The Relationship between Different Transmissible Plasmids Introduced by F into the Same Strain of *Escherichia coli* K12

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(Accepted for publication 4 May 1970)

SUMMARY

Plasmids containing the genetic determinants for resistance to tetracyclines (Tc), streptomycin (Sm) and sulphonamide (Su), colicine production (Col) and α -haemolysin production (Hly) were transferred from four wild strains of *Escherichia coli* to *E. coli* K12 by conjugation brought about by F factor. Linkage was demonstrated between F and Tc and between Sm and Su. No linkage was noted between Col, Hly, F and Tc, and Sm and Su, which were transmitted as four independent units in no predetermined order. F and Tc were eliminated together during acridine orange and sodium lauryl sulphate treatment; the other characters were not eliminated. After mating for prolonged periods, F and Tc were most frequently found in the recipients. Recipients of other characters in the absence of F and Tc did not transmit them.

INTRODUCTION

The genetic determinants for several characters of *Escherichia coli* are now known to be located in plasmids which can be transferred from one bacterium to another by conjugation (Watanabe, 1963); it is not unusual for wild strains to contain more than one of these plasmids. The genetic elements responsible for their transfer from cell to cell, referred to hereafter as transfer factors, are probably numerous and it is not always possible to differentiate one from another. Consequently, although one may be able to detect the presence of a transfer factor in a cell by the fact that it can donate genetic material, only in certain cases can it be shown that more than one is present (Romero & Meynell, 1969). This complicates any study of the relationships between different plasmid-carried genes which may be found together in a wild strain of *E. coli*. Anderson (1965) and Anderson & Lewis (1965) reported the frequent occurrence of determinants for antibiotic resistance in the absence of transfer factors. These could be mobilized by introduction into the host bacterium of transfer factors from other sources. Plasmid-determined characters such as colicinogeny or antibiotic resistance can be readily transmitted during conjugation brought about by ColI and other transfer factors (Fredericq, 1954; Smith, Ozeki & Stocker, 1963; Anderson, 1965) and it thus seemed worth while to use the F factor to introduce a number of apparently plasmid-determined characters from different wild strains of *Escherichia coli* into the same bacterium and then to observe the relationships of one to the other in a common environment. Thus the genetic determinants for tetracycline (Tc), streptomycin (Sm) and sulphonamide (Su) resistance and colicine (Col) and α -haemolysin (Hly) pro-

duction were transferred to a single strain of *E. coli* K12 and their relationships were assessed by their relative tendencies to associate with one another during subsequent transfer from their common host.

METHODS

Bacterial strains. Two strains of *Escherichia coli* K12 were used in the transmission studies. One of these, strain PROTO, was prototrophic and lactose-fermenting (*lac*⁺) and the other, strain AUXO, was auxotrophic and nonlactose-fermenting (*lac*⁻). When one was used as donor, a mutant of the other strain resistant to nalidixic acid or ampicillin was used as recipient. The mutants were prepared by inoculating 200 ml. of nutrient broth containing 50 µg. of nalidixic acid or ampicillin/ml. with all the *E. coli* K12 organisms that had grown on a 'medicine flat' bottle of nutrient agar that had been incubated at 37° for 24 h. The broth culture was then incubated at 37°; like all the other broth cultures in this work, it was not aerated during incubation. At daily intervals, one loopful of culture was removed, spread over a small area of a Petri plate containing MacConkey medium and a disc containing 50 µg. of the same drug that had been added to the broth was applied to the surface. The plates were incubated at 37° for 24 h. and examined for the presence of colonies growing in the immediate proximity of the disc. When such a colony was noted on any of the plates, it was removed and purified by the usual plating technique. Its resistance to the relevant drug was finally confirmed.

