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Studies of non auto-transferring plasmids
in Escherichia coli and salmonellae.

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Thesis submitted for the Degree of Doctor of Philosophy in the
Faculty of Medicine, University of London.
A number of non auto-transferring plasmids (NTP) coding for drug resistance or for colicin synthesis were examined in Escherichia coli and salmonellae. These plasmids form Class 2 transfer systems in which the resistance or colicin determinant(s) and the transfer factor are discrete plasmids, independent of each other in the host cell. The transfer frequency of the determinant depends on the type of transfer factor used for its mobilisation. For example, the streptomycin-sulphonamide resistance (SSu) determinant was transferred more efficiently by I-like transfer factors than by F-like plasmids. The plasmide NTP1 to NTP11, of which eight coded for drug resistance and three for colicinogeny, fell into at least six compatibility groups. A number of wild SSu-resistant strains of salmonellae and E. coli carried non-transferring determinants incompatible with the ampicillin-sulphonamide resistance (ASu) determinant. These SSu plasmids are probably phylogenetically related to the prototype SSu determinant from which ASu was derived. NTP1 to NTP11 consist of covalently closed circular DNA molecules with mean contour lengths between 2.22 and 4.53 μm. All are present in multiple copies per chromosome in E. coli K12.

A non-transferring kanamycin resistance determinant K reversed the "fertility inhibition +" (fi+) property of Salmonella typhimurium phage type 36, to make it fi−. K had a similar effect...
on at least fourteen other strains of S. typhimurium. Examination
of five $\text{fi}^+$ S. typhimurium strains and one $\text{fi}^-$ strain demonstrated
that the $\text{fi}^+$ property was dependent on the presence of a plasmid,
which was designated MP10. Molecular studies confirmed that K
was incompatible with MP10 and the two plasmids showed a high
degree of DNA homology. K was probably formed by recombination
between a kanamycin resistance determinant and the MP10 plasmid
of the original S. typhimurium host. The K plasmid is present as
approximately one copy per chromosome and it has a mean contour
length of 18.1 $\mu$m.

These results establish that there are at least two sorts of
non auto-transferring plasmids. Plasmids of the first type are
small, less than 5 $\mu$m in length, and exist in multiple copies per
chromosome. In contrast, members of the second type are usually
much larger in size and are present as approximately one copy per
chromosome.
This thesis is a report of research performed by the author in the Enteric Reference Laboratory, Central Public Health Laboratory, Colindale, and in the School of Pharmacy, University of London from 1970 to 1975.

Accounts of parts of this work have been published as follows:


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Symbols and Abbreviations used in this thesis

Plasmid-borne resistance to antibacterial agents:

A = ampicillin  
C = chloramphenicol  
K = kanamycin  
S = streptomycin  
Su = sulphonamides  
T = tetracycline

Chromosomal resistance:

Str$^R$ = streptomycin resistance  
Nal$^R$ = nalidixic acid resistance

Escherichia coli K12 = K12  
Salmonella typhimurium = S.typhimurium  

TP = Auto-transferring plasmid  
NTP = Non auto-transferring plasmid  
MP = S.typhimurium plasmid

p.f.u. = Plaque forming unit  
MCL = Mean contour length  
$W_1$ = Hydroxyapatite  
PB = Phosphate buffer
INTRODUCTION

Discovery of transferable drug resistance

Transferable drug resistance in bacteria was first demonstrated in Japan sixteen years ago and since then it has created increasing problems in treatment of bacterial diseases throughout the world.

A strain of Shigella flexneri isolated in Japan in 1955 was found to be resistant to several drugs: sulphonamides, streptomycin, chloramphenicol and tetracyclines. Subsequently, multiply resistant and sensitive strains with the same biochemical and antigenic properties were isolated, in some cases from the same patient. It was also observed that most cases infected with multiresistant Shigella also carried multiresistant Escherichia coli. Ochiai et al. (1959) and Akiba et al. (1960) demonstrated that the multiple drug resistance could be transferred from resistant E. coli to sensitive Shigella, and also from Shigella to E. coli. They also established that cell-to-cell contact was necessary for transfer of drug resistance.

Further experiments in Japan, mainly by Watanabe and his coworkers, established the extrachromosomal nature of transferable drug resistance. The transfer of resistance usually occurred without the transfer of chromosomal markers (Watanabe and Fukasawa, 1960, 1961a). The resistance (or R) factors could be eliminated or "cured" at low frequency by acridine dyes which were already known to eliminate extrachromosomal elements such as the classical
F factor (Hirota, 1960; Watanabe and Fukasawa, 1961b). Examination of the kinetics of resistance transfer demonstrated that the number of recipient cells acquiring resistance increased rapidly and it was suggested that the R factors replicated faster than the bacterial chromosome (Watanabe and Fukasawa, 1961a).

Resistance transfer systems

Many studies in Japan centred on an R factor isolated from a strain of Shigella flexneri 2b (No. 222) by Nakaya, Nakamura and Murata (1960). This R factor, which conferred resistance to chloramphenicol (C), streptomycin (S), sulphonamides (Su) and tetracyclines (T), was named 222 by Watanabe and Fukasawa (1961b), but was also referred to as R100 (Sugino and Hirota, 1962) or NR1 (Nakaya et al., 1960). Cells carrying 222 transferred the four resistances en bloc, and lines that had newly received the R factor could then transmit it with high frequency, a phenomenon similar to that of high frequency of transfer of the colicin Ib factor (Stocker, Smith and Ozeki, 1963). These resistance transfer cultures were termed high frequency of transfer (HFT) (Watanabe, 1965a). All four drug resistance markers could also be transduced by phage P1 in Escherichia coli K12 or by P22 in Salmonella typhimurium (Watanabe and Fukasawa, 1961c). P1 usually transduced the complete R factor and the transductants retained their transferability. In contrast, the T marker was segregated.
from CSSu in P22 transduction and the majority of transductants were unable to transfer their drug resistance.

Watanabe and coworkers concluded that R factors such as 222 consisted of a single linkage group, part of which was the region coding for drug resistance, while the remaining genes included those for transferability. This latter region was termed the resistance transfer factor (RTF). The CSSuT resistance markers of R factors such as 222 and the RTF were shown by conjugational genetics and by transduction to be on a single linkage group, which could be rectilinear or circular (Watanabe and Fukasawa, 1961c).

Studies of transferable drug resistance in *S. typhimurium* by Anderson and coworkers demonstrated another type of resistance transfer system. A strain of *S. typhimurium* phage type 29, RT1, was resistant to ampicillin (A), streptomycin, sulphonamides, tetracyclines and furazolidone (Fu). Ampicillin resistance was transferred to K12 at a frequency of $2 \times 10^{-2}$ in overnight crosses. Streptomycin and sulphonamide resistances appeared to be closely linked and always transferred together at a frequency of $10^{-2}$. Joint transfer of A and SSu occurred at a frequency of $10^{-3}$. The frequency of T transfer was $10^{-6}$ or less, and no transfer of furazolidone resistance was observed (Anderson and Lewis, 1965a,b). Examination of unselected recipient colonies after an overnight cross revealed that more than 50% had received a transfer factor (designated A) without drug resistance.
Further crosses with the resistant K12 progeny demonstrated that A and SSu transferred to *S. typhimurium* at approximately $10^{-2}$, whereas T was now transferred at $5 \times 10^{-1}$ in overnight crosses. When A or SSu progeny from interrupted crosses (usually 2 h) to *S. typhimurium* were examined, many of the lines could no longer transfer their resistances. However, introduction of a transfer factor such as A into these lines resulted in mobilization of the resistances (Anderson, 1965).

Anderson and Lewis (1965a,b) suggested that there were independent plasmids* responsible for the resistance genes and the transfer factor in the original *S. typhimurium* type 29. The plasmids identified were the A determinant, the SSu determinant, and the A transfer factor. It is still not known whether the tetracycline resistance determinant of RT1 is chromosomal in location or is a plasmid in the wild strain RT1. It was further postulated that A was necessary for transfer of the resistance determinants A and SSu. There might be transient linkage between the transfer factor and determinants during conjugation but A, A and SSu appeared to exist independently in the host cell. Transfer of T resulted from recombination of the tetracycline resistance determinant with A forming a single linkage group designated T-A (Anderson and Lewis, 1965b).

Transduction by phage P1ko of the A-mediated resistance transfer systems (Anderson and Natkin, 1972) supported these postulates, which were originally based on conjugation studies. The R factor T-A was

*The word "plasmid" is used to describe a genetic element able to exist stably in the extrachromosomal state (Lederberg, 1952).
transduced as a single unit and was transferable after transduction. From lines carrying $A$ (or SSu) and $\Lambda$, the resistance determinant was transduced independently of $\Lambda$ to recipient cells. After transduction $A$ (or SSu) was not transferable but introduction of $\Lambda$ into these transductant lines resulted in mobilisation of the resistance determinants.

These observations based on the *S. typhimurium* strain RT1 led to the definition of two classes of resistance transfer systems (Anderson, 1968; Anderson, 1969; Anderson and Threlfall, 1970; Anderson and Natkin, 1972). In Class 1 the resistance determinant(s) and the transfer factor form a covalently-bonded complex which is transferred as a single linkage group. This complex is postulated to have a unique membrane attachment site which is that of the transfer factor (Anderson *et al.*, 1968). The R factors, for example R222, discovered in Japan, were the first examples of this class. T-\(\Lambda\) also belongs to Class 1 (Anderson and Lewis, 1965b; Anderson, 1969).

In Class 2 transfer systems the resistance determinant(s) and the transfer factor are discrete plasmids independent of each other in the host cell. The transfer factor and resistance determinant can be transferred separately or together, the transfer factor usually being transmitted at a substantially higher frequency than the determinant. Examples of this second class are $A_{\Lambda}$ and SSu,\(\Lambda\) identified in *S. typhimurium* type 29. This classification can also be applied to other transfer systems. For example, the F prime factors
and the colicinogenic factor ColI belong to Class 1, while the transfer of the colicinogenic determinant ColE1 by the F factor forms a Class 2 transfer system.

Colicinogeny

The genetic determinants for colicin production (termed colicinogenic or colicin factors) were transferred to non-colicinogenic strains by cell contact (Fredericq and Betz Bareau, 1953; Fredericq, 1954a). There was no linkage to any known chromosomal markers in these experiments. Studies on various colicin factors (ColIV-K94, ColIa-CA53, ColE2-K317) in different Hfr and F" strains demonstrated that the frequency of transfer of colicinogeny depended on the colicin factor and not on the origin of the Hfr strain (Nagel de Zwaig, Antón and Puig, 1962). It was concluded that these colicin factors existed in an extrachromosomal or plasmid state. Later studies also established the plasmid nature of ColE1-K30 (Clowes, 1963; Nagel de Zwaig and Puig, 1964).

A comparative study of different colicin factors in S.typhimurium was performed by Stocker and coworkers (Ozeki, Stocker and Smith, 1962; Stocker et al., 1963; Smith, Ozeki and Stocker, 1963). The colicin factors were transferred to S.typhimurium LT2 and their transfer to a non-colicinogenic LT2 strain was then followed. ColB-K77 and ColIb-P9 transferred with high efficiency, while ColE1-K30, ColE2-P9 and ColK-K49 showed little or no transfer. However these latter colicin factors could be transferred after the introduction of ColB or ColIb. These experiments demonstrated
the existence of two different types of colicin factor. The first
group, exemplified by ColIb, ColV-K94 and ColB, were auto-
transferring, while the second group included the non auto-
transferring plasmids such as ColE1, ColE2 and ColK. Fredericq
(1954b) had previously found that F+ but not F- lines could transmit
the colicin factors ER (= ColE1) and S2 (= ColE2). In a study
of 314 colicinogenic strains Fredericq (1956) observed that most
strains did not appear to transfer their colicinogeny. However,
this could have resulted from the inability to detect low
frequencies of transfer. The mobilisation of non auto-transferring
colicin factors was not investigated in Fredericq's study. However,
it is clear that colicinogenic plasmids fall into classes 1 and 2 in
relation to transfer, as described in the preceding section.

Studies on the transfer kinetics of ColIb demonstrated that
newly-infected cells acted as efficient donors; this property was
termed high frequency transfer of colicinogeny (Stocker et al.,
1963).

Certain colicin factors can integrate into the K12 chromosome,
forming Hfr strains. ColVB-K260 gave rise to an Hfr with xyl as
the leading marker, but the strain produced only colicin B
(Fredericq, 1965, 1969). Kahn (1968) isolated 18 independently
derived Hfr strains from K12 (ColV-K94) but the site of
integration varied with different K12 derivatives.

Effect of plasmids on F fertility

Some R factors interfered with F-mediated fertility in K12
(Nakaya et al., 1960; Watanabe and Fukuasa, 1962). The
introduction of such plasmids into a K12F+ or Hfr strain reduced
the ability of these hosts to transfer the F factor and chromosomal markers. There was also loss of visible lysis by F-specific phages (Egawa and Hirota, 1962; Watanabe, Fukasawa and Takano, 1962), and abolition of agglutination by F-specific antiserum (Hirota et al., therefore 1964). R factors were divided into two types on the basis of their effect on F fertility. Those that inhibited F fertility were termed $\mathbf{\lambda}^+$ or $\mathbf{fi}^+$; R factors without any effect on F fertility were designated $\mathbf{fi}^-$ (Egawa and Hirota, 1962; Watanabe and Fukasawa, 1962; Watanabe et al., 1964). This subdivision was also applied to other types of transferable plasmid, such as colicin factors (Meynell and Datta, 1966b).

The inhibition of F fertility was attributed by Egawa and Hirota (1962) to a cytoplasmic repressor, encoded by the R factor, which acted on F. This interpretation was supported by Meynell and Datta (1965) who suggested further that the repressor also acted on the R factor itself, maintaining it in a repressed state. HFT cultures could be explained on this hypothesis, assuming that cells newly infected with an R factor would not have sufficient repressor for inhibition of transfer. The HFT state is analogous to the burst of $\beta$-galactosidase synthesis in K12 when the $\xi^+$ gene of the lac operon is transferred to a $\xi^-\mathbf{fi}^-$ host (Pardee, Jacob and Monod, 1959).

