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The relation of resistance transfer factors to the F-factor (sex-factor) of *Escherichia coli* K12

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1. INTRODUCTION

Resistance factors, or R factors, are extrachromosomal genetic elements which render their bacterial hosts resistant to one or more antibacterial agents. They also enable their hosts to conjugate with other strains which thereby acquire the R factor and in turn become drug-resistant. That part of an R factor which determines conjugation is known as the Resistance Transfer Factor (RTF), and an R factor may thus be thought of as a variable number of genes determining drug-resistance linked to an RTF. During conjugation brought about by an R factor, part of the chromosome of the R^+ donor cell may also occasionally be transmitted; thus, RTF resembles the sex-factor, F, of *Escherichia coli* K12, and bacterial plasmids like *col* factors, which determine the production of colicines.

One of the main problems associated with R factors is the nature of the RTF and its relation to F. RTF differs from F in that it is ordinarily transmitted from donor to recipient at a much lower frequency, and chromosomal transfer occurs rarely, when at all. Furthermore, the surface component produced by F^+ bacteria which is recognized as a new antigen (Ørskov & Ørskov, 1960) or as the receptor for a group of F-specific phages (Loeb & Zinder, 1961) cannot be detected in R^+ cultures either by agglutination of the bacteria by antiserum specific for F^+ strains or by lysis of the culture by F-specific phage. These differences between F and RTF would arise if the ability to conjugate were expressed in virtually every F^+ cell, but unexpressed in all but a minority of cells in an R^+ culture. In other words, the relevant structure might be the same in both F^+ and R^+ bacteria, but actually developed in far fewer of the latter due to a repressor made by RTF but not by F. That the conjugating function is indeed repressed in R^+ cultures is shown by the fact that bacteria which have newly received an R factor transmit it with greater efficiency than bacteria in which it has been established for many generations (Watanabe, 1963). This phenomenon, known as High Frequency Transfer (HFT) is also observed with transfer of *col* I (Stocker, Smith & Ozeki, 1963), where it is thought (Monk & Clowes, 1964) to be due to a repressor produced by *col* I itself. Transfer to a recipient without preformed repressor is followed by an interval in which the conjugating function is uninhibited until the repressor gene acquired with the factor has time to restore the repression. Some F phage-sensitive bacteria are indeed present in established R^+ cultures, and with newly-infected bacteria showing high frequency transfer, the proportion is enormously increased (Meynell & Datta, 1965).

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A functional relationship between RTF and F is indicated by the existence of a large class of R factors known as fi$^+$ (for Fertility Inhibition; Watanabe *et al.*, 1964), which inhibit F when introduced into F$^+$ bacteria. With such R$^+$F$^+$ bacteria, the frequency of conjugation is greatly diminished and the cultures are no longer agglutinated by specific F antiserum, nor lysed by F phage. F is still present, however, for its activity is seen to be restored in clones which lose the R factor. This effect of fi$^+$ R factors has been attributed to an epistatic effect of RTF suppressing the expression of F (Watanabe & Fukasawa, 1962) and to production by the R factor of a cytoplasmic repressor active against F (Egawa & Hirota, 1962); but it seems probable that the repression that RTF exerts on F is simply an extension of the repression which it ordinarily exerts on itself.

The present paper reports the results of testing thirty R factors chosen at random, which show that only those which are fi$^+$ confer sensitivity to the F phage. Thus, the RTFs associated with the two classes of R factor are dissimilar, although both establish a mechanism for conjugation.

2. MATERIAL AND METHODS

Bacterial strains. All were derivatives of *Escherichia coli* K12, and are listed in Table 1.

R factors. These are listed in Table 2. All came from *Salmonella* and were transferred to *E. coli* K12, strains 58.161, 58.161/*sp*, RC709 and RC711 directly from the salmonella strain in which they were first found; they were transferred to strain RC24 from RC709.