Identification of nontransmissible character in wild strains of Escherichia coli. Nutrient broth (Oxoid no. 2) in 10 ml. amounts was seeded with 0.02 ml. of a 24 h. broth culture of a wild strain of *Escherichia coli* resistant to one or more antibiotics or producing haemolysin or colicine, and an equal volume of one of the resistant mutants of *E. coli* K12 strain AUXO. The mixed culture was incubated at 37° for 24 h. and then plated on media containing 30 µg. nalidixic acid or ampicillin/ml., depending on which recipient mutant was used. This prevented growth of the wild *E. coli* strain so that only colonies of the strain AUXO could develop. Haemolysin production (Hly) was detected by using 'washed blood' agar (Smith & Halls, 1967). The plates were incubated at 37° for 24 h. and examined for haemolytic colonies. To detect colicine production (Col), nutrient agar plates inoculated by streaking were incubated at 37° for 24 h. A thin layer of melted nutrient agar at 50° containing the same antibiotic as the basal layer and a few drops of broth culture of the strain AUXO was poured carefully over the colonies on the surface of the plate and incubation continued for a further 24 h. The plates were then examined to determine whether any of the colonies were surrounded by zones of inhibition of growth of the overlaid strain AUXO. To detect transmission of antibiotic resistance, the mixed culture was centrifuged and the deposit inoculated heavily in MacConkey agar + nalidixic acid or ampicillin + 15 µg./ml. of one of the antibiotics to which the wild strain had been shown to be resistant. The use of MacConkey agar, which contains lactose and a pH indicator, allowed colonies of the *lac*⁻ AUXO strain to be distinguished from any mutant colonies of the wild strain which might arise from the large inoculum. Transfer of sulphonamide resistance was not directly tested. Strains from which Hly, Col or antibiotic resistance was not transmitted at first examination were retested, often with slight variation of technique, e.g. by varying the relative amount of the two strains used to inoculate the mixed culture

and by varying the composition of the culture medium and the time for which it was incubated. No strain was classified as unable to transfer these characters until it had been tested at least 80 times.

Mobilization by F factor. Nutrient broth, in 10 ml. amounts, inoculated with 0.02 ml. of a 24 h. broth culture of a wild strain of *Escherichia coli* containing a nontransmissible determinant and an equal volume of a broth culture of an F⁺ strain of *E. coli* K 12 was incubated at 37° for 24 h. Fresh broth was then inoculated with 0.2 ml. of this mixed culture and an equal volume of a broth culture of one of the resistant mutants of strain AUXO, and incubated at 37° for 24 h. Finally, this culture was examined by the methods given in the previous paragraph.

Transfer between Escherichia coli K 12 mutants. Essentially the same methods were used as in testing for transfer from the wild strains of *Escherichia coli*. Mixtures of the two *lac*⁺ and *lac*⁻ strains were chosen to act as donor and recipient such that the donor could be eliminated by one of the two antibiotics, nalidixic acid or ampicillin, to which the recipient was resistant. After incubation, the mixed cultures were streaked on MacConkey agar containing nalidixic acid or ampicillin and isolated colonies were examined for the various characters present in the donor strain. In some experiments in which Col transmission was being studied, a mutant resistant to the colicine was used as recipient. In some other cases, a colicine-sensitive strain was used and Col⁺ recipients were directly selected by incorporating colicine in the agar to prevent the growth of Col⁻ bacteria. The surface of a MacConkey agar plate without antibiotic was spread with a culture of the donor strain and incubated at 37° for 24 h., after which the bacteria were killed with chloroform vapour and covered with a thin layer of MacConkey agar containing nalidixic acid or ampicillin for inoculation of the mating mixture.

Generally, the mixture of donor and recipient was incubated at 37° for 18 h. before plating. To test the effects of shorter periods of mating, 18 h. broth cultures of the donor and recipient strains were diluted 1/10 in fresh broth and, after further incubation for 150 min., a mixture of equal parts of each was incubated for 5 min. and then diluted 1/100 in broth at 37° to prevent formation of additional cell to cell contacts. Samples were removed at intervals, mating was interrupted by agitation for 2 min. in an M.S.E. macerator and suitable volumes were plated on selective media, which contained the antibiotic to which the recipient was resistant and either the colicine or the antibiotic for which the donor possessed a resistance determinant.

