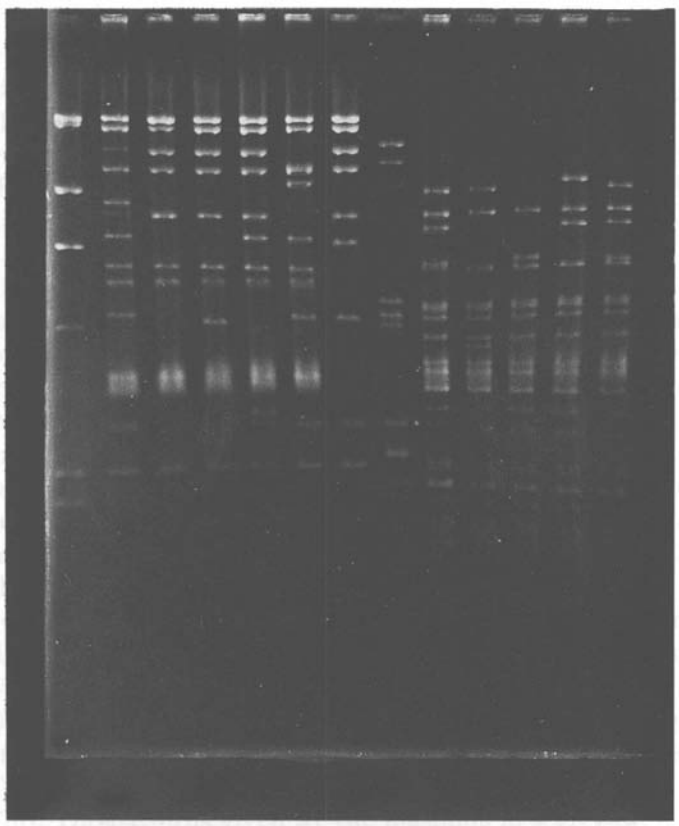

Fig. 1. Restriction enzyme digest of untypable *S. typhimurium*. Track 1, λ*Hin*d III; 2, P155251 *Hin*d III; 3, P155261 *Hin*d III; 4, P158409 *Hin*d III; 5, P156296 *Hin*d III; 6, P150201 *Hin*d III; 7, P163245 *Hin*d III; 8, λ*Pst* I; 9, P155251 *Pst* I; 10, P155261 *Pst* I; 11, P158409 *Pst* I; 12, *P*156296 *Pst* I; 13, P150201 *Pst* I; 14, 163245 *Pst* I.

Table 2. *Dice coefficients of similarity between* $F_I$me *plasmids in untypable* S. typhimurium *from poultry and humans expressed as a percentage*

Strain	P163245	P150201	P156296	P158409	P155261	P155251
P155251	80	85	72	70	73	100
P155261	70	66	84	94	100	
P158409	77	63	80	100		
P156296	80	76	100			
P150201	73	100				

six colonies that displayed loss of one or more of the resistance markers were fully R-typed and screened for plasmid DNA; 15 of these colonies that were representative of the different segregant types were phage typed, tested for the ability to produce aerobactin and their plasmid DNA digested with *Hind* III and *Pst* I. The phage types, R-types and plasmid profiles of these segregants are shown in Table 3.

In all segregants there was some loss of plasmid DNA, in six segregants apparent loss of the complete F_I*me* plasmid, and in two segregants loss of the

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Table 3. *Plasmid DNA, phage type and aerobactin production in spontaneous segregants of* S. typhimurium *strain P155251*

Segregant number	Phage type	R-type	Aerobactin production	Molecular weight of plasmid DNA (MDa)								
P155251/02	U	ACGKSSuTm	−					—				5·5
12	U	CGKSSuT	+						52			5·5
13	U	ACGKSSuTm	−					—				5·5
14	U	ACKSSuT	+			62						5·5
15	U	CGKSSuT	+						56			5·5
17	U	ACGKSSuTm	−					—				5·5
24	32	SSu	−					—				5·5
29	U	ACGKSSuTm	−					—				5·5
30	U	ACSSuT	+				58					5·5
31	U	KSSu	−							50		5·5
33	U	ACSSuT	+		68							5·5
36	U	CGKSSuTTm	+			67						5·5
42	U	ACSSuT	+					58				—
54	U	ASSuTTm	+				65					5·5
93	32	—	−					—				—

Resistance symbols: as Table 1.
U, untypable.

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5·5 MDa SSu plasmid. In two segregants (24 and 93) loss of the F_I*me* plasmid was accompanied by loss of all the resistance markers encoded by this plasmid in the wild-type strain and by loss of the ability to produce aerobactin, and resulted in conversion of the segregant to phage type 32. However in four segregants (02, 13, 17 and 29) loss of tetracycline resistance appeared to be correlated with the absence of any high MW DNA in the strain and with loss of the ability to produce aerobactin. However the other resistances encoded by the F_I*me* plasmid in the wild-type parental strain had been retained and the segregants all displayed the R-type ACGKSSuTTm and had not changed in phage type.