Table 1. *Bacterial strains*

Strain	Relevant characters	Reference
Hfr.H	From 58.161 F$^+$ *met*$_1^-$, in which F has become integrated near the *thr* locus .λ^s .λ^+.	Hayes (1953*a*)
Hfr.B1	From W1655 F$^+$ *met*$^-$, in which F has become integrated near the *pro* locus λ^r λ^-.	Lederberg & Lederberg (1953); Broda (1966)
58.161	F$^+$ *met*$^-$.*lac*$^+$.S^s	Lederberg (1947)
RC.709	J5-3 (*pro*$_1^-$.*met*$_2^-$.*lac*$^+$.S^s.λ^s.λ^+). Acridine-cured F$^-$	Clowes & Rowley (1954)
RC.711	J6-2 (*pro*$_2^-$.*his*$^-$.*try*$^-$.*lac*$^-$.S^s.λ^s.λ^+). Acridine-cured F$^-$	Clowes & Rowley (1954)
RC.24	Derived from W677 (*thr*$^-$.*leu*$^-$.*B1*$^-$.*lac*$^-$; F$^-$) *Fim*$^-$.S^r.	Strain 129 of Maccacaro, Colombo & Nardo (1959)
58.161/*sp*	58.161 (*met*$_1^-$.*lac*$^+$). Spontaneous F$^-$	Hayes (1953*b*)

Donor and recipient strains were grown together in broth and plated on a solid medium containing one of the drugs appropriate to the R factor, and on which only the recipient could grow, or on which donor and recipient were distinguishable. For instance, the fermentation (utilization) of lactose was used in transfer from *Salmonella* (*lac*$^-$) to *lac*$^+$ lines of K12, and the different nutritional requirements of the strains were used in transfers from one line of K12 to another. Each R factor was classed as fi$^+$ or fi$^-$ by looking for repression of F function after transfer to 58.161 F$^+$. This was tested by examination of the R$^+$F$^+$ culture for visible lysis on plating with F phage, which appeared to be a valid test for the fi character since, in a sample of ten different R factors, phage-insensitivity was always associated with reduced frequency of recombination.

High Frequency Transfer (*HFT*) *systems.* The same procedure was used that gave HFT with *col* I (Stocker, Smith & Ozeki, 1963), and with R factors (Watanabe, 1963). Broth cultures of an R$^+$ strain and the same strain without the R factor were mixed and incubated overnight. Next day, the mixture was diluted in fresh broth and incubated for 2 hours before testing.

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Tests for frequency of transfer. One volume of the culture to be tested (either an established R+ culture or an HFT mixture) was mixed with 9 volumes of another strain which was to act as recipient, and which could be distinguished from the donor. After 1 hour at 37°C., dilutions of the mixture were plated on media allowing growth of (*a*) the donor strain, (*b*) R+ members of the donor strain, (*c*) the recipient strain, and (*d*) members of the recipient strain which had received the R factor. The number of colonies on (*d*) compared with the number on (*b*) gave a measure of the frequency of transfer.

Table 2. *R-factors*

Number	Conferring resistance to:	Number	Conferring resistance to:	Number	Conferring resistance to:
R1	Ap Cm Km Su	R56	Tc Su	R136	Tc
R2	Tc Sm Su	R62	Tc Ap Sm	R142	Tc Sm Su
R27	Tc	R64	Tc Sm	R143	Tc Km
R28	Tc Sm Su	R73	Tc Sm Su	R144	Tc Km
R36	Tc	R77	Tc Sm Su	R145	Tc Km
R39	Tc	R82	Tc Sm Su	R163	Tc Km
R45	Tc Ap	R92	Sm Su	R183	Tc
R46	Tc Ap Sm Su	R114	Tc Sm Su	R192	Tc Sm Cm Su
R51	Tc	R124	Tc Ap	R196	Tc Sm Cm Su
R52	Tc Sm Su	R128	Tc Ap Su	R199	Tc Su

Factor R2 appeared in a strain of *Salmonella typhimurium* (Datta, 1962); factors R192, R196 and R199 came from strains of *S. typhimurium* kindly sent by Dr Manten of Utrecht; factor R1 came from a strain of *S. paratyphi B*, and all the others from strains of *S. typhimurium* sent to the Enteric Reference Laboratory, Central Public Health Laboratory, London, for phage-typing, and kindly provided by Dr E. S. Anderson so that one of us (N. D.) might examine them for transmissible drug-resistance.

Tc = Tetracycline; Ap = Ampicillin; Cm = Chloramphenicol; Km = Kanamycin; Sm = Streptomycin; Su = Sulphonamide.

F specific phage. This was MS2 (Davis, Strauss & Sinsheimer, 1961), an RNA phage whose particles are spheres about 250 Å in diameter (Loeb & Zinder, 1961; Bradley, 1964). The phages in this group are so generally similar, and so closely related serologically (Scott, 1965) that they are probably to be regarded as mutational derivatives of a common ancestor (Zinder, 1965). Stocks were prepared either on K12 Hfr.B1 or on K12 Hfr.H, and sterilized by chloroform after low-speed centrifugation. Titres of over 5×10^{11}/ml. plaque-forming particles were regularly obtained, and stocks could be passed through membrane filters (Millipore, Type HA, pore size 0·45 μ) without perceptible drop in titre.