Several mutant R factors which are no longer self-repressed have been described (Egawa and Hirota, 1962; Nishimura et al., 1967; Meynell and Datta, 1967; Hoar, 1970; Grindley et al., 1971; Silver and Cohen, 1972). The mutant of the $\mathbf{fi}^+$ R factor R100 (termed R100-1)
no longer inhibited chromosomal transfer in Hfr strains, and it was suggested that this mutant coded for a defective repressor (Egawa and Hirota, 1962). Strains carrying these "derepressed" R factors sex-transfer the plasmid at high frequency, are agglutinated by fimbrial-specific antisera, and are visibly lysed by F-specific phages. If the fertility of these $F^+$ R factors is regulated by a cytoplasmic repressor there should be at least two classes of derepressed mutant. There would be mutants lacking a functional repressor and those insensitive to inhibition, analogous to the repressor-minus (i$^-$) and operator-constitutive (o$^c$) mutations of the lactose operon (Jacob and Monod, 1961). These alternatives have been distinguished by examining strains carrying a mutant R factor and wild-type $F^+$ plasmid (Frydman and Meynell, 1969; Hoar, 1970; Grindley et al., 1973a).

Studies by Finnegan and Willetts (1971) suggested that the inhibition of F transfer by $F^+$ R factors required not only a product of the R factor but also a product encoded by F itself. Cells carrying the $F^+$ R factor R100, and newly infected with Flac, could retransfer Flac at high frequency. However when these cells carried both R100 and a second F factor (Fhis), retransfer of Flac was inhibited. The component specified by an R factor locus was designated $fin$ (= fertility inhibition) and the plasmid-specific product encoded by the F factor itself was termed $P_F$. By analogy with the inhibition of F transfer, it was also suggested that a $fin$ product and a plasmid-specific product encoded by an R factor ($= P_R$) were necessary for inhibition of R factor transfer. This hypothesis
has been supported by studies on several F-like plasmids (Finnegan and Willetts, 1972; Grindley et al., 1973a). Two $f_1^+$ R factors, $R_{100}$ and $240$, appear to code for the same or a very similar $P_R$ product, which is distinguishable from the $P_R$ product of another $f_1^+$ R factor $33^4 (= R1)$ (Grindley et al., 1973a).

**F-like and I-like classes**

Cells carrying the F factor produce specific hair-like appendages, "fimbriae" or "pili" (Crawford and Gesteland, 1964; Brinton, Gemski and Carnahan, 1964; Brinton, 1965; Duguid, Anderson and Campbell, 1966), which act as receptors for F-specific phages (Loeb, 1960; Loeb and Zinder, 1961). A number of such phages have been described: some, such as f2, Q8, MS2 and µ2 are isometric, and others, such as f1 and fd, are filamentous (review, Zinder, 1965). F fimbriae are necessary for conjugation, and Brinton (1965) suggested that the fimbriae acted as the conjugation bridge.

The relationship between F and $f_1^+$ R factors was explored by Meynell and Datta (1965, 1966a), who examined the ability of R factor-carrying strains to support multiplication of F-specific phages. Phage MS2 multiplied in many of these cultures, and the proportion of phage-sensitive bacteria was increased in HFT preparations. In contrast, no multiplication of the F-specific phages was observed with strains carrying $f_1^-$ R factors. Electron microscopy of HFT cultures demonstrated the presence of sex fimbriae closely resembling those detected in $F^+$ lines (Lawn, 1966; Datta, Lawn and Meynell, 1966). Both types of sex fimbriae acted as receptors for F-specific
phages. The incidence of bacteria with sex fimbriae in different HFT cultures was correlated with the frequency of resistance transfer, and with the proportion of cells sensitive to phage MS2. These results supported the hypothesis that the structural genes for fimbrial synthesis of \( f^+ \) plasmids were normally under the control of a cytoplasmic repressor which could also act on the F factor. Cells carrying derepressed mutants of \( f^+ \) R factors showed a high degree of sex fimbriation; these included lines carrying R100-1 (Nishimura et al., 1967) and R1dr (Maynell and Datta, 1967).

Some of the colicin factors also appeared to be very closely related to the F factor because lines carrying ColV-K94 or ColV-K30 were visibly lysed by F-specific phages (Macfarren and Clowes, 1967). The ColB factors for example, ColB-K77 and ColB-K98, were self-repressed and resembled many \( f^+ \) R factors (Maynell and Datta, 1966b).

Although the fimbriae coded for by F and related plasmids were similar both morphologically and serologically, in some cases differences were found when cross-absorbed sera were used (Lawn, 1966; Lawn et al., 1967; Lawn and Maynell, 1970). Differences in sex fimbriae can also be detected by inefficient adsorption of F-specific phages in some cases (Nishimura et al., 1967; Macfarren and Clowes, 1967; Willetts, 1971).

The \( f^- \) R factors are self-repressed but do not inhibit F fertility. HFT cultures can be produced, as had been first demonstrated with the \( f^- \) ColIb factor (Stocker et al., 1963). Derepressed mutants of \( f^- \) R factors were isolated by selecting clones
which transferred drug resistance markers at high frequency (>10^{-1} in a 30 min mating) (Meynell and Datta, 1967). Electron microscopy of HFT cultures carrying ColIb-P9 first demonstrated the presence of sex fimbriae which were morphologically distinct from F fimbriae (Meynell and Lawn, 1967). These 'I fimbriae' were also found on cells carrying R64dr and R144dr, which are derepressed mutants of fli R factors. Filamentous DNA-containing phages were isolated which visibly lysed strains carrying these derepressed mutants (Meynell, 1967; Meynell and Lawn, 1968). The two phages were designated If1 and If2.

As a result of these studies of sex fimbriae, plasmids were classified by their ability to confer on their hosts the property of F-specific or I-specific phage multiplication. Such plasmids were termed F-like or I-like respectively (Meynell, Meynell and Datta, 1968). Most of the fli plasmids determine the synthesis of F-like fimbriae, while many fli plasmids code for the synthesis of I-like fimbriae and consequent I-specific phage propagation. However a number of R factors have been described which are both fli and I-like (Romero and Meynell, 1969; Grindley and Anderson, 1971). Some plasmids did not confer the ability to propagate F- or I-specific phages on their hosts (Lawn et al., 1967; Meynell et al., 1968). It was suggested that these R factors were either of a different type, or that cells carrying them produced too few sex fimbriae to detect an increase in phage titre.

The F-like and I-like plasmids have been further investigated by isolation of transfer-deficient mutants (Cusin and Jacob, 1967)
Ohtsubo, Nishimura and Hirota, 1970; Willetts, 1970; Achtman, Willetts and Clark, 1971). However, only the transfer system of the F factor has been examined genetically. Twelve cistrons required for F transfer have been identified, and mutations in all twelve cistrons are recessive to the wild-type alleles (Ohtsubo et al., 1970; Achtman et al., 1971). Since strains carrying many of the mutants are resistant to all F-specific phages and lack F fimbriae, the products of these cistrons in the wild-type F factor may form a biosynthetic pathway responsible for synthesis, modification and assembly of the F-fimbriae (Willetts and Achtman, 1972). Other mutant lines which still produce fimbriae may lack products necessary for DNA metabolism in the donor cell which is associated with transfer.

Two F-like colicin factors ColV-K94 and ColV-trp appear to have transfer systems which are indistinguishable from that of F because they complemented all eleven F tra cistrons tested (Willetts, 1972a). The R factors R100-1 and R1-19 complemented mutants in ten of twelve tra cistrons (Willetts, 1971). The exceptions were the tra I cistron and the control tra J cistron and it was concluded that the products of these two cistrons were plasmid-specific. Two I-like R factors, R64-11 and Collbtd, did not complement any mutants in the F tra cistrons and therefore have a transfer system which is genetically unrelated to that of the F factor (Willetts, 1970).
Incompatibility and surface exclusion

The inability of certain pairs of plasmids to coexist stably in the same cell has been used in the classification of R factors and other bacterial plasmids. This phenomenon, termed incompatibility, was first demonstrated by Scaife and Gross (1962). They found that an Flac factor could not multiply in Hfr cells and they were unable to isolate lines carrying both F and Flac. Further studies showed that Fgal and Flac were incompatible with progeny inheriting either one factor or the other (de Haan and Stouthamer, 1963). Certain pairs of fi+ R factors were also incompatible but fi+ and fi- plasmids were stably maintained in the same cell (Watanabe et al., 1964). Superinfection experiments with the A-mediated transfer systems demonstrated that A and its derived R factor T-A cannot coexist stably (Anderson, 1966). Incompatibility has also been demonstrated for non auto-transferring plasmids such as the resistance determinants SSu and ASu (Anderson et al., 1968).

In the last few years several classes or "compatibility" groups* have been defined (Romero, 1970; Khatoon and Iyer, 1971; Hedges and Datta, 1971, 1972; Datta and Hedges, 1971; Chabbert et al., 1972; Grindley, Grindley and Anderson, 1972; Datta, 1975). In general, plasmids within a group exhibit similar kinetics of transfer. Plasmids belonging to groups N and P can also be identified by lysis of their host strains with sex-specific phages. The phage Ike has been isolated for strains carrying members of the N group (Khatoon, Iyer and Iyer, 1972), while phage PRR1 lyses strains carrying

*Members of each compatibility group can generally coexist stably with those of other groups, but are incompatible with each other.
P group R factors (Olsen and Shipley, 1973). With Ike and PRR1 there is visible lysis, in surface spot tests, of the appropriate indicator strains carrying the wild-type R factors. This contrasts with F-like and I-like plasmids where visible lysis is only observed with lines carrying plasmids derepressed for transfer, such as F or T-Adr1. Phages such as Ike and PRR1 thus provide a useful tool for the rapid identification of certain plasmids.

Studies in this laboratory have demonstrated that plasmids belonging to one compatibility group do not necessarily coexist stably with members of all other groups (Smith et al., 1973a). Four R factors were described which fell into a single compatibility group designated group H (Anderson and Smith, 1972a; Grindley et al., 1972). On transfer to K12F*, three of the plasmids usually displaced the F factor while the remaining R factor coexisted stably with F. DNA reassociation experiments showed that there is little or no homology between F and the four group H plasmids. The ability of three H group R factors to displace F cannot therefore be explained in terms of genetic similarity. The displacement of a plasmid of one group by an R factor belonging to another group has also been reported by Coetzee, Datta and Hedges (1972).

Two models have been proposed for plasmid replication and incompatibility. The first is based on the replicon hypothesis which postulates that replicons require attachment to a specific membrane site for both replication and uniform segregation of replicons into daughter cells at cell division (Jacob, Brenner and Cuzin, 1963). Incompatibility would result in competition between two plasmids for
the same site, leading to establishment of only one plasmid with loss of the other. On the other hand, compatible plasmids would occupy different sites. This model has been suggested by several groups to explain plasmid incompatibility (Kahn and Helinski, 1964; Watanabe et al., 1964; Anderson, 1966). Lines carrying incompatible plasmids usually show rapid loss of one plasmid or the other with only a minority of cells still carrying both plasmids. With suitable selection, recombinants of the two plasmids can be obtained. In some cases the incompatibility between plasmids is asymmetric (Macfarren and Clowes, 1967; Anderson et al., 1968; Frydman and Meynell, 1969; Grindley et al., 1972; Nordström, Ingram and Lundbäck, 1972). For example, ColV2-K94 is retained in preference to the F factor regardless of whether the colicinogenic factor is in the donor or recipient strain.

In the second model it is postulated that the plasmid codes for a specific inhibitor of replication which is synthesized immediately after initiation and is then diluted out during cell growth. The inhibitor would prevent replication of a closely-related superinfecting plasmid. This repressor hypothesis has been proposed for the incompatibility of F in Hfr strains (Dubnau and Maas, 1968; Pritchard, Barth and Collins, 1969). However, the integrated F factor may remain attached to the F maintenance site although this attachment may not be necessary for vegetative replication.

The property of surface or entry exclusion is often found in
crosses between strains carrying incompatible plasmids. There is a reduction in transfer frequency into the strain carrying the closely-related plasmid compared with transfer into the same recipient lacking a plasmid (Lederberg, Cavalli and Lederberg, 1952; Watanabe, 1963b; Watanabe et al., 1964). It is postulated that entry exclusion results from a change in the cell surface that is specified by the plasmid. This is supported by studies on "DNA-less minicells". The F factor is not excluded from minicells derived from an F− parent but is excluded from minicells originating from an F+ parent. F factor DNA was absent from these cells and very few cells possessed F fimbriae (Cohen et al., 1967; Sheehy, Orr and Curtiss, 1972).

Falkow and his coworkers have studied entry exclusion by measuring the incorporation of labelled plasmid DNA following its entry into a recipient cell. There was a reduction of 80 to 90% in the rate of 3H-thymine incorporation when the recipient carried a plasmid closely related to the R factor in the donor strain (Falkow et al., 1971; Leblanc and Falkow, 1973).

Mechanisms of resistance to antibacterial agents

Resistance of bacteria to antibiotics and other drugs could result from the following mechanisms.

1) The synthesis by the resistant bacteria of an enzyme inactivating the drug.

2) Loss of accessibility of the drug to the cell.

3) Alteration in the target of the antibacterial agent.
Transferable drug resistance appears to result from any of the three mechanisms.

**Penicillin resistance.** Studies on transferable ampicillin (and penicillin) resistance in *S. typhimurium* demonstrated the production of an enzyme which destroyed the antibiotic (Anderson and Datta, 1965). This enzyme was shown to be a β-lactamase (Anderson and Lewis, 1965a; Datta and Kontomichalou, 1965). A number of different β-lactamases have been identified by their substrate specificity, their immunological properties, electrophoretic mobilities and sensitivity to p-mercuribenzoate and cloxacillin (Jack and Richmond, 1970; Richmond *et al.*, 1971; Richmond and Sykes, 1973). The enzymes were grouped into five main classes, with the majority of R factors coding for enzymes with type III properties. The resistance level or enzymatic activity of the ampicillin resistance factors can change upon transfer from one host to another (Smith, 1969). Mutations in the host chromosome can also influence the expression of ampicillin and other resistances.

