Stable Coexistence of Three Resistance Factors (*fi*⁻) in *Salmonella panama* and *Escherichia coli* K12

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SUMMARY

During an epidemic, a strain of *Salmonella panama* resistant to ampicillin, kanamycin, tetracycline and which was colicinogenic was isolated. The three drug-resistance determinants were transferred singly, in pairs or *en bloc* to sensitive *Escherichia coli* K12 strains. When the recipient was an Hfr strain, it remained sensitive to F-specific phage f 2, so the transferred resistance factors can be classified as *fi*⁻. The three resistance characters were always independently transduced by phage P 1-b, and remained transferable in subsequent conjugations. In contrast to closely related R factors or Col factors which cannot coexist stably in the same cell, the three R factors described here do not specifically interfere with one another. They are maintained together in the naturally occurring strain of *S. panama* and after successive transfer to *E. coli* K12. These results support the hypothesis that three independent *fi*⁻ resistance factors, R (Am), R (Km), R (Tc) and a Col factor coexist in *S. panama* and *E. coli* K12 strains.

INTRODUCTION

Resistance factors (R factors) have been classified in two groups: *fi*⁺ (or *i*⁺) factors which inhibit the properties of the sex factor F, and *fi*⁻ (or *i*⁻) which do not inhibit these properties (Egawa & Hirota, 1962; Watanabe, Nishida, Ogata, Arai & Sato, 1964). When two R factors are harboured by a single bacterium, both elements coexist stably in the progeny when they do not belong to the same *fi*⁺ or *fi*⁻ class. When both R factors are *fi*⁺ or *fi*⁻, mutual exclusion occurs, and it has been assumed that the two compete for a single replication site (Watanabe *et al.* 1964).

During an epidemic in Paris, a *Salmonella panama* strain (LA 46. R 11), resistant to ampicillin (Am), kanamycin (Km), tetracycline (Tc) and harbouring a Col factor was isolated and this seems to behave differently from the previously described systems. Transfer of drug-resistance to sensitive *Escherichia coli* K12 was easily obtained (Chabbert, Baudens & Bouanchaud, 1967). The recipient bacteria rarely became resistant to all three drugs (Am, Km, Tc), but generally to only one or two of them. When any or all resistance characters were transferred to an *E. coli* K12 Hfr strain, the recipients remained sensitive to F-specific RNA phage f 2, thus the transferred resistance factors can be classified as *fi*⁻ (Chabbert, Baudens & Bouanchaud, 1969).

Spontaneous loss of resistance occurred at low frequency in *Salmonella panama* LA 46. R 11: 0.1 % for Am and Tc, and 2 % for the Km character. Growth at a sub-inhibitory concentration of acriflavine increased the percentage of Km sensitive bacteria to 41 % (Baudens & Chabbert, 1967). Similar results were obtained with

ethidium bromide, another 'curing' agent (Bouanchaud, Scavizzi & Chabbert, 1968). In contrast, the percentages of Am or Tc sensitive bacteria were not increased by these treatments. The present work is an attempt to determine whether the three resistance characters (Am, Km, Tc) belong to a single plasmid (R₁₁), or whether there are several independent *fi*⁻ factors, R₁₁₋₁ (Am), R₁₁₋₂ (Km), R₁₁₋₃ (Tc) and a Col factor which can stably coexist in *S. panama* LA 46 or in *Escherichia coli* K 12.

METHODS

Bacterial strains. *Salmonella panama* LA 46.R 11 (Am Km Tc) (ColIb), kindly supplied by Professor L. Le Minor (Centre National des Salmonelles, Institut Pasteur), was isolated during an epidemic in Paris. Colicin type was determined by Dr Y. Hamon (Institut Pasteur). *Escherichia coli* K 12 54117 F⁻ *lac*⁺ was also obtained from

Table 1. *Origin of strains*

<i>Escherichia coli</i> K 12	Drug-resistance pattern	Transfers donor × recipient		Transductions by P 1-b donor × recipient	
LA 117	Am Km Tc	LA 46	LA 115	—	—
LA 118	Am Tc	LA 46	54117	—	—
LA 119	Sm Cm Tc Su	LA 49	LA 116	—	—
LA 120	Am	LA 46	LA 115	—	—
LA 121	Tc	LA 117	LA 116	—	—
LA 122	Tc	LA 46	LA 115	—	—
LA 123	Am	LA 117	LA 116	—	—
LA 124	Km	—	—	LA 117	54117
LA 125	Km	—	—	LA 117	LA 116
LA 126	Tc	LA 141	LA 116	—	—
LA 129	Am	LA 139	LA 116	—	—
LA 139	Am	—	—	LA 117	54117
LA 141	Tc	—	—	LA 117	54117
LA 142	Km Tc	LA 126	LA 124	—	—
LA 143	Am Km Tc	LA 129	LA 142	—	—

