R FACTORS OF ENTERIC BACTERIA WITH SPECIAL REFERENCE TO THE Rfi⁻ FACTORS ISOLATED FROM SALMONELLA

by

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ABSTRACT

Studies with R factors isolated from clinical strains obtained in the Ottawa area indicated that whereas all the R factors (RM98, RM227, RM413, RM414 and RM430) from Salmonella typhimurium were fi^-, those from Aerobacter aerogenes were fi^+ (KR61, KR36, KR78, and KR68) except KR19 which lacked the RTF and could not be classified.

E.coli F^- strains bearing each of the Rfi^- factors, except RM98, showed propagation of phage If1. This led to the isolation of IKe, a phage specific for the sex factor of RM98. IKe was not propagated by the hosts of I type sex factors e.g. R64-11, but IKe-specific lysis or propagation could be observed with the strains bearing some other fi^- sex factors, including the unclassified R factors R45, R46 and R199. All the fi^- sex factors showing IKe specificity were, therefore, designated as the "IKe specific sex factors". Hosts of RM227, RM413, RM414 and RM430 also propagated IKe besides If1, indicating the possibility of a third kind of fi^- sex factor, intermediate between I and IKe-specific sex factors; or the possession of two different sex factors. The fi^+ R factors did not render their E.coli F^- hosts sensitive to F specific phages, resembling unclassified fi^+ R factors.

The unrelatedness of I- and IKe-specific sex factors was confirmed by the lack of entry exclusion and absence of incompatibility between R64-11 and RM98. Entry exclusion was observed between IKe-specific R factors, confirming their relatedness. Members of the

third group of fi^- sex factors (RM227, RM413, RM414 and RM430) exhibited relation to either RM98 or to R64-11 by the criteria of entry exclusion and incompatibility.

All the 9 isolated R factors showed little or no segregation and curing by acridine orange or ethidium bromide; did not restrict coli-phages T3, T7, ØII, λ, P1kc and BF23 or Salmonella phages SP6 and P22; those studied (RM98, RM227, RM413, RM414, RM430 and KR61) were not found to change the phage type of S. typhimurium LT2. None of the fi^- R factors harboured colicinogenic determinants; only RM413 and RM430 conferred a partial Col Ib resistance on their E.coli hosts. RM98 yielded a relatively high proportion of RTF^+ transductants in P22 transductions resembling R45, R46, R205 and N3; it did not effect chromosomal transfer between E.coli F^- strains, in contrast to R64-11. The resistance pattern of RM98 appears to be the most common among Salmonella-R factors isolated in North America.

The phage IKe was found to be filamentous and relatively more heat sensitive than Ifl. It was inactivated by chloroform, nagarse, pronase and trypsin. Antiserum against IKe did not cross neutralize Ifl or M13.

PART ONE

INTRODUCTION

Ever since their discovery in Japan in 1959 (Akiba, Ochiai et al.), genetic elements bringing about the transmission of antibiotic resistance (R factors) in bacteria have been the subject of intensive investigation. Whereas this has led to an enhanced understanding of their nature and behaviour in bacterial hosts, several gaps in our knowledge still exist, particularly in respect of the control of their infectious spread within bacterial populations. At least five excellent reviews on R factors have appeared in the literature (Watanabe, 1963, Falkow et al., 1967, Anderson, 1968, Meynell et al., 1968[a], Novick, 1969), which have attempted to elucidate their taxonomic relationships, interactions with other genetic elements of a similar nature, genetic organization, molecular nature and replication. The phenomenon of infectious antibiotic resistance which R factors mediate is of special interest to the microbial geneticist and of yet greater importance in clinical medicine to the epidemiologist. A bacterial cell becomes resistant to antibiotics by (1) spontaneous mutation and selection or (2) transfer of genetic information from a resistant bacterium to a sensitive one followed by selection. Three processes have so far been recognized to bring about transfer of genetic information: DNA-mediated transformation, bacteriophage-mediated transduction and sexual recombination which follows conjugation between bacteria of opposite mating types.

Early epidemiological studies with multiple drug resistant bacteria clearly indicated that cell to cell contact(conjugation) was required for transfer of multiple drug resistance(Mitsuhashi et al., 1960, Watanabe, 1963), although in some cases it could be transduced (Watanabe and Fukasawa, 1961[a]). In most cases, genetic determinants of multiple drug resistance were transferred together and independently of the chromosome of the donating bacterium(Watanabe and Fukasawa, 1961[b]). The multiple drug resistance was observed to be lost spontaneously or eliminated by treatment with acridine dyes(Mitsuhashi et al., 1961). These findings suggested the episomic nature of infectious drug resistance.

The R factors which were first discovered conferred resistance to one or more of the drugs: streptomycin, tetracycline, chloramphenicol and sulfonamide (Mitsuhashi et al., 1961, Watanabe, 1963). They were later found to accumulate other resistance determinants such as kanamycin, neomycin (Lebek, 1963) and penicillins (Anderson and Datta, 1965[c] , Voogd et al., 1968). Most frequent resistance patterns among R factors isolated from Salmonella include: SmTcSu, ApSmTcSu, ApTcSu and ApSmSu (Datta,1962, Anderson and Datta 1965[c], Pocurull et al, 1971), with the pattern ApSmTcSu predominating(Pocurull et al., 1971). R factors can infect most genera of the Enterobacteriaceae (Harada et al.,1960) including Serratia (Rownd et al., 1966) and certain other genera e.g. Vibrio (Baron and Falkow, 1961, Kuwabara et al., 1963) and Pasteurella(Ginoza and Matney, 1963).The frequency of R factor transfer varies greatly between different strains (Watanabe 1963).

Nature of R factors

Cells of enteric bacteria possess a chromosome which comprises a major linkage group containing most of the genes the cell has (Hayes, 1960, Taylor, 1970, Sanderson, 1970). Many bacteria, however, may possess additional linkage groups that are small, dipensable and physically unlinked to the chromosome (Jacob, Schaffer and Wollman, 1960). The latter are collectively referred to as extrachromosomal elements and are further subdivided into two groups (1) transferable extrachromosomal elements and (2) nontransferable extrachromosomal elements. Complete R factors belong to group (1) and confer on their hosts two outstanding functions (a) render them resistant to one or more antibacterial agents and (b) enable them to conjugally transmit this property to other bacteria. The recipient bacterium as a result becomes capable of functioning as donor in its turn. R factors as well as other extrachromosomal elements e.g. F factor of E.coli K-12 and Col Ib, etc., have also been referred to as plasmids or episomes at various times in the literature (Falkow et al., 1967, Novick, 1969). Episomes are the extrachromosomal elements which can establish themselves in the autonomous as well as in the chromosomally integrated state (Jacob and Wollman, 1958, 1960) while plasmids are those for which the chromosomally integrated state has not been demonstrated, but are stably inherited by the cell progeny (Novick, 1969). Since

an extrachromosomal element may behave as an episome in one host and as a plasmid in another, the two definitions are not rigid (Falkow et al., 1967). Both episomes and plasmids are considered as replicons.

Chemically the R factors consist of DNA like the chromosome (Falkow et al., 1966, Rownd et al., 1966). They may occur either as (1) single, closed linkage group consisting of a sex factor which brings about the transfer of antibiotic resistance (RTF) and antibiotic resistance (R) determinants or (2) the two entities could exist independently of each other. Whereas the studies of Watanabe et al.,(1961[a], 1962[b], 1964[b], 1968) involving transductions, conjugations and segregations favour the first model for R factor structure, the work of Anderson et al.,(1965a, 1965[b]) and later that of Mitsuhashi et al., (1969) indicates that R factors are probably made up of distinct, physically separate components, RTF and R determinants. Only RTF is conjugally transferable;R determinants may acquire transferability in association with the RTF. However, the model of Watanabe and the model of Anderson do not appear to be mutually exclusive. Biophysical studies seem to indicate that whereas the R factor may exist as separate units in Proteus, in E.coli it exists as a single linkage group (Cohen & Miller, 1970, Silver & Falkow, 1970).

Classes of sex factors mediating transfer of antibiotic resistance

In 1953 Cavali et al., observed that a factor " F " when present in E.coli strains conferred on them the property of donating genetic material to other strains that lacked F (and hence were F^- or female). Further studies in the field indicated that transfer

of genetic material (the F factor itself, or the bacterial chromosome) from F^+ to F^- cells probably occurred through special flilaments (F pili) found on the F^+ cells (Brinton, 1965). F pili can be differentiated from other cell appendages by several unique characteristics, the most obvious of which is the adsorption of F-specific phages (Brinton, 1965, Lawn, 1966). F is an episome in that it can recombine with the chromosome to yield a proportion of male cells showing a high frequency of recombination (Hfr males) for chromosomal markers when conjugated to F^- cells. When R factors were examined in male cells of E.coli (F^+R^+ or $HfrR^+$) it was found that the sex factor associated with the R factor was either able to inhibit or considerably reduce the F-associated functions (F pilus synthesis, conjugal fertility and ability to adsorb F-specific phages) or had no effect on these properties. The former were referred to as fi^+ (for possessing the property of fertility inhibition) and the latter as fi^- R factors (Watanabe et al., 1962[a], Watanabe et al., 1964[a]). Inhibition or reduction of F-associated functions by an R factor was attributed to the synthesis of a cytoplasmic repressor by the R factor (Egawa and Hirota, 1962) which in addition to inhibiting its own sex factor also repressed F (Meynell et al.,1968[a]). Rfi^- factors also synthesized a repressor which repressed their own sex factor, but had no effect on F (Meynell et al., 1968[a]). Ultraviolet irradiation was found to increase the donorability of R^+ strains (derepression); such R factors were referred to as derepressed (drd) R factors (Meynell and Datta, 1967[a])

and drd Rfi^+ factors no longer inhibited the F-associated functions.

When present in F^- E.coli cells, most Rfi^+ factors were found to determine the production of a sex pilus, antigenically and morphologically similar to that determined by F (Lawn, 1966, Datta et al., 1966, Nishimura et al., 1967, Lawn et al., 1967). F^- E.coli strains bearing Rfi^- factors, on the other hand, bore sex pili that were morphologically and antigenically different from F pili but closely resembled the pili (I pili) synthesized by strains bearing another plasmid Col Ib (Meynell and Lawn, 1967[b], Lawn et al., 1967). I pili of the strains bearing either Col Ib or the Rfi^- factors showed affinity for I-specific phages (Lawn et al., 1967, Meynell and Lawn, 1968[b]). This led to the classification of R factors into two major groups F-like and I-like (Lawn et al., 1967). The two groups can be differentiated on the basis of (1) adsorption of sex factor specific phages to the specific pili synthesized by plasmid bearing cells or indirectly by the ability of the host strain to show (2) visible lysis or (3) propagation of the specific phage. All the properties (1), (2) and (3) are based on the presence of specific sex pili which must have receptor sites for the sex factor specific phages (Crawford et al., 1964, Caro and Schnos, 1966, Lawn et al., 1967). Since demonstration of (1) is difficult in case of filamentous phages (Tzagoloff et al., 1964, Marvin and Hohn, 1969) and (2) can be observed only if the R factor is derepressed (Datta et al., 1966), (3) is the method of choice in most cases (Meynell and Datta, 1966[a], Lawn et al., 1967).

Propagation of I- or F-specific phages by bacterial strains occurs following the steps of adsorption (to the specific pili), penetration (of the phage genome into the cell) and replication of phage nucleic acid with the ultimate release of progeny. This can be observed in the form of lysis (plaque) if all or most of the cells are sensitive to the phage. In case where all the cells are not sensitive to the phage (strains bearing wild type or repressed R factors), propagation or increase in the plaque forming units (p.f.u) can be measured indirectly by assaying a broth lysate on a sensitive indicator. The known F-specific phages are either isometric or filamentous. The former include: MS2, R17, QB, μ2, f2, M12 etc. (Strauss and Sinsheimer, 1963, Paranchych and Graham, 1962, Overby et al., 1966, Dettori et al., 1961, Loeb and Zinder, 1961, Hofchneider, 1963) and are known to adsorb along the sides of F pili (Brinton et al., 1964, Crawford et al., 1964, Brinton and Beer, 1967). The latter include: M13, f1, fd, EC9, AE2 etc. (Hofschneider, 1963, Loeb, 1960, Zinder et al., 1963, Marvin et al., 1963, Dettori and Neri, 1965, Panter et al., 1966) and adsorb to the tips of F-type pili (Caro and Schnos, 1966, Marvin and Hohn, 1969). The I-specific phages known so far, include If1 and If2 (Meynell and Lawn, 1968), both of which are filamentous and are also collectively referred to as the "I phage" (Lawn et al., 1967). Their mode of adsorption is the same as that of the filamentous F phages i.e. to the tips of I-pili (Lawn et al., 1967).

The ability to synthesize sex pili, the ability to conjugate and sensitivity to sex factor specific phages were found to be highly correlated. When synthesis of the sex pilus was repressed, ability to conjugate and phage sensitivity were always coordinately repressed (Datta et al., 1966, Meynell and Datta, 1966[b]). These findings indicated that phage sensitivity and ability to conjugate were probably two different manifestations of the activity of one gene. This was probably the gene which determined the pilus synthesis (Datta et al., 1966).

A small group of R factors which included both Rfi^+ and Rfi^- factors was not found to confer sensitivity to I- or F-specific phages and hence could not be classified on the basis of phage propagation (Lawn et al., 1967, Meynell et al., 1968[a]). Lack of phage propagation was attributed to (1) the highly repressed state of the R factors (Lawn et al., 1967, Datta, personal communication)or (2) possession by these R factors of a third kind of sex factor (Lawn et al., 1967), which synthesized pili other than I- or F-type pili. If the latter was the case, there was a possibility that other phages, which were specific for the sex factors associated with such R factors existed.

Other criteria used for the classification of R factors include the phenomenon of superinfection immunity (comprising of entry exclusion and incompatibility: Novick, 1969). The reason for superinfection immunity between two related plasmids is not clearly understood. It can be observed at (1) the entry level:

when cells carrying one fi type of R factor reduce the conjugal entry of a superinfecting R factor of the same fi type, and (2) the post entry level: when R^+ cells superinfected by another R factor of the same fi type lose one of the R factors during replication or segregation (incompatibility), unless agents that would select both R factors are included in medium at all times (Mitsuhashi et al., 1960, 1962, Watanabe et al., 1964[a]). Under conditions where the loss of superinfecting R factor is prevented by applying selective pressure for both R factors, the two may recombine and then coexist stably without any further selective pressure (Mitsuhashi et al., 1962, Watanabe et al., 1964[a]). The phenomenon of incompatibility has been attributed to the competition between two related plasmids for the same "maintainence site" in the cell (Jacob and Monod, 1961, Novick, 1969). Attachment to the maintainence site, during cell division, is probably necessary for the plasmids to be distributed equally to cell progeny. Two unrelated plasmids (e.g. Rfi^- and Rfi^+ factors) do not exhibit entry exclusion against each other and can coexist as separate replicons within the same cell (Watanabe and Fukasawa, 1961[a], Watanabe et al., 1964[a]).

Restriction and modification of phages were first described by Watanabe et al., (1964[a]) as the properties associated with the presence of Rfi^- factors in E.coli hosts. Phage restriction was then found to be widely distributed among R factors irrespective of their fi character (Siccardi, 1966, Bannister and Glover, 1968). Patterns and degree of restriction of a number of test bacteriophages

can, therefore, be used to classify R factors into groups (Anderson, 1966, Guinee and Willem, 1967) other than fi groups (Bannister and Glover, 1968).

Criteria for the classification of R factors can be summarized as follows:

1. Ability (of the R factor) to diminish or repress F-associated fertility (Watanabe et al., 1964[a]).
2. Ability (of the R factor) to inhibit or decrease male phage adsorption by male E.coli cells (Watanabe et al., 1962[a]).
3. Synthesis of sex factor specific pili (by R^+F^- E.coli cells) that may show similarity to either F- or I-pili (Datta et al., 1966, Lawn, 1966, Lawn et al., 1967).
4. Ability of the R^+ hosts to adsorb, be lysed by or propagate sex factor (F or I) specific phages (Datta et al., 1966, Meynell and Datta, 1966[a], Lawn et al., 1967).
5. Superinfection immunity (entry exclusion and/or incompatibility) between two R factors (Mitsuhashi et al., 1962, Watanabe et al., 1964[a], Meynell et al., 1968[a], Novick, 1969).
6. Bacteriophage restriction (Guinee and Willem, 1967, Bannister and Glover, 1968).

The first two criteria divide R factors into fi^+ and fi^- groups; these two groups often correspond to the F- and I-groups of sex factors respectively, which can be defined on the basis of criteria 3 and 4. Classification by the 6th criterion does not correspond to the above groups. The presence of entry exclusion and/or incompatibility (criterion 5) between two plasmids indicates a

genetical relationship between them.

Purpose of the current investigation

Cohen (1971) has listed five potential approaches to the problem of control of transferable drug resistance in bacteria (1) search for new antimicrobials, (2) elimination of R factors by selective inhibition of R factor replication (3) interference with genetic expression of R factors (at the level of transcription or translation), (4) prevention of formation of new R factors and (5) inhibition of R factor transfer. The first approach is more or less empirical, whereas the rational use of the last four approaches depends on a fundamental understanding of the nature and types of R factors and the interactions they exhibit in genetical and biophysical experiments.

Available published information indicated that R factors assigned to the fi^- group comprised an interesting and possibly heterogenous group,especially the fact that there existed some Rfi^- factors(R45, R46, R199 etc.) that were different from the Rfi^- factors bearing I-like sex factor(R64-11, R144 etc.)(Lawn et al., 1967). P22 transduction experiments by Drabble and Stocker (1968) further indicated that R factors R45, R46 etc. were probably different than other R factors (with respect to the linkage relationships between R determinants and RTF). The more recent observation that Rfi^- factors could coexist stably in one host

(Bouanchaud and Chabbert, 1969) was indicative of the need to undertake a systematic study of Rfi^- factors. Accordingly the present studies were undertaken to isolate and characterize the Rfi^- factors using locally available bacterial strains (from Ottawa General Hospital and Laboratory of Hygiene, Department of National Health and Welfare). In these attempts some Rfi^+ factors were also isolated. However, the latter attempts led to the isolation of 5 Rfi^- factors, one of which (RM98) was found to belong to the unclassified group of Lawn et al., (1967). A phage IKe was isolated from sewage (obtained from the Green Creek Pollution Center, Ottawa) for the sex factor of RM98 and used for the classification of fi^- sex factors, besides other criteria.

PART TWO

MATERIALS AND METHODS

A. BACTERIAL, BACTERIOPHAGE STRAINS AND PLASMIDS

The bacterial strains used are listed in Table I. The bacteriophages and R factors (excluding those isolated during the present work) or other plasmids are presented in Tables 2 and 3 respectively.

B. MEDIA, ANTIBIOTICS AND OTHER REAGENTS

Media

Resistance determinations were made on MacConkey (M.A.) to which single antibiotics were added at the desired concentrations. Davis minimal agar (Difco) (M.M.) with appropriate supplements of amino acids (20 µg/ml); adenine, thymine (20 µg/ml) and/or thiamine (1 µg/ml) was used for contraselection of donors in conjugation experiments. In case of conjugations involving R factor transfer, M.M. was also supplemented with the appropriate concentration of the desired antibiotic. Penassay broth (P.B.) antibiotic medium 3 (Difco) and penassay agar (P.A.) were used for the growth of bacteria and as the basal medium when loss of resistance was scored after acridine or ethidium bromide exposure and also when determinations of antibiotic sensitivity were carried out by the disc method. P.A. was also used for making bacterial input counts in quantitative conjugation experiments. Oxoid nutrient broth 2 and oxoid blood agar base were used in the studies involving IKe

TABLE 1

Bacterial strains

Strain	Genetic markers or characteristics#	Source
Escherichia coli K-12 13-6a	F^- lac^- pro A^- pur^- trp^- met^- Sm^r $T6^r$ (a lac^+ mutant of 13-6a was also used)	A. Ahmad
Escherichia coli K-12 AB712	F^- lac^- leu^- thr^- thi^- pro^- Sm^r $T6^s$	E. Adelberg
Escherichia coli K-12 MA50	F^- lac^- thr^- lys^- cys^- thi^- Sm^s	B. Low
Escherichia coli K-12 AB2463	F^- lac^- thr^- leu^- pro^- his^- arg E^- thi^- rec $A13^-$	B. Low
Escherichia coli K-12 JE2571	F^- lac^- thr^- leu^- gal^- xyl^- mal^- Sm^r pil^- fla^-	Y. Nishimura
Escherichia coli K-12 AB301	HfrH lac^+ met^- Sm^s $T6^s$	E. Adelberg
Escherichia coli K-12 X407	HfrH lac^+ thi^- pro^- Sm^s $T6^s$	
Escherichia coli K-12 Hfr1	Hfr lac^+ met^- Sm^s $T6^s$ (used as indicator for all F specific phages)	H. Yamazaki (via S.R. Khan)
Escherichia coli K-12 X478	F^- lac z^- pro C^- pur E^- trp^- lys A^- thi^- met E^- ara^- xyl^- Sm^r $T6^r$	B. Low
Escherichia coli K-12 MA124	F^- lac^- leu^- his^- arg G^- met^- Sm^r $T6^s$	B. Low
Escherichia coli K-12 AB1157	F^- lac^- thi^- thr^- leu^- pro^- his^- arg E^- Sm^r gal^- ara^- xyl^- mtl^-	B. Low

cont. 2

TABLE 1 (cont.)

Strain	Genetic markers or characteristics#	Source
Escherichia coli K-12 AB2495	Same as AB1157 but thy^- trp^-	B. Low
Escherichia coli K-12 Row	Colicin sensitive indicator	P. Fredericq
Escherichia coli K-12 J5	F^- met^- pro^-	N. Datta
Escherichia coli K-12 264A*	MA50 + RM98	derived
Escherichia coli K-12 Der7A5*	AB712 + RM98	derived
Salmonella typhimurium LT2	F^- prototroph phage type 4	R. Middleton
Salmonella typhimurium	trp A512$^-$ (deletion), Sm^s	R. Middleton
Salmonella typhimurium M533	cys D36, Col E1a$^+$ (propagating strain and indicator for If1)	G. G. Meynell
Shigella Sonnei L53		
Salmonella typhimurium M98	Wild type, untypable Ap^r Sm^r Tc^r	
Salmonella typhimurium M227	Wild type, untypable Ap^r	R. Khakhria
Salmonella typhimurium M413	Wild type, untypable Ap^r	(Department of
Salmonella typhimurium M414	Wild type, untypable Ap^r	National Health and
Salmonella typhimurium M430	Wild type, untypable Ap^r	Welfare)

* 264A and Der7A5 could be used as indicators and propagating strains for IKe

cont. 3

TABLE 1 (cont.)