Transfer of antibiotic resistance was examined by selecting recipients on antibiotic-containing media or by replica-plating to MacConkey agar containing antibiotic. For sulphonamide, broth cultures were streaked up to strips of filter paper containing a high concentration of sulphadimidine on plates of D.S.T. agar (Oxoid no. CM 261). For Hly, colonies were inoculated as spots on 'washed blood' agar and examined for haemolysis after 18 h. incubation. To test for Col, colonies were similarly subcultured onto nutrient agar previously spread with a colicine-sensitive strain of *Escherichia coli* K 12 F⁻; colicine production was shown by the presence of a clear zone around the colony after incubation.

The presence and expression of F factor was recognized by susceptibility of cultures on nutrient agar to lysis by the F-specific phage MS 2 (Davis, Strauss & Sinsheimer, 1961).

Acridine orange treatment. *Escherichia coli* K 12 cultures were grown from small

inocula in broth containing 0.01 % (w/v) acridine orange at 37° for 24 h., a concentration that had a definite inhibitory effect.

Sodium lauryl sulphate treatment. This was done in a manner similar to that devised by Tomoeda, Inuzuka, Kubo & Nakamura (1968). Cultures were grown from small inocula in nutrient broth containing 10 % sodium lauryl sulphate at 37° for 24 h.

When the effect of passage in acridine orange and sodium lauryl sulphate was being studied, the cultures after each passage were inoculated onto Petri plates of MacConkey agar in a manner likely to yield widely separated colonies after incubation.

RESULTS

Nontransmissible antibiotic resistance and haemolysin- or colicine-production in wild strains of Escherichia coli: their transfer by means of F to a single strain of E. coli K12

Four wild strains of *Escherichia coli* were isolated that were resistant to tetracycline (Tc) or streptomycin (Sm) and sulphonamide (Su), or that produced colicine (Col) or α -haemolysin (Hly), and in which these characters were not transmissible until the strain had been infected by F factor. Using F to bring about conjugation, the five characters were transferred to a nalidixic acid-resistant mutant of *E. coli* K12 strain AUXO by two different procedures. In the first, each character was transferred to a different culture giving four F⁺ strains that were Tc⁺, Sm⁺Su⁺, Col⁺ or Hly⁺ respectively. Col was then transferred to the Tc⁺ strain to produce a Tc⁺Col⁺ strain. The latter was mated with the strain Sm⁺Su⁺ to produce a Tc⁺Col⁺Sm⁺Su⁺ strain which, in turn, was mated with the Hly⁺ strain to produce a strain Tc⁺Sm⁺Su⁺Col⁺Hly⁺. The determinants for all five characters were then transferred *en bloc* to an ampicillin-resistant mutant of *E. coli* strain PROTO; this and the *E. coli* AUXO donor are referred to subsequently as the M strains of K12 F⁺Tc⁺Col⁺Hly⁺Sm⁺Su⁺. The second procedure was to transfer each character in turn to the nalidixic acid-resistant mutant of *E. coli* strain AUXO from the four wild strains of *E. coli*. First, Tc was transferred, then Col, to produce a F⁺Tc⁺Col⁺ K12 strain. A K12 F⁺ strain Tc⁺Col⁺Sm⁺Su⁺ could not be produced by direct mating of an F⁺Tc⁺Col⁺ strain with the wild Sm⁺Su⁺ strain but was obtained by growing a mixed culture of the two strains with a further inoculum of the nalidixic acid-resistant mutant of *E. coli* strain AUXO. Employing the usual techniques, it was not possible to introduce Hly directly from the wild strain of *E. coli*; however, F, Tc, Col, Sm and Su could be transferred together to the wild strain and subsequent mating with the nalidixic acid-resistant mutant of strain AUXO resulted in the isolation of the required K12 F⁺Tc⁺Col⁺Hly⁺Sm⁺Su⁺ strain. All the characters were then simultaneously transferred to the ampicillin-resistant strain PROTO; these two are referred to as the G strains of *E. coli* K12 F⁺Tc⁺Col⁺Hly⁺Sm⁺Su⁺. Both M and G strains were fully susceptible to lysis by phage MS 2, showing that F was present and that its function continued to be expressed.