**Resistance to aminoglycoside antibiotics.** There are three different mechanisms by which the aminoglycoside antibiotics are known to be inactivated: acetylation of amino groups, phosphorylation of hydroxyl groups or adenylylation of hydroxyl groups. At least nine enzymes have been identified so far, three acetylating, four phosphorylating and two adenylylating enzymes.

Okamoto and Suzuki (1965) first demonstrated that a cell-free extract from an R factor-carrying strain of *E. coli* inactivated
kanamycin in the presence of acetyl coenzyme A. It was later shown that the antibiotic had been acetylated.

Two further acetylating enzymes have been characterised which can be distinguished by their substrate specificities (Brzezinska et al., 1972; Benveniste and Davies, 1973a).

Streptomycin can be inactivated by adenylylation (Umezawa et al., 1968; Yamada, Tipper and Davies, 1968). Strains carrying R factors that inactivated streptomycin by this mechanism were also resistant to spectinomycin which was inactivated in the same way (Benveniste, Yamada and Davies, 1970; Smith et al., 1970). A gentamicin adenylyltransferase has also been identified and partially purified (Benveniste and Davies, 1971). Streptomycin and other aminoglycosides can also be inactivated by phosphorylation (Ozanne et al., 1969) but the enzyme does not affect spectinomycin.

Chloramphenicol resistance. Chloramphenicol is inactivated by cell extracts from resistant strains in the presence of acetyl coenzyme A (Suzuki and Okamoto, 1967; Shaw, 1967). The enzyme chloramphenicol acetyltransferase has been purified from R factor-carrying strains and shown to be synthesised constitutively and located intracellularly (Shaw and Brodsky, 1968). The enzymes coded by several $\phi^+$ R factors were indistinguishable but the chloramphenicol acetyltransferase determined by the $\phi^-$ R factor R387 showed several differences from the enzyme coded by the $\phi^+$ R factors (Shaw, Sands and Datta, 1972). R factor-carrying strains which conferred resistance to chloramphenicol, but did not result in the inactivation of the drug, have been reported
(Baudens and Chabbert, 1967; Nagai and Mitsuhashi, 1972). The resistance was thought to result from a selective decrease in permeability.

**Resistance to other antibacterial drugs.** The biochemical mechanism for resistance to tetracyclines is not clearly understood, but seems to involve a decreased uptake of the drug by R+ strains (Izaki and Arima, 1963; Franklin and Godfrey, 1965). Tetracycline resistance encoded by R factors is inducible (Franklin, 1967; Franklin and Higginson, 1967) so that the level of resistance is increased after exposure to subinhibitory concentrations of the drug. Mutants which appear to be constitutive and resistant to a high level of tetracycline have been isolated (Franklin and Cook, 1971). As an explanation for these findings it has been suggested that there is induced synthesis of an inhibitor of transport of tetracycline.

Plasmid-mediated sulphonamide resistance may result from a reduced permeability of the membrane to the drug (Akiba and Yokota, quoted by Watanabe, 1963b).

**Trimethoprim resistance encoded by R factors** has recently been explained in terms of an altered target site of the antibacterial agent. The R factor-carrying strain synthesises an altered dihydrofolate reductase which has a larger molecular weight than the wild-type enzyme and is much less susceptible to trimethoprim (Amyes and Smith, 1974; Sköld and Widh, 1974).
Molecular studies of bacterial plasmids

One of the first direct demonstrations that plasmids were composed of DNA was the investigation of an Flac factor in Serratia marcescens (Marmur et al., 1961). Analytical ultracentrifugation in caesium chloride showed that Flac was present as a separate satellite band of DNA which was readily distinguished from the host chromosomal DNA. Similar experiments involved Proteus mirabilis strains carrying F factors, colicin factors and R factors (Wohlhieter et al., 1964; Falkow et al., 1964a, b, 1966; DeWitt and Helinski, 1965).

The procedure first used for preparation of plasmid DNA resulted in breakage of the DNA into fragments of less than 10 x 10^6 molecular weight (Marmur, 1961). A later method involved formation of spheroplasts and lysis with the detergent sodium lauryl sulphate. Protein was removed with phenol and the resulting preparation was centrifuged in caesium chloride to separate the plasmid DNA from the chromosomal DNA. These experiments were performed in hosts where there was a buoyant density difference between the plasmid and chromosomal DNA. Electron microscopy revealed that plasmid molecules were detected to a substantial extent in a covalently-closed circular DNA configuration (Roth and Helinski, 1967; Hickson, Roth and Helinski, 1967; Freifelder, 1968a). This form has now been demonstrated for a large number of R factors and other plasmids (reviews, Glovers, 1972; Helinski and Clewell, 1971; Helinski, 1973). Closed-circular molecules
have a supercoiled configuration which provides the molecules with decreased viscosity and is less sensitive to shear. Therefore a supercoiled molecule sediments faster than an open circular form or linear DNA. A mixture of chromosomal and plasmid DNA can be subjected to conditions under which the chromosomal DNA can be fragmented while plasmid DNA is left intact because of its smaller size and covalently-closed circular form. This is achieved by shearing the DNA and then by brief exposure to high temperature or alkali which results in denaturation of the DNA. If the pH is returned to neutrality the covalently-closed DNA renatures and can be separated by passage through a nitrocellulose column which retains the alkali-denatured and single-stranded material (Cohen and Miller, 1969). Lysates which have been sheared and denatured with alkali can also be subjected to alkaline sucrose gradient centrifugation (Freifelder, 1968a, b). In this state the closed-circular DNA sediments three to four times more rapidly than linear or open circular DNA (Weil and Vinograd, 1963; Vinograd et al., 1965).

The purification of closed-circular DNA molecules was much improved by a procedure using the intercalating dye ethidium bromide (Radloff, Bauer and Vinograd, 1967; Bauer and Vinograd, 1968). When DNA preparations are centrifuged to equilibrium in ethidium bromide-cassium chloride density gradients, the covalently-closed circular DNA bands at a higher density than open circular or linear DNA. This occurs because there is less binding of ethidium bromide by the closed-circular form, and consequently a
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smaller decrease in density of these closed-circular molecules. This dye-buoyant density procedure is particularly useful in cases where the circular plasmid DNA has a buoyant density identical to the chromosomal DNA. Plasmids have now been shown to exist as covalently-closed circles within the cells but a small proportion of molecules may be present in the open circular form or as the linear duplex form (Freifelder, Folkmanis and Kirschner, 1971). The isolation procedure employed obviously affects the amount of plasmid DNA recovered as covalently-closed circular DNA molecules. This is clearly demonstrated by the presence of the DNA of certain plasmids in the form of a supercoiled DNA-protein relaxation complex (Clewell and Helinski, 1969). Treatment with pronase, Sarkosyl, ethidium bromide or certain other agents results in the conversion of the supercoiled DNA existing as relaxation complex to an open circular form which bands with linear chromosomal DNA in a density gradient.

A number of plasmids including F, ColE1, ColE2, A and R6K have been found mainly in the relaxation complex form (Clewell and Helinski, 1969; Kline and Helinski, 1971; Humphreys, Grindley and Anderson, 1972; Helinski, 1973). For ColE1, ColE2 and F, the open circular DNA has a single nick in the "Crick" strand (Clewell and Helinski, 1970; Blair et al., 1971). The protein in the relaxation complex may be a latent strand-specific endonuclease that is activated by proteases and other agents. This nicking enzyme may be involved in replication and possibly the transfer of plasmids (Clewell and Helinski, 1969; Helinski et al., 1973).
Other procedures for isolation of plasmid DNA do not depend on the closed-circular nature of plasmid molecules. Gentle lysis with non-ionic detergent Brij 58 and sodium deoxycholate followed by low speed centrifugation results in sedimentation of more than 99.5% of the chromosomal DNA (Godson and Sinsheimer, 1967; Clewell and Helinski, 1969). The plasmid DNA remains in the supernatant which is termed the "cleared lysate".

Another procedure involves the use of E. coli "minicells" which lack chromosomal DNA. These can be formed in large numbers from the abnormal cell division of a mutant K12 strain (Adler et al., 1967). Minicells can be separated from the parental cells by differential centrifugation, followed either by successive sucrose gradients or by growth in penicillin (Adler et al., 1967; Levy, 1970). The resulting fraction of parental cells to minicells is approximately 1 in $10^6$; these purified minicells can be lysed by standard methods. Several plasmids segregate efficiently into minicells including F-like and I-like R factors and the non-transferring ColE1 (Inselburg, 1970; Levy and Norman, 1970; Levy, 1971a). However, the F factor segregates with very low efficiency (<1%) into minicells (Kass and Yarmolinsky, 1970).

The molecular studies in E. coli indicate that most plasmids fall into one of two groups. Transferable plasmids have molecular weights exceeding $20 \times 10^6$ daltons, and are usually present as approximately one copy per chromosome. On the other hand, non-transferring plasmids have a lower molecular weight ($<10 \times 10^6$) and exist as multiple copies per chromosome. However, the R factor R6K, with a molecular weight of $26 \times 10^6$ daltons, is autotransferable but
is present in multiple copies (13 - 38) per chromosome (Kontomichalou, Mitani and Clowes, 1970).

The division of transfer systems into Classes 1 and 2 is supported by molecular studies in E. coli. Watanabe and coworkers concluded that R factors were composed of two linked units: the resistance transfer factor (RTF) and the drug resistance genes. Studies of R222 (CSSuT) established linkage relationships between the genetic markers which could be located on a circular genetic map (Watanabe, 1963b). The molecular weights of a number of segregants of R222 were estimated by electron microscopy. It was established that the C, S and Su genes were closely linked, whereas the T marker was located in a separate but adjacent segment of the circular molecule (Nisioka, Mitani and Clowes, 1970).

Molecular studies on the plasmids of the A transfer systems confirmed the postulates based on the genetic evidence. The resistance determinants A and SSu and the transfer factor A exist as independent covalently-closed circular DNA molecules in K12. The molecular weights of A, SSu and A are approximately 5.6, 5.7 and 59 x 10^6 daltons respectively (Smith, Anderson and Clowes, 1970; Humphreys, Grindley and Anderson, 1972; Smith, Humphreys and Anderson, 1974). Cells carrying T-A yield only one class of plasmid molecule with a molecular weight of 62 x 10^6 daltons. The size of this recombinant R factor would be expected if the T determinant region of T-A were about the same size as the A and SSu determinants. A and SSu are present as multiple copies per chromosome whereas there is approximately only one copy of A (or T-A)
Dissociation of R factors

The physical properties of certain R factors have also been studied in *Proteus mirabilis*. In this host the R factors R222, R1 and R6 are found as three size classes of covalently-closed circular DNA molecules. For R222 the molecular weights are $68 \times 10^6$, $54 \times 10^6$ and $12 \times 10^6$ with buoyant densities of 1.711, 1.709 and 1.717 respectively (Nisioka, Mitani and Clowes, 1969). It has been concluded that the largest species, which is the form predominantly found in *E.coli* K12, is a composite of the two smaller molecules. Cohen and Miller (1969, 1970a) examined R1 DNA in *E.coli* K12 and found that about 5% of the total number of molecules were small, with a molecular weight of $10 \times 10^6$. K12 lines carrying the transfer factor of R1, but lacking all the resistance markers, were isolated as a result of spontaneous loss and by transfer from *P.mirabilis* carrying R1 (Cohen and Miller, 1970b; Silver and Falkow, 1970; Haapala and Falkow, 1971). The transfer factor had a molecular weight of $54 \times 10^6$ and a density of 1.709, corresponding to one of the DNA species detected in *P.mirabilis* carrying R1. It was concluded that in *P.mirabilis* certain R factors dissociate into two components corresponding to the (resistance) transfer factor and the component that codes for drug resistances, termed the *r* unit (Falkow, Haapala and Silver, 1969; Nisioka et al., 1969; Cohen and Miller, 1970a).

When *P.mirabilis* carrying R100 (= R222) (CBS47) is grown for several generations in chloramphenicol or streptomycin there is a
marked increase in the component of density 1.717 (Rownd, 1969; Falkow et al., 1969). This phenomenon is reversible when the cells are transferred to a medium lacking antibiotics. In the presence of antibiotics the level of resistance to chloramphenicol and streptomycin (but not tetracycline) is much increased (Rownd et al., 1973). It has been suggested that under these conditions there is incorporation of many copies of the \( r \) determinant into the complete \( R \) factor with selection for those cells with the greatest number of copies of the \( r \) determinant. After transfer to an antibiotic-free medium it is postulated that there is a dilution of any dissociated \( r \) determinant by cell division (Rownd et al., 1973).

Dissociation of \( R \) factors in \( P. mirabilis \) has been studied by other workers who suggested an alternative explanation to that of Rownd (Kopecko and Punch, 1971; Punch and Kopecko, 1972). These authors postulated that the presence of antibiotics results in the relaxed replication of the \( r \) determinant in both monomeric and multiple-circular DNA forms. Recombination could occur between the transfer factor and the different forms of the \( r \) determinant.

The dissociation of \( R100 \) into three DNA species in a minicell-producing strain of \( S. typhimurium \) has also been described (Sheehy et al., 1973). Studies in this laboratory have shown that several \( R \) factors of different compatibility groups dissociate in \( S. typhimurium \) type 36. \( R1-19 \) (FII), \( TP123 \) (H1), \( TP116 \) (H2) and \( TP125 \) (B) dissociate in this \( S. typhimurium \) host but usually form a single molecular species in K12 (Humphreys, Willsaw and Anderson, 1974).
DNA reassociation has been measured between various plasmid DNAs, using hydroxyapatite to separate single-stranded from double-stranded DNA. Guerry and Falkow (1971) found 74% homology between the two $\text{fi}^+$ F-like R factors R1 and R222 and that 38% of R1 was homologous with the F factor. However, R1 has little or no homology with two I-like plasmids and an N group R factor. Reassociation studies in this laboratory with members of several R factor compatibility groups showed that incompatible plasmids usually have a high degree of homology with each other (Grindley, Humphreys and Anderson, 1973b). Little or no DNA homology was found between plasmids of different compatibility groups. However, one R factor, TP116, belonging to group H, has minimal homology with the other members of the group which are closely related to each other. It was also interesting that there was no detectable homology between plasmids of groups I1 and I2. Members of these groups enable their host strains to propagate the I-specific phage If1 but belong to two distinct compatibility groups (Grindley et al., 1972).