Strain	Genetic markers or characteristics#	Source
Aerobacter aerogenes 61-OGH	Wild type Ap^r Km^r Nm^r Sm^r Tc^r	M. B. Graves (Ottawa General Hospital)
Aerobacter aerogenes 36-OGH	Wild type Ap^r Cm^r Sm^r Tc^r	
Aerobacter aerogenes 78-OGH	Wild type Ap^r Cm^r Sm^r Tc^r	
Aerobacter aerogenes 68-OGH	Wild type Ap^r Cm^r Sm^r Tc^r	
Aerobacter aerogenes 19-OGH	Wild type Ap^r Cm^r Sm^r Tc^r	

Ap^r, Cm^r, Km^r, Nm^r, Sm^r, Tc^r= resistance to ampicillin, chloramphenicol, kanamycin, neomycin, streptomycin and tetracycline respectively.

$T6^r$= resistance to phage T6.

pil^-, fla^-= unability to synthesize pili and flagella respectively. The nutritional markers are according to Taylor 1970.

TABLE 2

Bacteriophages

Phage	Characteristics	Source
ø II, T3, T7	Female specific coliphages	W. C. Summers
T6	Donor-eliminating phage	R. S. Edgar
BF23	Coliphage	P. Fredericq
λ	Coliphage	S. R. Palchoudhary
P1kc	E.coli transducing phage	L. S. Baron
P22	Salmonella transducing phage	R. Middleton
SP6	Female specific Salmonella phage	N. Zinder
MS2	F specific coliphage	N. Datta
μ2	F specific coliphage	R. Dettori
R17	F specific coliphage	H. Yamazaki(via S.R. Khan)
Qβ	F specific coliphage	L. R. Overby
M13	F specific coliphage	V. N. Iyer
If1	Phage specific for the I sex factor	E. Meynell

TABLE 2 Continued

Foot notes:

Hosts for the phages T3, T7, BF23 and P1kc were *E.coli* strains AB712, 13-6a or JE2571; for Ø II, AB712 or 13-6a; for P22 and SP6, *S.typhimurium* LT2; for T6, *E.coli* B/5 or AB712; for MS2, X407; for R17 and μ2 , *E.coli* Hfr1; for M13, *E.coli* K37 or Hfr1; for QB, *E.coli* Q13 or Hfr1 and for If1, *S.typhimurium* M533.

TABLE 3

R factors and other plasmids

Plasmid	Genetic markers or character*	Original host	Source
N3	Sm Tc Su	E.coli W1485	T. Watanabe (via S. Falkow)
R45	Ap Tc Su	E.coli J5	
R46	Ap Sm Tc Su	J5	
R199	Tc	J5	
R48	Ap Sm Tc Su	E.coli J5÷3	N. Datta
R205	Ap Tc Su	J5÷3	
R300	Sm Su	J5	
R305	Sm Su	J5	
R310	Sm Su	J5	
RP4	Ap Km Tc	J5	
R64-11	Sm Tc Su	E.coli 2395	E. Meynell
R144-3	Km	E.coli J5	S. Falkow
F'(KLF1)	F^+ leu^+ thr^+	E.coli AB2463	B. Low (via S.R. Palchoudhury)

cont.

TABLE 3 (cont.)

Plasmid	Genetic markers or character*	Original host	Source
Col Ib-P9	Col^+	*Shigella sonnei* P9	P. Fredericq
Col E2	Col^+ (from P9)	*E.coli* TR23	P. Fredericq
Col E1-30	Col^+ (from *E.coli* K-30)	*E.coli* TR15	P. Fredericq
Col Ib	Col^+ (from P9)	*S. typhimurium* cysD36	D. Helinski
Col Ia	Col^+	*E.coli* CA53	D. Helinski
Col E1a	Col^+	*S. typhimurium* M533	G.G. Meynell
Sex factors	Col^+, mob^+	*E.coli* E10A, E2c E193B, E47, E66, E92	S. Falkow
Sex factors	mob^+	*E.coli* E108B, E178, E160A, E194B, E147B, E181, E258, E251c, E2B, E107A, E136B, E84B, E8C, E238A, E11, E10C	S. Falkow

* Ap= ampicillin, Km= kanamycin, Sm= streptomycin, Tc= tetracycline
Su= sulfonamide, Col^+= colicin production, mob^+= ability to mobilize a nontransferable reistance.

isolation. Later, cultivation of IKe and of other phages was carried out by the agar layer method using L broth, L agar and L soft agar (0.6% agar) overlays. The L agar contained 1% Bacto tryptone (Difco), 0.1% yeast extract, 0.8% NaCl and 1% agar. Glucose (0.1%) and CaCl 2 (0.002 M.) were added to the medium immediately before use. The bacterial host was grown in Trypticase soy broth (T.S.B.) (Difco) and plated on Trypticase soy agar (T.S.A.) (Difco) for the agar layer cultivation of If1. In conjugation experiments where phages were used for elimination of donor or in transduction experiments, L broth was used for the cultivation of bacteria. Triple sugar iron agar or T.S.I. (Difco) was used for the identification of exconjugants in Salmonella E.coli crosses.

Antibiotics

The antibiotics used and their sources were as follows: ampicillin trihydrate (Ap), streptomycin sulfate (Sm), Kanamycin sulfate (Km) and tetracycline phosphate (Tc), Bristol Laboratories Inc., Syracuse, N.Y.; chloramphenicol (Cm), Parke, Davis, and Co., Detroit, Mich., and neomycin sulfate (Nm), The Upjohn Co., Kalamazoo, Mich. The antibiotic sensitivity discs were from Difco.

Buffers and solutions

Buffers and other solutions used for studies on IKe were as follows:

1. Dilution buffer (Salivar, Tzagoloff and Pratt, 1964), or phosphate buffered saline.

Na_2HPO_4	7 g.
KH_2PO_4	3 g.
NaCl	4 g.
Distilled water	1000 c.c.
pH	7

2. Phosphate buffer (0.01 M)

Na_2HPO_4	2.68 g.
KH_2PO_4	1.36 g.
Distilled water	1000 c.c.
pH	7

3. Pucks' saline

NaCl	8 g.
KCl	0.4 g.
Dextrose	1 g.
$NaHCO_3$	0.35 g.
Distilled water	1000 c.c.
pH	7

4. Tris buffer (Sigma)

0.05 M , pH 7.5

Tris buffer was prepared by mixing the following two reagents in appropriate proportion:

i. Trizma base (T-1503); Tris (hydroxymethyl) aminomethane.

ii. Trizma HCl (T-3253); Tris (hydroxymethyl) aminomethane hydrochloride.

Both of the reagents were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Other reagents

Amino acids, ribonuclease and trypsin were from Nutritional Biochemical Corp., Cleveland, Ohio; bovine serum albumin (B.S.A.) was purchased from Sigma Chemical Co., St. Louis, Mo .; pronase and ethidium bromide (2,7 diamino-10-ethyl-9-phenyl phenan-thridium bromidium)

were from Cal biochem, Los Angeles, California; acridine orange (A.O.) was from Fisher Scientific Co., Fair Lawn, N.J.; cesium chloride (CsCl) was from Harshaw Chemical Co., Solon, Ohio; and Nagarse was from Enzyme Development Corp., N.Y.

C. CULTIVATION OF BACTERIA AND BACTERIOPHAGES

I. Bacterial strains

a. Stock cultures

Bacterial strains to be stored for long periods were grown overnight at 37 C with aeration (in a water bath shaker: Warner-Chilcot Labs Model 2156-1) in penassay broth (P.B.) which was supplemented with antibiotics in case of R factor bearing strains. Sterilized glycerol was then added to a final concentration of 5% and cultures were distributed in small vials which were kept frozen at -20 C. These cultures remained viable for one year or more depending on the strain.

Cultures used routinely were maintained on penassay agar (P.A.) slopes which were kept refrigerated at 4 C.

b. Cultures for use

Bacterial strains were grown with or without antibiotics in 5 ml P.B. contained in 125 ml or 25 ml Erlenmayer flasks overnight at 37 C in a water bath shaker. Overnight cultures were diluted 50-fold into fresh broth and regrown for one or two hours, depending on the experiment, under similar conditions. Unless otherwise indicated antibiotics were not added to the freshly grown cultures which were usually

grown in L broth for the experiments involving bacteriophages or for the conjugal crosses requiring a phage for donor elimination. For those conjugation experiments requiring other means of donor elimination, P.B. was used. Cultures to be used in the studies involving phage Ifl were grown in trypticase soy broth (T.S.B.).

II. Bacteriophages

a. Cultivation

i. Agar layer method

Bacteriophages were usually cultivated by the agar layer method of Adams (1959). A log phase culture of the propagating bacterial host and an appropriate dilution of bacteriophage preparation known to give confluent lysis were mixed in equal volumes (0.1 ml each) in molten soft agar overlays (held at 42 C); this was mixed and poured on top of the L agar plates. Cultivation of Ifl was carried out using T.S.A. Approximately 15 plates were sufficient for the cultivation of small batches. For large batches 100 or 200 plates were required. Following overnight incubation at 37 C, the top layer was scraped off with 1 or 1.5 ml L broth per plate using a sterile bent glass rod. The scraped growth was collected in flask or centrifuge tubes which were vortexed for one minute for even mixing and then centrifuged at low speed (3000 R.P.M.) to remove bacteria and agar. The supernatant was either first treated with chloroform and aerated to remove chloroform or filtered directly (in case of IKe & Ifl) through the millipore filters (Millipore Corporation. Type HAWP, pore size 0.45 μm) contained in micro-syringe filter holders

(Millipore Corp. Catalogue No: XX 30 025 00). Unless the lysate was too viscous, approximately 10-15 ml could be passed through these filters fitted with the prefilter type AP 25 (Millipore Corp.).

ii. Liquid cultivation

Small batches of certain phages, such as MS2, T6 and other lytic phages could be grown by the lysis of broth cultures. A log phase broth culture of the propagating strain was infected at a multiplicity of $1x10^{-1}$ or less, followed by overnight incubation at 37 C. The lysate was either first chloroformed or centrifuged directly to remove bacteria and filtered through millipore filters.

Filtered phage preparations were used directly for most experiments, except those involving studies on the phage itself, in which case further purification was required.

b. Purification

Large batches of the phages IKe and If1 were grown by the agar layer method (100 L agar or T.S.A. plates/phage) using the hosts 264A and M533 respectively and sterilized by millipore filtration. The phage IKe was concentrated from crude lysates by addition of polyethylene glycol before purification in cesium chloride density gradient (Iyer, R., unpublished). Phage If1 was concentrated by differential centrifugation (Sedimentation of the phage occurred at 60,000 g, in 36 hrs at 5 C), and purified on a preformed 5 step cesium chloride density gradient (Yamamoto et al., 1970). Dialysis of the purified preparation was carried out in 0.05 M tris buffer (pH 7.5). The purified preparation of IKe yielded $9x10^{13}$ p.f.u./ml; that of If1

yielded $2x10^{13}$ p.f.u/ml. Purified phages were used for studies in Section I, II, III, and IV (Materials & Methods). Purified M13 was kindly provided by V.N. Iyer.

c. Phage assays

i. Agar layer method

Filtered phage preparations were assayed for plaque forming units (p.f.u.). After making a series of ten-fold dilutions from $1x10^{-1}$ to $1x10^{-10}$ in broth, 0.1 ml of the desired dilutions were mixed with 0.1 ml of the indicator bacterium (grown to log phase) in separate test tubes. Soft agar overlays (held at 42 C) were mixed with each of these tubes, agitated and the mixture was poured on L agar or T.S.A. plates. It was necessary to pour assay plates and age them for 24 hr at room temperature before use. Plaque counts were made after overnight incubation at 37 C and the p.f.u/ml of the preparation was calculated.

ii. Spot method

Under certain circumstances (for the assay of If1 and of the phages used in propagation studies) it was expedient to assay the phages by depositing 0.02 ml of each dilution on the plates already containing the indicator strain seeded in the soft agar overlays. Four dilutions (0.02 ml each) could be deposited easily on the same plate with 0.1 ml or 0.2 ml pipettes and the drops were dried before incubation. The method was described by Meynell and Lawn (1968[b]).

D. ANTIBIOTIC RESISTANCE AND SENSITIVITY OF STRAINS

I. Screening for resistance -- Antibiotic disc method

Wild type antibiotic resistant strains from the Ottawa General Hospital and the Laboratory of Hygiene (Department of National Health and Welfare) were screened for resistance using filter paper discs impregnated with: Ap, Cm, Km, Nm, Sm and Tc (Difco). A late log phase culture of a test strain (0.1 ml) was poured onto a P.A. plate using a 2 ml P.A. overlay (held at 42 C). After about ten minutes, antibiotic discs were dispensed on the solidified overlay with a Difco disc dispensor. Following overnight incubation at 37 C, sensitivity or resistance to the antibiotics was scored, absence of a zone of inhibition indicating resistance.

II. Resistance levels -- Velvet replication

Where required, antibiotic resistant donor strains (capable of transferring their resistance to recipients in conjugal crosses in separate experiments) were replicated to determine the level of each antibiotic they were resistant to. For this purpose, a broth culture of the test strains was plated on M.A. to obtain isolated colonies. Approximately 50 individual colonies were picked onto a master plate, grown overnight and replicated on M.A. plates containing desired concentrations of each of the test antibiotics. Sterile velveteen was stretched on a wooden block by means of a metallic ring, and used for replication. The highest concentration of an antibiotic showing growth of all replicated clones was

considered as the resistance level of the strain for that particular antibiotic.

III. Sensitivity levels of recipients -- Minimal inhibitory concentrations of the antibiotics

Overnight P.B. cultures of the recipient strains were plated in 0.1 ml amounts on M.A. containing known graded concentrations of each antibiotic to determine the minimal inhibitory concentration of the latter capable of totally annihilating the recipient cells (MIC). Growth (or its absence) was recorded after 24 as well as 48 hours of incubation at 37 C.

Antibiotic stock solutions (10 mg/ml) were prepared in distilled water (chloramphenicol was dissolved in alcohol) and sterilized by millipore filtration (Type HA, pore size 0.45 μm). Stock solutions were maintained at -20 C. Solutions for daily use were refrigerated (4 C).

E. BACTERIAL CONJUGATIONS

I. Conjugation experiments

a. Broth or tube conjugations

Overnight cultures of donor and recipient cells were diluted 50-fold in P.B. or L broth, depending on the donor eliminating agent to be used for a particular cross (see donor elimination below), and grown with shaking at 37 C for 2 hours. At the end of this period, viable counts of the donor/recipient were made if required. Donors were then mixed with recipients in the proportion of 1:10 and incubated at 37 C without shaking for the desired length of time

(2 hours in case of R factor donors). Conjugation was terminated by contraselecting donor cells by means of an appropriate donor eliminating agent (see below) and challenging the surviving cells on nutrient or minimal media containing appropriate antibiotic and/or nutritional markers to contraselect recipients. Control platings of unmated donor and recipient cells were always made.

Exconjugant colonies arising on the plated media were purified twice on the selective medium used and replicated to check for the donated markers. Additional tests were performed on exconjugants, where required, to confirm their recipient nature.

i. Biparental crosses

These were set up as follows:

Donor	Recipient
Hfr $\pm$ R	F^-
F $\pm$ R	F^-
F^- + R	F^-
R + prototroph	F^-, F^+ or Hfr

The third type of cross was used to determine the ability of an R factor to mediate its own transfer.

ii. Triparental crosses

These were performed using a primary donor containing a sex or transfer factor which was crossed to an intermediate recipient bearing a non-transmissible plasmid of known antibiotic resistance. The final recipient was chosen so it could be easily selected for on a medium on which both primary donor and intermediate

recipient failed to grow. The selective medium contained the antibiotic to which the intermediate receipient was resistant.

iii. Interrupted matings

These were biparental crosses performed in a manner similar to that described by Pearce and Meynell (1968). Cultures of donor and recipient strains were grown overnight in P.B. The donor was regrown (after diluting 1:10 in P.B.) to late log phase and mixed with the overnight grown recipient in the proportion of 1:10. Following static incubation for 5 minutes at 37 C, the mixture was diluted (1:100) in fresh, warm P.B. maintained at 37 C in a water bath. At desired intervals, 0.1 ml samples were withdrawn and mixed with 2 ml molten unsupplemented agar (held at 42 C). This was violently agitated on an interruptor for 10 seconds, to separate mating pairs before being poured onto the selective medium.

For quantitative biparental crosses, the frequency of transfer of a genetic marker was calculated per hundred donors (see below), unless otherwise specified.

$$\text{Frequency of transfer/100 donors} = \frac{\text{No. of exconjugants/ml of conjugation mixture}}{\text{No. of (viable) donor cells/ml of conjugation mixture}} \times 100$$

b. Replication method

This was most convenient to quickly check if the segregant, derepressed or transduced R factors borne by an auxotrophic host strain carried an RTF. Several (ca. 100/plate) colonies were transferred to one or more master plates on a medium usually containing

a suitable antibiotic. After overnight growth at 37 C these were replicated onto a lawn of recipient bacteria spread on minimal agar supplemented with recipient auxotrophic markers and containing an antibiotic whose transfer was being studied. Growth of recipient-type exconjugants was indicative of RTF in the donor clone.

II. Derivation of exconjugants

a. Donor elimination

Following conjugation, donor cells were eliminated from mating mixtures by (a) plating on media containing high levels of streptomycin (to which the recipient was resistant), (b) challenging with a bacteriophage known to specifically lyse the donor and to which the recipient was insensitive or (c) in the case of auxotrophic strains, use of appropriate supplements in minimal medium. Recipients were contraselected with the addition of an appropriate antibiotic (in case of R factor transfer) or withholding an auxotrophic marker when its donation was being studied.

(a) Elimination with streptomycin was possible in most cases where the donor was streptomycin sensitive. Donor strains bearing R factor mediated low level streptomycin resistance could be also eliminated when the R^- recipient was streptomycin resistant (high level chromosomal resistance, usually more than 3000 µg./ml).

(b) Elimination with phage was the method of choice when wild type strains bearing R factors were the donors (see below). For these crosses donor-eliminating phages (to which recipients were

resistant) were isolated from sewage and used. In most crosses between strains of E.coli and involving R factor transfer, phage T6 elimination was efficiently used. In $F^+ \times F^-$ crosses, recipients were contraselected by pretreating the mating mixture with the phage ØII. Phage, when used, was added at a multiplicity of 100 to 1000 to obtain donor elimination. The growth medium for mating cells was L broth. A 30 min. incubation at 37 C following phage addition was found to be effective in most cases.

Donor-eliminating phages used in crosses with wild type donors

R factor donor	Phage	Elimination
61-OGH	P61	Very effective
36-OGH	P36	Fairly effective
19-OGH	P19	Fairly effective
78-OGH	P36	Not very effective
68-OGH	P43b	Not very effective

(c) When auxotrophic parent strains were used in conjugating mixture, the latter were washed in saline or minimal broth before plating on the appropriate minimal medium.

b. Identification of exconjugants and transductants

A recipient bacterium, which has received from a donor bacterium genetic material which codes for certain phenotypically recognizable properties, is referred to as an exconjugant. An exconjugant from a bacterial mating will therefore possess all the chromosomally determined properties of the recipient in addition to the donated properties transferred to it during conjugation.

In some experiments donor elimination was not very effective. This necessitated the use of confirmatory tests, such as sugar fermentation, reaction on T.S.I. agar and sensitivity to bacteriophages (P1kc, P22, T6, ØII, T3, T7, SP6 or MS2 etc.). Whenever possible, the auxotrophic markers of potential exconjugants were checked by replication. In case of R factor transfer, exconjugants and transductants were replicated on M.A. plates containing desired antibiotics to determine the pattern of resistances acquired. Conjugal transferability of a transduced R factor was checked by replication method (Section E, I, b, Materials and Methods). The U.V. sensitivity of rec^- recipients was checked by exposing several colonies of the test strain (gridded on a solid medium) to U.V. from a Westinghouse sterilamp (G36T6H) at a distance of 15 cm for different periods of exposure (1-15 sec.), always using a rec^+ control at the same time. Usually an exposure of 5 sec. was lethal for the rec^- strain, whereas a rec^+ control survived 15 sec. of exposure.

F. TRANSDUCTION IN ESCHERICHIA COLI AND SALMONELLA TYPHIMURIUM

I. P1kc transduction

In E.coli, transductions were performed using the phage P1kc. Transducing lysate of the phage was prepared on the donor strains by confluent lysis using the agar layer technique. P1kc grown by this method yielded $1x10^9$ - $1x10^{11}$ p.f.u/ml.

A log phase culture of the recipient strain was prepared in L broth and infected with the transducing lysate at a multiplicity

of 0.5-1.0. Following static incubation at 37 C for 1 hr., the transducing mixture was centrifuged, the pellet resuspended in 5 ml P.B. and reincubated for 2 hr. at 37 C. The mixture was then plated on M.A. plates containing a selective antibiotic. Transductants usually appeared in 24-48 hrs. Normally, transductant clones were purified twice on appropriate selective media. This eliminated, to a certain extent, the phage particles associated with the transductants and also unstable transductants.

II. P22 transduction

Phage P22 was the transducing phage for S. typhimurium. Transductions were carried out by a similar method as described for P1kc except that in this case the multiplicity of infection was 1-10 and the initial incubation of recipient cells with the P22 transducing lysate was 20 mins - 1 hr.

G. CHARACTERIZATION OF R FACTORS

I. Curing and spontaneous segregation

Curability

Acriflavin or acridine orange (A.O.) at concentrations (10 µg - 20 µg/ml) which partially inhibit bacterial growth have been reported to bring about complete or partial loss of R factor borne resistances (Mitsuhashi et al., 1961, Watanabe et al., 1961[c]). Such losses were found to occur at greater frequencies when Shigella or Salmonella host strains were used (Mitsuhashi et al., 1961, Watanabe et al., 1961[c]). Bouanchand et al., (1969) reported more

effective R factor losses by using ethidium bromide. Ultraviolet irradiation of host-cells prior to the treatment with A.O. or EBr has been found to further increase the rate of R factor losses (Mitsuhashi et al., 1961, Watanabe et al., 1961[c], Iyer and Iyer, 1969).

The method used here was similar to the method used by Watanabe et al., (1961[c]). Wild type E.coli or S. typhimurium hosts bearing an R factor were used. Penassay broth (P.B.) (pH 7.6) was used in all these experiments. Resistant cultures grown to log phase were U.V.-irradiated to 1% survival followed by an incubation of 1 hr. at 37 C in the dark. P.B. tubes containing graded concentrations of A.O. or of EBr were inoculated with irradiated or unirradiated log phase cultures to give a 50-fold-dilution. A control tube without dyes was always included. All the tubes were incubated overnight at 37 C in the dark. The contents of the control tube and of the tube showing partial inhibition of growth were plated on P.A. to obtain isolated colonies. Approximately 100-400 colonies per plating were gridded onto master (M.A.) plates. After overnight incubation at 37 C, these were replicated on antibiotic containing plates. In some cases the presence of RTF was also checked.