Assay of phage. Phage was assayed by the agar layer method with incubation at 42°C., which leads to clearer and more numerous plaques than incubation at 37°C. (Dettori, Maccacaro & Turri, 1963). An Hfr strain was used as indicator in preference to an F+ to minimize any chance of transfer of F to the bacteria under test. Strain Hfr.B1 was used initially, but was later abandoned when it was found that Hfr.H gave a higher efficiency of plating (1/0·7), and also that in parallel cultures of the two strains which had taken up the same amount of phage (*c.* 75%, added at a multiplicity of 5 particles/bacterium), the proportion of infected bacteria (i.e. antibody-resistant infective centres) was greater with Hfr.H (50–80%) than with Hfr.B1 (10–20%).

Antiphage serum. This was prepared against another F-specific phage, μ2 (Dettori, Maccacaro & Piccinin, 1961), but neutralized phage MS2 at the same rate, with a K value of about 10,000. Antibacterial antibodies were absorbed to prevent agglutination during tests on bacterial suspensions for phage sensitivity. The antiphage activity of the serum remained unaltered.

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Culture media. Bacteria were grown in Oxoid Nutrient Broth No 2, or on the same broth solidified with 1·25% Davis N.Z. agar. The liquid medium (TYECa) used with phage MS2 contained Oxoid Tryptone 10 g., yeast extract 1 g., and NaCl 8 g. dissolved in 1 l. distilled water and adjusted to pH 7·2. After sterilization, glucose was added to 0·15%, and $CaCl_2$ to 0·002 M, which is required for phage penetration (Loeb & Zinder, 1961), and possibly for attachment (Valentine & Strand, 1965). Phage was assayed on solid medium consisting of Oxoid Tryptone 10 g., NaCl 8 g., glucose 1 g. and Difco Bacto agar 10 g. dissolved in 1 l. distilled water and adjusted to pH 7·2. For transfer of the R factors, either a mineral salts medium (Tatum & Lederberg, 1947) was used with the omission of asparagine, solidified with Davis N.Z. agar 1·5%, and supplemented so as to allow growth only of the recipient strain; or else McConkey bile salt lactose indicator agar was used, which allows *lac*$^+$ and *lac*$^-$ colonies to be distinguished.

Tests of cultures for the presence of phage-sensitive bacteria. The technique evolved was designed to measure the proportion of phage-sensitive bacteria in the culture. Since only few bacteria were likely to be infected, the proportion of unadsorbed phage was expected to be considerable. Thus, high titre antibody would be needed to neutralize it quickly enough to allow plating of the bacteria within the latent period of phage multiplication. Residual antibody had then to be removed while maintaining a bacterial concentration high enough to detect small numbers of phage-sensitive cells. Therefore the bacteria were grown to $2{\cdot}5\text{–}5 \times 10^8$/ml. and phage MS2 added at a multiplicity of 2–100, usually 20. After 8 min. at 37°C. for adsorption (a period shown to allow up to 90% of phage to be taken up by cultures of Hfr.H, with infection of up to 90% of the bacteria), 0·5 ml. of the mixture was added to 2 ml. of a 1/500 dilution of the antiphage serum (a dilution which neutralized 99·99% of phage in 10 min.). This was held at 37°C. for 10 min.; about 10 ml. of TYECa was then added and the whole amount poured on a Millipore filter (Type HA), which was sucked almost to dryness before being washed through with another 10 ml. of TYECa. The filter was then transferred to 10 ml. of TYECa, rinsed well to release the bacteria, and then discarded. (Control tests on recovery from the filter showed that 70–90% of bacteria, Hfr, F$^+$ or F$^-$, were recovered as colony-formers, and over 50% of infected bacteria of strain Hfr.H were recovered as plaque-formers.) The culture was immediately assayed for phage (Initial Plaques in Table 3), and assayed again after incubation for $1\frac{1}{2}$ or 2 hours at 37°C. Increase in phage titre over this period is given in Table 3.

3. RESULTS AND DISCUSSION

A number of the R factors tested enabled F$^-$ bacteria to support multiplication of the F-specific phage, MS2 (Table 3). The ability to grow in R$^+$ cultures was not due to mutation of the phage, for phage recovered from infected R$^+$ cultures behaved in the same way as the parent stock in its ability to infect and multiply in such cultures.