Electron microscopy techniques can be used to determine the extent and relative location of homology in heteroduplexes formed by the denaturation and renaturation of a mixture of two different plasmid molecules (Sharp et al., 1972; Sharp, Cohen and Davidson, 1973). In the case of $\text{fi}^+$ F-like R factors virtually all the sequences present in R222 were also found in R6, while R1 and R6 showed less homology with each other. No homology was found between
the $f^{+}$ F-like R factors and the $f^{-}$ I-like plasmid R64. Heteroduplex analysis of the F factor revealed that 90% of the nucleotide sequences in one half of F were present in both R1 and R6 molecules, thus indicating the localisation of genes responsible for fertility of F and F-like R factors.

**Maintenance and replication of plasmids**

The replicon hypothesis proposes that discrete genetic elements which are independent units of replication are attached to specific membrane sites which govern replication and segregation (Jacob et al., 1963). There is evidence for the association of the growing point of the replicating chromosome of E.coli (and B.subtilis) with rapidly sedimenting cellular material with membrane properties (reviews, Helinski and Clewell, 1971; Pato, 1972). A similar observation has been found with CoLE1 DNA (Helinski, 1973). Multiple points of association between DNA and the membrane have been suggested from studies of plasmid DNA in minicells (Shull et al., 1971; Levy, 1971b) and for the E.coli chromosome (Rosenberg and Calvalieri, 1968).

There are a number of conflicting reports regarding the timing of replication of various plasmids in relation to chromosomal replication and the cell cycle. However, a mechanism that coordinates cell division with plasmid DNA replication is required to account for the observed stability of most plasmids. The replicon hypothesis proposes that there is a unit of segregation and this is supported by the observation that a temperature-sensitive F'ac mutant always
segregated with a specific strand of the chromosome at cell division over many generations when the cells were grown at 42°C (Cuzin and Jacob, 1967; Hohn and Korn, 1969).

Transfer of plasmids

Only one of two DNA strands of F, F-like or I-like factors is transferred to the recipient during conjugation in E.coli (Vapnek and Rupp, 1970; Vapnek, Lipman and Rupp, 1971). In the case of each of these types of plasmid it is the "heavy" strand that is transferred with the 5' end first by an asymmetric (possibly rolling circle) type of mechanism. The untransferred strand is conserved in the donor and replicated during conjugation. The origin of transfer of F lac has now been localised between the traJ and ϕII R loci (Willetts, 1972b).

Replication of plasmid DNA appears to occur both in the donor and recipient, but it is not known if this replication is necessary for conjugation (reviews, Curtiss, 1969; Brinton, 1971). Experiments with temperature-sensitive E.coli mutants show that the transfer of F occurs at the restrictive temperature, where chromosomal DNA synthesis is inhibited, thus indicating plasmid-specific replication during transfer (Marinus and Adelberg, 1970; Vapnek and Rupp, 1971). In contrast, F transfer is inhibited at the restrictive temperature in a chromosomal DNA initiation mutant of S.typhimurium (Spratt and Rowbury, 1971). Temperature-sensitive chromosomal mutants of E.coli that are normal in chromosomal DNA replication but defective in ColE1
replication at the restrictive temperature are also defective in ColE1 transfer (Kingsbury and Helinski, 1973). Initiation of transfer possibly involves the synthesis or activation of a plasmid-determined endonuclease specific for the "origin" of transfer present on a particular strand of the plasmid DNA.

The transfer of an R factor has been analysed by following specifically labelled newly-transferred R1 DNA (Falkow et al., 1971). It was suggested that after transfer the single-stranded DNA is bound to the membrane and converted sequentially to the linear double strand, the open circular DNA form and then after release from the membrane, to covalently-closed circular DNA. The formation of this latter circular form may involve cohesive ends or recombination between repeating regions in a transferred linear molecule of greater than unit length (Ohki and Tomizawa, 1968; Matsubara, 1968).

The mechanism by which a transfer factor mobilises an independent genetic determinant is not clear. It has been suggested that the determinants may pass through the conjugation tube without any physical linkage between it and the transfer factor. Alternatively, a limited amount of hydrogen bonding may occur between the transfer factor and the resistance (or other) determinant while both are in the single-stranded state (Anderson, Mayhew and Grindley, 1969).
Scope of this thesis

Bacterial plasmids can be classified on the basis of whether or not they can promote their own transfer. Most studies on the classification of plasmids have concentrated on those which are auto-transferring. A number of non auto-transferring plasmids were therefore investigated in an attempt to subdivide them on a genetic and molecular basis.

Most of the plasmids were identified in wild strains of enterobacteria sent to the Enteric Reference Laboratory. The plasmids code for antibiotic resistance, colicinogeny or the ability to inhibit the fertility of the F factor, that is, the $fi^+$ character. One of the most useful criteria for investigating interrelationships between plasmids is that of incompatibility, since closely related plasmids are unable to coexist stably in the same cell. Compatibility tests were performed to define groups of non auto-transferring plasmids.

Molecular studies included measurement of contour lengths of the plasmids for determination of molecular weights. For plasmids of known molecular weight the number of copies of the plasmid per chromosome was calculated from the percentage of total labelled DNA that was recovered as plasmid DNA. DNA reassociation experiments were performed to examine the degree of DNA homology between some of the plasmids.
Materials and Methods

Materials

1. **Standard bacterial strains and plasmids.** The standard strains and plasmids used in this thesis are shown in Tables 1, 2 and 3.

2. **Plasmids and wild strains studied.** These are listed in Tables 4 and 5. Most of the plasmids were isolated from wild strains sent to the Enteric Reference Laboratory for phage-typing or studies of drug resistance.

3. **Bacteriophages.** The F-specific phages were φ2 (Dettori, Maccacaro and Piccinin, 1961) and fd (Marvin and Hoffman-Berling, 1963). The I-specific phage was If1 (Meynell and Lawn, 1968). K12 strains carrying plasmids were also tested with the "female-specific" phage φ2 (Cuzin, 1965). The DNA of phage φX174 was used as the standard for contour length measurements (see Section II). The Enteric Reference Laboratory phages used in testing strains of *S. typhimurium* were mainly as described by Callow (1959). The salmonella O1 phage (Felix and Callow, 1943) was used to eliminate donor strains in conjugation experiments.

All the phages were prepared by the agar-overlay method (Adams, 1959) and the preparations were sterilized of bacteria with toluene (Anderson and Felix, 1953) or in the case of O1 phage by incubating at 57°C for 40 min. The strains used for
propagation and the titre of the phage preparations were as follows:

<table>
<thead>
<tr>
<th>Phage</th>
<th>Strain</th>
<th>Titre (pfu/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>μ2</td>
<td>K12-983 Hfr Broda 10</td>
<td>$10^{11}$</td>
</tr>
<tr>
<td>fd</td>
<td>K12-983 Hfr Broda 10</td>
<td>$10^{12}$</td>
</tr>
<tr>
<td>If1</td>
<td>24R468 S. typhimurium 36 T-Δdrp1</td>
<td>$10^9$</td>
</tr>
<tr>
<td>φ2</td>
<td>1R713 K12 F&quot;</td>
<td>$10^{10}$</td>
</tr>
<tr>
<td>O1</td>
<td>S. typhi O1</td>
<td>$10^{12}$</td>
</tr>
</tbody>
</table>

4. **Media**

**Nutrient broth.** This contained 20g/litre of "Bacto" dehydrated nutrient broth (Difco) and 8.5g/litre of sodium chloride (pH = 6.8).

**Nutrient agar.** Nutrient broth was solidified with 13g/litre of New Zealand powdered agar. 0.4% nutrient agar was used to pour layers in colicinogeny and phage experiments.

**L broth.** This contained 10g/litre Bacto-tryptone, 5g/litre Difco yeast extract and 5g/litre of sodium chloride (pH 7.0).

**L agar.** L broth was solidified with Davis agar (1%) 2,3,5-triphenyl-2H-tetrazolium chloride (20 μg/ml) and lactose (1%) were added for differentiation between lactose and non-lactose fermenting bacteria.

**MacConkey agar.** Oxoid MacConkey agar No. 3 was used to differentiate between lactose and non-lactose fermenting bacteria.

"Laked-blood" agar. 5 ml of lysed horse blood was added to 100 ml nutrient agar. Sulphathiazole (100 μg/ml) was added to the molten agar in the preparation of plates for testing sulphonamide resistance.
Minimal medium. 3 g of Davis New Zealand agar was dissolved in 175 ml distilled water. 20 ml of a salts solution, 2 ml of 25% glycerol and 1 ml of 4% Mg SO₄ were added to the molten agar. L-amino acids were also added as required at a final concentration of 20 μg/ml. The salts solution contained per litre:

- \( \text{Na}_2 \text{HPO}_4 \cdot 2\text{H}_2\text{O} \) 35 g
- \( \text{KH}_2 \text{PO}_4 \) 30 g
- NaCl 5 g
- \( (\text{NH}_4)_2 \text{SO}_4 \) 10 g
- Fe \( \text{SO}_4 \) 0.005 g

5. Antibacterial agents. Antibiotics and other drugs were added in solution to molten agar before pouring the plates. Details of these chemicals and the concentrations used in plates are as follows:

<table>
<thead>
<tr>
<th>Name</th>
<th>Source</th>
<th>Concentration in plates (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin (Penbritin)</td>
<td>Glaxo Laboratories</td>
<td>100</td>
</tr>
<tr>
<td>Benzylpenicillin B.P. (Crystapen)</td>
<td>Glaxo Laboratories</td>
<td>100</td>
</tr>
<tr>
<td>Chloframphenicol B.P. (Chloromycetin)</td>
<td>Parke, Davis &amp; Co.</td>
<td>20</td>
</tr>
<tr>
<td>Kanamycin sulphate B.P.C. (Kannasyn)</td>
<td>Bayer Products Co.</td>
<td>20</td>
</tr>
<tr>
<td>Malidixic acid (Negram)</td>
<td>Winthrop Laboratories</td>
<td>40</td>
</tr>
<tr>
<td>Paromomycin sulphate</td>
<td>Parke, Davis &amp; Co.</td>
<td>60</td>
</tr>
<tr>
<td>Spectinomycin dihydrochloride pentahydrate</td>
<td>Upjohn Ltd.</td>
<td>100</td>
</tr>
<tr>
<td>Streptomycin sulphate B.P.</td>
<td>Glaxo Laboratories</td>
<td>20, 40 or 500</td>
</tr>
<tr>
<td>Sulphathiazole B.P.C.</td>
<td>May &amp; Baker Ltd.</td>
<td>100</td>
</tr>
<tr>
<td>Tetracycline hydrochloride (Tetracyn)</td>
<td>Pfizer Ltd.</td>
<td>5 or 10</td>
</tr>
</tbody>
</table>
6. Storage of strains. Strains were stored on wax-sealed Dorset egg slopes at room temperature. All standard strains and those with unstable characteristics were also freeze-dried.
## Table 1. Standard strains

<table>
<thead>
<tr>
<th>ERL No.</th>
<th>Original No.</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1R713</td>
<td>K12-703</td>
<td>Escherichia coli K12 F&lt;sup&gt;−&lt;/sup&gt; prototrophic</td>
<td>Prof. W. Hayes</td>
</tr>
<tr>
<td>14R525</td>
<td>.</td>
<td>1R713 Nal&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Dr. M. J. Lewis</td>
</tr>
<tr>
<td>38R666</td>
<td>.</td>
<td>1R713 Str&lt;sup&gt;R&lt;/sup&gt;</td>
<td>.</td>
</tr>
<tr>
<td>14R683</td>
<td>.</td>
<td>14R525 ColE&lt;sup&gt;R&lt;/sup&gt;</td>
<td>.</td>
</tr>
<tr>
<td>24R597</td>
<td>.</td>
<td>14R525 ColI&lt;sup&gt;R&lt;/sup&gt;</td>
<td>.</td>
</tr>
<tr>
<td>1R716</td>
<td>K12-712</td>
<td>K12 F&lt;sup&gt;−&lt;/sup&gt; pro lac trp his Str&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Prof. W. Hayes</td>
</tr>
<tr>
<td>14R519</td>
<td>K12-711</td>
<td>K12 F&lt;sup&gt;−&lt;/sup&gt; pro lac trp his Nal&lt;sup&gt;R&lt;/sup&gt;</td>
<td>.</td>
</tr>
<tr>
<td>22R380</td>
<td>CL142</td>
<td>K12-KOW. Colicin indicator strain</td>
<td>Prof. P. Fredericq</td>
</tr>
<tr>
<td>44R610</td>
<td>.</td>
<td>E.coli C</td>
<td>.</td>
</tr>
<tr>
<td>42R366</td>
<td>.</td>
<td>Salmonella typhimurium phage type 36 plasmid free</td>
<td>.</td>
</tr>
<tr>
<td>42R500</td>
<td>.</td>
<td>S.typhimurium phage type 36 plasmid free</td>
<td>.</td>
</tr>
<tr>
<td>34R99</td>
<td>.</td>
<td>S.typhimurium phage type 36 Nal&lt;sup&gt;R&lt;/sup&gt; plasmid free</td>
<td>.</td>
</tr>
<tr>
<td>18R613</td>
<td>.</td>
<td>S.typhimurium phage type 36 Str&lt;sup&gt;R&lt;/sup&gt;. Carries the fi&lt;sup&gt;R&lt;/sup&gt; plasmid MP&lt;sup&gt;10&lt;/sup&gt;<code>&lt;sub&gt;36</code>&lt;sup&gt;+&lt;/sub&gt;</td>
<td>.</td>
</tr>
</tbody>
</table>

<sup>a</sup> 42R366 and 42R500 are derivatives of two independently isolated strains of S.typhimurium phage type 36. These strains do not carry the fi<sup>R</sup> plasmid MP<sup>10</sup>`<sub>36`<sup>+</sub> present in the wild strains.