Spontaneous segregation

Spontaneous segregation of RM98 was examined in E.coli F^-, rec^- and S. typhimurium LT2 hosts. Strains bearing RM98 were grown in antibiotic free broth through 2-3 transfers; dilutions were then plated on M.A. to obtain isolated colonies. Some 300-400 colonies were gridded to master plates. These were replicated to

check for losses of resistance determinants and RTF.

II. Grouping into fi type

a. Fertility inhibition

R factors have been broadly classified into the fi^+ or fi^- types (fi= fertility inhibition) depending on their ability to inhibit or reduce the conjugal fertility function of E.coli strains bearing the classical sex factor F (Watanabe et al., 1964[a]). The fi type of an R factor can thus be inferred by comparing the frequency of donation of an early chromosomal marker in isogenic crosses of the type: Hfr R^- x F^-

Hfr R^+ x F^-

All the R factors being examined were separately transferred to AB301 Hfr met^-. The recipient was AB712 (F^-, leu^-, thr^-, pro^-, thi^-). The frequency of donation of leucine was scored using each of these donors and compared to that of the control cross.

b. Reduction/inhibition of MS2 adsorption

The inhibition of F fertility by an Rfi^+ type of factor has been attributed to the production of a specific repressor which limits the synthesis of the F pilus (Egawa and Hirota, 1962). In established lines of E.coli F^+ Rfi^+, one may therefore expect repression of F pilus synthesis and of adsorption of the F-specific phage MS2 (Watanabe et al., 1962[a], Meynell et al., 1968[a]).

Accordingly R^+ AB301 strains were repeatedly cultivated (to ensure repressor synthesis) through 10 transfers in P.B. + selective

antibiotic. Log phase cultures of each of these R^+ strains and the controls AB301 R^- and another F^- strain were grown up in L broth (c.a. $1x10^9$ cells/ml) and MS2 added to each at a multiplicity of 10^{-5}. Incubation was for 10 min. at 37 C. The contents of each tube were rapidly filtered through millipore filters (Type HA, pore size 0.45 µm; contained in Microsyringe filter holder, Millipore Corp., XX 30 025 00), and the filtrate assayed for unadsorbed phage on the indicator E.coli Hfrl by the agar layer method. The extent of phage adsorbed was measured and expressed as:

$$\text{Percent of adsorbed phage} = \frac{\text{(phage input-phage unadsorbed)}}{\text{phage input}} \times 100$$

c. Lysis by/propagation of sex factor specific phages

Two classes of sex factors, F and I have been differentiated in the Enterobacteriaceae (Lawn et al., 1967). The F-type sex factors including those of the F factor (of E.coli) and several Rfi^+ factors, produce a sex pilus called F-type pilus which makes their hosts sensitive to F-specific phages (Lawn et al., 1967). These include the (1) isometric RNA phages: MS2, R17, QB, µ2,etc. and (2) filamentous DNA phages: M13, fd,etc. The I-type sex factors including those of Col Ib, Col E1a and several Rfi^- factors, make their hosts sensitive to I phages (of which only filamentous phages If1 and If2 are known). R factors can therefore be classified into F or I types depending on whether the hosts bearing them can be lysed by or propagate sex factor specific phages (Lawn et al., 1967). Since lysis can be observed using only derepressed (drd) plasmids (Datta et al., 1966),

Propagation is the method of choice in most cases (Meynell et al., 1968[a]).

Spot lysis

For spot lysis, log phase cultures of the strains were prepared in L broth. The culture (0.1 ml) was mixed with molten 2 ml soft agar which was overlaid on the L agar plates. After the top layer of soft agar had solidified, one drop of the phage preparation known to give confluent lysis (approximately $1x10^5$ p.f.u./ml), was deposited on the surface. Several drops could be deposited separately on the same plate if the host was to be tested for several phages. The drops were dried before incubating the plates at 37 C. Lysis was recorded after overnight incubation.

Propagation of IKe and If1

Log phase cultures of the test strains were grown in L broth or T.S.B. and infected with IKe or If1 at a multiplicity of approximately $1x10^{-4}$, followed by an overnight incubation at 37C without shaking. Isogenic R^- and RM98+ strains served as negative and positive controls for IKe respectively. In case of If1, the positive control was M533. The bacteria were removed from the mixture by centrifugation and the supernatents were assayed on suitable indicators (Der7A5 or 264A for IKe; M533 for If1). When the strain to be tested was colicinogenic, the supernatent was usually filtered before assay to avoid false positive results. Broth treated millipore filters (Type HA, pore size 0.45 μm) contained in microsyringe filter holders without prefilters were used for this purpose.

Propagation of QB, μ2 and M13

E.coli JE 2571 bearing each test R factor was used to determine if phages QB, μ2 and M13 could be propagated. The positive and negative controls were JE 2571/F and JE 2571 respectively. Three separate sets of cultures were inoculated with the three phages at a multiplicity of approximately $1x10^{-4}$. After 5 hours incubation at 37 C, bacteria-free supernatants were assayed on E.coli Hfr1.

MS2 propagation

The ability of strains bearing single test R factors to support MS2 propagation was studied in E.coli F^- hosts by a method similar to that described by Meynell and Datta (1966[a]). Isogenic R^-, and F^+ strains served as negative and positive controls respectively. The cultures were infected with MS2 at a multiplicity of $3x10^{-5}$ and following an 8 min incubation at 37 C, 2 ml of the mixture was treated with 2 ml of anti-MS2 antiserum known to neutralize the added phage in 10 min. After further incubation for 10 min at 37 C, the mixture was filtered through millipore filters and washed twice with 5 ml cold L broth to remove excess antiserum. Bacteria were eluted from the filter by resuspending in 4 ml L broth and incubated at 37 C. Phage assays were made at 0,2,4 hr. and after overnight incubation.

III. Bacteriophage restriction

Phage restriction by R factor bearing bacteria is a general phenomenon, independent of the fi property of R factors (Siccardi,

1966, Bannister & Glover, 1968). R factors can, therefore, be classified on the basis of extent and pattern of restriction of a number of test bacteriophages (Guinee and Willems, 1967, Bannister & Glover, 1968).

Strains of E.coli or S. typhimurium containing single R factors or other plasmids and isogenic controls lacking the plasmid were grown to log phase in L broth. A bacteriophage to be tested for restriction was plated (by the agar layer method) on isogenic R^+ and R^- strains. Restriction was measured as a decrease in efficiency of plating (e.o.p.) of a test bacteriophage on R^+ host as compared to the e.o.p. of the same phage preparation on R^- control. Where restriction was noted, plaque size and morphology were also often affected. Bacteriophages used in the restriction studies included T3, T7, ØII, BF23, λ, P1kc, P22 and SP6. Bacterial hosts for testing the restriction of phages T3, T7, ØII, BF23 or P1kc were AB712, JE2571 or 13-6a; for λ, X407; and for P22 and SP6, S. typhimurium LT2.

IV. Superinfection immunity (Entry exclusion and compatibility)

a. Entry exclusion

Bacterial cells carrying one fi type of R factor resist superinfection by another R factor of the same fi type (Mitsuhashi et al., 1962, Watanabe et al., 1964[a]). This is observed as a reduction in transfer frequency of a donated R factor to an R^+ recipient relative to the isogenic R^- recipient. The phenomenon has been referred

to as entry exclusion and has been used to establish the extent of relatedness between plasmids (Meynell et al., 1968[a], Novick, 1969).

Entry exclusion of a donated R factor (R1) by a resident R factor (R2) was studied in E.coli F^- crosses (with isogenic donors and isogenic recipients) set up as under:

$$R1^+F1^- \times R2^+F2^- \quad \ldots\ldots\ldots(1)$$

$$R1^+F1^- \times R^-F2^- \quad \ldots\ldots\ldots(2)$$

Conjugations were carried out as described earlier and the exconjugants were selected on minimal agar containing a donor resistance marker. If necessary, the presence of the resident R factor was checked by replication. Reduced transfer frequency of the donated R factor (R1) in cross (1) compared to that in cross (2) indicated entry exclusion of the donated factor by the resident (R2) factor.

b. Compatibility

Two R factors belonging to different fi types can coexist as two independent entitities inside the same bacterial cell (compatibility); while those of the same fi types do not (incompatibility), unless selective pressure is applied for both. In the latter situation, the 2 R factors may recombine and will then coexist without selective pressure (Mitsuhashi et al., 1962; Watanabe et al., 1964[a]; Meynell et al., 1968[a]).

To study compatibility between pairs of factors, strains infected with 2 R factors were constructed by conjugally infecting an R^+ recipient with another R factor. Presence of the 2 R factors was confirmed by replication. Doubly infected strains were then grown in antibiotic-free P.B. through 20 consecutive transfers.

These strains were checked to see (1) if they had sustained a loss of either or both R factors and (2) if both R factors coexisted, whether they had undergone recombination. Approximately 200 individual colonies of the doubly infected strains were gridded onto master plates and replicated to detect the resistance genes they carried.

Conjugation and transduction experiments were performed to investigate whether recombination had occurred between two coexisting R factors (Romero and Meynell, 1969, Mitsuhashi et al., 1962). Independent coexistence of two R factors was inferred (1) if segregation of the resistance traits carried by 2 R factors occurred in conjugal crosses and (2) if the resistance determinants carried by the 2 factors were not cotransduced by P1kc. Confirmatory tests wherever available were used to further characterize the segregants and transductants. The latter were also checked for their conjugal transferability.

V. Colicin production and colicin resistance

General techniques were the same as those described by Fredericq (1957). A strain to be tested for colicin production was stabbed in the middle of a P.A. plate. Following growth for 24-48 hrs, the culture was killed by chloroform vapour and overlaid by a known colicin sensitive indicator (E.coli Row), seeded in the molten soft agar. Zone of inhibition surrounding the stabbed culture, following incubation for 24 hrs at 37 C, indicated colicin production by the stabbed culture. Colicin resistance of a strain was studied by stabbing a known colicinogenic strain in a P.A. plate. Following the

necessary incubation and chloroform sterilization, the plate was overlaid by soft agar containing the strain to be tested for colicin resistance. Absence of inhibition zone around the stabbed culture was attributed to colicin resistance of the overlaid culture.

VI. Derepression

Soon after acquiring R factors, bacterial hosts transfer them with high frequency (HFT state); the rate of trasfer gradually decreases over a period of a few generations and remains low in established cultures (repressed state). This is attributed to the production of a cytoplasmic repressor by the R factors. Ultraviolet irradiation has been found to increase the donorability of R^+ strains. The phenomenon is referred to as derepression and the R factors obtained by this method as derepressed (drd) R factors (Meynell and Datta, 1967[a], Meynell et al., 1968[a]).

Derivatives of E.coli 13-6a containing single R factors were grown to log phase in P.B. Bacterial cells were then centrifuged and the pellet resuspended in unsupplemented minimal broth and plated (0.1 ml) on appropriately supplemented minimal medium. After the surface of the medium was reasonably dry, the plates were exposed to ultraviolet irradiation for 20 second at a distance of 54 cm from a Westinghouse sterilamp model G36T6H. Microclones arising on the plates after 16 hrs incubation at 37 C in the dark were replicated on lawns of E.coli AB712 spread on the supplemented minimal medium containing a selective antibiotic to select for ex-

conjugants. After 18-48 hrs incubation at 37 C, exconjugants arising on the plates were purified twice on their respective selective media and checked for their frequency of transfer by the tube conjugation method.

H. ISOLATION OF BACTERIOPHAGES FROM SEWAGE

I. Treatment of sewage

Unchlorinated sewage (2 liters) was obtained from the Green Creek Pollution Control Center, Ottawa, in a sterile flask. Calcium chloride (0.002 M) was added to it, followed by centrifugation at 3000 r.p.m. at 4 C, to remove the debris. The supernatant was filtered through broth-treated Whatman filter paper (No. 1) and centrifuged again at 35,000 g. for 3 hr at 5 C (L2 Beckman ultra-centrifuge, rotor 21). More than half of the supernatant was decanted from each tube; the lower portions were pooled and re-centrifuged at 120,000 g. for 4 hr at 5 C (rotor 50). The upper halves of the supernatants from each tube were again decanted, the lower halves were pooled and centrifuged at low speed (3000 r.p.m.) to remove residual bacteria and aggregated particles. The super-natant from this step was filtered through broth treated millipore filters and used as described below.

II. Isolation of donor-specific phages

Phages were isolated from sewage to eliminate wild type R factor donors used in the conjugal crosses. Two methods for phage isolation were employed (1) spot lysis (2) broth lysis.

Spot lysis

This was similar to the one described in section C II c ii above. The plates were seeded with wild type donor strain for which a lytic phage was to be isolated. A drop of the treated sewage was deposited on the solidified lawns. Following an incubation at 37 C, areas of lysis or individual plaques were isolated by means of a pasteur pipette, in 1-2 ml of L broth. This was chloroformed (to kill bacteria), aerated (to remove chloroform), and used for further cultivation of the phage.

Broth lysis

A culture of the desired bacterium was grown to log phase in L broth. Two or three drops of treated sewage were mixed with 1-2 ml of the broth culture followed by incubation at 37 C. Observations were made for clearing (lysis) at hourly intervals for 8 hr. The cultures not showing lysis were left for overnight, while those which showed lysis were chloroformed, aerated and used for further cultivation of the bacteriophage.

Both spot lysis and broth lysis were simultaneously used for each desired donor strain. Some strains that did not show lysis by the first method were lysed in broth and vice versa. The isolated phages are listed in section E II a.

III. Isolation of phage IKe

The method used was basically the same as that described for the isolation of I phages (Meynell and Lawn, 1968[b]). One ml of the overnight culture of S. typhimurium LT2 bearing the R factor

RM98 and 1 ml of treated sewage were mixed with 9 ml of oxoid nutrient broth. Following overnight incubation at 37 C, the mixture was centrifuged to remove bacteria and filtered through broth-treated millipore filters (type HAWP, pore size 0.45 µm). Nine 10-fold dilutions (10^{-1} - 10^{-9}) of the filtrate were made and assayed by spot lysis on each of four indicator strains: LT2, LT2 RM98^{+}, JE2571 and JE2571 RM98^{+}, using oxoid nutrient agar. After overnight incubation at 37 C all four indicators were examined for lysis. Only those plaques that were common to both the R^{+} hosts, but were absent from the respective R^{-} strains were of interest and were carefully picked into L broth.

I. STUDIES WITH IKe

I. Conditions for cultivation

Although IKe was isolated using oxoid medium, it was later successfully grown and assayed on L agar. Addition of $CaCl_2$ to the medium was not found necessary for phage growth and its presence did not affect its plaque forming ability. Small batches of IKe (5 or 10 ml) could be cultivated overnight in L broth by infecting E.coli RM98^{+} cultures at a multiplicity of 10^{-4}, which yielded about 10^{9} to 10^{11} p.f.u./ml. Several attempts to grow large batches of IKe by liquid cultivation were unsuccessful in yielding high-titer lysates. These included aeration, different multiplicities of infection and the use of nutrient as well as defined liquid media. For this reason IKe was grown by the confluent lysis (Agar layer

method) using L agar. Incubation was at 37 C. E.coli or Salmonella strains bearing RM98 were used as propagating as well as indicator strains. However, plaques were clearer and slightly more numerous when E.coli MA50 was the host strain. IKe preparations grown by the agar layer method yielded about 10^{12} p.f.u/ml.

II. Shape and size of the phage

a. Electron microscopy

Specimen grids were prepared by depositing a drop of IKe suspension (10^{10} - 10^{11} p.f.u/ml) made in 0.02% BSA on carbonized formvar-coated grids of 200 or 400 mesh. Staining was done by adding a drop of 2% potassium phosphotungstate (pH 7) for 30 sec. Excess of the mixture was removed by means of filter paper, the grids dried and examined in the Philips EM 300.

b. Photographic techniques

Electron micrographs were taken by 35mm camera or plate camera. The film was developed by D-19 developer and rapid fixer of Kodak. Enlargements from the negatives could be made on Kodabromide or polycontrast paper (Kodak) and developed using the usual photographic techniques.

c. Measurement of the particle

Length of the phage was calculated by measuring 102 individual particles with a Minerva curvimeter and dividing these lengths with the total magnification of the print (Negative mag. x print mag). The width was obtained by measuring several particles occuring in parallel array. This was then divided by the total print magnification to arrive at the actual diameter.

III. Effect of various physical and chemical agents

a. Chloroform

2-3 drops of chloroform were added to 2 ml of phage lysate ($5x10^9$ p.f.u/ml). The mixture was kept at 4 C and titered for p.f.u/ml after 1 hr, 3 hr and about 18 hr.

b. Heat

To study the kinetics of heat inactivation, IKe was added to L broth (to a final titer of $3x10^{10}$ p.f.u/ml) maintained at 82 C in a water bath. This was gently shaken at intervals and 0.5 ml samples were removed at 0, 10, 20, 30, 40, 50, 60, 70 and 80 min. All the samples were assayed to obtain the number of surviving phage particles (p.f.u/ml).

c. Freezing and thawing

A suspension of IKe in dilution buffer (pH 7) containing $4x10^4$ p.f.u/ml was frozen at -20 C. Repeated freezing and thawing were carried out 10 times and phage survivors assayed after each alternate thawing.

d. Drying

0.5 ml of a dilution buffer suspension of IKe ($4x10^4$ p.f.u/ml) was dried in a desiccator. After complete desiccation (5 days), it was resuspended in 0.5 ml dilution buffer and assayed for surviving phage particles.

e. Enzymes

i. Nagarse

Two test tubes containing equal volumes of dilution buffer,

to one of which 100 µg/ml of nagarse was added were maintained at 37 C in a water bath. IKe was added to both tubes at a final concentration of $6x10^7$ p.f.u/ml. At 5, 15, 30, 45 and 60 minutes, 0.3 ml samples were withdrawn and assayed for the phage survivors.

ii. Pronase

Four test tubes containing 0.01 M phosphate buffer alone or with 10, 100 and 1000 µg/ml pronase were maintained in a water bath at 37 C. The pronase less tube served as the control. IKe was added to each of them to a final concentration of $3x10^7$ p.f.u/ml. Samples were withdrawn at 2, 4 and 8 hr and assayed for the plaque forming particles.

iii. Trypsin

Two tubes, one containing Pucks' saline (control tube) and the other containing an equal volume of Pucks' saline + 0.25% trypsin were maintained at 37 C in a water bath. IKe was added ($3x10^7$ p.f.u/ml) and mixed well. Samples were removed after 2 and 4 hour and assayed for plaque forming units.

iv. RNAse

Presence of small amounts of RNAse (1-50 µg/ml) in the top agar layer is known to inhibit plaque formation by RNA phages (Zinder, 1963, Bradley, 1964). To study the effect of RNAse, a preparation of IKe, known to give confluent lysis (10^5 p.f.u/ml) was plated on an appropriate indicator by the agar layer method. Two to three drops of RNAse solutions (50 µg/ml and 500 µg/ml) were immediately deposited on the surface and plates incubated at 37 C

overnight. A control RNA phage MS2 was also treated in a similar way.

IV. Serological properties

a. Preparation of antiserum

Rabbits were inoculated through the marginal ear vein with 1 ml of an L broth suspension of purified IKe, containing 9×10^{11} p.f.u/ml. Injections were made each alternate day for a period of two weeks, separated by an interval of one week. The rabbits were bled by heart puncture; blood was collected in separate vials and individual antisera assayed. The antisera were then pooled, absorbed with the host bacterium (264A) and reassayed. Samples of normal sera, obtained from the blood withdrawn before phage inoculation were also pooled and absorbed in a similar way. Antiserum and normal serum were finally sterilized by filtration through millipore filters.

b. Determination of K-value

The method was similar to that described by Adams (1950). IKe antiserum was diluted in L broth to give five 10-fold dilutions (10^{-2} - 10^{-6}). These were distributed in 0.9 ml volumes in small tubes held at 37 C in a water bath. A purified preparation of IKe (0.1 ml of 2×10^{7} p.f.u/ml), in L broth, was added to the above tubes and shaken gently. 0.1 ml samples were withdrawn from each tube after 5, 10, 15, 20, 25 and 30 minutes and diluted in 9.9 ml L broth (to stop the antigen-antibody reaction) and phage assays

performed. Controls were set up in a similar way by treating IKe with (1) L broth alone and (2) normal serum. The K value was calculated as follows:

$$K = 2.3\ D/t \times \log P^o/P$$

P^o = phage assay at 0 time

P = phage assay at time t minutes

D = reciprocal of dilution factor of serum.

c. Cross reactions of IKe antiserum with other filamentous phages

Cross reaction of IKe antiserum with the phages M13 and If1 was performed as described above (see K-value determinations). The highest dilution (10^{-5}) of antiserum showing neutralization of IKe and three lower dilutions, 10^{-4}, 10^{-3} and 10^{-2}, were used for this purpose. Both phages were added to a final titer of 2×10^7 p.f.u/ml. The dilution 10^{-4} of anti-IKe antiserum was also tested against an input of M13 and If1 at 2×10^4 p.f.u/ml.

TABLE 4

R factors, their sources and other properties

R factor	Resistance pattern*	Level of Resistance (μg/ml)	Source
RM98	Ap Sm Tc	Ap 30, Sm 30, Tc 30	Salmonella typhimurium strains, Dept. of National Health and Welfare-Ottawa
RM227	Ap	Ap 5000	
RM413	Ap	Ap 5000	
RM414	Ap	Ap 20,000	
RM430	Ap	Ap 10,000	
KR61*	Ap Km Nm Sm Tc	Ap 800, Km 400, Nm 300, Tc 500, Sm 250	Aerobacter aerogenes strains, Ottawa General Hospital
KR36	Cm Sm Tc	Cm 250, Sm 100, Tc 300	
KR68	Cm Sm Tc	Cm 100, Sm 100, Tc 200	
KR78	Cm Sm Tc	Cm 100, Sm 200, Tc 200	
KR19	Cm Tc	Cm 100, Sm 50	

* KR61 segregated to yield two patterns; conjugal transferability was associated only with Km Nm Sm Tc.

* Ap = ampicillin, Cm = chloramphenicol, Km = kanamycin, Nm = neomycin, Sm = streptomycin, Tc = tetracycline.

PART THREE

RESULTS AND DISCUSSION

A. ISOLATION AND CHARACTERIZATION OF R FACTORS

R factors (Table 4) were isolated by conjugating wild type, antibiotic resistant strains to auxotrophic E.coli recipients that had been pre-tested for their sensitivity levels to ampicillin, chloramphenicol, kanamycin, neomycin, tetracycline and in some cases also for streptomycin. Since transfer of genetic material between 2 E.coli strains is highly suggestive of the presence of a sex factor in the donating strain (Watanabe, 1963, Novick, 1969); sex factor activity of the R factors was tested in conjugal crosses between R^+F^- donors and F^- recipients. All the R factors, except KR19, were found to be transferable; these were therefore inferred to have a sex factor (RTF). Transfer frequencies in conjugal crosses varied depending on the donating host and the recipient used. After being transferred to auxotrophic E.coli strains, the R factors were studied further for other plasmid-associated properties.