No F$^-$ strain gave any increase of the phage. Some increase was, however, observed with strain 58·161/*sp* which, although it has been generally regarded as F$^-$, has not in fact lost F (Meynell & Datta, 1966). The second assay on F$^-$ strains showed a considerable decrease in plaque count, which was probably due to carry-over of antibody, for a decrease occurred with control suspensions of the phage alone treated in the same way as the mixtures, while suspensions made simply by dilution were perfectly stable.

The first part of Table 3 shows that the proportion of phage-sensitive bacteria in R$^+$ cultures was greatly increased in HFT systems, where repression of the conjugating function is lifted. Release from repression was very variable with the different R factors, as well as depending to a lesser extent on the conditions used. Details will be published elsewhere, but HFT donors showed transfer frequencies 10–5000-fold higher than established R$^+$ cultures. The number of phage-infected bacteria in the HFT cultures could

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Table 3. *Phage MS2-sensitivity conferred by R-factors*

R-factor	fi	In strain	Number of tests	Initial plaques	Increase
Negative controls		Phage in TYECa	4	20 –$7{\cdot}2\times10^{2}$	None
		Heat-killed F^-	5	$2{\cdot}0\times10^{2}$–$4{\cdot}6\times10^{3}$	None
Phage-lysed cultures		Hfr.H	6	$3{\cdot}5\times10^{6}$–$1{\cdot}5\times10^{7}$	×2000–50,000
		Hfr.B1	4	$8{\cdot}4\times10^{5}$–$2{\cdot}0\times10^{6}$	×20,000–100,000
–		RC.709	18	$2{\cdot}4\times10^{2}$–$1{\cdot}0\times10^{4}$	None
R1	+	,,	6	$1{\cdot}6\times10^{3}$–$4{\cdot}7\times10^{4}$	×600–1500
HFT		,,	2	$1{\cdot}0\times10^{5}$; $6{\cdot}0\times10^{5}$	
R2	+	,,	1	$1{\cdot}7\times10^{3}$	×110
R27	?	,,	1	$1{\cdot}6\times10^{3}$	None
R28	+	,,	1	$1{\cdot}7\times10^{3}$	×100
R36	+	,,	2	$3{\cdot}6\times10^{3}$; $2{\cdot}6\times10^{3}$	×13; 15
R46	–	,,	1	$4{\cdot}6\times10^{2}$	None
R124	+	,,	1	$1{\cdot}1\times10^{5}$	×8000
R142	–	,,	1	$1{\cdot}4\times10^{3}$	None
R145	–	,,	1	$2{\cdot}4\times10^{3}$	None
R62	?	,,	1	$2{\cdot}6\times10^{3}$	None
R128	?	,,	1	$1{\cdot}0\times10^{3}$	None
–	.	RC.711	9	$3{\cdot}0\times10^{2}$–$9{\cdot}0\times10^{3}$	None
R1	+	,,	4	$1{\cdot}5\times10^{3}$–$3{\cdot}0\times10^{3}$	×100–2900
HFT		,,	2	$3{\cdot}3\times10^{5}$; $3{\cdot}7\times10^{5}$	×85,000
R2	+	,,	2	$1{\cdot}2\times10^{3}$; $3{\cdot}0\times10^{3}$	×154; 850
HFT		,,	1	$3{\cdot}0\times10^{3}$	×1700
–		RC.24	15	$3{\cdot}0\times10^{2}\times9{\cdot}2\times10^{3}$	None
R1	+	,,	1	$3{\cdot}8\times10^{3}$	
HFT		,,	1	$2{\cdot}4\times10^{5}$	
R27	?	,,	3	$1{\cdot}6\times10^{3}$–$3{\cdot}0\times10^{3}$	None
R39	–	,,	1	$4{\cdot}8\times10^{3}$	None
R45	–	,,	1	$5{\cdot}0\times10^{2}$	None
R51	+	,,	2	$1{\cdot}5\times10^{3}$; $2{\cdot}5\times10^{3}$	×30; 50
R52	+	,,	2	$5{\cdot}6\times10^{3}$; $5{\cdot}7\times10^{3}$	×7; 10
R56	–	,,	1	$2{\cdot}6\times10^{3}$	None
R62	?	,,	2	$2{\cdot}2\times10^{3}$; $4{\cdot}9\times10^{3}$	None
R64	–	,,	1	$3{\cdot}0\times10^{2}$	None
R73	+	,,	1	$2{\cdot}8\times10^{3}$	×260
R77	+	,,	1	$2{\cdot}0\times10^{3}$	×1000
R82	+	,,	2	$1{\cdot}0\times10^{3}$; $3{\cdot}9\times10^{3}$	×26; 40
R92	–	,,	1	$2{\cdot}4\times10^{3}$	None
R114	+	,,	2	$1{\cdot}0\times10^{3}$; $2{\cdot}9\times10^{3}$	×30; 94
R128	?	,,	2	$7{\cdot}7\times10^{2}$; $4{\cdot}6\times10^{3}$	None
R136	+	,,	1	$2{\cdot}4\times10^{3}$	×100
R143	–	,,	1	$1{\cdot}9\times10^{3}$	None
R144	–	,,	1	$6{\cdot}5\times10^{2}$	None
R163	–	,,	1	$3{\cdot}6\times10^{3}$	None
R183	–	,,	1	$2{\cdot}0\times10^{3}$	None
R192	+	,,	1	$1{\cdot}1\times10^{3}$	×170
R196	+	,,	2	$6{\cdot}0\times10^{3}$; $2{\cdot}0\times10^{4}$	×16; 22
R199	–	,,	1	$3{\cdot}0\times10^{2}$	None
–		58.161/*sp*	6	$3{\cdot}2\times10^{2}$–$4{\cdot}0\times10^{3}$	×77–500
R1	+	,,	4	$2{\cdot}2\times10^{3}$–$4{\cdot}0\times10^{3}$	×135–6200
R1 eliminated by acridine		,,	2	$1{\cdot}1\times10^{3}$; $1{\cdot}2\times10^{3}$	×150