<sup>b</sup> 42R366, 34R99 and 18R613 are derived from the same parent strain of S.typhimurium phage type 36, RT576.
Table 2. Standard plasmid-carrying strains

<table>
<thead>
<tr>
<th>ERL No.</th>
<th>Original No.</th>
<th>Description</th>
<th>Plasmid-determined antibiotic resistance</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>21R306</td>
<td>K12-501</td>
<td>K12 F&lt;sup&gt;am&lt;/sup&gt;lac&lt;sup&gt;T&lt;/sup&gt;</td>
<td>-</td>
<td>Prof. W. Hayes</td>
</tr>
<tr>
<td>16B399</td>
<td>W1655(Flag)</td>
<td>K12 Flac</td>
<td>-</td>
<td>Prof. W. Hayes</td>
</tr>
<tr>
<td>30B893</td>
<td></td>
<td>14R519 carrying FlacT&lt;sup&gt;a&lt;/sup&gt;</td>
<td>T</td>
<td>Prof. W. Hayes</td>
</tr>
<tr>
<td>32R1000</td>
<td></td>
<td>K12 Hfr Hayes prototrophic Nal&lt;sup&gt;T&lt;/sup&gt;</td>
<td>-</td>
<td>Anderson &amp; Lewis (1965b)</td>
</tr>
<tr>
<td>18B312</td>
<td></td>
<td>1R713, Δ</td>
<td>T</td>
<td>N. D. F. Grindley</td>
</tr>
<tr>
<td>26B844</td>
<td></td>
<td>1R713, T-Δ</td>
<td>A</td>
<td>Meynell &amp; Cooke (1969)</td>
</tr>
<tr>
<td>20B770</td>
<td></td>
<td>1R713, T-Adrp&lt;sup&gt;b&lt;/sup&gt;</td>
<td>A</td>
<td>Meynell &amp; Cooke (1969)</td>
</tr>
<tr>
<td>22B869</td>
<td></td>
<td>1R713, A-Adrp&lt;sup&gt;b&lt;/sup&gt;</td>
<td>A</td>
<td>Meynell &amp; Cooke (1969)</td>
</tr>
<tr>
<td>30B587</td>
<td></td>
<td>1R713, R144-3&lt;sup&gt;b&lt;/sup&gt; (KColIB)</td>
<td>K</td>
<td>Meynell &amp; Cooke (1969)</td>
</tr>
<tr>
<td>28B781</td>
<td></td>
<td>1R713, 33&lt;sup&gt;b&lt;/sup&gt;Adrp&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ACSSu</td>
<td>Meynell &amp; Cooke (1969)</td>
</tr>
<tr>
<td>18B951</td>
<td></td>
<td>1R716 carrying the fi&lt;sup&gt;b&lt;/sup&gt; F-like transfer factor X</td>
<td>-</td>
<td>Anderson, Pitton &amp; Mayhew (1968)</td>
</tr>
<tr>
<td>34B621</td>
<td></td>
<td>1R713, TP110 (KColIB)</td>
<td>K</td>
<td>Anderson &amp; Smith (1972b)</td>
</tr>
<tr>
<td>20B675</td>
<td>C1 136</td>
<td>K12-ROW, ColE&lt;sup&gt;1&lt;/sup&gt; Colicin indicator strain</td>
<td>-</td>
<td>Prof. P. Fredericq</td>
</tr>
<tr>
<td>20B676</td>
<td>C1 137</td>
<td>K12-ROW, ColE&lt;sup&gt;2&lt;/sup&gt;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>46R160</td>
<td></td>
<td>14B525, ColE&lt;sup&gt;3&lt;/sup&gt;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>22B82</td>
<td>C1 223</td>
<td>K12-ROW, ColIA-CA53</td>
<td>&quot;</td>
<td>Prof. P. Fredericq</td>
</tr>
<tr>
<td>22B83</td>
<td>C1 232</td>
<td>K12-ROW, ColIB-P9</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Flac<sup>T</sup> is a recombinant between Flac and the tetracycline resistance marker of the I-like factor T-Δ (Anderson & Smith, 1972b).

<sup>b</sup> R<sup>144-3</sup> is a derepressed mutant of the I-like R factor R<sup>144</sup>.

<sup>c</sup> 33<sup>b</sup>Adrp<sup>c</sup> is a derepressed mutant of the F-like R factor 33 isolated from S.paratyphi B 7268 (also known as R1).
Table 3. Reference plasmids of defined compatibility groups

<table>
<thead>
<tr>
<th>Plasmid No.</th>
<th>Compatibility group</th>
<th>Resistances and other markers</th>
<th>Origin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>F₂-lac</td>
<td>F₅</td>
<td>Lac⁺</td>
<td>S. typhi</td>
<td></td>
</tr>
<tr>
<td>TP114</td>
<td>I₂</td>
<td>K</td>
<td>E. coli</td>
<td>Scotland, 1967</td>
</tr>
<tr>
<td>TP118</td>
<td>N</td>
<td>AS</td>
<td>S. enteritidis</td>
<td>England, 1969</td>
</tr>
<tr>
<td>TP125</td>
<td>B</td>
<td>CSSuT</td>
<td>Shigella dysenteriae</td>
<td>Central America, 1969</td>
</tr>
<tr>
<td>TP117</td>
<td>H₁</td>
<td>T</td>
<td>S. typhimurium</td>
<td>England, 1961</td>
</tr>
<tr>
<td>TP116</td>
<td>H₂</td>
<td>CSSu</td>
<td>S. typhi</td>
<td>Spain, 1969</td>
</tr>
<tr>
<td>S-a</td>
<td>W</td>
<td>CKSSu</td>
<td>Shigella sp.</td>
<td>Japan</td>
</tr>
</tbody>
</table>

a All these plasmids are auto-transferring.
<table>
<thead>
<tr>
<th>Plasmid No. or designation</th>
<th>Antibiotic resistance or colicinogeny</th>
<th>Species of original host strain</th>
<th>Phage type of original host strain</th>
<th>Year and place of origin</th>
<th>Source or ERL No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTP1</td>
<td>A</td>
<td>Salmonella typhimurium</td>
<td>29</td>
<td>England, 1964</td>
<td>RT1</td>
</tr>
<tr>
<td>NTP2 a</td>
<td>SSu</td>
<td>Salmonella typhimurium</td>
<td>29</td>
<td>England, 1964</td>
<td>RT1</td>
</tr>
<tr>
<td>NTP3</td>
<td>A5u</td>
<td>Salmonella typhimurium</td>
<td>29</td>
<td>ERL, 1967</td>
<td>9R314</td>
</tr>
<tr>
<td>NTP4</td>
<td>ASSu</td>
<td>Salmonella typhimurium</td>
<td>29</td>
<td>ERL, 1968</td>
<td>13R135</td>
</tr>
<tr>
<td>NTP5</td>
<td>T</td>
<td>Salmonella typhimurium</td>
<td>49</td>
<td>Scotland, 1969</td>
<td>9M7779</td>
</tr>
<tr>
<td>NTP6</td>
<td>A</td>
<td>Salmonella typhimurium</td>
<td>168</td>
<td>England, 1972</td>
<td>12H52</td>
</tr>
<tr>
<td>NTP7</td>
<td>ASSu</td>
<td>Salmonella typhimurium</td>
<td>157</td>
<td>Argentina, 1972</td>
<td>12R364</td>
</tr>
<tr>
<td>NTP8</td>
<td>Colicin E1</td>
<td>Escherichia coli</td>
<td>-</td>
<td>-</td>
<td>K30</td>
</tr>
<tr>
<td>NTP9</td>
<td>Colicin E2</td>
<td>Shigella sonnei</td>
<td>-</td>
<td>-</td>
<td>P9</td>
</tr>
<tr>
<td>NTP10</td>
<td>Colicin E3</td>
<td>Escherichia coli</td>
<td>-</td>
<td>-</td>
<td>CA38</td>
</tr>
<tr>
<td>NTP11</td>
<td>K</td>
<td>Salmonella virchow</td>
<td>-</td>
<td>England, 1974</td>
<td>44R376</td>
</tr>
<tr>
<td>K (= fi-K)</td>
<td>K</td>
<td>Salmonella typhimurium</td>
<td>29</td>
<td>England, 1965</td>
<td>5W3436</td>
</tr>
<tr>
<td>fi'K</td>
<td>K</td>
<td>Salmonella typhimurium</td>
<td>29</td>
<td>England, 1965</td>
<td>&quot;</td>
</tr>
<tr>
<td>MP1036</td>
<td>-</td>
<td>Salmonella typhimurium</td>
<td>36</td>
<td>England, 1964</td>
<td>RT576 (4M3543)</td>
</tr>
<tr>
<td>MP10LT2</td>
<td>-</td>
<td>Salmonella typhimurium</td>
<td>4</td>
<td>English, 1964</td>
<td>LT2b (= 42B93)</td>
</tr>
<tr>
<td>MP108</td>
<td>-</td>
<td>Salmonella typhimurium</td>
<td>8</td>
<td>England, 1956</td>
<td>21B341</td>
</tr>
<tr>
<td>MP1014</td>
<td>-</td>
<td>Salmonella typhimurium</td>
<td>14</td>
<td>Wales, 1948</td>
<td>21B337</td>
</tr>
<tr>
<td>MP1074</td>
<td>-</td>
<td>Salmonella typhimurium</td>
<td>74</td>
<td>Kenya, 1960</td>
<td>21B340</td>
</tr>
</tbody>
</table>

\[a\] Strains carrying NTP2, NTP3, NTP4 and NTP7 are resistant to streptomycin but not to spectinomycin.

\[b\] Strain LT2 of *S. typhimurium* was described by Lilleengen (1948).
Table 5. Wild strains resistant to streptomycin and sulphonamides

<table>
<thead>
<tr>
<th>ERL No.</th>
<th>Species of host strain. Phage type of S. typhimurium or Salmonella O group</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>3x0635</td>
<td>E. coli&lt;br&gt;E. coli</td>
<td>Animal, England, 1973&lt;br&gt;Animal, Ireland, 1970</td>
</tr>
</tbody>
</table>

* All these strains are sensitive to spectinomycin, that is, the streptomycin resistance is probably caused by a phosphorylating enzyme (Ozanne et al., 1969).
Table 5. Wild strains resistant to streptomycin and sulphonamides

<table>
<thead>
<tr>
<th>ERL No.</th>
<th>Species of host strain. Phage type of S.typhimurium or Salmonella O group</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>8M3993</td>
<td>S.typhimurium 32 &quot; 1 &quot; 44 &quot; 6 &quot; 12a &quot; 12a &quot; 56 &quot; 95 &quot; Untypable &quot; 104 &quot; 156</td>
<td>Human, Scotland, 1968</td>
</tr>
<tr>
<td></td>
<td>S.agona B</td>
<td>Human, England, 1970</td>
</tr>
<tr>
<td></td>
<td>S.brandenburg B</td>
<td>Human, Wales, 1970</td>
</tr>
<tr>
<td>42R652</td>
<td>S.bredeney B</td>
<td>Human, England, 1970</td>
</tr>
<tr>
<td></td>
<td>S.derby B</td>
<td>Human, England, 1970</td>
</tr>
<tr>
<td></td>
<td>S.montevideo C</td>
<td>Human, England, 1970</td>
</tr>
<tr>
<td>42R653</td>
<td>S.newport C</td>
<td>Human, England, 1970</td>
</tr>
<tr>
<td></td>
<td>S.stanley B</td>
<td>Human, England, 1970</td>
</tr>
<tr>
<td></td>
<td>S.chester B</td>
<td>Human, England, 1970</td>
</tr>
<tr>
<td></td>
<td>S.indiana B</td>
<td>Human, England, 1970</td>
</tr>
<tr>
<td>EC4240</td>
<td>E.coli</td>
<td>Human, England, 1970</td>
</tr>
<tr>
<td>EC4316</td>
<td>E.coli</td>
<td>Human, England, 1970</td>
</tr>
<tr>
<td></td>
<td>E.coli</td>
<td>Animal, England, 1973</td>
</tr>
<tr>
<td>3EC635</td>
<td>E.coli</td>
<td>Animal, England, 1973</td>
</tr>
<tr>
<td></td>
<td>E.coli</td>
<td>Animal, Ireland, 1970</td>
</tr>
</tbody>
</table>

*All these strains are sensitive to spectinomycin, that is, the streptomycin resistance is probably caused by a phosphorylating enzyme (Ozanne et al., 1969).*
General experimental techniques

Resistance typing. Broth cultures of the test strains and the controls were streaked with a wire loop across a nutrient-agar plate. The appropriate control strains were placed in the middle of the cultures to be typed. A strip, impregnated with an antibiotic, was laid at right angles to the cultures on the plate. Plates were incubated overnight at 37°C. Cultures which were fully resistant to the antibiotic grew to the edge of the strip, whereas with sensitive strains there was an area where growth was inhibited. This technique of resistance typing also allowed the detection of partial resistance where the cultures were less inhibited than the control sensitive strain, but did not grow to the edge of the strip (see Plate 1).

Plate 1. Resistance typing of bacterial strains

The plate shows testing for resistance to tetracycline. The strains are derivatives of K12F" (1R713).

- Lines 1 and 5: K12(T-Δ) - Full resistance
- Lines 2 and 6: K12(NTP2) - Sensitive
- Lines 3 and 7: K12(NTP5) - Partial resistance
- Lines 4 and 8: K12F" - Sensitive control
Sulphonamide resistance testing. Broth cultures were diluted $10^{-4}$ in saline and spotted on laked-blood agar plates with and without sulphathiazole. Resistant and sensitive control strains were included on each plate. All cultures grow on the control plate lacking sulphathiazole, whereas only strains resistant to sulphonamides can grow on the plate containing sulphathiazole.