I. Curing and spontaneous segregation

Extra chromosomal elements, including R factors, may be lost spontaneously from the host cell because of some errors in replication or segregation (Novick, 1969). These losses (elimination or curing) can be increased by treating the host cells with certain physical and chemical agents such as acridine dyes and ethidium bromide

TABLE 5

Spontaneous segregation and curing of wild type RM98 (ApSmTc RTF^{+} IKe^{S}) in Escherichia coli

Host	Eliminating agent	Conc. of EBr or A.O. µg/ml of broth	No. of colonies tested	No. of colonies with lost marker(s)	Markers* lost	% Loss or curing
E.coli F^{-} (AB 712 or JE 2571)	---	---	150	1	Tc(Sm)RTF	0.7
	A.O.	5	150	---	---	0.0
	"	10	100	---	---	0.0
	"	15	100	---	---	0.0
	"	20	250	---	---	0.0
	"	25	100	---	---	0.0
	"	40	100	---	---	0.0
	U.V. to 1% survival and EBr	4	200	1	Complete loss	0.5
	"	40	200	---	---	0.0
E.coli F^{-}, rec^{-}, (AB 2463)	---	---	481	122.	Tc	10-55
	EBr	10	200	116	Tc	58
	"	15	200	128	Tc	64
	"	20	200	134	Tc	64
	"	25	200	58 1	Tc Complete loss	29 0.5
	"	30	200	56	Tc	19-37

* Tc=resistance to tetracycline, (Sm)=loss of plasmid borne streptomycin resistance was uncertain because of the high level chromosomal streptomycin resistance of the host strains, RTF= resistance transfer factor Complete loss= loss of Ap(ampicillin resistance), Tc, Sm, RTF and IKe^{S}(sensitivity to IKe)

(EBr) with or without prior ultraviolet irradiation (Mitsuhashi et al., 1961, Watanabe et al., 1961c, Bouanchaud et al., 1969, Iyer and Iyer, 1969). Since chromosomally integrated episomes are generally incurable (Hirota, 1960), the loss of a plasmid is considered to be a criterion for its extrachromosomal nature (Novick, 1969).

Spontaneous segregation and curing of the R factors

RM98, RM227, RM413, RM414, RM430, KR61 and KR36 were studied in E.coli F^- hosts. RM98 was additionally studied in E.coli F^- rec^- and S. typhimurium LT2. Treatment of E.coli strains bearing RM227, RM413, RM414, RM430 or KR36 with acridine orange (A.O.) (20 µg/ml) indicated that loss of resistance genes had not occurred. Spontaneous losses of resistance genes were also not observed when 150 colonies per strain were tested. Similarly, no spontaneous segregation or curing was observed for KR61 despite combining U.V. pretreatment with up to 300 µg/ml of EBr; 300 colonies were tested after the U.V. + dye treatment and 300 for spontaneous segregation. RM98 had a low frequency of segregation and curing in E.coli (Table 5) as well as in Salmonella hosts (Table 6). Tc marker appeared to be unstable in rec^- host (Table 5), although Ap was just as stable as in the other hosts and so were the RTF and sensitivity to IKe.

The extent of spontaneous loss or segregation is a property of a particular plasmid (Mitsuhashi et al., 1961). Sus-

TABLE 6

Spontaneous segregation and curing of wild type

RM98 (ApSmTc RTF^{+} IKe^{S}) in S. typhimurium LT2

Eliminating agent	Conc. of EBr µg/ml of the broth	No. of colonies tested	No. of colonies with lost markers	Marker(s) lost	Loss or curing %
—	—	400	2	Sm	0.5
U.V.(1% survival)	—	300	5	Sm	1.7
			1	Complete loss	0.3
U.V. and EBr	50	231	2	Sm	0.9
			2	Tc	0.9
U.V. and EBr	100	300	5	Sm	1.7
			1	Tc	0.3

TABLE 7

Effect of R factors on leu transfer from AB301 ± R to AB712 F^-,R^-

R factor	Frequency (per 100 donors) of leu transfer	Reduction in the transfer of leu (Fertility inhibition)
-- (Control)	50 - 70	
KR61	0.02 - 0.04	+
KR36	0.02 - 0.08	+
KR78	0.04	+
KR68	0.01	+
RM98	60	-
RM227	75	-
RM413	40	-
RM414	50	-
RM430	75	-

ceptibility to a curing agent also varies among plasmids; some plasmids are not cured by any of the curing agents used here. (Bouanchaud et al., 1969, Derylo et al., 1970). Since episomes integrated into the bacterial chromosome are generally resistant to curing (Hirota, 1960), the possibility that the R factors owed their stability to chromosomal integration, could not be completely eliminated. However, cotransfer of chromosomal markers with resistance genes, in routine conjugation experiments and P1kc transductions was never detected.

Further characterization of the R factors included their classification into fi groups, studies on their phage restriction ability and their colicinogeny and colicin resistance etc.

II. Grouping into fi type

a. Fertility inhibition

When present in male strains of E.coli, some R factors inhibit or considerably reduce F-associated fertility while others do not; the former comprise the Rfi^+ (fertility inhibition positive) and the latter are Rfi^- type of factors (Watanabe et al., 1964[a]).

Only those R factors which had their own RTF were studied to see if they showed inhibition of F associated fertility (Table 7). For this purpose AB301 Hfr R^+ x AB712 F^-R^- crosses were set up. Frequency of leu (an early chromosomal marker) transfer by AB301 was found to be drastically reduced by each of the R factors KR61, KR36, KR78 and KR68. The other R factors had no effect on

TABLE 8

Effect of R factors on MS2 adsorption* by E.coli AB301 (HfrH) ± R factors

R Factor	MS2 (p.f.u/ml) recovered in supernatent	% MS2adsorption	Inhibition of MS2 adsorption
—	8×10^2	92	
# 13-6a F^-,R^-	1×10^4	0.0	
KR78	1×10^4	0.0	+
KR68	1×10^4	0.0	+
KR36	1×10^4	0.0	+
KR61	1×10^4	0.0	+
RM414	1.2×10^3	88	-
RM227	1×10^3	90	-
RM413	6.7×10^2	93.3	-
RM430	9.4×10^2	90	-
RM98	3.8×10^2	96	-

* phage input was 1×10^4 p.f.u/ml.

\# E.coli 13-6a served as a negative control.

this property. Hence KR61, KR36, KR78 and KR68 were designated as fi^+ while RM98, RM227, RM413, RM414 and RM430 as fi^- R factors.

b. Inhibition of MS2 adsorption

F^+ or Hfr strains of E.coli bearing Rfi^+ but not Rfi^- factors become insensitive to F specific phages (Watanabe et al., 1962[a]) due to the repression of F pilus synthesis among other F-associated properties which become coordinately repressed (Datta et al., 1966). It was of interest to examine whether or not F-specific phage adsorption by AB301 was inhibited due to the presence of the indicated R factors (Table 8). AB301 strains separately bearing each of the R factors KR61, KR36, KR78 and KR68; as well as those bearing RM98, RM227, RM413, RM414 and RM430 were tested for their ability to adsorb the isometric phage MS2. Isometric phages are well suited for these studies, since their adsorption rate is faster. More than 90% of input phage can be adsorbed in 5 minutes at 37 C (Davis and Sinsheimer, 1963).

As expected, all the R factors designated fi^+ (KR61, KR36, KR78 and KR68) by the criterion of fertility inhibition, also inhibited MS2 adsorption by AB301 (Table 8) while those designated as fi^- (RM98, RM227, RM413, RM414 and RM430) did not.

c. Adsorption, propagation of or lysis by sex factor specific phages

Another property of the Rfi^+ factors is to confer, on their E.coli F^- hosts, the ability to synthesize F type pili which can be detected directly by adsorption of F-specific phages (Lawn, 1966,

Datta et al., 1966, Nishimura et al., 1967, Lawn et al., 1967) or indirectly by (1) propagation of F-specific phages by the R^+ host (Meynell and Datta, 1966[a]) and (2) visible lysis of the host by these phages. Visible lysis is not usually observed when the R factors are in their repressed state (Datta et al., 1966, Meynell et al., 1968[a]).

E.coli F^- strains bearing each of the R factors: RM98, RM227, RM413, RM414, RM430, KR61, KR36, KR78 and KR68 were tested for adsorption of MS2 and spot lysis by F-specific phages: MS2, R17, QB, μ2 and M13. Although strains bearing Rfi^- factors did not adsorb MS2 and were not lysed by any of the male-specific phages as expected (thus proving that they were indeed Rfi^-, not naturally drd Rfi^+ factors), those bearing Rfi^+ factors also failed to adsorb MS2 and resisted lysis by F-specific phages. This could be due to repressed state of Rfi^+ factors or because they produced pili which had no affinity for F-specific phages. To examine the first possibility, propagation of F-specific phages was examined.

Propagation of MS2 (F-specific phage) has been demonstrated in E.coli $F^-R^+fi^+$ strains (Meynell and Datta, 1966[a]) and can also be observed when the R factors are in their repressed state (Meynell et al., 1968[a]). Also, the large burst sizes of RNA phages (Davis and Sinsheimer, 1963, Zinder, 1965) should allow one to detect the presence of a small number of phage propagating cells. However, none of the above R factors (fi^+ or fi^-) showed propagation of QB, μ2 or M13 in E.coli JE2571; the control strain JE2571 F^+ propagated all

three phages. Similar results were obtained with MS2 when the host was AB712. These findings although confirmed the original observations regarding the fi^- nature of RM98, RM413, RM414 RM227 and RM430 did not provide any additional information on KR61, KR36, KR78 and KR68, all of which were fi^+ . The hosts bearing these R factors may synthesize pili unable to adsorb male specific phages. There is at least one report in the literature (Lawn et al., 1967) on the exceptional Rfi^+ factors which did not render their E.coli F^- hosts sensitive to the male specific phages.

RM98, RM227, RM413, RM414 and RM430 recognized so far as belonging to fi^- group on the basis of negative criteria (1) absence of fertility inhibition and absence of inhibition of male phage adsorption by their male E.coli hosts and (2) unability to confer the property of male phage propagation, on their E.coli F^- hosts, were further characterized on the basis of some positive criteria. One of the properties of most Rfi^- factors is to confer on their E.coli F^- hosts, the ability to synthesize I type pili(Lawn et al.,1967). These pili, like F type pili can be detected either directly by the adsorption of I specific phage, or indirectly by (1) lysis of the Rfi^- factor bearing E.coli or S. typhimurium

R205	E.coli AB712	5×10^4	4×10^4	7×10^4	1×10^{10}	-	+
R199	E.coli J5			7×10^4	3×10^9	-*	+
N3	E.coli J5	5×10^4	6×10^4	4×10^4	1×10^7	-	+
Colicin	E.coli E2C	1×10^4	9.5×10^3	7×10^4	4×10^8	-	+
Colicin	E.coli E10A	1×10^4	1×10^3	7×10^4	2×10^7	-	+
Colicin	E.coli E193B	1×10^4	1×10^4	7×10^4	5×10^6	-	+
Col E1-30	E.coli K30(TR15)	1×10^4	2×10^3	7×10^4	5×10^4	-	-
R300	E.coli J5			7×10^4	1×10^5	-*	-
R310	E.coli J5			7×10^4	2×10^5	-*	-
R305	E.coli J5			7×10^4	1×10^5	-*	-
RP4	E.coli J5			7×10^4	1×10^5	-*	-
R61 (KmNmSmTc segregant)	E.coli AB712			4×10^4	1×10^4		-
R36	E.coli AB712			4×10^4	1×10^4		-
R78	E.coli AB712			4×10^4	1×10^4		-
R68	E.coli AB712			4×10^4	1×10^4		-
Colicins	E.coli E47, E66 and E92			7×10^4	1×10^4	?	-
Sex factors	E.coli E108B, E178, E160A, E194B, E147B, E181, E258, E251C, E2B, E107A, E136B, E84B, E8C, E238A, E11, E10C			7×10^4	$1\text{-}5 \times 10^4$	?	-

* Data from Nature, 216: 343-346 (1967)

TABLE 9

Plasmid specific propagation of phages IKe and If1 by E.coli or S. typhimurium hosts

Plasmid	Host strain and strain No.:	If1 Input	If1 Recovery	IKe Input	IKe Recovery	Propagation If1	Propagation IKe
F	E.coli AB712 (F1 from KLF1)			4×10^4	8×10^3	-	-
Col Ib-P9	S. typhimurium	1×10^4	1×10^9	7×10^4	1×10^4	+	-
Col E1a	S. typhimurium M533	2×10^3	5×10^{10}	7×10^4	5×10^4	+	-
Col Ia	E.coli CA53	1×10^4	1×10^5	7×10^4	1×10^4	±	-
R64-11	E.coli AB712	1×10^4	5×10^7	4×10^4	4×10^3	+	-
R144-3	E.coli J5	1×10^4	1×10^8	7×10^4	5×10^4	+	-
RM227	E.coli AB712	1×10^4	9×10^5	4×10^4	2×10^7	+	+
RM413	E.coli AB712	1×10^4	9×10^5	4×10^4	2×10^7	+	+
RM414	E.coli AB712	1×10^4	9×10^5	4×10^4	1×10^7	+	+
RM430	E.coli AB712	1×10^4	9×10^5	4×10^4	6×10^6	+	+
RM98	E.coli AB712	1×10^4	8×10^3	4×10^4	3×10^{11}	-	+
R45	E.coli J5			7×10^4	1.5×10^{11}	-*	+
R46	E.coli J5			7×10^4	1.5×10^{11}	-*	+
R48	E.coli AB712	5×10^4	6.5×10^4	7×10^4	1.5×10^{10}	-	+

strain or (2) propagation by the host strain, of I-specific phage (Lawn et al., 1967).

The I-specific phages isolated so far, include If1 and If2 both of which are filamentous (Meynell and Lawn, 1968[b]). Adsorption of filamentous phages in relatively difficult to demonstrate, but lysis by or propagation of such phages can be demonstrated using host strains bearing some Rfi⁻ factors e.g. R64-11 (Lawn et al., 1967). None of the E.coli F⁻ strains bearing RM98, RM227, RM413, RM414 or RM430 showed lysis by If1. Only those bearing the last four factors propagated this phage. Whereas these findings indicated that RM227, RM413, RM414 and RM430 probably shared similarities with R64-11 (an R factor with I like sex factor), RM98 possessed a different type of sex factor.

Phage IKe, which was specific for RM98 and isolated on strains bearing it (section D), was tested against the strains bearing RM227, RM413, RM414 or RM430. A control strain bearing R64-11 was also included. While none of these strains was lysed by IKe, only those bearing RM227, RM413, RM414 and RM430 showed its propagation. Further studies with the propagation of If1 and IKe, using strains bearing R factors (and other plasmids) from various sources indicated that three divisions or groups of Rfi⁻ factors (and other plasmids) could be recognized (Table 9):

(1) Rfi⁻ factors propagating If1 alone (or the I sex factor of Lawn et al., 1967).

(2) Rfi⁻ factors propagating IKe alone

(3) Rfi⁻ factors propagating both IKe and If1.

Group (1) has already been described by Lawn et al., (1967) and is represented by the Rfi⁻ factors (and other plasmids) with I-like sex factors (e.g. R34-11, R144-3, Col Ib, Col E1-a etc.). Group (2) is represented by RM98 and other R factors or plasmids propagating only IKe (Table 9). Some R factors (R46, R45 and R199) belonging to this group were previously described as unclassified R factors (Lawn et al., 1967) and the absence of If1 propagation by their hosts was attributed to the highly repressed state of these R factors (Lawn et al., 1967, Datta, personal communication). Group (3) is represented by only four Rfi⁻ factors, RM227, RM413, RM414 and RM430. These R factors may either possess a kind of sex factor intermediate between groups (1) and (2) or probably the strains carrying these R factors harbour more than one plasmid having different phage specificities.

The detection of groups (2) and (3) was made possible because of the isolation of phage IKe. Whether or not IKe infects its hosts following adsorption to specific pili in the manner most filamentous phages are known to adsorb is not yet known; but if it does, then hosts of RM98 (and of other related plasmids) must synthesize pili different from I pili. By the same criterion, hosts bearing R factors of group (3), should these be one composite R factors, may synthesize pili of intermediate kind between I and IKe-specific pili, with an affinity for both phages.

It is interesting that R factors belonging to group (2) either confer on their host strain (a) the ability of visible IKe

lysis as well as IKe propagation, or (b) the ability of IKe propagation alone. The former include RM98, R45, R46, R48, R205 and R199; the latter N3, Col E2C, Col E10A and Col E193B. Whether the difference observed is due to the extent of R factor (or plasmid) repression is not clear at the moment. As far as RM98 is concerned, all its hosts (*E.coli* strains, F^-, F^+ or Hfr; and *S. typhimurium* strains) showed visible lysis with the phage IKe and no difference was observed by the extent of repression of the R factor. One may argue that RM98 was a naturally derepressed R factor; this seems unlikely in view of the HFT state often observed with newly infected hosts (Section A V). Visible IKe lysis, exhibited by RM98-bearing strains may also be due to the relative genetical stability of RM98 (Section A I) and its presence in most cells of a population.

The next question to ask was whether IKe sensitivity was associated with the sex factor of RM98; only then, IKe could be a sex factor specific phage and could be used for the classification of sex factors. For this purpose genetical analysis of RM98 was carried out and the results are presented in section C.

III. Bacteriophage restriction

Restriction of phages is a property found associated with some R factors, and is independent of their fi type (Siccardi, 1966; Bannister and Glover, 1968). Plasmids other than R factors are also found to specifically restrict certain phages. Examples are restriction of phages T3, T7 and ØII etc. by F^+ *E.coli* hosts

(Schell et al., 1963, Makela et al., 1964, Linial and Malamy, 1970) and restriction of BF23 by strains bearing Colicin Ib or Colicin E2 (Strobel and Nomura, 1966). Specific pattern and extent of phage restriction exhibited by R factor-bearing strains can be used to classify R factors (and other plasmid) into separate groups (Guinee and Willem, 1967, Bannister and Glover, 1968). A relation between R factors and other plasmids is therefore reflected by a similarity of phage restriction pattern and could perhaps indicate a recombination event between the R factor and that particular plasmid (Siccardi, 1966).

To characterize the R factors on the basis of their phage restriction patterns, each of the R factors: RM98, RM227, RM413, RM414, RM430, KR61, KR78, KR36 and KR68, was tested in E.coli hosts against phages ØII, T3, T7, BF23, P1kc and λ. None of the R factors was found to restrict any phages. The first six R factors were also studied in S. typhimurium LT2 for the restriction of phages SP6 and P22 and to observe changes, if any, in the phage type of the host. None of the R factors altered the phage type of S. typhimurium LT2 (Khakhria, personal communication) or restricted the phages SP6 and P22. R64-11 (which has an I-like sex factor) was found to restrict phages T3, T7, ØII and BF23 (as indicated by a decreased e.o.p. and/or reduced plaque size compared to R^- controls) in E.coli hosts; and phages P22 and SP6 in Salmonella host. When present in S. typhimurium LT2, R64-11 also altered its phage type from 4 to 6 (Khakhria, personal communication).

E.coli strains bearing the Rfi⁻ factors R46, R45, R48 and R205 (related to RM98 on the basis of IKe propagation) did not restrict phages T3, T7 and ØII, whereas strain bearing R199 was found to restrict ØII.

From these findings therefore RM98, RM227, RM413, RM414, RM430, KR61, KR36, KR78 and KR68 appear to belong to a large group of R factors, which remained unclassified because of the lack of phage restriction (Bannister and Glover, 1968). Additionally, restriction of ØII by R199 compared to the lack of restriction by RM98, R45, R48, R46 and R205 indicates that IKe specific Rfi⁻ factors may show differences in their patterns of phage restriction.

IV. Colicin production and Colicin resistance

R factor bearing strains may occasionally exhibit colicin production or colicin resistance (Siccardi, 1966, Novakova *et al.*, 1969). Colicin production by R^+ strains may result from the coexistence of a Col factor or probably by association of a Col determinant(s) with the R factor. Colicin resistance of a strain is due to (1) mutation in a chromosomal gene (2) colicin production or (3) R factor borne colicin resistance (Siccardi, 1966). In the absence of (1) and (2), colicin resistance of an R^+ strain is likely to be associated with the R factor.

E.coli (AB712) bearing each of the R factors: RM98, RM227, RM414, RM413 and RM430 was tested for colicinogeny and resistance to Col Ib-P9. None of the R factors conferred colicinogeny (tested against a colicin sensitive indicator Row) on AB712. The hosts

bearing first three R factors were lysed by Col Ib-P9 to the same extent as the R^- control and hence have no resistance to Col Ib-P9. AB712 strains bearing RM413 and RM430 exhibited reduced zones of lysis by colicin Ib-P9 as compared to the control AB712 R^-. Host bearing RM98 was also tested for, and found to be sensitive to colicins E1-30 and E2.

These findings indicated that although none of the R factors conferred colicinogeny on their E.coli host, RM413 and RM430 conferred a low degree of resistance to Col Ib-P9. The latter could also be due to the coexistance of another plasmid (without recognizable markers) in the hosts carrying RM413 and RM430.

RM98 does not appear to carry determinants for the Salmonellin like substance (Atkinson, 1966), because 3 Salmonella cultures examined were not lysed by AB712 + RM98.

V. Effect of derepression

Bacterial strains freshly infected with an R factor transfer it with high frequency (HFT state) in conjugal crosses, exhibit synthesis of specific pili and show visible lysis with sex factor specific phages (Meynell et al., 1968[a]). After several growth cycles, the transfer frequency of the R factor is decreased (repressed state) with a concomittant loss or reduction of the other R factor associated properties (Meynell et al., 1968[a]). The repressed state is presumably because of the synthesis, by the R factor, of a cytoplasmic repressor. Synthesis of the latter can be prevented by U.V. irradiation(derepression)of the host strain

TABLE 10

Transfer frequencies± of R factors in repressed, derepressed and HFT states

Exp.*	R factor	Transfer frequency in repressed state	derepressed state	HFT state
1	RM98	$5 \times 10^{-2} - 3 \times 10^{-1}$	$5 \times 10^{0} - 1 \times 10^{1}$	$1 \times 10^{1} - 1 \times 10^{2}$
2	RM430	3×10^{-1}	3×10^{-1}	
3	RM227	1×10^{-1}	1×10^{1}	$5 \times 10^{0} - 1 \times 10^{1}$
4	RM413	1×10^{-1}	1×10^{1}	$6 \times 10^{0} - 1 \times 10^{1}$
5	RM414	4×10^{-2}	3×10^{-1}	
6	KR61	6×10^{0}	1×10^{1}	1×10^{1}
7	KR36	1×10^{-1}	5×10^{-1}	3×10^{-1}
8	KR78	2×10^{-1}	5×10^{-1}	2×10^{-1}
9	KR68	4×10^{-2}	4×10^{-1}	2×10^{-1}

± Expressed as per hundred donors; AB712 and 13-6a were the donor and recipient strains respectively in all crosses

* Selection for exp. 1, 2, 3, 4, 5 was Ap 30 µg/ml, that for no. 6, Km 30 µg/ml and for exp. no. 7, 8 and 9, Tc 30 µg/ml.