Long-term mating of Escherichia coli K12 F⁺Tc⁺Col⁺Hly⁺Sm⁺Su⁺ with E. coli K12 F⁻

Table 1 shows the properties of colonies of *Escherichia coli* K12 F⁻, and of colicine-resistant mutants, obtained after mating for 18 h. with M and G strains. F was always found with Tc, and Sm with Su in the recipients; no other consistent associations were noted. Of the recipients that were F⁻, none transmitted their characters to another F⁻

strain; these could however, as before, be transferred by conjugation when F was introduced by mating with *E. coli* K12 F⁺. By contrast, F⁺ recipients transmitted the characters which they had received at a high rate.

Despite their different methods of preparation, there was no obvious difference between the M and G strains in the fashion in which their determinants were transmitted, except possibly in the degree of Sm and Su transfer. In all the experiments in which the colicine-resistant mutant of K12 F⁻ was used as recipient, the most common class was that carrying simply F and Tc. Although Table 1 shows more recipients of Hly after mating with the G strain than with the M strain, this difference was not consistently found. In the experiments in which the recipient was colicine-sensitive, all

Table 1. *The transfer of characters from the M* and G* strains of Escherichia coli K12 F⁺Tc⁺Col⁺Hly⁺Sm⁺Su⁺ during 18 h. mating with E. coli K12 F⁻*

Character	No. of 100 colonies of the prospective recipient strain that acquired the character during mating					
	PROTO K12 F ⁻ <i>col-r</i> and donor AUXO		PROTO K12 F ⁻ and donor AUXO		AUXO K12 F ⁻ and donor PROTO	
	M	G	M	G	M	G
F	54	62	93	81	59	55
Tc	54	62	93	81	59	55
Col	26	25	100	100	100	100
Hly	6	17	38	51	69	55
Sm	8	4	45	18	36	9
Su	8	4	45	18	36	9
None	44	37	0	0	0	0

Character pattern						
F	Tc	Col	Hly	Sm	Su	
+	+	+	+	+	+	0
+	+	+	+	+	-	3
+	+	+	-	+	+	6
+	+	-	-	+	+	1
+	+	+	-	-	-	15
+	+	-	+	-	-	1
+	+	-	-	-	-	28
-	-	+	+	+	+	1
-	-	+	-	+	+	0
-	-	+	+	-	-	1
-	-	+	-	-	-	0
-	-	-	+	-	-	0
-	-	-	-	-	-	44
						37
						16
						12
						20
						3
						18
						29
						26
						27
						26
						4
						1
						2
						0
						0
						0
						12
						23
						0
						0
						0
						0
						9
						0
						6
						4
						8
						14
						25
						9
						12
						16
						0
						0
						0

AUXO = auxotrophic; PROTO = prototrophic; *col-r* = mutant resistant to the colicine produced by the donor.

* M and G are K12 F⁺ Tc⁺ Col⁺ Hly⁺ Sm⁺ Su⁺ donor strains in which the characters were introduced by different procedures (see text). The presence or absence of F was determined by MS2 phage sensitivity.

colonies were Col⁺, a probable consequence of killing of Col⁻ bacteria by colicine present in the mixed-mating culture. The distribution of the other characters in these Col⁺ colonies resembled mainly their distribution in the experiments in which the colicine-resistant mutant was the recipient. The types of colony obtained with the colicine-resistant mutant selected for Tc⁺, Col⁺, Hly⁺ or Sm⁺ after mating with the M and G strains of K12 F⁺ are shown in Table 2 in which the results for the M and G

strains are pooled because they were similar. Again, complete correlation was found between the presence of F and of Tc and between Sm and Su, but between no other pairs of characters. Of 160 colonies examined, 152 had F and Tc, while the others were transmitted much less commonly.