Spectinomycin resistance. Cultures were diluted about 10,000 fold in saline and spotted on to nutrient agar plates containing spectinomycin (100 µg/ml).

Colicinogeny testing. This was carried out by the method of Fredericq (1957). Broth cultures of the test strains were stabbed on to nutrient agar plates with a straight wire. There were usually up to twelve strains, including two controls, on each plate, which were incubated overnight at $37^\circ$ C. The plates were inverted over watch glasses containing chloroform for 10 to 15 min and then left open to dry for 30 min. 5 ml of 0.4% nutrient agar, containing 0.3 ml of a broth culture of the sensitive indicator strain, was poured on each plate. After overnight incubation colicin production was detected by inhibition zones in the lawn of the sensitive indicator strain (Plate 2). The type of colicin was identified by use of standard indicators which are strains carrying standard colicin factors or strains resistant to known colicins (Tables 1 and 2).
Each culture was stabbed on to a nutrient agar plate in duplicate. The control non-colicinogenic strain is shown on each side of the test strains. The indicator was K12-ROW.

Detection of colicin-producing colonies in a recipient population after a mating experiment.
Plate 2. Colicinogeny testing of bacterial strains

Each culture was stabbed on to a nutrient agar plate in duplicate. The control non-colicinogenic strain is shown on each side of the test strains. The indicator was K12-ROW.

Detection of colicin-producing colonies in a recipient population after a mating experiment.
Plate 2. Colicinogeny testing of bacterial strains

Each culture was stabbed on to a nutrient agar plate in duplicate. The control non-colicinogenic strain is shown on each side of the test strains. The indicator was K12-ROW.

Detection of colicin-producing colonies in a recipient population after a mating experiment.
Broth cultures of donor and recipient strains were grown on a Luckham Rotatetet shaker at 37° C to exponential phase and contained approximately $2 \times 10^8$ organisms per ml. For short crosses, the cultures were mixed in a ratio of 1:10 and the mating was interrupted by blending on a Fison's Whirlimixer. The duration of these crosses was either 30 min, 1 h or 2 h. Donor and recipient cultures were mixed in equal quantities for overnight crosses (usually 18 h). After each cross decimal dilutions of the mixtures were prepared in phosphate buffer, and either 0.01 ml or 0.1 ml quantities of the undiluted cross and each dilution were plated in duplicate. 0.01 ml volumes were streaked in linear fashion with a standard wire loop and 0.1 ml amounts were plated with glass spreaders. Crosses were plated on either MacConkey, nutrient or L agar containing suitable concentrations of the appropriate antibiotics.

Counter-selection against the donor strains was exercised by the following methods. If the donor strain was sensitive to nalidixic acid or streptomycin, a strain chromosomally resistant to one of these agents was used as recipient. In some experiments it was necessary to eliminate K12 strains with colicin E2 by spreading 0.3 ml into each plate. Counter-selection against S.typhimurium strains was effected with the salmonella 01 phage of Felix and Callow (1943). These techniques for the detection of resistance transfer have been described previously (Anderson and Lewis, 1965b).

All crosses were incubated overnight at 37° C and suitable
plates were scored with a colony counter. The frequency of transfer in interrupted crosses was expressed as the proportion of resistant progeny per donor cell, while the frequency in overnight crosses was calculated as the proportion per recipient cell. Subcultures of colonies were picked from the plates and tested for the presence of resistance or colicinogeny markers as described previously. When a larger sample of colonies was screened for drug resistance, suitable plates were replicated on to nutrient agar containing the appropriate antibiotics. For detection of colicinogeny, plates were replicated on to plain nutrient agar and the colonies subsequently tested for colicin production as described previously.

Compatibility experiments

Compatibility between two plasmids was tested by introducing one into a strain carrying the other, and examining the progeny for the presence of both plasmids. When both were present, segregation was studied by growing picks of the respective clones for 5 h in broth at 37°C, followed by plating on nutrient agar plates. These master plates were replicated on to nutrient agar containing the relevant antibiotics. In experiments with the non-transferring colicin factors, one plasmid was mobilised by an R factor into a strain carrying the other colicin factor. Selection was exercised for the resistance coded by the R factor and progeny were examined for the presence of the two colicin factors. Master plates were prepared as above, replicated on to nutrient agar, and the colonies
In all experiments at least three hybrid clones were plated and usually more than 100 colonies of each were replicated. Compatible plasmids showed a rate of segregation no higher than the rate of spontaneous loss of either parent factor.

When pairs of plasmids appeared to be compatible, strains were examined for independent transfer of the two plasmids to a new host. Interrupted crosses were usually performed with separate selection for the resistances encoded by the two plasmids and progeny were tested for the presence of both plasmids. In experiments with non-transferring plasmids it was necessary to test for possible recombination between the determinant and the transfer or R factor used for mobilisation. After interrupted crosses progeny carrying non-transferring plasmids were examined for the transfer (or R) factor. Determinants can usually be separated from transfer factors by short crosses especially when the transfer is from K12 to *S. typhimurium*.

**Mobilisation of non auto-transferring plasmids**

When there was no direct transfer of a plasmid the strains were examined for mobilisation. This was performed as follows. In a triparental cross for determinant mobilisation (Anderson, 1965) equal quantities of broth cultures of the donor strain carrying a transfer factor and the intermediate strain with the non-transferring plasmid were incubated together at 37°C for at least 2 h. The
plasmid-free final recipient was then added, using the same volume as before, and the mixture incubated overnight at 37° C. The cross was plated on a medium which selected for the resistance coded by the plasmid in the intermediate strain, but counter-selected against both donor and intermediate strains. A control mixture of the intermediate and final recipients was tested to establish that no transfer of the plasmid to be mobilised took place in the absence of a transfer factor.

The mobilisation procedure was performed in two steps in certain experiments. A transfer factor or R factor was introduced into the strain carrying the non-transferring plasmid and lines carrying the two plasmids were identified. The mobilisation of the non-transferring plasmid could then be measured by further crosses. A number of different transfer or R factors were employed in these mobilisation tests.

Inhibition of F-mediated fertility

1. K12 F+. The plasmids were transferred to K12 F+ and progeny were tested for visible lysis by the F-specific phage u2 in surface spot tests. These tests were performed by spreading a loopful (0.01 ml) of a broth culture over an area of about 1.5 cm diameter on a nutrient agar plate. About 0.01 ml of the phage was spotted on the centre of the inoculated area using a loop or a pipette. The plates were incubated at 37° C for about 5 h (Pitton and Anderson, 1970).

2. K12 HfrH. Lines of HfrH carrying the plasmid were examined for visible lysis by u2, and for frequency of pro transfer to
K12F\(^{-}\) (1R716) in 1 h crosses. L-broth cultures of donor and recipient strains were mixed in a ratio of 1:10 and Pro\(^{+}\) recombinants were selected on minimal medium supplemented with histidine and tryptophan, with glycerol as the carbon source. Streptomycin (500 \(\mu\)g/ml) was used to counterselect against the HfrH donor strain. The frequency of pro transfer was expressed as the number of Pro\(^{+}\) recombinants per donor cell. Fertility inhibition caused by the plasmid in HfrH was detected by a reduction in frequency of pro transfer compared with that from HfrH itself.

**Phage multiplication experiments**

The ability of strains carrying plasmids to support multiplication of sex specific phages was tested as described by Grindley and Anderson (1971). Cultures were grown in broth to late exponential phase and 1 ml of each strain was diluted into 8 ml of nutrient broth. One ml of the phages \(\mu\)2, fd or If1 (titre \(5 \times 10^{5}\) pfu/ml) was added to each culture so the phage:bacterium ratio was about 1:1000. This mixture, and a control using a plasmid-minus strain, were incubated overnight at 37\(^{\circ}\) C, and then centrifuged at 3000 rpm for 15 min in an MSE Minor Angle centrifuge. Phage in the supernatant was titrated in agar layer with the indicator strain either K12F\(^{+}\) (for \(\mu\)2 and fd) or K12 T-Adr1 (for If1). This derepressed mutant of the R factor T-\(\Delta\) was isolated by N. D. F. Grindley in the Enteric Reference Laboratory. The plates were incubated overnight, the plaques counted and the titre of the phage was calculated for each test and control experiment.
Detection of β-lactamase production

Release of β-lactamase was examined by the technique of Anderson and Lewis (1965a) except that minimal agar was used instead of nutrient agar. Cultures were plated on minimal agar containing 0.2% soluble starch and required supplements and the plates were incubated overnight. Suitable plates were flooded with a solution containing 3 mg/ml of iodine, 15 mg/ml of potassium iodide and 50 mg/ml of benzyl penicillin in phosphate-buffered saline of pH 6.4. The agar was coloured blue-black because of formation of the starch-iodine complex. Clear colourless zones surrounded colonies indicating the release of β-lactamase. Decolorization resulted from removal of iodine from the starch-iodine complex by penicilloic acid liberated by the action of β-lactamase on benzyl penicillin. Colonies that were sensitive to ampicillin (and benzyl penicillin) were stained brown by iodine, and no zone of decolorization was produced (see Plate 3). This method was used to detect loss of ampicillin resistance from resistant plasmid-carrying strains.
Plate 3. Detection of β-lactamase production

The strains were all derivatives of K12F⁻ (1R713). The cultures were spotted in duplicate.

1  K12 (NTP1)
2  K12 (NTP3)
3  K12 (NTP4)
4  K12 (NTP6)
5  K12 (NTP7)
6  K12 (A-Δ)

The control spots were stained brown with no clear halo. These are shown on each side of the test strains.
The strains were all derivatives of K12F™ (K713). The cultures were spotted in duplicate.

1  K12 (NTP1)
2  K12 (NTP3)
3  K12 (NTP4)
4  K12 (NTP6)
5  K12 (NTP7)
6  K12 (A-Δ)

The control spots were stained brown with no clear halo. These are shown on each side of the test strains.
SECTION I. RESULTS

Preliminary characterisation of the plasmids NTP1 to NTP11. A number of the non auto-transferring plasmids studied in this investigation and listed in Table 4 have been described previously. The A (NTP1) and SSu (NTP2) resistance determinants were both present in strain RT1 of *S. typhimurium* phage type 29 (Anderson and Lewis, 1965a,b). The ASu determinant was produced by ultraviolet irradiation of RT1 (Anderson *et al.*, 1968; Anderson, 1969). The ASSu determinant NTP4 arose by recombination between SSu and ASu (Anderson, 1969). These four determinants of the Δ-mediated transfer systems were transferred from *S. typhimurium* strains to the standard K12 strains 1R713 and 14R525 by crosses interrupted at 30 min and lines carrying the determinants alone were isolated.

The standard colicinogeny determinants ColE1, ColE2 and ColE3 were transferred to K12 from the wild colicinogenic strains. Colicin E-resistant mutants of K12 were used in these experiments. In the case of ColE1, this was first transferred to K12 HfrH from the wild strain *E. coli* K30. An HfrH line carrying ColE1 was then mated for 1 h with K12 F− and colonies carrying ColE1 alone were identified. For the transfer of ColE2 and ColE3, the R factor T-Δ was introduced into the respective wild colicinogenic strains. Lines carrying ColE2 or ColE3 and T-Δ were crossed with *S. typhimurium* type 36 and tetracycline-resistant progeny were tested for ColE2 or ColE3. *S. typhimurium* ColE2, T-Δ and *S. typhimurium* ColE3, T-Δ were mated overnight with K12 F− NalR and unselected K12 progeny were tested.
for colicinogeny. Lines carrying ColE2 alone and ColE3 alone were identified.

Detection of non-transferring plasmids in wild strains

A strain of *S. typhimurium* type 49, 9M3779, was resistant to tetracycline and produced colicin Ia. Both drug resistance and colicinogeny were transferable to K12 and the results of conjugation experiments are shown in Table 6. Both selected and unselected recipient progeny were examined for resistance, colicinogeny and transfer. All resistant K12 lines were ColIa⁺ and transferred tetracycline resistance. Nine out of 20 unselected picks carried a ColIa factor, but were drug-sensitive. A K12 line which carried T and ColIa was then mated with *S. typhimurium* 36 for 2 h. Five out of 20 tetracycline-resistant progeny were ColIa⁻ and did not transfer T to K12. Unselected picks of an overnight cross to *S. typhimurium* 36 showed that 8 of 20 lines tested were colicinogenic and drug-sensitive. These results establish that this is a Class 2 resistance transfer system in which the tetracycline resistance determinant, designated NTP5, is transferred by a transfer factor that is linked to a ColIa determinant.
Table 6. Crosses with the tetracycline resistance determinant NTP5

<table>
<thead>
<tr>
<th>Donor</th>
<th>X</th>
<th>Recipient</th>
<th>Selection</th>
<th>Time of cross</th>
<th>Frequency of transfer</th>
<th>Analysis of progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. typhimurium</em> (9M3779)</td>
<td>K12F&lt;sup&gt;-&lt;/sup&gt; (24R357)</td>
<td>Tetracycline</td>
<td>2 h</td>
<td>4 × 10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>20/20 T, ColIa</td>
<td></td>
</tr>
<tr>
<td>9M3779</td>
<td>24R357</td>
<td>Tetracycline</td>
<td>18 h</td>
<td>5 × 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>20/20 T, ColIa</td>
<td></td>
</tr>
<tr>
<td>9M3779</td>
<td>24R357</td>
<td>Unselected</td>
<td>18 h</td>
<td>.</td>
<td>9/20 ColIa; 11/20 T&lt;sup&gt;+&lt;/sup&gt; ColIa&lt;sup&gt;−&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>K12(NTP5, ColIa)</td>
<td><em>S. typhimurium</em> 36 (42R366)</td>
<td>Tetracycline</td>
<td>2 h</td>
<td>2 × 10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>5/20 T; 15/20 T, ColIa</td>
<td></td>
</tr>
<tr>
<td>K12(NTP5, ColIa)</td>
<td>42R366</td>
<td>Tetracycline</td>
<td>18 h</td>
<td>1 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>20/20 T, ColIa</td>
<td></td>
</tr>
<tr>
<td>K12(NTP5, ColIa)</td>
<td>42R366</td>
<td>Unselected</td>
<td>18 h</td>
<td>.</td>
<td>8/20 ColIa; 12/20 T&lt;sup&gt;+&lt;/sup&gt; ColIa&lt;sup&gt;−&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>
Strains carrying the Colla plasmid propagated the I-specific phage If1 about $10^3$ fold, whereas lines carrying NTP5 alone did not. The compatibility of this ColIA factor was examined by transferring the I, R factor A-Adrpl to K12 carrying NTP5 and Colla. All 20 colonies selected on ampicillin, or ampicillin and tetracycline, lost the ability to produce colicin but retained NTP5. This confirmed that NTP5 and Colla are independent plasmids, of which Colla is a member of group I.