Professor Le Minor. *E. coli* K 12 C 600 F⁻ *lac*⁻ *thr*⁻ *leu*⁻ *bio*⁻ was kindly supplied by Dr E. L. Wollman (Institut Pasteur). *E. coli* K 12 LA 115, mutant resistant to sodium azide, derived from *E. coli* K 12 54117 and selected on gradient plates (Szybalski, 1952). *E. coli* K 12 LA 116, mutant resistant to rifampicin derived from *E. coli* K 12 C 600. *E. coli* LA 49.R 15 (Sm Cm Tc Su)* *fi*⁺, serotype 111.B 4, was isolated from clinical material. Other *E. coli* K 12 strains were obtained in transfer or transduction experiments and are listed in Table 1. All resistant strains derived from *S. panama* LA 46.R 11 harboured a *fi*⁻ resistance factor.

Culture media and drugs. Tryptic soy broth (0370 Difco) and Mueller Hinton solid medium (0252-01 Difco) were routinely used. Fermentation of lactose was observed on Drigalski medium. Phage P 1-b was propagated on bacteria grown in pancreatic peptone broth (Peptone P.T.V. obtained from Ets. Réactifs, 75-Gentilly, France).

Ampicillin was obtained from Laboratoires Delagrangé, Paris; kanamycin sulphate from Laboratories Bristol, Paris; sodium azide from Laboratoires Rhône-Poulenc, Paris; tetracycline hydrochloride from Laboratoires Pfizer-Clin, Paris. Rifampicin

* Sm, streptomycin; Cm, chloramphenicol; Su, sulphonamides; Tc, tetracycline.

was a gift from Laboratoires Lepetit, Paris. Antibiotic resistance was determined with sensitivity discs from the Institut Pasteur.

Conjugation. Multiply resistant donor strains (*Salmonella panama* LA 46.R 11 and *Escherichia coli* LA 143) were cultivated for 4 hr on cellophan membranes lying on nutrient agar plates supplemented with the following concentrations ($\mu\text{g./ml.}$) of antibiotics: ampicillin, 20; kanamycin, 12.5; tetracycline, 16; to obtain entirely multi-resistant populations (Chabbert & Patte, 1960). The donor bacteria were suspended in broth (2.5×10^8 bacteria/ml.) and mixed with the recipient strain. In other cases, conjugation conditions were as described by Watanabe & Fukasawa (1961*a*). Twenty minutes later, the mixture was blended and diluted in broth containing methionine ($20 \mu\text{g./ml.}$) (Mitsuhashi, 1965); 0.2 ml. of each dilution were spread on Drigalski medium containing sodium azide ($300 \mu\text{g./ml.}$) or rifampicin ($100 \mu\text{g./ml.}$), and the antibiotic selecting for the R factor ($\mu\text{g./ml.}$): ampicillin, 20; kanamycin, 12.5; tetracycline, 12. Frequencies of transfers were expressed per donor bacterium in the mixture.

Transduction. Phage P 1-b, a mutant of phage P 1-kc was kindly supplied by Professor F. Jacob (Institut Pasteur; originally from Dr S. Brenner). Phage P 1-b was propagated in resistant donor bacteria. Preparations containing about 10^8 p.f.u./ml. were obtained and sterilized with chloroform. The recipients were infected (multiplicity of infection = 1) and transductants were selected on gradient plates containing the selecting antibiotic (Szybalski, 1952). Trypsin ($200 \mu\text{g./ml.}$) was added to the phage suspension when the donor bacteria were colicinogenic.

RESULTS

Transfers in 20 min. from Salmonella panama LA 46.R 11 to Escherichia coli K 12 LA 115 and from E. coli K 12 LA 143 to E. coli K 12 LA 116.

Salmonella panama LA 46.R 11 (Am Km Tc) (collb) was grown on agar supplemented with the three antibiotics to obtain an entirely multi-resistant population. Before conjugation, 396 colonies were isolated and tested for drug-resistance: no sensitive segregant was observed on replica plates. The same results were obtained with *E. coli* K 12 LA 143 (Am Km Tc) (*col*⁻). The transfers observed in 20 min. are reported in Table 2. It can be seen that some recipients became resistant to all three drugs, but transfer of single or pairs of resistance characters (Am Km, Am Tc, Km Tc) was also obtained, and that all combinations of transfer were possible. The frequencies of transfer from *S. panama* to *E. coli* K 12 LA 115 varied from 4×10^{-4} to 1.2×10^{-6} . The isolated characters Am and Tc were transferred at the highest frequency, and the character Km at the lowest. Each pair of characters was transferred at about the same frequency, and the complete combination of three at lower frequency. Similar results were obtained in transfers from *E. coli* K 12 LA 143 to *E. coli* K 12 LA 116, and the frequencies of transfers varied from 3.1×10^{-6} to 2×10^{-7} . Transfer of colicin factor was frequently observed, but not with all the drug-resistant recipient bacteria. This transfer from *S. panama* was always associated with transfer of Km character (alone or with other characters). In contrast, colicin production was not always observed in recipients resistant to Am, Tc or Am Tc.