Table 2. *The characters possessed by recipient colonies of a colicine-resistant mutant of Escherichia coli* $\kappa 12$ F^- mated with *E. coli* $\kappa 12$ $F^+Tc^+Col^+Hly^+Sm^+Su^+$ for 18 h. and selected for different characters

Character selected	Characters possessed by 40 selected recipient colonies					
	F	Tc	Col	Hly	Sm	Su
Tc	40	40	19	14	6	6
Col	39	39	40	16	22	22
Hly	39	39	30	40	9	9
Sm	34	34	33	19	40	40

Additional evidence that an F^+ strain donated Tc much more frequently than Sm and Su was obtained when two $\kappa 12$ F^+ strains, one with Tc and the other with Sm and Su, were mated with one another. The two strains could be differentiated by resistance to nalidixic acid or ampicillin. After mixed culture for 18 h., there were many more organisms of the $\kappa 12$ $F^+Sm^+Su^+$ strain with Tc than of the $\kappa 12$ F^+Tc^+ strain which had acquired Sm and Su. Both of these $F^+Tc^+Sm^+Su^+$ strains, although they had received Tc and Sm and Su in a different order, transmitted Tc with F very much more frequently than Sm and Su to an F^- strain. The same relative frequencies of transmission were observed after the donor had received Tc and Sm and Su directly from the wild strains of *Escherichia coli* irrespective of the order in which the characters had been received.

Short-term mating of Escherichia coli $\kappa 12$ $F^+Tc^+Col^+Hly^+Sm^+Su^+$ with *E. coli* $\kappa 12$ F^-

The results of mating the G strain of *Escherichia coli* $\kappa 12$ $F^+Tc^+Col^+Hly^+Sm^+Su^+$ for short periods with $\kappa 12$ F^- are shown in Tables 3 and 4. There was no great difference between the numbers of Tc^+ , Sm^+ and Col^+ recipients at 5 min. (Table 3). As with mating for 18 h. all those that were Tc^+ were also F^+ and all which were Sm^+ were also Su^+ ; otherwise, only the selected character was usually present. After a further 20 min. of mating, additional characters appeared (Table 4) and it was noteworthy that the proportion of colonies containing Hly was approximately the same whether selection was

Table 3. *The numbers of viable organisms of Escherichia coli* $\kappa 12$ F^- that acquired Sm Tc and Col during short-term mating with the G strains of *E. coli* $\kappa 12$ $F^+Tc^+Col^+Hly^+Sm^+Su^+$

Character	Approximate no. of recipient organisms per ml. of culture that acquired the character during mating	
	5 min.	5 + 20 min. *
Tc	1,200	60,000
Sm	750	20,000
Col	1,000	80,000

* Five min. mating followed by dilution and further incubation for 20 min. before plating.

for tetracycline resistance, streptomycin resistance or colicine production. The proportions of Tc⁺ and of Sm⁺ colonies which produced colicine were also approximately the same, as were the proportions of Tc⁺ and of Col⁺ colonies which were Sm⁺. None of the recipients that were F⁻ could transmit their characters to another F⁻ strain.

Table 4. *The characters acquired by colonies of Escherichia coli* $\kappa 12$ F⁻ after short-term matings with the G strain of *E. coli* $\kappa 12$ F⁺Tc⁺Col⁺Hly⁺Sm⁺Su⁺

Character	No. of 100 colonies that acquired the character during mating					
	5 min. characters selected			5 + 20 min.* characters selected		
	Tc ⁺	Sm ⁺	Col ⁺	Tc ⁺	Sm ⁺	Col ⁺
F	100	0	0	100	11	13
Tc	100	0	0	100	11	13
Sm	0	100	3	7	100	11
Su	0	100	3	7	100	11
Col	1	6	100	33	46	100
Hly	0	3	3	38	39	33

Character pattern											
F	Tc	Col	Hly	Sm	Su						
+	+	-	-	-	-	99	0	0	44	0	0
-	-	-	-	+	+	0	91	0	0	37	0
-	-	+	-	-	-	0	0	94	0	0	57
+	+	+	-	-	-	1	0	0	16	0	3
-	-	-	+	+	+	0	3	0	0	13	0
-	-	+	-	+	+	0	6	3	0	18	4
-	-	+	+	-	-	0	0	3	0	0	23
+	+	-	+	-	-	0	0	0	20	0	0
+	+	-	-	+	+	0	0	0	1	4	0
+	+	+	+	-	-	0	0	0	13	0	6
-	-	+	+	+	+	0	0	0	0	21	3
+	+	-	+	+	+	0	0	0	2	0	0
+	+	+	-	+	+	0	0	0	1	2	3
+	+	+	+	+	+	0	0	0	3	5	1

* Five min. mating followed by dilution and further incubation for 20 min. before plating.