(Meynell and Datta, 1967[a]). Such derepressed R factors exhibit high transfer frequencies and make their hosts sensitive to visible lysis by R factor-specific phages.

E.coli strains with the indicated R factors (Table 10) were treated with ultraviolet irradiation to examine whether (1) transfer frequencies of the R factors were increased and (2) the Rfi^+ bearing hosts were made sensitive to male specific phages. It will be noticed that transfer frequencies of some R factors were either not altered or only slightly affected, whereas the others did show increased transfer frequencies. However, none of the strains bearing Rfi^+ factors KR61, KR36, KR78 or KR68 was made sensitive to MS2 by this treatment. Further irradiation with ultraviolet light was found to decrease, instead of increasing their transfer frequencies. Similarly the transfer frequency of RM430 was also decreased by further irradiation.

RM98 showed increased transfer frequency on derepression. In the HFT state the R factor was occasionally transferred with 100% frequency. Transfer frequencies varied with different donor and recipient combinations used. E.coli strains bearing RM98 in either repressed, derepressed or HFT state showed visible lysis with the phage IKe. Quantitative differences in the e.o.p. of IKe relative to the state of R factor were not examined. From these findings it appears that RM98 had either not reached the highly repressed state where visible lysis was not obtained or IKe

lysis was independent of the state of the R factor. Other Rfi$^-$ factors RM227, RM413 and RM414 did not show visible IKe lysis in either repressed or derepressed state.

B. INTERACTIONS WITHIN THE fi$^-$ GROUP OF R FACTORS

Although different types of R factors (fi$^+$ and fi$^-$) can coexist (without recombination) in a cell, the presence of a particular type of R factor confers on that cell an ability to resist infection by another R factor of the same fi type. This 'immunity' can operate both at the entry level (entry exclusion) or on events subsequent to entry, such as replication, resulting in the ultimate loss of the superinfecting or resident R factor (incompatibility). When this loss is prevented by applying selective pressure (addition of appropriate antibiotics to the growth medium), the superinfecting R factor may acquire stability through recombination with the resident R factor (Mitsuhashi et al., 1962, Watanabe et al., 1964[a]).

A recent report on the stable coexistence of three Rfi$^-$ factors (Bouanchaud and Chabbert, 1969) indicated that some Rfi$^-$ factors can coexist together (without recombination) in the same cell. Heterogeneity within fi$^-$ group of R factors was also observed by differences in (1) phage propagation (Section A II c), (2) phage restriction (Section A III) and (3) sensitivity to Col Ib-P9 (Section A IV). To examine whether subdivisions within Rfi$^-$ factors inferred earlier on the basis of phage (IKe and Ifl) propagation could be supported by data from superinfection immunity experiments, studies were undertaken to demonstrate entry exclusion,

TABLE 11
Entry exclusion of R factors

Exp.*	Donor	Recipient	Frequency of R factor transfer‡
A	AB712 + R64-11	13-6a R^-	1.2×10^{-1}
		13-6a + RM227	1.2×10^{-1}
		13-6a + RM413	2.5×10^{-2}
		13-6a + RM414	2.0×10^{-3}
		13-6a + RM430	6.2×10^{-2}
B	13-6a + RM98	AB712 R^-	5.0×10^{-2}
		AB712 + R64-11	2.5×10^{-2}
C	AB712 + R48	13-6a R^-	2.5×10^{0}
		13-6a + RM98‡	1.2×10^{-2}
D	AB712 + R199	13-6a R^-	5.0×10^{0}
		13-6a + RM98‡	7.0×10^{-2}
E	AB712 + R205	13-6a R^-	8.0×10^{0}
		13-6a + RM98‡	1.0×10^{-1}
F	AB712 + R45	13-6a R^-	4.2×10^{0}
		13-6a + RM98‡	2.0×10^{-1}
G	AB712 + R46	13-6a R^-	1.2×10^{1}
		13-6a + RM98‡	4.0×10^{-1}
H	AB712 + RM98	13-6a R^-	3.5×10^{-1}
		13-6a + RM227	2.0×10^{-1}
		13-6a + RM413	3.5×10^{-1}
		13-6a + RM414	4.5×10^{-1}
		13-6a + RM430	4.5×10^{-1}

* Selection for experiments A,C,D,E,F,G and H was Tc (30 µg/ml); Selection for experiment B was Ap (30 µg/ml).

‡ Derivative of RM98 with the markers $ApSmRTF^{+}IKe^{S}$.

‡ Expressed as frequency per hundred donors and selected on a donor marker.

compatibility or recombination between pairs of Rfi$^-$ factors (Khatoon and Iyer, 1971).

I. Exclusion at the entry level

Frequencies of transfer of donor R factors (R64-11, RM98, R48, R199, R205, R45 or R46) to recipient strains bearing the indicated factors or lacking one were compared (Table 11). As indicated by the reduction in the frequency of donated R factor by the R$^+$ recipient compared to R$^-$ recipient, entry exclusion was observed between the pairs: 64-11:RM413, 64-11:RM414, 64-11:RM430 and all the R factor pairs of experiments C,D,E,F and G. No entry exclusion was found between 64-11:RM227 and the pairs of experiments B and H, (Table 11).

Lack of entry exclusion between RM98 and R64-11 (Table 11, Cross B) shows that the two R factors are unrelated thus confirming the inference drawn from phage (IKe and Ifl) propagation (Section A II c). Entry exclusion by RM98 of R48, R199, R205, R45 and R46 (Table 11, Crosses C,D,E,F and G) indicates that all these R factors are related by this criterion in addition to being able to propagate IKe. The third group of R factors which propagated both IKe and Ifl (Section A II c), appears to be heterogenous by the criterion of entry exclusion. RM413, RM430 and RM414 appear to be related to R64-11 (an R factor with I like sex factor), whereas RM227 represents a different type.

II. Compatibility

E.coli strains bearing pairs of R factors, obtained from

RM98; RM414	Ap(l)SmTc Ap(h)	Ap	Ap(h)Tc
		Tc	Ap(h)Tc; Tc
		Ap⁂+Tc	Ap(h)Tc
RM98; RM430	Ap(l)SmTc Ap(h)	Ap	Ap(l)Tc; Ap(l)
		Tc	Ap(l)Tc; Tc
		Ap⁂+Tc	n.o.

* Donor and recipient strains in all transductions were 13-6a or AB712.

\+ Ap and Tc were used at 30 µg/ml, unless otherwise specified.

‡ Plasmid-borne Sm resistance was masked by high-level chromosomal Sm resistance of both donor and recipient strains.

Ap(l) = 30 µg/ml; Ap(h) = 2000 µg/ml.

⁂ Ap was used at 2000 µg/ml.

Note: n.o., cotransduction of Ap and Tc not obtained.

TABLE 12

Transduction analyses for recombination between R factors

R factors in donor*	Resistance genes‡	Selection†	Resistance of transductants
R64-11; RM227	SmTc Ap(h)	Ap Tc Ap+Tc	Ap(h) Tc n.o.
R64-11; RM413	SmTc Ap(h)	Ap Tc Ap+Tc	Ap(h) Tc n.o.
R64-11; RM414	SmTc Ap(h)	Ap Tc Ap+Tc	Ap(h) Tc n.o.
R64-11; RM430	SmTc Ap(h)	Ap Tc Ap+Tc	Ap(h) Tc n.o.
R64-11; RM98	SmTc Ap(l) SmTc	Ap Tc Ap+Tc	Ap(l); Ap(l)Tc Tc; Ap(l)Tc Ap(l)Tc
RM98; RM227	Ap(l)SmTc Ap(h)	Ap Tc Ap* +Tc	Ap(l)Tc; Ap(h)Tc Ap(l)Tc; Ap(h)Tc; Tc Ap(h)Tc
RM98; RM413	Ap(l)SmTc Ap(h)	Ap Tc Ap*+Tc	Ap(l)Tc; Ap(h) Ap(l)Tc n.o.

cont. 2

crosses A,B and H (Table 11) failed to yield segregants on repeated transfers in antibiotic-free P.B. and were capable of stably maintaining both R factors. It was, therefore, of interest to find out if the stability of the coexisting factors was due to a recombination event between them or whether they existed independently of each other.

a. Transduction

En bloc transduction of antibiotic resistence or other markers by P1kc has been used as a means of inferring whether one is dealing with a single R factor or with multiple R factors harboured by a host strain. In the latter case segregation of resistance genes will occur (Mitsuhashi et al., 1962, Romero and Meynell, 1969, Watanabe et al., 1964[a]). Transduction analyses of the doubly-infected strains were performed; the data are presented in Table 12. Co-transduction was never observed between the first four pairs of R factors when either single resistance markers or a combination of two markers (borne by the 2 R factors) was selected for. This constituted clear evidence for the absence of recombination between any of these pairs of R factors (Mitsuhashi et al., 1962, Watanabe et al., 1964[a], Romero and Meynell, 1969). Doubly infected host bearing RM98 and R64-11 yielded transductants having resistance to Ap and Tc. This could be due to the transduction of RM98 alone or a recombination event between the two R factors. The latter possibility could be inferred if the transductants had the phage restriction property associated with R64-11. However, since P1kc-

TABLE 13

Conjugation analyses for recombination between R factors

Donor	R factors in donor	Resistance pattern*	Recipient	Selection‡	Resistance transferred	ØII restriction※
AB712	R64-11; RM98	SmTc Ap(l)SmTc	13-6a	Ap Tc Ap+Tc	Ap(l)Tc Ap(l)Tc Tc Ap(l)Tc	- - + -
AB712	R64-11; RM413	SmTc Ap(h)	13-6a	Ap Tc Ap+Tc	Ap(h) Tc Ap(h)Tc	- + +
AB712	R64-11; RM414	SmTc Ap(h)	13-6a	Ap Tc	Ap(h) Ap(h)Tc Tc Ap(h)Tc	- +
AB712	R64-11; RM430	SmTc Ap(h)	13-6a	Ap Tc	Ap(h) Ap(h)Tc Tc Ap(h)Tc	- +
13-6a	RM98 RM227	Ap(l)SmTc Ap(h)	AB712	Ap# Tc	Ap(h);Ap(h)Tc Ap(h)Tc	
13-6a	RM98 RM413	Ap(l)SmTc Ap(h)	AB712	Ap# Tc	Ap(h);Ap(h)Tc Ap(h)Tc;Ap(l)Tc	
13-6a	RM98 RM414	Ap(l)SmTc Ap(h)	AB712	Ap# Tc	Ap(h);Ap(h)Tc Ap(h)Tc	
AB712	RM98 RM430	Ap(l)SmTc Ap(h)	13-6a	Ap# Tc	Ap(h)Tc Ap(l)Tc	

* Ap(l) = 30 µg/ml; Ap(h) = 5000 ug/ml

\# Ap was used at 2000 µg/ml

‡ Ap and Tc were used at 30 µg/ml unless indicated otherwise

※ ØII restriction is R64-11 specific

specific restriction was operative and imposed a limitation on the interpretation this approach would have provided, this question was resolved by conjugation experiments.

Analyses of hosts bearing RM98 and the factors conferring high Ap resistance indicated that whereas RM227 and RM414 owed their stability to recombination with RM98; RM413 and RM430 co-existed stably without undergoing recombination.

Transductants arising from all the unrecombined pairs of R factors were transferable in subsequent conjugal crosses showing that the resistance genes were probably associated with their own sex factors. The sex factors of RM413 and RM227 in transductants, could be easily differentiated from that of R64-11 on the basis of their characteristic transfer frequencies; sex factors of RM414, RM430 and RM98 were differentiated (from that of R64-11) by the ability of their hosts to propagate IKe.

b. Conjugation

The presence of two or more R factors in a host has been inferred on the basis of segregation of donor markers yielding two or more distinct resistance patterns in conjugation experiments (Romero and Meynell, 1969).

Results of the conjugation analyses performed to support transduction analyses of doubly infected strains are presented in Table 13. The segregation of resistance markers and ØII restriction (a property associated with R64-11) indicated that recombination had not occurred between RM98 and R64-11. The pairs: R64-11:RM413,

R64-11:RM414, R64-11:RM430 and RM98:RM413 also showed occasional segregation of resistance markers borne by the respective parent R factors thus confirming the results obtained by transduction analysis. Data on the conjugation analyses of the pairs RM98: RM227, RM98:RM414 and RM98:RM430 were not conclusive.

Once again, the differences observed between RM98 and R64-11 (by phage propagation, Section A II c; and entry exclusion, Section B I) were confirmed by their compatibility within the same host.

RM413, RM414 and RM430 were found to be compatible with R64-11. This observation did not support the inference based on data from entry exclusion experiments that RM413, RM414 and RM430 were related to R64-11. Dissimilarity inferred between RM227 and R64-11 by the absence of entry exclusion was also confirmed by their compatibility with each other. Surprisingly, the R factors RM227 and RM414 which appeared to differ from RM98 (no entry exclusion was observed) underwent recombination with it. The data on superinfection immunity are summarized in Table 23.

These findings, therefore, indicate that I-specific and IKe-specific sex factors represented as groups (1) and (2) respectively on the basis of phage propagation (Section A II c), are totally distinct from each other (by all the criteria employed). On the contrary, group (3) R factors which mediate the propagation of IKe and Ifl by their host strains represent a heterogenous population of sex factors. Some members of this group share similarities

with the members of group (1) e.g. entry exclusion between pairs R64-11:RM413, R64-11 :RM430 and R64-11:RM414 (Table 23) , while others share similarities with members of group (2) e.g. recombination between pairs RM98:RM227 and RM98:RM414 (Table 23). Still other members share similarities with both groups (e.g. RM414 which excludes R64-11 and recombines with RM98, see above). Whether hosts bearing RM227, RM413, RM414 and RM430 carry a single sex factor is not yet known.

C. RM98

I. Genetical analysis

Linkage order of the genes on an R factor can be inferred by studying patterns and frequencies of the R factor-borne markers in transduction, conjugation and segregations (induced or spontaneous) (Mitsuhashi, 1971, Watanabe and Lyang, 1962[b], Watanabe, 1963). Transduction with the E.coli phage P1kc is important to demonstrate that all the markers in question are indeed on the same R factor and form a single linkage group, since this phage is usually known to transduce an R factor as a whole (Watanabe and Fukasawa, 1961[a], Watanabe et al., 1968). The Salmonella phages P22 and E[15] can transduce only segments of an R factor (Watanabe and Fukasawa, 1961[a], Harada et al., 1963) which often results in the segregation of resistance markers and the sex factor (RTF). Difference between transduction by P1kc and P22 is attributed to the amount of DNA which can be packaged by the two phages (Falkow et al., 1967) and is usually equivalent to the lengths of their genomes(P1kc=

TABLE 14

Transduction of RM98 by P1kc in Escherichia coli K-12

Exp. No.*	Selection#	No. of colonies examined	No. of colonies exhibiting a particular property						No. of transductants with a particular pattern±
			Ap^r	Sm^r	Tc^r	lac^+	RTF^+	IKe^S	
I	Ap	16	16	16	16	-	16	16	Ap Sm Tc RTF^+ IKe^S (16)
	Sm	10	10	10	10	-	10	10	Ap Sm Tc RTF^+ IKe^S (10)
	Tc	84	82	82	84	2	84	82	Ap Sm Tc RTF^+ IKe^S (82) Tc lac^+ RTF^+ IKe^r (2)
2	Ap	61	61	61	61	-	60	60	Ap(Sm)Tc RTF^+ IKe^S (60) Ap(Sm)Tc RTF^- IKe^r (1)
	Tc	104	104	104	104	-	104	104	Ap(Sm)Tc RTF^+ IKe^S (104)

* Exp. No: 1 : donor 13-6a, recipient MA50; Exp. No: 2 : donor and recipient AB712.

\# Selection was at the level of 30 µg/ml for each antibiotic.

± Ap, Sm, Tc = resistance to ampicillin, streptomycin and tetracycline respectively; lac^+ = lactose positive; RTF^+ = conjugally transferable; IKe^S = sensitive to the phage IKe.
(Sm) denotes chromosomally determined high level streptomycin resistance.
IKe^S was determined by spot lysis or propagation.
RTF was detected by replicating the transductants on recipient lawns spread on appropriate media.

mol wt $6x10^7$, P22= mol wt $3x10^7$).

A report by Drabble and Stocker(1968) on the P22 transduction of R factors:R46, R48, R45, and R205(or R Brighton, R Bradford, R Enfield and R Utrecht respectively) indicated that all resistance markers (ApSmTc or ApSmTcSu) of these R factors were cotransduced when Tc selection was used;10% of the transductants were also conjugally transferable(RTF^+). Similar findings were also reported by Watanabe et al. (1968) for some other R factors including N3. These findings were in contrast to the earlier reports on the P22 transduction of some R factors(Watanabe and Fukasawa 1961^a) which showed a segregation of resistance genes and RTF. No explanation of the above findings was available, although Drabble and Stocker assumed that P22 was able to transduce the entire R factor.

Genetical analysis of RM98 was undertaken to (1) locate the determinant for IKe sensitivity (2) examine the order of resistance genes and (3) to examine whether RM98 behaves like R46, R48, R45, R205 and N3 in P22 transductions, since it appears related to these R factors by some other criteria(IKe propagation and entry exclusion).

a. Transductions

i. P1kc transduction in E.coli

The results of P1kc transduction of RM98 are presented in Table 14. P1kc was found to transduce all five phenotypically recognizable markers (ApSmTc= resistance to ampicillin, streptomycin and tetracycline, RTF= resistance transfer factor, IKe^S= IKe sensitivity) associated with RM98 together, this being similar to observations following conjugal crosses. The markers ApSmTc RTF^+ IKe^S,

TABLE 15

Transduction of RM98 by P22 in Salmonella typhimurium LT2

Exp. No.*	Selection#	No. of colonies examined	No. of colonies exhibiting a particular property Ap^r	Sm^r	Tc^r	RTF^+	IKe^S	No. of transductants with a particular pattern*
1	Ap	150	150	(149)	3	145	145	Ap Sm RTF^+ IKe^S (143) Ap(Sm) RTF^- IKe^r (3) Ap(Sm)Tc RTF^- IKe^S (2) Ap Sm Tc RTF^+ IKe^S (1) Ap RTF^+ IKe^S (1)
	Tc	65	65	(65)	65	7	7	Ap(Sm)Tc RTF^- IKe^r (58) Ap Sm Tc RTF^+ IKe^S (7)
2	Ap	17	17	12	0.0	17	17	Ap Sm RTF^+ IKe^S (12) Ap RTF^+ IKe^S (5)
	Tc	89	89	86	89	43	43	Ap Sm Tc RTF^- IKe^r (44) Ap Sm Tc RTF^+ IKe^S (42) Ap Tc RTF^- IKe^r (2) Ap Tc RTF^+ IKe^S (1)
	Sm	7	7	7	0.0	7	7	Ap Sm RTF^+ IKe^S (7)

Exp. No: 1 : donor and recipient S. typhimurium LT2 (prototroph); Exp. No: 2: donor S. typhimurium LT2 (prototroph), recipient S. typhimurium trp A 512.

Selection was at the level of 30 µg/ml for each antibiotic.

* Ap, Sm, Tc = resistance to ampicillin, streptomycin and tetracycline respectively; RTF^+ = conjugally transferable; IKe^S = sensitive to the phage IKe.

Markers underlined were capable of being lost spontaneously or during transfer. Presence of the markers in brackets was uncertain due to the high level chromosomal resistance to streptomycin of the recipient.

therefore, comprised a single linkage group (Watanabe and Fukasawa, 1961[a], Watanabe et al., 1968). Chromosomal markers of the host strains were not found to be cotransduced along with the resistance markers in routine P1kc transductions. Out of a total of 110 transductants only 2 bore the pattern $Tclac^{+}RTF^{+}IKe^{r}$. Transduction frequencies on Ap and Sm selections were approximately $1x10^{-7}$, whereas that on Tc selection was $6x10^{-7}$, calculated on the basis of an input of 100 plaque forming units (p.f.u). Patterns $Tclac^{+}$ $RTF^{+}IKe^{r}$ and $Ap(Sm)TcRTF^{-}IKe^{r}$ constituted approximately 2% of the total transductants on Tc and Ap selections respectively. In subsequent separate conjugal crosses the transductant $Tclac^{+}RTF^{+}IKe^{r}$ was found to have a transfer frequency of $4.5x10^{-2}$ as compared to $5x10^{0}$ for RM98 and the P22 transductant $ApSmRTF^{+}IKe^{s}$. Entry exclusion was not observed between $Tclac^{+}RTF^{+}IKe^{r}$ and RM98. The lac^{+} character of the former segregated spontaneously or on repeated P1kc transduction. A small percentage of P1kc transductants inherited a mucoid character, which was also occasionally observed during conjugal transfers of RM98. This character was unstable and easily lost.

ii. P22 transduction in S. typhimurium

A total of seven different patterns were observed when Ap and Tc selections were used (see Table 15, fig. 1). Transduction frequencies were $2x10^{-9}$ with Ap and Sm selection and $5x10^{-10}$ with Tc selection, calculated on an input of 100 p.f.u. of P22. The major pattern on Ap selection was $ApSmRTF^{+}IKe^{s}$ which could segregate to yield $ApRTF^{+}IKe^{s}$ either spontaneously or through further transduction

by P1kc in E.coli. Transductant ApSmRTF$^+$IKe^s and RM98 had equal transfer frequencies in homologous conjugal crosses and exhibited entry exclusion for each other. As expected, ApSmRTF$^+$IKe^s did not show entry exclusion towards Tclac$^+$RTF$^+$IKe^r (P1kc transductant). Nontransferable transductants ApSmTcRTF$^-$IKe^r could be made conjugally transferable by infecting them with ApSmRTF$^+$IKe^s but not with Tclac$^+$ RTF$^+$IKe^r; the latter was also unable to mobilize ApSmRTF$^-$IKe^r. E.coli lac$^-$ strains often showed an unstable inheritance of lac$^+$ marker after being conjugally infected by the transductant patterns ApSmTcRTF$^+$IKe^s and ApTcRTF$^+$IKe^s. Such E.coli strains formed red sectored colonies on MacConkey's medium. Two or three further cultivations resulted in the disappearance of red sectors from the colonies. A small percentage of P22 transductants also showed mucoid colonies, irrespective of the selective marker used.

b. Segregations

The findings on the segregation of RM98 are presented under characterization of R factors (see curing and spontaneous segragation). In S. typhimurium LT2, spontaneous segregation of Sm was 0.5% (Table 6). The loss of Tc was found to be 0.3-0.9% under the action of ultraviolet and EBr. Complete loss of all the markers was 0.3-0.5% in Salmonella and E.coli hosts including E.coli rec$^-$ F$^-$ (Tables 5,6). The most usual segregation patterns of RM98 obtained in E.coli and Salmonella were ApSmRTF$^+$IKe^s and ApTcRTF$^+$IKe^s which correspond well to the patterns obtained in P22 transduction. Since pattern ApRTF$^+$IKe^s represented the smallest segment of RM98 (Table 15) it was used to study if

TABLE 16

Segregation of RM98 (Ap RTF$^+$ IKe^S)* in S. typhimurium LT2

Eliminating agent	No. of clonies tested	Pattern(s) obtained after treatment	No. of colonies with a particular pattern	Loss %
-----	300	Ap RTF$^+$ IKe^S	291	0.0
		complete loss	9	3
Ultra violet irradiation to 1% survival	260	Ap RTF$^+$ IKe^S	259	0.0
		complete loss	1	0.4

* ApRTF$^+$IKe^S was a P22 transductant of RM98.

segregation between Ap and RTF$^+$IKe^s occurred (Table 16). Under the conditions used, no segregation was observed; all the markers were lost together.

c. RM98 and the lac$^+$ property

E.coli lac$^-$ recipients, conjugally infected with RM98 donated by lac$^+$ or lac$^-$ E.coli hosts did not manifest any change in their lac$^-$ property, except for the presence of occasional lac$^+$ centres. Similarly transductants of P1kc (with the exception of Tclac$^+$RTF$^+$IKe^r) did not alter the lac$^-$ property of their E.coli hosts. On the other hand, transductants of P22 with the patterns ApSmTcRTF$^+$IKe^s and ApTcRTF$^+$IKe^s when conjugally transferred to E.coli lac$^-$ strains, conferred on them a partial lac$^+$ character. Such E.coli strains formed red sectored colonies (on MacConkey's medium), which rapidly segregated to yield normal lac$^-$ colonies without affecting the other markers. The lac$^+$ character associated with P1kc transductant Tclac$^+$RTF$^+$IKe^r was relatively stable. It segregated to give lac$^-$ colonies, with a low frequency and on prolonged cultivations or through subsequent P1kc transduction. Transferability of the Tc marker was not affected by the loss of lac$^+$ character. Three lac$^-$ segregants of Tclac$^+$RTF$^+$IKe^r were not found to transfer lac$^+$ character in conjugal crosses from E.coli donors that were Tclac$^-$RTF$^+$IKe^r/F$^-$lac$^+$ to R$^-$F$^-$lac$^-$ recipients, although Tc was transferred. This appeared to indicate an absence of gene pick up from lac region of the donor cell chromosome by Tclac$^-$RTF$^+$IKe^r.