For further genetic and molecular studies a K12 line carrying NTP5 alone was detected after a 30 min mating between K12 strains.

In an examination of some resistant S.typhimurium strains, ampicillin resistance was not transferable from 12M521 which is a strain of S.typhimurium type 168. This line was tested for mobilisation of A resistance by T-Adrpl in a triparental cross with K12 F" as the final recipient. The ampicillin resistance determinant, designated NTP6, was easily mobilised by T-Adrpl. A K12 line carrying NTP6 and T-Adrpl was mated with S.typhimurium 36 for 2 h. Eight of 10 lines selected on penicillin did not carry T-Adrpl and the A resistance was non-transferring. The plasmids had formed a Class 2 transfer system; separation of the two components was most easily demonstrated in short crosses from K12 to S.typhimurium 36. However, a K12 line carrying NTP6 alone was isolated after a 30 min mating between S.typhimurium 36(NTP6, T-Adrpl) and 18713.

The S.typhimurium strain 12M361^, isolated in Argentina, carried the resistance markers ACKSSuT and Nal^R. All resistances were transferable to K12 except that to nalidixic acid, which was
presumably chromosomal in origin. Selection on ampicillin yielded lines carrying A, S and Su; the resistances were all transferable together to further recipients. An overnight cross from K12 ASSu performed; to *S. typhimurium* 36 was 18 of 20 resistant progeny did not transfer ASSu to K12. This ASSu determinant NTP7 was then mobilised by T-A from *S. typhimurium* 36. After a 30 min mating between *S. typhimurium* 36 and K12, 3 of 30 resistant colonies examined carried ASSu alone, while the remaining lines carried ASSu and T-A.

One of the lines of *S. typhimurium* 36 which transferred ASSu, without introduction of T-A, was tested for propagation of If1. There was an increase in phage titre of approximately 100-fold compared with the plasmid-free control, indicating that the original transfer factor from 12M3614 which mobilised ASSu was I-like. *S. typhimurium* 36(NTP7, TF*) was mated with K12F− overnight and 40 unselected recipient colonies were examined. Five colonies carried ASSu and the transfer factor, 26 were drug sensitive but carried the transfer factor, which was identified by mobilisation of SSu in triparental crosses. The remaining 9 lines were drug sensitive and possessed no transfer factor.

A K12 line carrying ASSu and the transfer factor, designated TP151, was used in compatibility tests with standard I-like plasmids. T-Adrp1 (I1) and TP114 (I2) were transferred to this strain and to K12F− (Table 7). There was no surface exclusion of T-Adrp1 or TP114, and the resulting progeny were stable for ASSu and either T-Adrp1 or TP114. It was not known whether the transfer factor TP151 was
still present. Table 7 also shows the results of transferring NTP7 from a strain carrying NTP7 and TP151 to K12(T-Δ) or K12(TP114). There was a 30-fold reduction in transfer with K12(TP114) as recipient, but no difference with K12(T-Δ). Progeny selected on ampicillin were all stable for ASSu and T-Δ or TP114. These results suggest that TP151 belongs to group I_2, but do not prove it, since there was no method of testing for the presence of TP151 in the progeny of these crosses.

Table 7. Compatibility tests with the I-like plasmid TP151

<table>
<thead>
<tr>
<th>Plasmids in donor strain</th>
<th>Plasmids in recipient strain</th>
<th>Time of cross</th>
<th>Selection</th>
<th>Frequency of transfer</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-Adrpl</td>
<td>NTP7, TP151</td>
<td>1 h</td>
<td>T</td>
<td>9 x 10^{-1}</td>
</tr>
<tr>
<td>I_4</td>
<td>-</td>
<td>1 h</td>
<td>T</td>
<td>3 x 10^{-1}</td>
</tr>
<tr>
<td>TP114</td>
<td>NTP7, TP151</td>
<td>1 h</td>
<td>K</td>
<td>1 x 10^{-3}</td>
</tr>
<tr>
<td>I_2</td>
<td>-</td>
<td>1 h</td>
<td>K</td>
<td>5 x 10^{-4}</td>
</tr>
<tr>
<td>NTP7, TP151</td>
<td>T-Δ</td>
<td>18 h</td>
<td>A</td>
<td>2 x 10^{-1}</td>
</tr>
<tr>
<td></td>
<td>TP114</td>
<td>18 h</td>
<td>A</td>
<td>3 x 10^{-3}</td>
</tr>
</tbody>
</table>

^a All lines selected on ampicillin were also resistant to streptomycin and sulphonamides.

A strain of S.virchow resistant to ampicillin, kanamycin, streptomycin and sulphonamides, isolated in a hospital outbreak of salmonellosis, was investigated. All four resistances were transferable to K12, but in a 30 min cross selection on kanamycin yielded 9 out of 10 recipient lines resistant to kanamycin alone. K was not transferable from these lines but was mobilisable by Δ and the F-like R factor 240. It appeared from these experiments that kanamycin resistance was coded for by a non-transfering determinant termed NTP11.
Spectrum of drug resistance encoded by the plasmids

The drug resistances encoded by the plasmids NTP1 to NTP11 are listed in Table 4 (page 48). The following properties were also investigated.

1) Strains carrying plasmids coding for ampicillin resistance were tested for β-lactamase production as described in Materials and Methods. As shown in Plate 3 (page 59) all six plasmid-bearing strains produced β-lactamase, whereas the control host strain showed no zone of decolorization. There were differences in the size of the ring possibly reflecting differences in the amount of β-lactamase produced. The smallest zone was observed with K12(A-Δ) in which there is approximately one copy of the plasmid per chromosome (Humphreys et al., 1972). In contrast, the non-transferring plasmids NTP1, 3, 4, 6 and 7 all exist in multiple copies per chromosome (see Section II). The difference in β-lactamase production by strains carrying multiple-copy determinants such as A (NTP1) and ASu (NTP3), as compared with those carrying single-copy plasmids such as A-Δ, is reflected in the penicillin MIC of such strains: about 3000 μg/ml in strains carrying A or ASu; and about 300 μg/ml in those carrying A-Δ (Anderson et al., 1968; Anderson, 1969).

2) R factor-mediated resistance to streptomycin occurs by two different enzymatic mechanisms, adenylylation and phosphorylation (see Introduction). The adenylate synthetase also inactivates spectinomycin, whereas the streptomycin phosphotransferase does not. Strains carrying NTP2, NTP4, and NTP7 were resistant to streptomycin but not to spectinomycin, which suggests that these plasmide code for a streptomycin phosphotransferase.

3) Kanamycin can be inactivated by three different mechanisms by R factor-carrying strains: acetylation, phosphorylation and adenylylation. The phosphorylating enzyme also inactivates paromomycin, whereas the other
types of enzyme do not. K12 strains carrying the kanamycin resistance determinant NTP11 were resistant to kanamycin and paromomycin. Thus, NTP11 probably codes for a phosphorylating enzyme.

Inhibition of F fertility. The plasmids NTP1 to NTP11 were introduced into K12 HfrH with the fl^- R factor T-A. The resulting progeny were tested for sensitivity to the F-specific phage μ2 in surface spot tests and for their frequency of transfer of pro* to K12F'. HfrH strains carrying each of the eleven plasmids were fully sensitive to phage μ2 and transferred pro* at a similar frequency to that of HfrH alone, that is approximately 10^-7 in a 1 h cross. NTP1 to NTP11 are therefore fl^- plasmids.

Compatibility experiments (plasmids NTP1 to NTP11).

Pairs of non-transferring plasmids were tested for compatibility by transferring one plasmid with a suitable transfer factor into a strain carrying the other. In experiments with the colicin factors ColE1, ColE2 and ColE3, S.typhimurium strains or colicin-resistant K12 strains were employed. The results of experiments with donor strains carrying A (NTP1), SSu (NTP2) and ASu (NTP3) are shown in Table 8. The A determinant NTP1 was identified in the same S.typhimurium type 29 strain, RT1, as SSu, and these two determinants coexisted stably in the same cell (Anderson and Lewis, 1965a,b). NTP1 was also compatible with NTP5, ColE1, ColE2, ColE3 and NTP11. Since NTP1 and NTP6 code for resistance to ampicillin only, it has been impossible to test for compatibility between them.
The SSu determinant was incompatible with ASu; this was first demonstrated by Anderson et al. (1968). ASSu (NTP7) arose by recombination between SSu and ASu. Thus SSu, ASu and ASSu all belong to the same compatibility group, of which SSu is the prototype. SSu was compatible with T (NTP5), A (NTP6) and K (NTP11), and the three colicin factors.

The results of compatibility experiments with T (NTP5), A (NTP6), and K (NTP11), are shown in Table 9. These three resistance determinants were compatible with each other and with ColE1, ColE2 and ColE3.

The ASSu determinant NTP7 was tested for compatibility with SSu (NTP2) by transfer to S. typhimurium 36 already carrying SSu, selection being exercised for ampicillin resistance. The progeny were examined for segregation by replica plating and the results are given in Table 10. Up to 78% loss of ampicillin resistance was detected in some clones indicating that the incoming ASSu plasmid was incompatible with SSu. Lines that were stable for A, S and Su were mated with 148525, selecting for streptomycin resistance only. If both ASSu and SSu were present in the stable donor strain there should be independent transfer of the plasmids, so that a proportion of colonies selected on streptomycin should be sensitive to ampicillin. However, all the progeny selected on streptomycin carried ASSu only. The original cross had evidently resulted in the displacement of SSu by ASSu. ASSu (NTP7) thus belongs to the SSu compatibility group.

In all these experiments lines which carried two compatible resistance determinants were examined for independent transfer of the two plasmids to a new host after a short mating. Separate transfer of the two determinants by the transfer factor was demonstrated in all cases.
Table 8. Compatibility experiments with A (NTP1), SSu (NTP2) and ASu (NTP3).

<table>
<thead>
<tr>
<th>Non-transferring plasmid in donor strain</th>
<th>Non-transferring plasmid in recipient strain</th>
<th>Selection</th>
<th>Analysis of progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (NTP1)</td>
<td>A</td>
<td>A</td>
<td>A/792</td>
</tr>
<tr>
<td></td>
<td>SSu (NTP2)</td>
<td>A</td>
<td>A, SSu 0/343</td>
</tr>
<tr>
<td></td>
<td>T (NTP5)</td>
<td>A</td>
<td>A, T 0/354</td>
</tr>
<tr>
<td></td>
<td>ColE1</td>
<td>A</td>
<td>A, ColE1 1A^- 0ColE1^-/519</td>
</tr>
<tr>
<td></td>
<td>ColE2</td>
<td>A</td>
<td>A, ColE2 0/469</td>
</tr>
<tr>
<td></td>
<td>ColE3</td>
<td>A</td>
<td>A, ColE3 0/432</td>
</tr>
<tr>
<td></td>
<td>K (NTP11)</td>
<td>A</td>
<td>A, K 0A^-1K^-/768</td>
</tr>
<tr>
<td>SSu (NTP2)</td>
<td>S</td>
<td>SSu</td>
<td>0/435</td>
</tr>
<tr>
<td></td>
<td>T (NTP5)</td>
<td>S</td>
<td>SSu, T 0/428</td>
</tr>
<tr>
<td></td>
<td>A (NTP6)</td>
<td>S</td>
<td>SSu, A 0/803</td>
</tr>
<tr>
<td></td>
<td>ColE1</td>
<td>S</td>
<td>SSu, ColE1 0/620</td>
</tr>
<tr>
<td></td>
<td>ColE2</td>
<td>S</td>
<td>SSu, ColE2 0/319</td>
</tr>
<tr>
<td></td>
<td>ColE3</td>
<td>S</td>
<td>SSu, ColE3 0/783</td>
</tr>
<tr>
<td></td>
<td>K (NTP11)</td>
<td>S</td>
<td>SSu, K 0/544</td>
</tr>
<tr>
<td>ASu (NTP3)</td>
<td>A</td>
<td>ASu</td>
<td>0/214</td>
</tr>
<tr>
<td></td>
<td>SSu (NTP2)</td>
<td>A</td>
<td>ASu/SSu 50A^-3188^-/614</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>ASu/SSu</td>
<td>1125A^-1058^-/2269</td>
</tr>
</tbody>
</table>
Table 9. Compatibility experiments with T (NTP5), A (NTP6) and K (NTP11)