Superinfection immunity and stability. Conjugal transfers were carried out between *Escherichia coli* K 12 donor strains, resistant to one drug and non-colicinogenic, and

recipient *E. coli* K 12 strains, resistant to one of the other drugs or sensitive. It can be seen in Table 3 that the frequencies of transfer in 20 min. were the same to sensitive or resistant recipients. In mixtures tested after overnight incubation, the frequencies of transfer were as usual increased, but again did not differ with sensitive or resistant recipients.

Table 2. *Frequencies of transfers of drug-resistance in 20 minutes*

Selecting antibiotic	Transferred characters	Frequencies of transfer/donor bacterium	
		<i>Salmonella panama</i>	<i>Escherichia coli</i> K 12
		LA 46	LA 143*
		$\times$ <i>E. coli</i> K 12 LA 115	$\times$ <i>E. coli</i> K 12 LA 116
Am	Am	8.4×10^{-6}	2×10^{-6}
	Am Km	2.4×10^{-6}	3.1×10^{-6}
	Am Tc	1×10^{-6}	2×10^{-7}
	Am Km Tc	6×10^{-6}	2.9×10^{-6}
Km	Km	1.2×10^{-6}	1.3×10^{-6}
	Am Km	1.2×10^{-6}	2×10^{-7}
	Am Km Tc	2×10^{-6}	—
Tc	Tc	1.1×10^{-4}	7×10^{-7}
	Km Tc	4×10^{-4}	—
	Am Km Tc	8×10^{-6}	—

* Origin of this strain can be seen in Table 1.

Table 3. *Frequencies of transfers in superinfection experiments*

Donor strains	Recipient strains	Selecting antibiotics	Frequencies of transfer/donor bacterium in 20 min.
LA 120 (Am)	LA 121 (Tc)	Am	1.4×10^{-6}
	LA 116		1.5×10^{-6}
LA 121 (Tc)	LA 120 (Am)	Tc	2×10^{-4}
	LA 115		1×10^{-4}
LA 122 (Tc)	LA 123 (Am)	Tc	1×10^{-6}
	LA 116		8×10^{-7}
LA 124 (Km)	LA 121 (Tc)	Km	8×10^{-6}
	LA 123 (Am)		1.2×10^{-6}
	LA 116		4×10^{-7}

Twenty-three recipient colonies resistant to Am and Tc obtained in these transfers were picked at random, grown for 24 hr in antibiotic-free medium and streaked on nutrient agar; the colonies were then tested for drug-resistance. All colonies (2300) on replica plates were still resistant to both antibiotics. Five were used as donors in transfers, and recipient bacteria resistant to one or two drugs were obtained. Similar stability and separate transfers were observed with bacteria resistant to Am and Km, or to Km and Tc obtained in superinfection experiments.

Transduction by phage P 1-b. Phage P 1-b was propagated on two resistant *Escherichia coli* K 12 strains obtained as described in Table 1: *E. coli* K 12 LA 117 (Am Km Tc) and (coIb), and *E. coli* K 12 LA 118 (Am Tc). As a control, phage P 1-b was also propagated on *E. coli* K 12 LA 119 carrying a *fi*⁺ R factor usually transferred *en bloc*

(Bouanchaud, Scavizzi & Chabbert, 1968). The results of the transduction in *E. coli* K 12 54117 and *E. coli* K 12 LA 116 can be seen in Table 4 (*a*, *b*). The entire *fi*⁺ R factor was transduced by phage P 1-b and remained transferable in subsequent transfers. In contrast, phage P 1-b transduced only isolated resistance characters from *E. coli* K 12 LA 117 to *E. coli* K 12 54117. The single Tc character was transduced from *E. coli* K 12 LA 118. The Km and Tc characters were separately transduced from *E. coli* K 12 LA 117 to *E. coli* K 12 LA 116. All the tested transductants were effective donors at the usual frequencies in subsequent transfer experiments. No transduction of groups of resistance characters or of colicinogeny was observed in these experiments.

Table 4.