Fig. 1. Structure of RM98

Patterns by P22 transduction

1. ApSmRTF^{+}IKe^{s}
2. ApRTF^{+}IKe^{s} (arises after loss of Sm from No. 1)
3. ApSmRTF^{-}IKe^{r}
4. ApSmTcRTF^{-}IKe^{r}
5. ApSmTcRTF^{+}IKe^{s}
6. ApTcRTF^{+}IKe^{s} (arises after loss of Sm from No. 5)
7. ApTcRTF^{-}IKe^{r} (arises after loss of Sm from No. 4)

Patterns by P1kc transduction

1. ApSmTcRTF^{+}IKe^{s}
2. Tclac^{+}IKe^{r}

Patterns by segregation

1. ApSmTcRTF^{+}IKe^{s}
2. ApTcRTF^{+}IKe^{s}

Pattern in conjugation

1. ApSmTcRTF^{+}IKe^{s}

Fig : 1

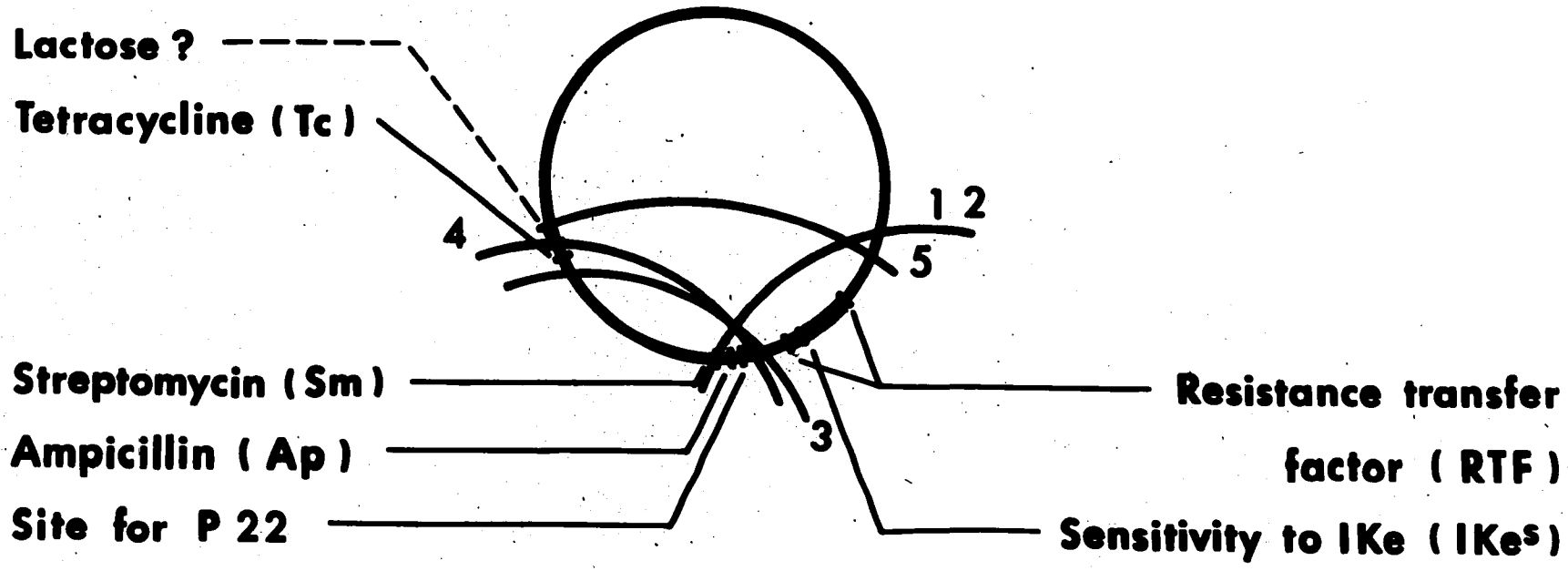
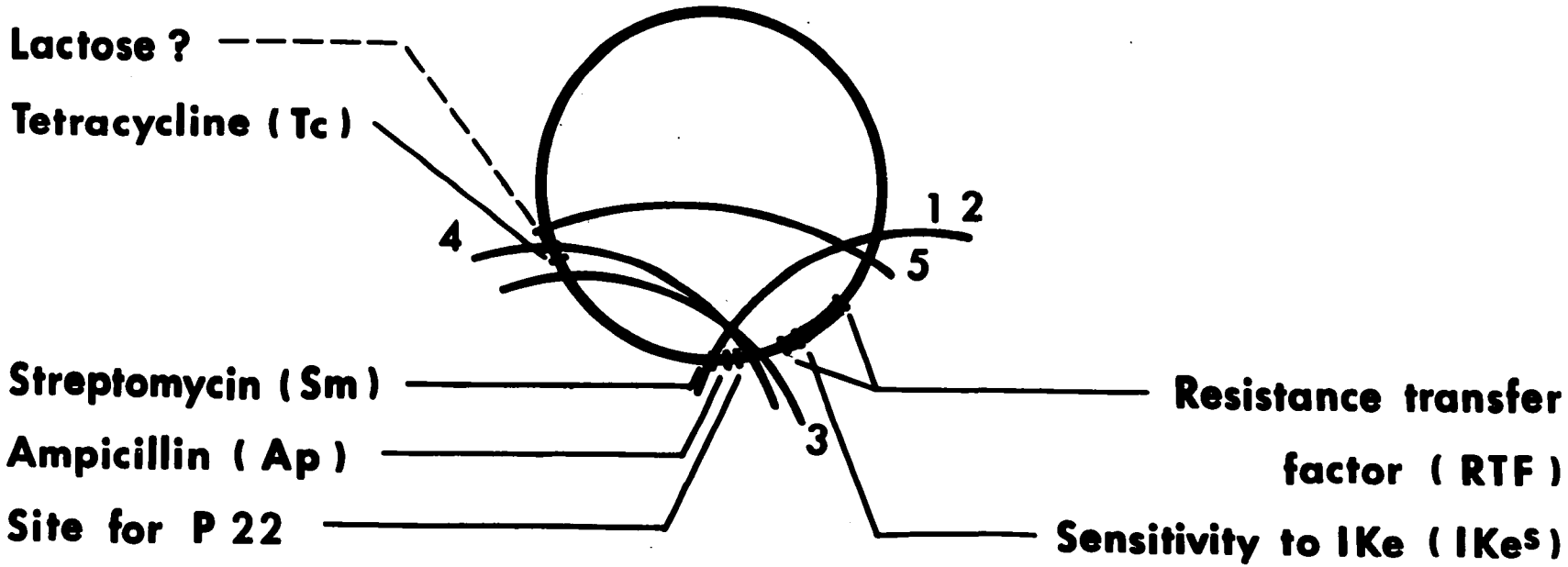

Lactose ?
Tetracycline (Tc)
Streptomycin (Sm)
Ampicillin (Ap)
Site for P 22
Resistance transfer factor (RTF)
Sensitivity to IKe (IKe^{s})
1 2
3
4
5

Fig : 1


Lactose ?
Tetracycline (Tc)
Streptomycin (Sm)
Ampicillin (Ap)
Site for P 22
Resistance transfer factor (RTF)
Sensitivity to IKe (IKe^{s})
1 2
4
5
3

d. Reconstitution of RM98

Patterns ApSmRTF^{+}IKe^{S} obtained by P22 transduction and Tclac^{+}RTF^{+}IKe^{r} (or Tclac^{-}RTF^{+}IKe^{r} segregant) obtained by P1kc transduction were used to reconstitute the original R factor (ApSmTcRTF^{+}IKe^{S}) by introducing them conjugally into the same E.coli host. Subsequent P1kc transduction indicated that the two plasmids had not undergone recombination in the doubly infected host. Ultraviolet irradiation of the doubly infected host, prior to making the P1kc lysate also indicated absence of recombination between the two transductant patterns. The two patterns ApSm RTF^{+}IKe^{S} and Tc(lac^{+} or lac^{-}) RTF^{+}IKe^{r} may, therefore, be either two different entities altogether or had suffered a loss of homologous regions, indispensable for recombination.

e. A tentative gene order for RM98

From the evidence presented above, a tentative gene order of the recognizable markers associated with RM98 can be deduced (fig. 1). The position of lactose remains unfixed. Almost every pattern arising through transduction, segregation and conjugation events can be explained by the suggested map. It will be noticed that whereas selection favours the appearance of definite transductant patterns with P22 as the transducing agent, the phage P1kc transduces all the five RM98 markers with the three selections used. The following observations may be made, on the basis of which the inference is drawn that P22 has an affinity for a specific site on the plasmid, which is most likely to be located between Ap and RTF.

(1) All the P22 transductants carry Ap resistance irrespective of the selection used.

(2) Frequency of RTF^+ transductants was 97% on Ap selection compared to 33% on Tc selection.

(3) Following patterns were never observed (on Sm or Tc selections): (a) $TcRTF^-IKe^r$

(b) $SmTcRTF^-IKe^r$

(c) $SmTcRTF^+IKe^s$

(d) $TcRTF^+IKe^s$

Results of the P22 transduction of RM98 are very similar to the findings of Drabble and Stocker with the R factors: R45, R46, R48 and R205 (1968). Out of the transductants tested, 67% were found to be RTF^+ and only 15% of them carried all the five markers ($ApSmTcRTF^+IKe^s$) compared to 99% of P1kc transductants. This implies that RM98 genome is probably longer than P22 genome. It is then of interest to ask why none of the patterns indicated in (3) above were obtained? One would expect at least the first two of these patterns if P22 transduced portions of the R factor without any affinity for a particular site. The fact that patterns listed in (3) were never observed and that the Ap locus was found associated with all the transductants irrespective of the selection used, strongly suggests that P22 has an affinity for a region on RM98, which appears to be a site situated between Ap and RTF. As indicated by (2) above, RTF appears to be much closer to Ap than Tc. From the spontaneous segregation of $ApSmRTF^+IKe^s$ to yield Ap

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$RTF^{+}IKe^{S}$, Ap appears to be located between Sm and RTF. This order of genes would explain the pattern $ApSmTcRTF^{-}IKe^{r}$.

The patterns $ApTcRTF^{+}IKe^{S}$ and $ApTcRTF^{-}IKe^{r}$ could have arisen from the patterns $ApSmTcRTF^{+}IKe^{S}$ and $ApSmTcRTF^{-}IKe^{r}$ respectively after the loss of Sm. Spontaneous segregation of RM98 in S. typhimurium (Table 6) indicated a loss of 0.5% for the Sm locus. Spontaneous segregation losses appear to be greater in transductants as indicated by the increased loss (from 0.5 to 3%) of $ApRTF^{+}IKe^{S}$ (Table 16).

IKe sensitivity seems to be closely associated with the RTF. All the P22 transductants inheriting the RTF were also sensitive to the phage IKe and conversely, those that were RTF^{-} were IKe resistant. A segregant of RM98 which had lost its transferability ($RTF^{+} \longrightarrow RTF^{-}$) was also found to have become IKe^{r} ($IKe^{S} \longrightarrow IKe^{r}$).

The pattern $Tclac^{+}RTF^{+}IKe^{r}$ obtained during P1kc transductions still remains unexplained. Two possibilities may be considered (a) it was a different plasmid altogether and existed as a covert factor with RM98 or (b) it was derived from RM98, but had lost some parts of the genome that were essential to recombination (see reconstitution of RM98) and such losses also rendered it IKe resistant as well as compatible with RM98. Reduced transfer frequency of $Tclac^{+}RTF^{+}IKe^{r}$ as compared to RM98 also supports the possibility (b). In addition, inheritance of lac^{+} character with

P22 tranductants ApSmTcRTF$^+$IKe^S and ApTcRTF$^+$IKe^S indicates the presence of lactose genes on RM98 itself; the genes may complement or be complemented by the lactose genes of the host strain. Further investigations of the lactose genes on RM98 (in hosts well defined with respect to the lactose locus) may throw some light on the origin of Tclac$^+$RTF$^+$IKe^S.

To summarize the findings, genetical analysis of RM98 indicates that (1) IKe sensitivity and RTF are closely associated, (2) markers Ap and Sm are situated close to each other and both of them are closer to RTFIKe^S than Tc is and (3) phage P22 probably has a special affinity for some site on the R factor, situated close to the Ap locus.

As indicated by P22 transductions, the linkage relationship between R determinants and RTF may be similar among RM98, R45, R46, R48, R205 and N3.

II. Interaction with the host chromosome

R factors resemble the fertility factor F of E.coli K-12 in conferring on their hosts the ability to donate the factor by conjugation to other bacteria (Watanabe, 1963). F factor when integrated into the chromosome (Hfr state) can bring about transfer of chromosomal markers in Hfr x F$^-$ E.coli crosses. Some R factors (both fi$^+$ and fi$^-$ type) can also bring about transfer of chromosomal markers in crosses of the type R$^+$F$^-$ x F$^-$ (Pearce and Meynell, 1968, Cook and Meynell, 1969). R64-11 and R144 belong to this class of R factors (Cooke and Meynell, 1969).

Resistance genes of R factor may sometime integrate into the bacterial chromosome without affecting its transfer (Harada

TABLE 17

Absence of transfer of chromosomal markers

in crosses of the type $RM98^{+}F^{-}$ x F^{-}

Donor	Recipient	Conjugation time	Selective marker
MA124 + RM98	X478	2 hr., 4 hr.	pro C, pur E, trp, lys
13-6a + RM98	MA50	2 hr., 4 hr.	cys C, lys
13-6a + RM98	AB1157	18 hr.	leu, lac, pro, arg E
MA50 + RM98	AB1157	2 hr., 4 hr.	pro, his, arg E
MA50 + RM98	AB2495	2 hr., 4 hr.	trp, thy
MA50 + RM98	AB2495	18 hr.	trp

et al., 1967, Iyobe et al., 1969, 1970). Since chromosomal transfer from Hfr strains is polarized and proceeds from a fixed origin, positions of such integrated genes can be mapped on the chromosome by interrupted matings of the type $HfrR^+ \times F^-$ in E.coli strains (Harada et al., 1967).

The R factor RM98 was studied in $F^-R^+ \times F^-R^-$ crosses to examine whether it can bring about transfer of chromosomal markers like some other R factors, including R64-11. In the crosses described (Table 17) recombinants for the following loci were never obtained leu, lac, proC, purE, trp, his, cysC, lysA, thy and argE. RM98, therefore, does not seem to affect the transfer of chromosomal markers in $F^-R^+ \times F^-R^-$ crosses and hence differs from those R factors which exhibit this property.

Since P22 transductants of RM98 conferred an unstable lac^+ character on the lac^- E.coli hosts and P1kc transductant $Tclac^+RTF^+IKe^r$ also exhibited lac^+ character (Section C I) it was of interest to find out whether RM98 was integrated on the E.coli chromosome near the lactose locus. For this purpose $HfrH\ RM98^+ \times F^-$ cross was performed between E.coli strains (Table 18). From the results of interrupted mating experiments, RM98 does not appear to have a stable integrated position near the lac locus. Although recombinants obtained on proC (a locus close to lac locus) showed 91% co-inheritence of lactose, only 1% co-inherited Ap and Tc (RM98 associated markers). Absence of co-

TABLE 18

Co-transfer of RM98 with chromosomal markers

in the cross AB301 (HfrH) $R98^+$ x X478 (F^-)

Mating interval (min.)	Selective marker	No. of colonies tested	Co-transferred markers Ap	Tc	lac	pro
10	leu	100	-	-	-	-
20	pro	100	1	1	91	100
30	pro	100	1	1	91	100
	pur E	100	-	-	72	

Fig. 2. IKe as seen under electron microscope (EM300).

Fig. 3. Distribution of lengths of IKe particles.