be estimated from the initial plaque count, although this was impossible with established R^+ cultures because of the high background of plaques observed in the controls. However, the fact that the phage increased showed that some phage-sensitive bacteria were present in the established R^+ cultures, and the proportion in HFT cultures probably

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exceeded 5%. It may be concluded that the phage receptor determined by these R factors is the same as that determined by F, but is ordinarily present on only a few cells: when repression is lifted, the increased frequency of conjugation is associated with an increased proportion of phage-sensitive cells. The phage receptor on F^+ bacteria is a specialized fimbria (Crawford & Gesteland, 1964), or pilus (Brinton, Gemski & Carnahan, 1964), and the same morphological structure acts as the receptor on F^-R1^+ bacteria (Datta, 1965; Lawn, Meynell & Datta, in preparation).

Of the thirty different R factors, fourteen proved to be unambiguously fi^+, and Table 3 shows that the F phage multiplied in all the cultures carrying these factors. There were thirteen fi^- R factors, and none of these conferred F phage sensitivity. The fi^+ and fi^-RTFs therefore differ fundamentally from each other. The remaining three R factors, R27, R62 and R128, behaved aberrantly in a number of ways concerned with their stability, rate of transfer and fi character, and are being further investigated.

The failure of fi^- R factors to inhibit the function of F could indicate that this class of R factor either made no repressor, or else a repressor without action on F. If the former were the case, the efficiency of transfer of fi^- R factors might be expected always to be high, and certainly not to be increased with bacteria which have newly acquired the factor. However, of the thirty R factors tested, the fi^- were not more efficiently transferred by established R^+ cultures than were the fi^+; and, moreover, when bacteria with newly-acquired R factors were examined, HFT systems were as readily produced with fi^- as with fi^+ factors. It therefore appears that fi^- R factors are associated with repression, just like fi^+, and that the failure of their repression to extend to F is due to the two being unrelated agents. On the other hand, both fi^+ RTF and F determine the synthesis of the same structure, subject to the same regulatory mechanism, which suggests a close relationship between them.

The F factor of *E. coli* K12 may be exceptional in expressing its conjugating function so freely that its presence could be originally detected simply by the fertility of its bacterial host. While freely expressed conjugation factors like F itself are so rare as to be practically unique, unexpressed conjugation factors of the same sort may be widespread, but detected only if they carry genes which alter some other property of the culture. These may be the agents which act as RTFs and give rise to R factors when they acquire drug-resistance genes. Density gradient centrifugation of extracts of bacteria of various species with no known plasmid function has shown minor bands of DNA (Rownd, personal communication) resembling those found in strains carrying F or an R factor (Falkow *et al.*, 1965). Such components may perhaps represent conjugation factors.

SUMMARY

The genetic elements promoting conjugation associated with transmissible drug resistance are of two or more kinds. Experiments with phage specific for F^+ bacteria have shown that one large class differs from F essentially only in the production of a cytoplasmic repressor which limits the synthesis of the specific F pilus, whether by the RTF itself or by F when this is present in the same cell.

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