(*a*) Transduction by phage P 1-b from *Escherichia coli* K 12 LA 117, LA 118 and LA 119 to *E. coli* K 12 54117

Donor strains	Resistance pattern	Selecting antibiotics	Drug-resistance of transductants	No. of transductants obtained and tested	*Frequency of transduction
LA 117	Am Km Tc	Am	Am	6/6	1×10^{-7}
		Km	Km	3/3	1×10^{-8}
		Tc	Tc	40/40	7×10^{-6}
LA 118	Am Tc	Am	—	—	—
		Tc	Tc	8/8	1.3×10^{-7}
LA 119	Sm Cm Tc Su	Cm	Sm Cm Tc Su	11/11	1×10^{-6}
		Tc	Sm Cm Tc Su	31/31	3×10^{-5}

(*b*) Transduction by phage P 1-b from *Escherichia coli* K 12 LA 117 to *E. coli* K 12 LA 116

LA 117	Am Km Tc	Am	—	—	—
		Km	Km	2/2	3×10^{-8}
		Tc	Tc	14/14	2×10^{-7}

* Frequency of transduction as no. transductants/p.f.u. phage.

DISCUSSION

Transferable resistance in a *Salmonella panama* strain isolated in Paris is different from previously described R⁺ strains. Instead of being transferred *en bloc*, the three resistance determinants (Am, Km, Tc) are usually transferred either singly or in pairs such as Am Km, Am Tc or Km Tc. The complete combination can be transferred but generally at a lower frequency to sensitive *Escherichia coli* K 12. This might be explained by frequent spontaneous segregation, but such segregation has been mainly observed when contacts between donor and recipient bacteria have been maintained for a long time (Walton & Fulton, 1967). During growth of *S. panama* LA 46.R 11 spontaneous segregants appeared rarely: 0.1 % for Am and Tc and 2 % for the Km character in 18 hr. Resistant recipients grown in drug-free medium also showed only a low frequency of spontaneous segregation. Thus it can be assumed that those recipients resistant to one or two drugs are not segregants of bacteria originally resistant to all three drugs.

In superinfection experiments performed with a donor carrying an R factor conferring resistance to one of the drugs, the frequencies of transfer were not decreased

when the recipient bacteria harboured an R factor conferring resistance to one of the other drugs. After transfer the recipients remained resistant to both drugs for at least 10 generations in antibiotic-free medium. Nevertheless, the two resistance characters were still independently transferred in subsequent conjugations.

The observed transfers with *Salmonella panama* LA 46.R 11 might be compared with those described by Anderson and colleagues with *S. typhimurium* phage-type 29. But in the *S. typhimurium* system, some resistance characters, when present singly, are not in themselves transferable, and their transfer has to be mediated by an independent transfer factor (Anderson & Lewis, 1965; Anderson, 1966; Anderson, 1967; Anderson & Perret, 1967). In other systems, a non-transferable colicin factor can be transferred when the host bacterium is superinfected with a transferable extrachromosomal element (Fredericq, 1956; Ozeki, Stocker & Smith, 1962). The *S. panama* system seems to be different, each element was in itself transferable, and remained transferable after transduction.

Transduction experiments were made with phage P1-b, a mutant of phage P1-kc which has been assumed to transduce entire resistance factors between *Escherichia coli* K 12 strains (Watanabe & Fukasawa, 1961*b*). Phage P1-b was shown to be able to transduce the entire R 15 (*fi*⁺) factor, and all the transductants which were tested were effective donors in subsequent transfers. In contrast, when phage P1-b was propagated on *E. coli* K 12 LA 117 or on *E. coli* K 12 LA 118, only single resistance characters were transduced. Transductants resistant to two or three drugs were never observed. All the transductants which were tested were effective donors at usual frequencies in subsequent transfers. Colicinogeny was not co-transduced with drug-resistance in these experiments, although the two properties were frequently co-transferred; so it can be suggested that the colicin determinant and the drug-resistance determinants are carried by independent plasmids. R factors governing the production of colicin I have been described, but in these strains, drug-resistance and colicinogeny were co-transduced (Siccardi, 1966; Meynell, Meynell & Datta, 1968; Romero & Meynell, 1969).

The results reported here suggest that several discrete genetic elements responsible for drug-resistance can stably coexist with a Col factor in *Salmonella panama* and *Escherichia coli* K 12. It follows that there is no competition for a unique site of replication between these elements: R 11-1 (Am), R 11-2 (Km), R 11-3 (Tc) and ColIb. Stable coexistence of different plasmids have been often reported, but not of 'homologous' *fi*⁻ resistance factors. Coexistence of these three replicons supports the hypothesis that genetic replication and pili formation (and type) are wholly dissimilar processes (Meynell, Meynell & Datta, 1968).

The fact that the plasmids harboured by *Salmonella panama* are independent is not incompatible with frequent simultaneous transfers, and such types of transfers have been previously reported (Dubnau & Stocker, 1964; Hardy & Nell, 1967). It should be of interest to investigate whether the fact that the plasmids described here have closely related sex factors can explain their frequent co-transfer.

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