Fig.2

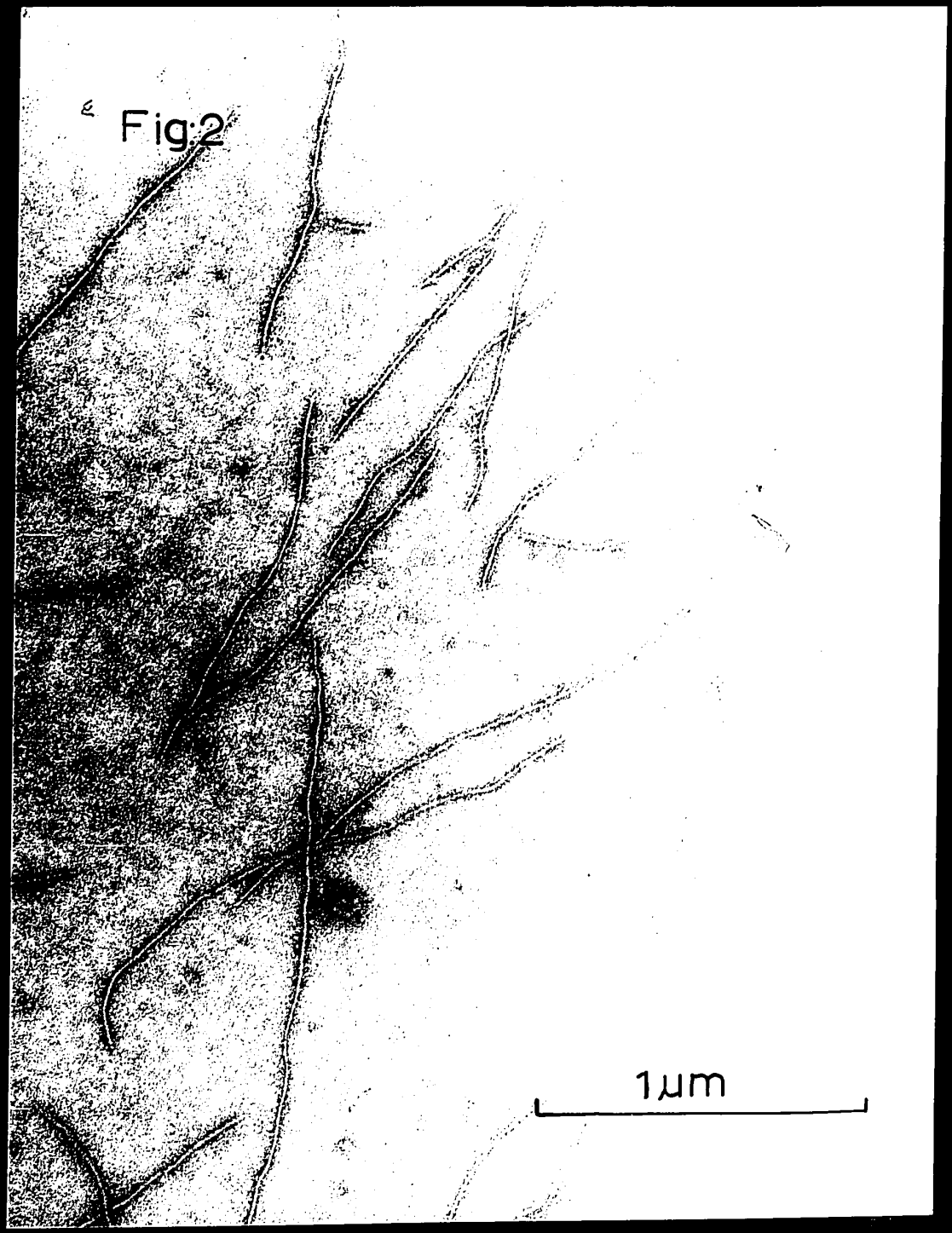


Fig : 3

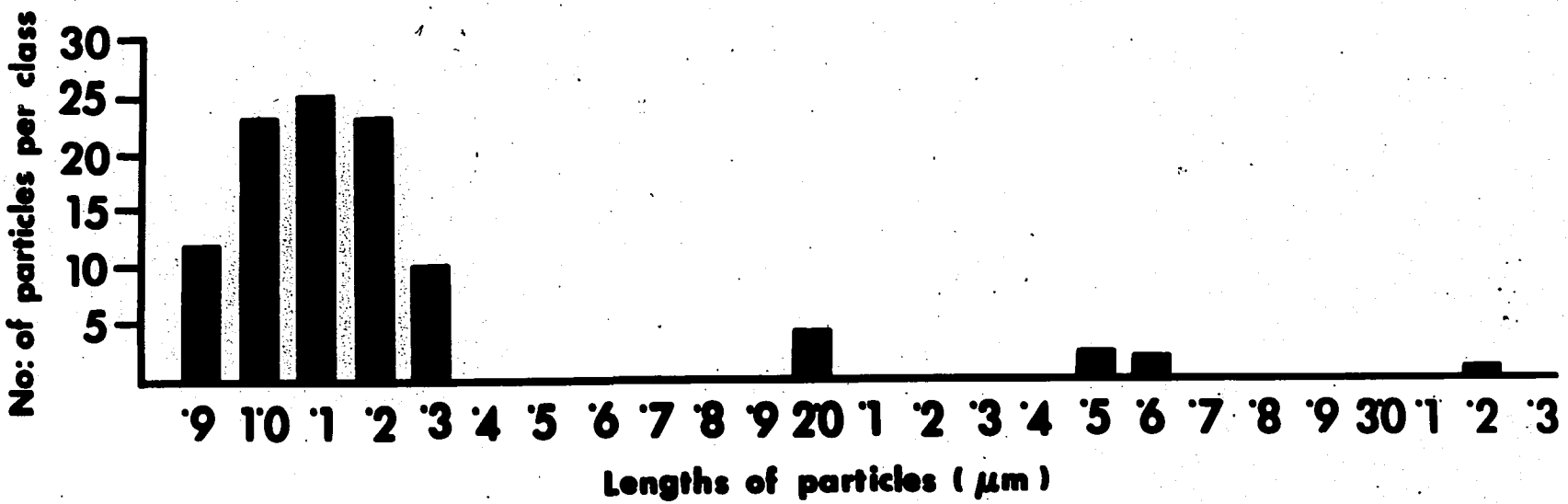
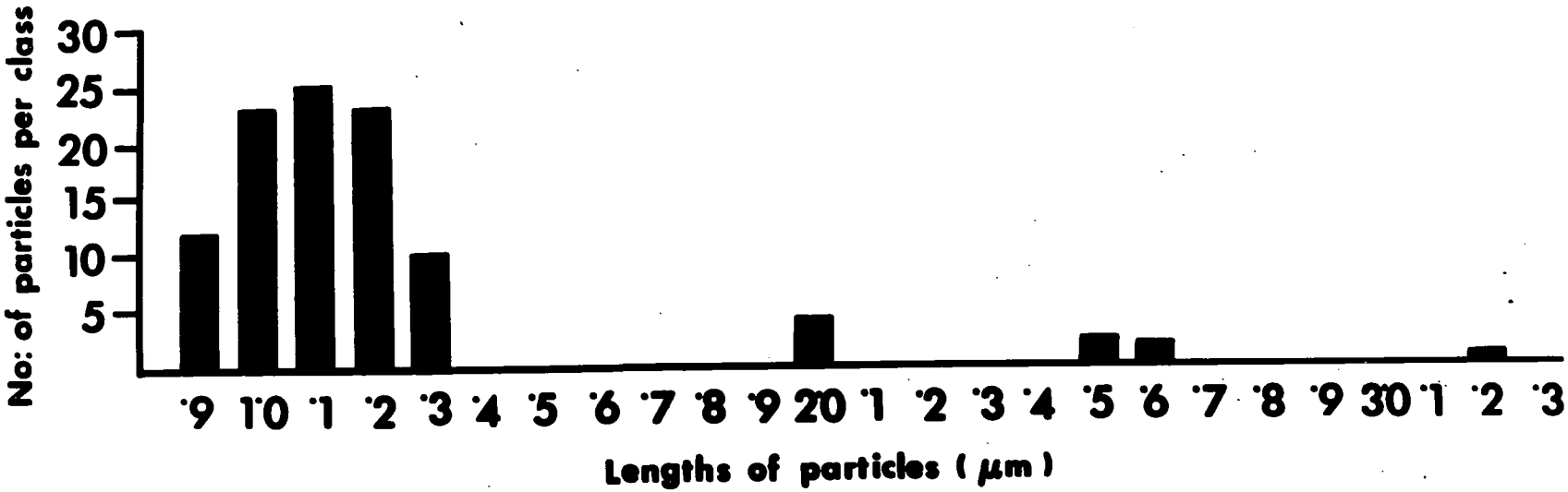

No: of particles per class
30
25
20
15
10
5
·9 1·0 ·1 ·2 ·3 ·4 ·5 ·6 ·7 ·8 ·9 2·0 ·1 ·2 ·3 ·4 ·5 ·6 ·7 ·8 ·9 3·0 ·1 ·2 ·3
Lengths of particles (μm)

Fig : 3


No: of particles per class
30
25
20
15
10
5
·9 1·0 ·1 ·2 ·3 ·4 ·5 ·6 ·7 ·8 ·9 2·0 ·1 ·2 ·3 ·4 ·5 ·6 ·7 ·8 ·9 3·0 ·1 ·2 ·3
Lengths of particles (μm)

inheritence of ApTc with leu and purE also indicated that RM98 was not integrated into 90-15 min region of the E.coli chromosome.

D. STUDIES WITH IKe, THE PHAGE SPECIFIC FOR RM98

I. Size and morphology of plaques

IKe lysate produced plaques of varying sizes ranging from pinpoint to 1 mm in diameter. Lysates yielding relatively numerous plaques of the same size were obtained by several single plaque isolations, always selecting for the larger size. Any variations in the conditions of cultivation, host strain and its concentration, extent of desiccation of bottom agar and increase in the agar percentage of the top layer affected the plaque size and morphology to a great extent. Optimal conditions for obtaining best plaques included using 24 hr old L agar plates and 0.6% agar in the top layer with 264A (MA50/RM98) as the indicator. IKe plaques were slightly turbid and did not have a sharp edge. Sharpness of the plaques could either be increased by increasing the host cell concentration in the top agar or by addition of 0.04% Giemsa in the bottom layer.

II. Shape and size of the phage particle

IKe is a filamentous phage as seen in the electronmicrograph (fig. 2) where a purified phage preparation was stained with 2% potassium phosphotungstate. Lengths of the individual particles were measured and found to vary from 0.92 μm to 1.3 μm with a mean

of 1.1 μm (fig. 3). The mean diameter was found to be 6.6 nm. Particles that were 2.5-2.6 μm and 3.2 μm in length were also encountered which could perhaps represent double or triple lengths of the individual particles.

III. Host range

Strains of S. typhimurium and E.coli carrying RM98 were the original hosts for IKe. E.coli strains bearing several other plasmids also showed lysis by or propagation of IKe. The results are presented elsewhere (please see isolation and characterization of R factors, Table 9). Lysis by IKe of strains bearing RM98 could also be observed when these strains were co-inhabited by other plasmids like: F factor of E.coli, Col Ela or other R factors (e.g. R64-11 etc.).

IV. Effect of various physical and chemical agents

a. Chloroform

Chloroform has been widely used for the sterilization of phage lysates from bacteria. Some filamentous phages have been reported to be inactivated by this treatment (Meynell and Lawn, 1968[b], Marvin and Hohn, 1969). IKe resembled these phages in being completely inactivated by chloroform treatment in one hour.

b. Heat

Kinetics of heat inactivation of IKe was studied at 82 C. IKe was found to be much more heat sensitive than filamentous phages Ifl and EC9 (fig. 4). After 30 min exposure at 82 C, there

Fig. 4. Kinetics of heat inactivation of phages: IKe (●), If1 (■) and EC9 (▲). The data for If1 and EC9 are from the ref.: Meynell et al., 1968[b] (Nature 217: 1184, 1968).

Fig. 5. Effect of Nagarse (100 µg/ml) on IKe.

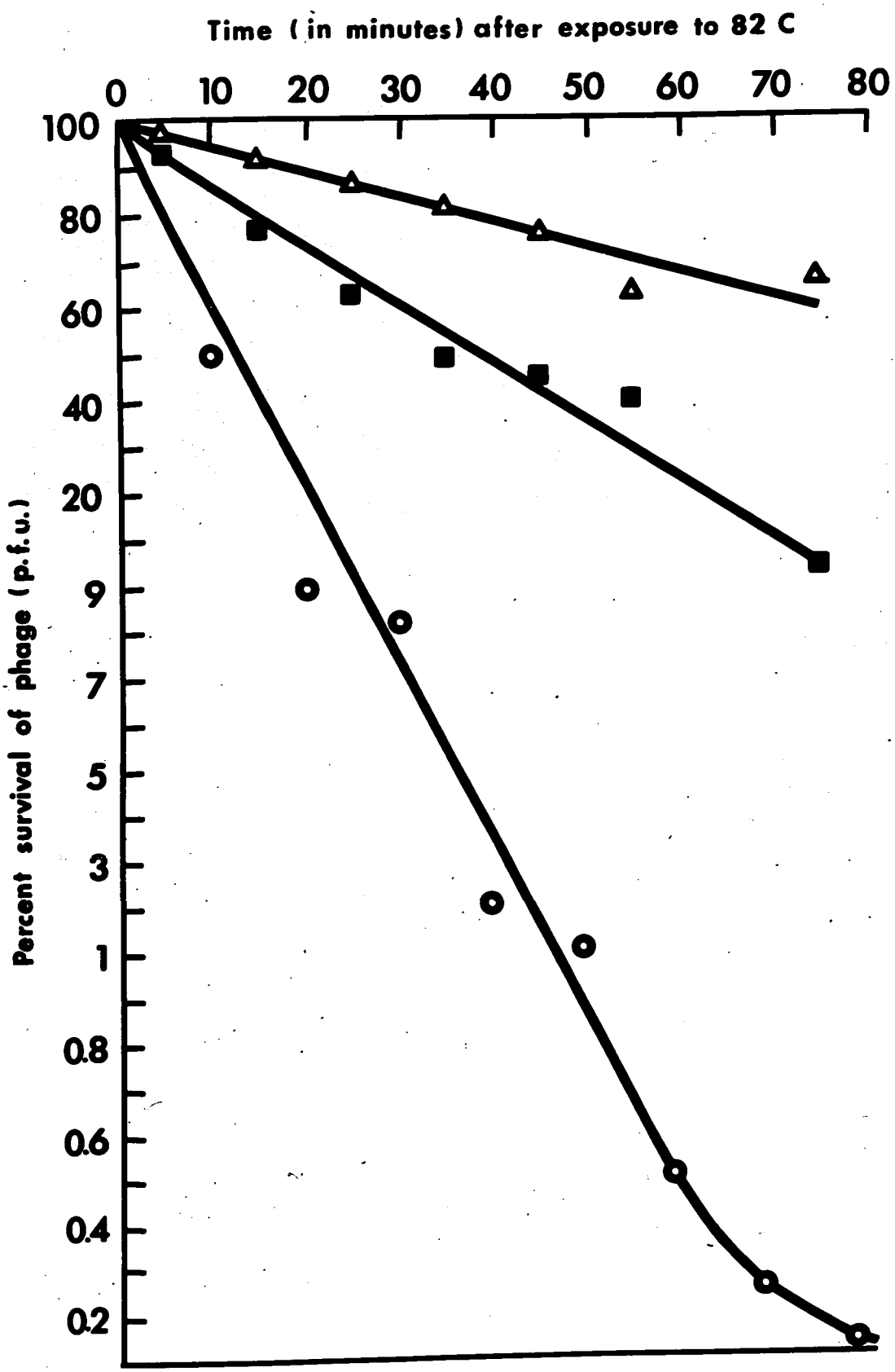

Time (in minutes) after exposure to 82 C
0
10
20
30
40
50
60
70
80
100
80
60
40
20
9
7
5
3
1
0.8
0.6
0.4
0.2
Percent survival of phage (p.f.u.)

Fig : 4

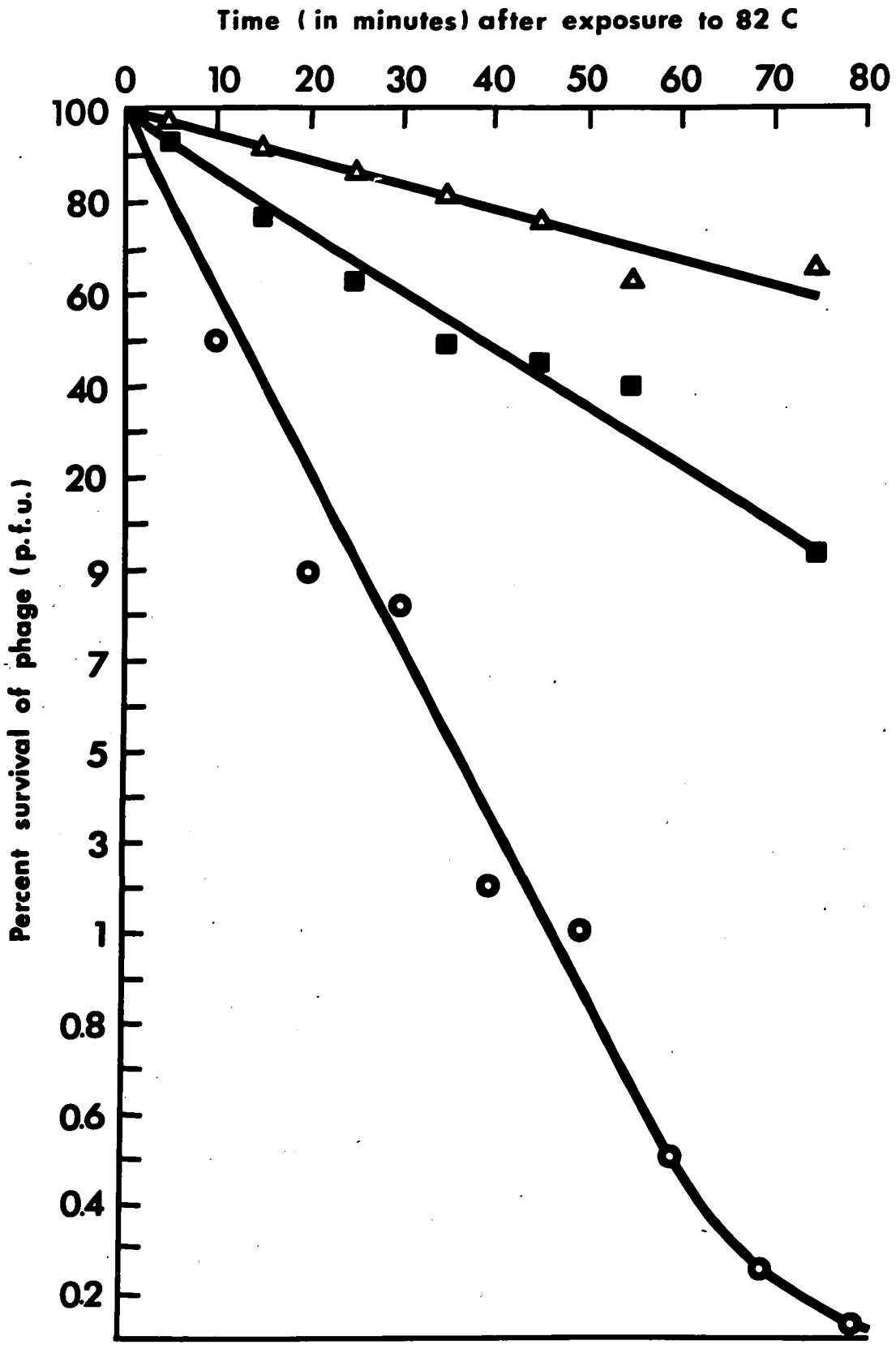

Time (in minutes) after exposure to 82 C
0
10
20
30
40
50
60
70
80
100
80
60
40
20
9
7
5
3
1
0.8
0.6
0.4
0.2
Percent survival of phage (p.f.u.)

Fig : 4

Fig:5

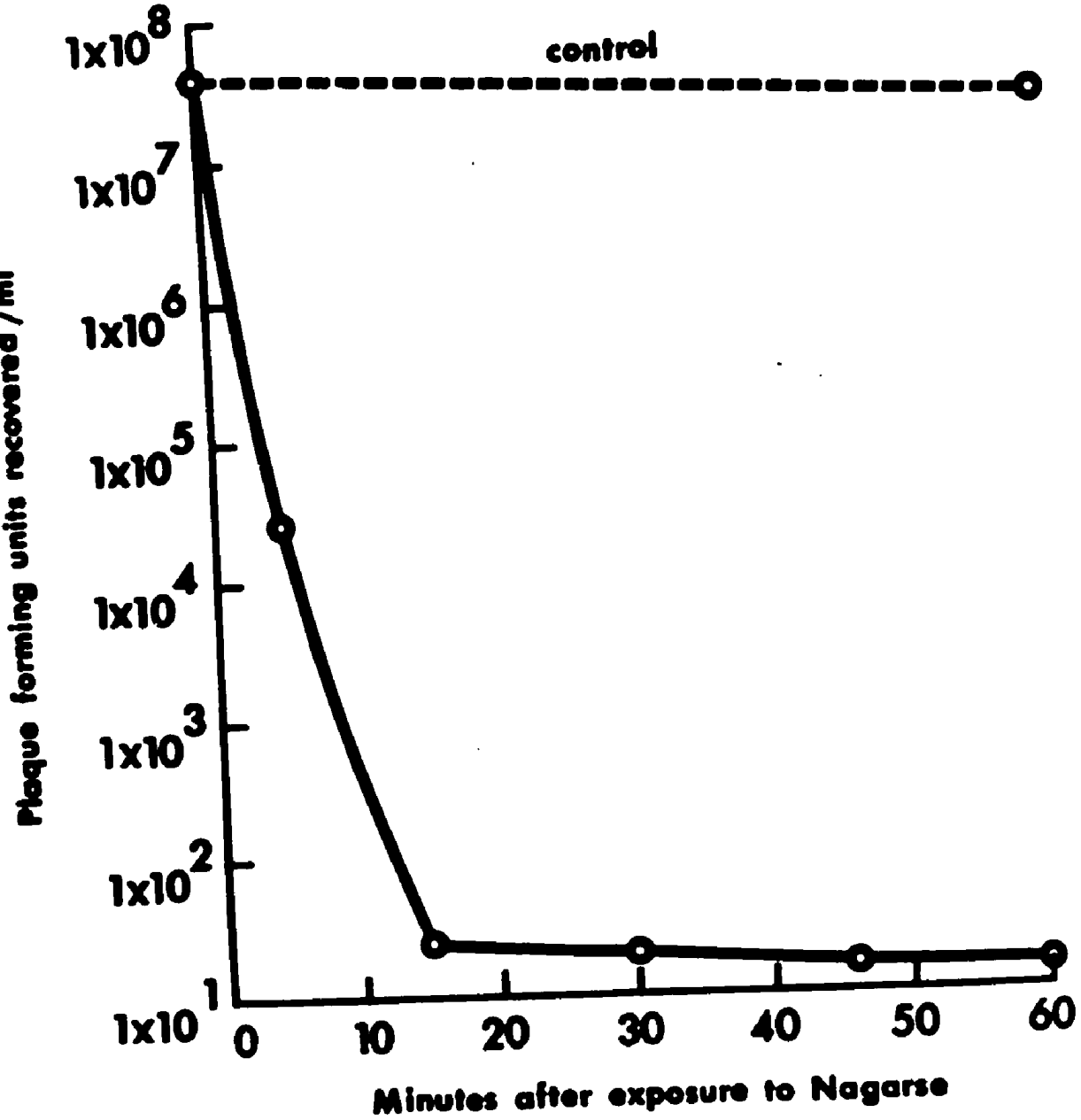

control
1x10^8
1x10^7
1x10^6
1x10^5
1x10^4
1x10^3
1x10^2
1x10^1
Plaque forming units recovered /ml
0
10
20
30
40
50
60
Minutes after exposure to Nagarse

TABLE 19

Effect of freezing and thawing# on IKe

No.: of treatments*	IKe p.f.u/ml recovered	% Loss of infectivity
0.0	4×10^4	0.0
2nd	4×10^4	0.0
4th	4×10^4	0.0
6th	3×10^4	25
8th	3×10^4	25
10th	1×10^4	75

freezing was by storage at -20 C; thawing was at 37 C.

* one treatment comprises freezing followed by thawing.

were only 7.5% surviving IKe particles as compared to 62% and 84% of survivors with If1 and EC9 respectively.

c. Freezing and thawing

IKe was found to be considerably resistant to freezing and thawing in dilution buffer (pH 7). There was no decrease in p.f.u/ml after four consecutive freezings and thawings (Table 19); further freezings and thawings, however, reduced the infectivity to 75% of the initial value after 10 such treatments.

d. Drying

Under conditions used for drying (please see Materials and Methods), the plaque forming ability of IKe was reduced from an initial titer of $4x10^4$ to $8x10^1$/ml. This effect may be due to drying as well as storage at room temperature.

e. Enzymes

Filamentous phages have been reported to be inactivated by the bacterial proteolytic enzyme nagarse (Salivar et al., 1964, Marvin and Hohn, 1969) but are relatively resistant to the enzymes pronase and trypsin (Marvin and Hohn, 1969). RNA phages have been reported to be sensitive to hydrolysis by ribonuclease (RNAse) during infection (Zinder, 1963). The nucleic acid of the DNA phages is not exposed during the infective process (Marvin and Hohn, 1969). Inactivation by RNAse of the infectivity (ability of plaque formation) of an unknown phage can be used to determine whether or not the phage contains RNA (Bradley, 1964).

Like some other filamentous phages (Salivar et al., 1964, Marvin and Hohn, 1969), IKe was found to be rapidly inactivated by nagarse. More than 99% of the infectivity was lost in 5 min (fig. 5).

Treatment of IKe with 10 µg/ml pronase for 2 hr also resulted in 99% loss of infectivity. The phage was completely inactivated by 100 µg/ml of pronase within 2 hr. Similarly, complete inactivation resulted by a 2 hr exposure to 0.25% trypsin. RNAse was not found to reduce or inhibit plaque formation by IKe, indicating that the phage probably contained DNA. Plaque formation of the control RNA phage was almost inhibited by the same RNAse preparations.