The 5·5 MDa SSu plasmid was absent in two segregants (41 and 93). One of these (41) had retained the F_I*me* plasmid but with loss of the gentamicin and trimethoprim resistance genes, and also with loss of kanamycin resistance; the second (93) had lost the complete F_I*me* plasmid. Although kanamycin resistance did not appear to be plasmid-encoded, 6 of the 15 segregants had lost resistance to this antimicrobial. In segregant 31 (KSSu), about 30% of DNA had been lost from the F_I*me* plasmid together with the ability to code for aerobactin and with all of the drug resistance encoded by the plasmid in the parental strain. However this segregant had retained both kanamycin resistance and the 5·5 MDa SSu plasmid, and showed no change in phage type.

Digestion with *Hind* III and *Pst* I of plasmid DNA in the nine segregants which had retained the F_I*me* plasmid resulted in nine different fragmentation patterns for each enzyme. Of these, the fragmentation patterns of the plasmids in segregant 33 (ACSSuT) were identical to those of the plasmids in P156296 (R-type ACSSuT). Likewise the fragmentation patterns of the plasmids in segregant 36 (CGKSSuTTm) were identical with those of the plasmids in P155261 (CGKSSuTTm). The patterns given by the plasmids in segregant 54 (ASSuTTm) were almost identical to those of the plasmid in the human isolate P163245 (ASSuTTm).

DISCUSSION

These studies have demonstrated that F_I*me* plasmids in the poultry isolates of *S. typhimurium*, although coding for different patterns of resistance, were closely related in respect of their restriction endonuclease fingerprints and their ability to code for the production of the aerobactin siderophore. F_I*me* plasmids in the two human isolates of R-types ASSuTTm and ACGKSSuTTm were also either closely related (ASSuTTm) or identical (ACGKSSuTTm) to those in the poultry isolates. Furthermore, phage typing analysis of poultry and human isolates from which the F_I*me* plasmids had been displaced either by the introduction of F-*lac* or by spontaneous loss after growth in unselective media demonstrated not only that the F_I*me* plasmids were phage type-determining in their wild-type hosts, but also that their parental strains belonged to the same *S. typhimurium* phage type, phage type 32. These results demonstrate that all the poultry isolates and the two human isolates had been derived from progenitor strains of phage type 32 at some stage in their evolutionary history.

Some plasmids of the F_I*me* incompatibility group have been reported to lose antimicrobial drug resistance both on transfer and on storage. This property has been observed with F_I*me* plasmids in *S. typhimurium* phage type 208 [4], in

S. johannesburg [22] and in *S. wien* [23]. The demonstration of rapid loss of antimicrobial resistance determinants from F_I*me* plasmid in P155251 when antibiotics were not included in the growth medium probably represents another example of this instability and may provide an explanation for the diversity of resistance spectra in the plasmid-carrying poultry isolates of *S. typhimurium*. It may also be significant that two spontaneous segregants of P155251 had the same R-types (ACSSuT and CGKSSuTTm) and identical restriction endonuclease fingerprints as those of two naturally-occurring isolates. Although it is impossible to determine retrospectively whether the isolates with different R-types had evolved within the poultry flocks from a single strain of R-type ACGKSSuTTm, the finding that at least two of the isolates with different R-types could be derived from a strain of R-type ACGKSSuTTm after overnight growth in non-selective media does indicate that the necessary evolutionary changes could have occurred over a relatively short period of time. Likewise, the observation that the SSu plasmid could also be lost from P155251 indicated that strains without this plasmid could also be derived from a strain of R-type ACGKSSuTTm. Analysis of spontaneous segregants of P155251 provided further evidence of the instability of these F_I*me* plasmids, since segregants exhibiting 11 different R-types in which loss of resistance determinants from the F_I*me* plasmid had seemingly occurred at random were readily obtained. Indeed, one segregant, P155251/31, had lost all the antimicrobial resistance and aerobactin production genes from the F_I*me* plasmid, and had retained only that portion of the plasmid which did not code for drug resistance.

Four segregants of R-type ACGKSSuTTm had lost the ability to produce aerobactin in addition to losing the genes coding for tetracycline resistance. However, although the 5·5 MDa SSu plasmid was present in these segregants, there was no evidence of high-molecular-weight plasmid DNA and the strains had not reverted to phage type 32. Since resistance to ACGKSSuTm had been retained and there had been no change in phage type, it is probable that the ACGKSSuTm resistance genes and the typing phage restriction genes had become chromosomally integrated. Chromosomal integration of plasmid-mediated multiple drug resistance has been reported in *S. dublin* isolated in Germany and Britain [24, 25]; the integration of plasmid-mediated drug resistance genes into the *S. typhimurium* chromosome, as reported here, could be another example of the ability of the bacterial cell to conserve plasmid-mediated antimicrobial resistance genes in the absence of selective pressure.

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