Elimination by acridine orange and sodium lauryl sulphate

Table 5 shows the results of passaging the G strain of *Escherichia coli* $\kappa 12$ F⁺Tc⁺Col⁺Hly⁺Sm⁺Su⁺ daily in nutrient broth containing acridine orange or sodium lauryl sulphate and examining 50 colonies obtained from each passage. Both agents eliminated Tc. No colonies were found that had lost Col, Hly, Sm or Su. Most of the 50 colonies examined after the first passage had lost sensitivity to lysis by phage MS2. Those still Tc⁺ were able to transmit Tc and also Col, Hly, Sm and Su, but at a very much lower rate than the untreated cultures. Twenty-five MS2-insensitive cultures were passaged several times in broth, when they regained susceptibility to lysis as well as high rate of donor ability. Another culture of Tc⁺ MS2-insensitive organisms which had been treated with lauryl sulphate remained MS2-insensitive and continued to transmit at low frequency after 20 passages in broth. However, cultures of other strains that had received Tc from the Tc⁺ MS2-insensitive strain showed the normal

high degree of MS2 susceptibility and donor activity. No colonies were found that had lost Tc while retaining sensitivity to lysis by phage MS2. Twenty-five Tc-sensitive MS2-insensitive colonies were also passaged 20 times in broth but did not become susceptible to lysis nor regain the ability to transfer Col, Hly, Sm or Su.

Table 5. *Elimination of characters from the G strain of Escherichia coli*
κ12 F⁺ Tc⁺ Col⁺ Hly⁺ Sm⁺ Su⁺

*Passage no.	No. of colonies containing				
	Tc		Col Hly Sm Su		
	Expt. 1	Expt. 2	Expt. 1	Expt. 2	
0	50	50	50	50	
1	45	2	50	50	
2	36	0	50	50	
3	16	N.T.	50	N.T.	
4	6	N.T.	50	N.T.	
5	1	N.T.	50	N.T.	
6	0	N.T.	50	N.T.	
16	0	0	50	50	

* Passage was continued daily in broth containing 10 % sodium lauryl sulphate (expt. 1) or 0.01 % acridine orange (expt. 2).

N.T.: not tested.

DISCUSSION

Although the M and G strains of *Escherichia coli* $\kappa 12$ F⁺Tc⁺Col⁺Hly⁺Sm⁺Su⁺ were prepared in different ways, the behaviour of the plasmids during subsequent mating was not influenced greatly by the order in which they had been introduced into the donor strain. The colicine had been identified as colicine E1 by Professor P. Fredericq, and ColE has been shown to require an independent transfer factor with which it is not necessarily associated in transfer (Fredericq, 1954; Smith *et al.* 1963; Watanabe, 1963). The only evidence of linkage between the characters was between F and Tc and between Sm and Su, which contrasts with the various associations of plasmid-determined characters observed to occur with another transfer factor, Δ , observed by Anderson (1968). The present results, together with those of Anderson (1965, 1968) and Anderson & Lewis (1965), indicate that plasmid-determined antibiotic resistance unlinked to a transfer factor is more common and of greater significance than was at first envisaged (see Watanabe, 1963).

Approximately equal numbers of F⁺Tc⁺ only, Col⁺ only, and Sm⁺Su⁺ only, recipients were found after mating with the M and G donor strains for 5 min., indicating the absence of any predetermined sequence of transmission. This strongly suggests that, given the presence of a transfer factor to provide the necessary connecting link between the bacteria, transfer is either completely automatic, or, if not, it is the individual plasmid itself that determines how it shall be transferred.

We are grateful to Miss Diane Poulton and Miss Carole Smith for their capable technical help. Our thanks are also due to Dr Elinor Meynell for her help in preparing this manuscript and to Professor P. Fredericq, Mrs Esther Johnson and Dr K. C. Sellers for assistance in various ways. The work was financed in part by Pfizer Limited. It was done when one of us (C. L. G.) was a Research Fellow of the Canadian Medical Research Council.

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