V. Serology

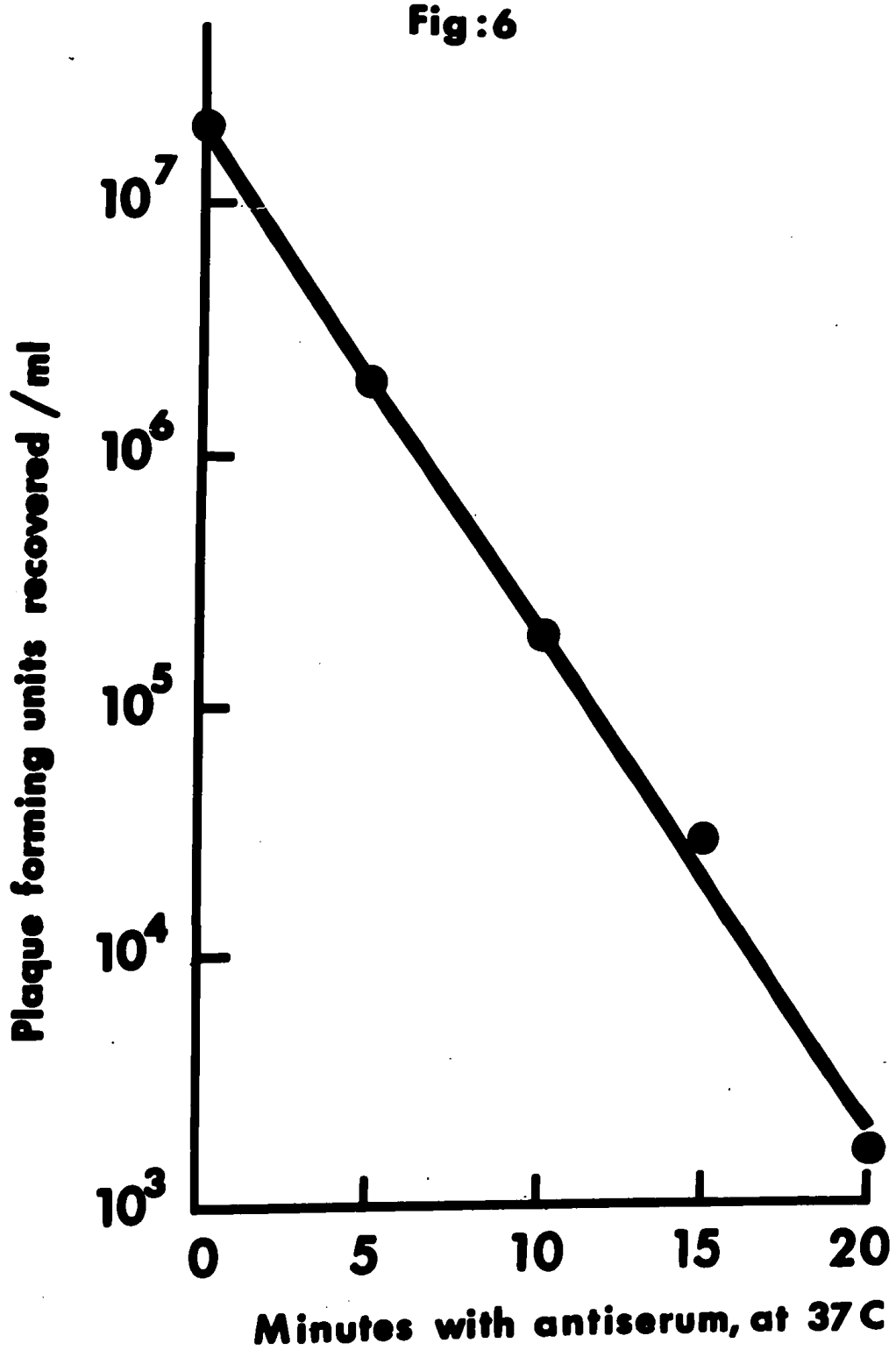
Neutralization of IKe with homologous antiserum followed the curve presented in fig. 6. A 99% loss of infectivity was observed within 10 min and the neutralizing antiserum had a K value of 4.6×10^4.

Normal serum did not reduce the infectivity of IKe. Cross-neutralization of purified preparations of M13 and If1 was not observed with IKe antiserum thus indicating that these phages though morphologically alike were serologically unrelated.

From the findings so far, IKe appears to share with the I-specific phage If1 (Meynell and Lawn, 1968[b]) (1) the ability to form small plaques (2) its filamentous shape and (3) its sensitivity

Fig. 6. Neutralization of IKe with IKe antiserum.

Fig : 6


Plaque forming units recovered / ml
10^7
10^6
10^5
10^4
10^3
0
5
10
15
20
Minutes with antiserum, at 37 C

to chloroform. It differs from Ifl in (1) being relatively heat sensitive (2) having a different host range and (3) yielding an antiserum that does not cross neutralize Ifl. IKe, therefore, constitutes the second phage isolated so far which displays an affinity for strains bearing the Rfi$^-$ factors.

TABLE 20

Transfer of KR61 from 61-0GH to *E.coli* and *Shigella*

Recipient strain and strain No.	Selective* marker	Resistance pattern transferred	Frequency of transfer per hundred donors
E.coli K-12 (F^-) 13-6a	Ap	Ap	1.8×10^{-2}
	Ap, Km, Nm or Tc	Ap Km Nm Sm Tc	4.2×10^{-1}
	Km, Nm or Tc	Km Nm Sm Tc	$8 \times 10^{0} - 1.1 \times 10^{1}$
E.coli K-12 (Hfr) AB301	Ap	Ap	8×10^{-3}
	Ap, Km, Nm, Sm or Tc	Ap Km Nm Sm Tc	2×10^{-2}
	Km, Nm, Sm	Km Nm Sm Tc	$8 \times 10^{-2} - 1 \times 10^{-1}$
Shigella L-53	Ap	Ap	1×10^{-2}
	Ap, Km, Nm, Sm or Tc	Ap Km Nm Sm Tc	3×10^{-2}
	Km, Nm, Sm or Tc	Km Nm Sm Tc	1×10^{-1}

* Antibiotics were used at the concentration of 30 µg/ml of the medium.

E. Rfi$^+$ GROUP OF FACTORS

The Rfi$^+$ factors include KR61, KR36, KR78 and KR68, which were isolated from Aerobacter aerogenes strains obtained from Ottawa General Hospital. KR19, also isolated from A. aerogeneses did not possess an RTF and is discussed below with the Rfi$^+$ factors.

I. KR61

61-OGH, the wild type A. aerogenes strain carrying resistances to Ap, Km, Nm, Sm and Tc when conjugated to E.coli and Shigella (L-53) recipients yielded the following resistance patterns:

1. Ap
2. ApKmNmSmTc
3. KmNmSmTc

Transfer frequencies and details on the selective markers used are presented in the Table 20. The resistance pattern 2 (above) always segregated to yield patterns 1 and 3 in subsequent conjugal crosses and P1kc transductions in E.coli. The segregation was much more pronounced in conjugal crosses from E.coli R$^+$ donors to S. typhimurium LT2 R$^-$ recipients than was in crosses from R$^+$ to R$^-$ E.coli strains. ApKmNmSmTc phenotype, therefore, appears to arise by the coexistence of Ap and KmNmSmTc in the same cell.

E.coli F$^-$ strains that had conjugally acquired Ap from 61-OGH could not transfer it in subsequent conjugal crosses to other E.coli F$^-$ strains and apparently did not possess the sex

E. Rfi$^+$ GROUP OF FACTORS

The Rfi$^+$ factors include KR61, KR36, KR78 and KR68, which were isolated from Aerobacter aerogenes strains obtained from Ottawa General Hospital. KR19, also isolated from A. aerogeneses did not possess an RTF and is discussed below with the Rfi$^+$ factors.

I. KR61

61-0GH, the wild type A. aerogenes strain carrying resistances to Ap, Km, Nm, Sm and Tc when conjugated to E.coli and Shigella (L-53) recipients yielded the following resistance patterns:

1. Ap
2. ApKmNmSmTc
3. KmNmSmTc

Transfer frequencies and details on the selective markers used are presented in the Table 20. The resistance pattern 2 (above) always segregated to yield patterns 1 and 3 in subsequent conjugal crosses and P1kc transductions in E.coli. The segregation was much more pronounced in conjugal crosses from E.coli R$^+$ donors to S. typhimurium LT2 R$^-$ recipients than was in crosses from R$^+$ to R$^-$ E.coli strains. ApKmNmSmTc phenotype, therefore, appears to arise by the coexistence of Ap and KmNmSmTc in the same cell.

E.coli F$^-$ strains that had conjugally acquired Ap from 61-0GH could not transfer it in subsequent conjugal crosses to other E.coli F$^-$ strains and apparently did not possess the sex

TABLE 21

Transfer* of KR36, KR78, KR68 and KR19 from wild type donors to E.coli recipients

Donor	Recipient*	Resistance pattern transferred	Frequency of transfer per hundred donors
36-OGH	X_{407}	CmSmTc	9×10^{-3}
	13-6a	Cm(Sm)^{x}Tc	2.3×10^{-2}
78-OGH	X_{407}	CmSmTc	1×10^{-4}
	13-6a	Cm(Sm)Tc	5×10^{-3}
68-OGH	X_{407}	CmSmTc	7×10^{-5}
	13-6a	Cm(Sm)Tc	5×10^{-4}
19-OGH	X_{407}	CmTc	1×10^{-4}
	13-6a	Cm(Sm)Tc	2×10^{-4}

* Selected on Tc 30 µg/ml of the medium

* X 407 was Hfr and 13-6a F^{-} E.coli

x Plasmid borne(Sm) resistance was masked by chromosomal Sm resistance of 13-6a.

factor (RTF). However, Ap could be mobilized in these crosses by infecting the donor with the segregant KmNmSmTc which had its own RTF. Other Rfi^+ factors KR68, KR36 or KR78 and the Rfi^- factor R64-11 were not able to mobilize Ap. A male strain of E.coli (X407) bearing Ap alone was able to conjugally transfer it to E.coli 13-6a (F^- strain); although X407 R^- was not efficient in yielding chromosomal recombinants with 13-6a.

The segregant pattern KmNmSmTc was transferable in E.coli crosses of the type R^+F^- x R^-F^-, showing that an RTF was associated with these resistances . . It could also be transduced as one entity by P1kc. Presence of Ap in F^+ hosts bearing KmNmSmTc was not found to affect Rfi^+ property of KmNmSmTc (i.e. fertility inhibition, or inhibition of MS2 adsorption Section A II a,b). The sex factor (RTF) of KmNmSmTc appears to be closer to Tc; a segregant which had lost the Tc marker had also lost conjugal transferability.

II. KR36, KR78, KR68 and KR19

Aerobacter strains 36-OGH, 78-OGH, 68-OGH and 19-OGH bearing resistances to Ap, Cm, Sm and Tc transferred either Cm Sm Tc or Cm Tc when conjugated to E.coli strains (Table 21). The R factors were designated as KR36 (CmSmTc), KR78 (CmSmTc), KR68 (CmSmTc) and KR19 (CmTc) respectively, according to the numbers of their original hosts. Transfer of Ap resistance was not observed from the wild type hosts when Ap selection was used; similarly Sm was not transferred by 19-OGH.

Except KR19, all the R factors were transferable in E.coli crosses: R^+F^- x R^-F^- and hence possessed their own sex factors (RTFs). The indicated resistances were transferred together in conjugal crosses when selection was made on Cm, Sm or Tc, transfer frequency being higher on Tc. None of the R factors: KR68, KR78 or KR36 were conjugally transferable from E.coli R^+ donors to S. typhimurium LT2 R^- recipients.

KR36, KR68 and KR78 were classified as Rfi^+ factors on the basis of F mediated fertility inhibition and inhibition of MS2 adsorption by AB301 strains bearing them (Section A II a,b). KR19, which did not possess an RTF, was found to be transferable in conjugations from E.coli X407 R^+ to 13-6a (F^-).

The transfer of Ap (KR61 segregant) and of CmTc (KR19) from X407 donors to 13-6a is interesting. X407 although sensitive to MS2 was highly inefficient in yielding chromosomal recombinants (for pro, trp, met etc.) with 13-6a recipients. Hence it appears unlikely that Ap or Cm Tc were transferred along with the chromosome (if one presumes that they had integrated into the chromosome). Resistance markers of the R factors are known to become transferable by recombination with the F factor of E.coli (Watanabe and Fukasawa, 1962[c], Harada et al., 1964, Watanabe and Ogata, 1966). Whether F factor of X407 (presumably an Hfr) existed in the extrachromosomal state or some other mechanism was available for the mobilization of Ap and Cm Tc needs further investigation. In view of the findings that Ap was also mobilized by KmNmSmTc (KR61 segregant) but not by

KR36, KR78, KR68 or R64-11; X 407 and *E.coli* F⁻ hosts bearing KmNmSmTc should have similar mechanisms for mobilizing Ap.

TABLE 22

Characteristics of isolated R factors

R factor	fi type	Phage propagation MS2	If1	IKe	Phage restriction*	Colicin production	Colicin resistance colIb	colE1	colE2
RM98	-	-	-	+	-	-	-	-	-
RM227	-	-	+	+	-	-	-		
RM413	-	-	+	+	-	-	+		
RM414	-	-	+	+	-	-	-		
RM430	-	-	+	+	-	-	+		
KR61	+	-		-	-				
KR36	+	-		-	-				
KR78	+	-		-	-				
KR68	+	-		-	-				
KR19		-		-	-				

* T3, T7, ØII, λ, P1kc and BF23 in E.coli hosts; P22 and SP6 in S. typhimurium hosts.

PART FOUR

(1) SUMMARY AND CONCLUSIONS

R factors isolated from the clinical strains of Aerobacter aerogenes (obtained from Ottawa General Hospital) and Salmonella typhimurium (obtained from Department of National Health and Welfare, Ottawa) were studied and characterized.

All of the 5 R factors isolated from S. typhimurium (RM98, RM227, RM413, RM414 and RM430) were classified as fi^- by the criterion of absence of fertility inhibition of male E.coli. When E.coli F^- strains bearing these R factors were examined for I phage (If1) propagation, RM98 appeared to possess a sex factor different from the I sex factor (Lawn et al., 1967). RM98 bearing hosts were unable to propagate If1 (Table 22). A phage (IKe) specific for the sex factor of RM98 was isolated from sewage and was found to be unrelated to the phage If1 (Sections C I and D V; Results and Discussion). Studies involving propagation of phages If1 and IKe using several R factor borne and other sex factors revealed that three groups of the fi^- sex factors might exist:

(1) Sex factors propagating If1 alone (or I sex factor group)

(2) Sex factors propagating IKe alone (IKe sex factor group)

(3) Sex factors propagating If1 and IKe both.

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TABLE 23

Superinfection immunity among Rfi⁻ factors

Pairs of R factors	Entry exclusion*	Recombination	Compatibility*
RM98 : R64-11	-	-	+
RM98 : RM227	-	+	-
RM98 : RM414	-	+	-
RM98 : RM413	-	-	+
RM98 : RM430	-	-	+
RM98 : R45	+	n.d.[x]	n.d.
RM98 : R46	+	n.d.	n.d.
RM98 : R48	+	n.d.	n.d.
RM98 : R199	+	n.d.	n.d.
RM98 : R205	+	n.d.	n.d.
R64-11 : RM227	-	-	+
R64-11 : RM413	+	-	+
R64-11 : RM430	+	-	+
R64-11 : RM414	+	-	+

* A plus sign = reduction in transfer frequency

* A plus sign = co-exist stably as independent replicons through 20 transfers in medium lacking antibiotics

x n.d. = not done

Sex factors belonging to group (1) were identified by Lawn et al., (1967) as the sex factors which when present in F⁻ E.coli cells produced I like pili, making their hosts sensitive to the phage If1. This group included several sex factors, such as the sex factors of: R64-11, R144-3, Col Ib-P9 and Col E1a etc.

The group (2) as revealed by IKe propagation included sex factors associated with: RM98, some R factors from other sources (R45, R46, R48, R205, R199 and N3) and plasmids other than R factors (Col E2C, Col E10A, Col E193B). The R factors: R45, R46 and R199 which now fall in group (2) were previously among the unclassified R factors (Lawn et al., 1967). Inability to propagate If1 by their hosts was attributed to either the repressed state of these R factors or that the sex factor they bore represented a third kind and was different from the recognized I and F sex factors (Lawn et al., 1967). The present studies indicate that R45, R46 and R199 are related to RM98 not only by the visible IKe lysis exhibited by their hosts, but also by the criterion of entry exclusion (Table 23); hence the inability to propagate If1 by their hosts does not seem to be a result of the repressed state of these R factors. Since RM98 appears to be unrelated to R64-11 (which bears an I-like sex factor) (see Table 23), R45, R46 and R199 may also be unrelated to R64-11. In conclusion all the sex factors of group (2) appear to be related to each other by at least two criteria (a) their ability to confer IKe sensitivity on their hosts (b) entry exclusion (Table 23).

RM98 was found to yield a relatively large proportion of P22 transductants bearing RTF and all the resistance determinants; this observation was similar to the reports on P22 transduction of R45, R46, R48, R205 (Drabble and Stocker, 1968) and N3 (Watanabe et al., 1968). RM98, R45, R46, R48, R205 and N3, therefore, appear to be different from the earlier reported R factors which showed a constant segregation of resistance determinants and RTF in P22 transductions (Watanabe et al., 1961[a], 1964[b]). Resistance pattern of RM98 appears to be similar to the most common resistance pattern (ApSmTcSu) encountered in the Salmonella R factors of North America (Pocurull et al., 1971). Although a correlation is not necessary between the resistance pattern and the kind of sex factor, most of the R factors related to RM98 were found to carry resistances ApSmTcSu or ApTcSu. R48 and R205 which were selected for the present studies merely on the basis of their resistance pattern and their behaviour in P22 transduction, turned out to be related to RM98 by the criteria of entry exclusion and visible lysis of their hosts by IKe. This observation seems to indicate that IKe sex factor may not be uncommon among Salmonella R factors of North America. By its low level resistance to all antibiotics (Ap, Sm, Tc) and in transferring all these resistances together in conjugal crosses, RM98 resembles the R factors isolated from S. typhimurium phage type 1a (Anderson and Lewis, 1965[a]). RM98 was not found to effect chromosomal transfer in E.coli crosses of the type R^+F^- x R^-F^-,

this being in contrast to the report on R64-11 (Cooke and Meynell, 1969).

The group (3) included only four R factors: RM413, RM227, RM430 and RM414, which exhibited similarities to RM98 and/or to R64-11 in genetical studies (Table 23). It was not possible to study entry exclusion between members comprising this group because of the lack of differential markers (all were found to confer high level Ap resistance on their hosts). RM413 and RM430 also conferred, on their E.coli hosts, a low level resistance to Col Ib, similar to the Col Ib insensitivity conferred by some other R factors (Siccardi, 1966). This group of R factors, therefore, appears to be heterogenous. All the 4 R factors conferred high level Ap resistance on their hosts thus resembling the R factors isolated from phage type 29 of S. typhimurium (Anderson and Lewis, 1965a).

Whatever the route of infection, IKe seems to be specific for the sex factor of RM98 and hence is a sex factor specific phage. The classification of fi⁻ sex factors into groups (2) and (3) would, therefore, be on the basis of phage propagation (until the adsorption site of IKe is revealed) rather than on the basis of specific pili (Lawn et al., 1967).

There is no conclusive evidence as to the transfer of genetic material through the specific pili, although indirect evidence (Ou et al., 1970) coupled with the observation that

specific pili are always found on sex factor bearing cells strongly suggests that either pili constitute the conjugation tubes through which DNA is transferred from the donor to the recipient cells or they help in gene transfer by stabilizing mating pairs. Genes controlling the sensitivity to sex factor specific phages are closely associated with those controlling the synthesis of a specific pilus. However, presence of the pilus as such, may not confer sensitivity to all the specific phages (Ohtsubo et al., 1970). If a pilus is synthesized by the IKe-specific group of sex factors, it may differ from the I pilus either completely (antigenically, morphologically and physiologically) or only with respect to the phage receptor sites. Whatever is the case, positions of groups (1) and (2) remain unchanged because of the lack of entry exclusion and incompatibility between members of the two groups. The group (3) sex factors may synthesize a pilus having affinity for both phages IKe and If1, or the strains bearing these R factors are probably co-inhabited by two different sex factors, one belonging to group (1) and the other to group (2).

Strains bearing some fi^- R factors (R300, R310, R305 and RP4) were not found to propagate either If1 or IKe. It is not impossible that sex factors of these plasmids have yet different phage specificities.

Out of a total of 5 R factors isolated from A. aerogenes, 4 (KR61, KR36, KR78 and KR68) were found to be fi^+ by the criterion of fertility inhibition of male E.coli. KR19 did not possess an

RTF and hence could not be classified (Table 22). None of the R factors was found to confer ability to propagate F-specific phages on E.coli F^- hosts. Since phage propagation can be observed in the repressed state (Meynell et al., 1968[a]), the absence of F phage propagation by the hosts of KR36, KR61, KR78 and KR68 does not appear to be due to their repressed state. These R factors may possess sex factors similar to those of exceptional Rfi^+ factors (which failed to confer the ability for F phage propagation on their F^- hosts) reported by Lawn et al., (1967). Attempts to isolate specific phages for these R factors were not successful. This could be due to one of the following reasons: (1) the sewage samples used did not have a proper sex factor specific phage (2) strains bearing these R factors either did not synthesize specific phage receptor sites or synthesized defective receptor sites or (3) in the absence of (1) and (2), detection of a new sex factor specific phage would also be impossible if the R^+ strains only propagated the phage without showing visible plaques. F^- E.coli strains bearing the nontransferable plasmid KR61 Ap, were found to conjugally transfer it when superinfected by KR61 (KmNmSmTc) but not when superinfected by KR36, KR78, KR68 or R64-11. Similarly KR61 Ap was conjugally transferable from a male E.coli donor (X407). This may reflect some functional similarity between KR61 (KmNmSmTc) and F factor of E.coli K-12.

(2) AREAS FOR FURTHER INVESTIGATION

Investigations are needed to further study the following points arising from the present work:

(i) Whether or not a pilus is synthesized by the IKe specific sex factors: and the site of IKe adsorption.

(ii) Reasons for the heterogeneity in group (3) of Rfi^- sex factors.

(iii) Origin of $Tclac^+RTF^+IKe^r$ (Section C I; Results and Discussion).

(iv) Reasons for genetical stability of the R factors (Section A I; Results and Discussion).

(v) Reasons for the lack of F-specific phage propagation by F^- hosts of KR61, KR36, KR78 and KR68.

(vi) Mechanism of mobilization of R61 Ap.

(vii) Classification of the sex factors which do not propagate any sex factor specific phages (Section A IIc; Results and Discussion, Table 9).

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APPENDIX

List of abbreviations

Ap	Ampicillin
A.O.	Acridine orange
B.S.A.	Bovine serum albumin
Cm	Chloramphenicol
EBr	Ethidium bromide
e.o.p.	Efficiency of plating
hr	Hour(s)
IKe^s	Sensitivity to the phage IKe
M.A.	MacConkey's agar
MIC	Minimal inhibitory concentration
M.M.	Minimal medium
M.O.I.	Multiplicity of infection
Nm	Neomycin
P.A.	Penassay agar
P.B.	Penassay broth
p.f.u.	Plaque forming units
RTF	Resistance transfer factor (or the sex factor of an R factor)
RTF^+	Presence of an RTF
RTF^-	Absence of an RTF
Sm	Streptomycin
Su	Sulfonamide
RNAse	Ribonuclease

Tc	Tetracycline
T.S.A.	Trypticase soy agar
T.S.B.	Trypticase soy broth
T.S.I.	Triple sugar iron agar
U.V.	Ultraviolet irradiation