<table>
<thead>
<tr>
<th>Non-transferring plasmid in donor strain</th>
<th>Non-transferring plasmid in recipient strain</th>
<th>Selection</th>
<th>Analysis of progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>T (NTP5)</td>
<td>A (NTP6)</td>
<td>T</td>
<td>T, A</td>
</tr>
<tr>
<td></td>
<td>ColE1</td>
<td>T</td>
<td>T, ColE1</td>
</tr>
<tr>
<td></td>
<td>ColE2</td>
<td>T</td>
<td>T, ColE2</td>
</tr>
<tr>
<td></td>
<td>ColE3</td>
<td>T</td>
<td>T, ColE3</td>
</tr>
<tr>
<td></td>
<td>K (NTP11)</td>
<td>T</td>
<td>T, K</td>
</tr>
<tr>
<td>A (NTP6)</td>
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<td>A</td>
<td>A, ColE1</td>
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<td></td>
<td>ColE1</td>
<td>A</td>
<td>A, ColE1</td>
</tr>
<tr>
<td></td>
<td>ColE2</td>
<td>A</td>
<td>A, ColE2</td>
</tr>
<tr>
<td></td>
<td>ColE3</td>
<td>A</td>
<td>A, ColE3</td>
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<tr>
<td></td>
<td>K (NTP11)</td>
<td>A</td>
<td>A, K</td>
</tr>
<tr>
<td>K (NTP11)</td>
<td>K</td>
<td>K</td>
<td>K, ColE1</td>
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<td></td>
<td>ColE1</td>
<td>K</td>
<td>K, ColE2</td>
</tr>
<tr>
<td></td>
<td>ColE2</td>
<td>K</td>
<td>K, ColE3</td>
</tr>
<tr>
<td></td>
<td>ColE3</td>
<td>K</td>
<td>K, ColE3</td>
</tr>
</tbody>
</table>

R-type or colicinogenicity Segregation of clones (pooled data)

0/648 0/628 0/456 0/402 0/746 0/1002 0/436 0/375 0/343 0/496 0/329 0/317 0/600 0/396 0/273
Table 10. Compatibility test with ASSu (NTP7)

<table>
<thead>
<tr>
<th>Non-transferring plasmid in donor strain</th>
<th>Non-transferring plasmid in recipient strain</th>
<th>Selection</th>
<th>Analysis of progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASSu (NTP7)</td>
<td>SSu (NTP2)</td>
<td>A</td>
<td>Colony 1</td>
</tr>
<tr>
<td>A</td>
<td></td>
<td></td>
<td>0/674</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td>34/170</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td>0/147</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td>4/122</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td>51/99</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td>0/168</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td>150/191</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td>0/125</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td>13/176</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td>0/101</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>34/182</td>
</tr>
</tbody>
</table>

a All colonies selected for ampicillin resistance were also resistant to streptomycin and sulphonamides.
Compatibility experiments with the three colicin E factors

The three colicin factors ColE1, ColE2 and ColE3, shown to be compatible with the resistance determinants, were tested against each other. *S. typhimurium* 36 and a colicin E-resistant mutant of K12 were employed as the hosts in these experiments. The R factor T-λ was used to mobilise the colicin factors and colonies selected on tetracycline were examined for colicin production. The colicin indicator strains are listed in Table 2. K12 (ColE1) is sensitive to colicins E2 and E3 but resistant to E1; K12 (ColE2) is sensitive to E1 and E3 but resistant to E2, while K12 (ColE3) is sensitive to E1 and E2 but resistant to E3. ColE1 was compatible with ColE2 and ColE3 as shown in Table 11. Progeny were selected after 2 h and 18 h matings in some experiments and lines from both types of cross were examined in segregation tests.

Compatibility tests with ColE2 and ColE3 indicated that there was usually a low degree of incompatibility between these two colicin factors (Table 12). As these plasmids show about 80% of DNA homology (Inselburg, 1973) they might be expected to belong to the same compatibility group. Loss was most marked (about 10%) with some of the progeny from a 2 h cross after transfer of ColE3 into a strain carrying ColE2; there was a predominant loss of the incoming colicin factor in these experiments. A low rate of loss of either ColE2 or ColE3 was detected from some of the progeny selected after 18 h matings. Lines which appeared to be stable for ColE2 and ColE3 were examined for recombination between the two colicin factors. Six separate lines showed independent transfer of
ColE2 and ColE3, which suggested that the donor strains did not carry only recombinants of ColE2 and ColE3. However, recombination could have occurred to some extent and this would not be detected in these experiments.

The results of the compatibility experiments with the plasmids NTP1 to NTP11 are summarised in Table 13. These resistance and colicin determinants have been provisionally assigned to six groups. These groups appear to be distinct from the compatibility groups of auto-transferring plasmids.
ColE2 and ColE3, which suggested that the donor strains did not carry only recombinants of ColE2 and ColE3. However, recombination could have occurred to some extent and this would not be detected in these experiments.

The results of the compatibility experiments with the plasmids NTP1 to NTP11 are summarised in Table 13. These resistance and colicin determinants have been provisionally assigned to six groups. These groups appear to be distinct from the compatibility groups of auto-transferring plasmids.
Table 11. Compatibility experiments with ColE1, ColE2 and ColE3.

<table>
<thead>
<tr>
<th>Colicin factor in donor strain</th>
<th>Colicin factor in recipient strain</th>
<th>Selection a</th>
<th>Duration of cross</th>
<th>Analysis of progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>ColE1</td>
<td>-</td>
<td>T</td>
<td>18 h</td>
<td>29/105 ColE1</td>
</tr>
<tr>
<td>ColE2</td>
<td>T</td>
<td>2 h</td>
<td></td>
<td>19/20 ColE1, ColE2</td>
</tr>
<tr>
<td>ColE2</td>
<td>T</td>
<td>18 h</td>
<td>Colicinogeny</td>
<td></td>
</tr>
<tr>
<td>ColE3</td>
<td>T</td>
<td>2 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ColE3</td>
<td>T</td>
<td>2 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ColE1</td>
<td>-</td>
<td>T</td>
<td>18 h</td>
<td>10/10 ColE3</td>
</tr>
<tr>
<td>ColE3</td>
<td>T</td>
<td>2 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ColE1</td>
<td>T</td>
<td>18 h</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Colicinogeny and Segregation of clones (pooled data):
- ColE1
- ColE2
- ColE3

Coliogeny:
- ColE1
- ColE2
- ColE3

Segregation of clones:
- ColE1
- ColE2
- ColE3

*a Colonies were selected on tetracycline and examined for colicin production.
Table 12. Compatibility experiments with ColE2 and ColE3.

<table>
<thead>
<tr>
<th>Colicin factor in donor strain</th>
<th>Colicin factor in recipient strain</th>
<th>Selection ( a )</th>
<th>Duration of cross</th>
<th>Analysis of progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>ColE2</td>
<td>-</td>
<td>T</td>
<td>2 h</td>
<td>6/10 ColE2</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>T</td>
<td>18 h</td>
<td>6/10 ColE2</td>
</tr>
<tr>
<td>ColE3</td>
<td>T</td>
<td>2 h</td>
<td>9/10 ColE2, ColE3</td>
<td>8ColE2(^{-}) 4ColE3(^{-}) /1610</td>
</tr>
<tr>
<td>ColE3</td>
<td>T</td>
<td>18 h</td>
<td>2/10 ColE2, ColE3</td>
<td>0ColE2(^{-}) 4ColE3(^{-}) /281</td>
</tr>
<tr>
<td>ColE3</td>
<td>-</td>
<td>T</td>
<td>2 h</td>
<td>40/40 ColE3</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>T</td>
<td>18 h</td>
<td>23/35 ColE3</td>
</tr>
<tr>
<td>ColE2</td>
<td>T</td>
<td>2 h</td>
<td>31/40 ColE2, ColE3</td>
<td>5ColE2(^{-}) 24ColE3(^{-}) /2366</td>
</tr>
<tr>
<td>ColE2</td>
<td>T</td>
<td>18 h</td>
<td>24/70 ColE2, ColE3</td>
<td>4ColE2(^{-}) 12ColE3(^{-}) /1706</td>
</tr>
</tbody>
</table>

\( a \) Colonies were selected on tetracycline and examined for colicin production.
Table 13. Compatibility groups of the non-transferring plasmids

<table>
<thead>
<tr>
<th>Group</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>Not yet grouped</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmids</td>
<td>A (NTP1)</td>
<td>SSu (NTP2)</td>
<td>T (NTP5)</td>
<td>ColE1</td>
<td>ColE2</td>
<td>K (NTP11)</td>
<td>A (NTP6)</td>
</tr>
<tr>
<td></td>
<td>ASu (NTP3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ColE3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ASSu (NTP4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ASSu (NTP7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Compatibility tests with wild strains resistant to streptomycin and sulphonamides

The wild enterobacterial strains carrying streptomycin-resistant Table 5 (page 49) and sulphonamide resistance listed in Table 7 were examined in order to investigate the incidence of SSu resistance determinants which were related to the prototype SSu (NTP2). Four strains (S. typhimurium 6, S. chester, E. coli EC4240 and EC4316) carried transfer factors, with that in the S. chester strain also coding for colicin I. SSu was non-auto-transferring from the remaining 22 strains.

ASu was transferred by T-Δ to the wild strains, selecting for ampicillin or ampicillin and streptomycin resistances. Progeny resistant to A, S and Su were examined for segregation after growth for 6 h in drug-free nutrient broth. The results of these compatibility tests are shown in Table 14. Incompatibility was detected in 19 of the 26 strains which comprised ten different phage types of S. typhimurium, ten salmonella serotypes and five independent E. coli strains. In these 19 cases ASu or SSu were lost at high frequency, with often less than 20% of the colonies retaining both plasmids. These colonies which were ASu/SSu hybrids also showed segregation after growth in drug-free medium. The strains in which incompatibility was found probably carry SSu resistance determinants which are identical with, or closely related to, the SSu determinant of RT1.

The ASu determinant was compatible with the streptomycin-sulphonamide resistance in seven of the strains tested: one
*S. typhimurium* (8M3993); and three other salmonella serotypes, *S. bredeney* (42R652), *S. newport* (42R653) and *S. panama* (42R654).

Three *E. coli* strains showed ASu/SSu stability: EC424O, EC4316 and 3EC635. In these experiments the rate of segregation was no higher than the rate of spontaneous loss of either parent factor.

The wild strains were tested for mobilisation of SSu by $\Delta$ and the F-like transfer factor X (Table 14). The SSu determinants in the 19 strains demonstrating incompatibility with ASu were mobilisable with both transfer factors. No mobilisation of SSu was detected in these tests with five strains showing ASu/SSu stability. The remaining two strains (EC424O and EC4316) possessed transfer factors which directly transferred streptomycin-sulphonamide resistance to *S. typhimurium* 36.
Table 14. Compatibility and mobilisation tests with wild strains resistant to streptomycin and sulphonamides

<table>
<thead>
<tr>
<th>Wild strain</th>
<th>Triparental crosses for determinant mobilisation</th>
<th>Segregation of clones after transfer of ASu into wild SSu strains</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>by A by transfer factor X</td>
<td>No. of clones tested</td>
<td>Loss of ASu</td>
</tr>
<tr>
<td>S. typhimurium 32 8M3993</td>
<td>-       -</td>
<td>1741</td>
<td>1</td>
</tr>
<tr>
<td>S. typhimurium 1</td>
<td>+       +</td>
<td>156</td>
<td>84</td>
</tr>
<tr>
<td>S. typhimurium 44</td>
<td>+       +</td>
<td>502</td>
<td>182</td>
</tr>
<tr>
<td>S. typhimurium 6 *</td>
<td>+       +</td>
<td>439</td>
<td>105</td>
</tr>
<tr>
<td>S. typhimurium 12a</td>
<td>+       +</td>
<td>235</td>
<td>122</td>
</tr>
<tr>
<td>(Human)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. typhimurium 12a</td>
<td>+       +</td>
<td>171</td>
<td>130</td>
</tr>
<tr>
<td>(Animal)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. typhimurium 56</td>
<td>+       +</td>
<td>268</td>
<td>168</td>
</tr>
<tr>
<td>S. typhimurium 95</td>
<td>+       +</td>
<td>147</td>
<td>94</td>
</tr>
<tr>
<td>S. typhimurium Unotypical</td>
<td>+       +</td>
<td>98</td>
<td>59</td>
</tr>
<tr>
<td>S. typhimurium 104</td>
<td>+       +</td>
<td>713</td>
<td>132</td>
</tr>
<tr>
<td>S. typhimurium 126</td>
<td>+       +</td>
<td>317</td>
<td>109</td>
</tr>
<tr>
<td>S. agona</td>
<td>+       +</td>
<td>428</td>
<td>146</td>
</tr>
<tr>
<td>S. brandenburg</td>
<td>+       +</td>
<td>752</td>
<td>199</td>
</tr>
<tr>
<td>S. bredensky 42R652</td>
<td>+       +</td>
<td>1117</td>
<td>5</td>
</tr>
<tr>
<td>S. derby</td>
<td>+       +</td>
<td>580</td>
<td>73</td>
</tr>
<tr>
<td>S. montevideo</td>
<td>+       +</td>
<td>650</td>
<td>248</td>
</tr>
<tr>
<td>&amp; newport 42R653</td>
<td>+       +</td>
<td>812</td>
<td>0</td>
</tr>
<tr>
<td>S. panama 42R654</td>
<td>+       +</td>
<td>677</td>
<td>3</td>
</tr>
<tr>
<td>S. stanley</td>
<td>+       +</td>
<td>459</td>
<td>189</td>
</tr>
<tr>
<td>S. chester *</td>
<td>+       +</td>
<td>580</td>
<td>228</td>
</tr>
<tr>
<td>S. indiana</td>
<td>+       +</td>
<td>558</td>
<td>217</td>
</tr>
<tr>
<td>E.coli EC4240 *</td>
<td>+       +</td>
<td>211</td>
<td>0</td>
</tr>
<tr>
<td>E.coli EC4318 *</td>
<td>+       +</td>
<td>338</td>
<td>0</td>
</tr>
<tr>
<td>E.coli 3C451</td>
<td>+       +</td>
<td>353</td>
<td>19</td>
</tr>
<tr>
<td>E.coli 3C635</td>
<td>+       +</td>
<td>1622</td>
<td>0</td>
</tr>
<tr>
<td>E.coli EC4201</td>
<td>+       +</td>
<td>318</td>
<td>25</td>
</tr>
</tbody>
</table>

* These wild strains carry a transfer factor.
Further investigation of \textit{S. typhimurium} 32 (8M3993)

The ASu determinant was compatible with the SSu resistance of 8M3993. Further attempts were made to mobilise SSu from this line of \textit{S. typhimurium}. T-A and the F-like R factor 240 were transferred separately to 8M3993, and three progeny lines from each cross were tested for transfer of SSu to K12F\(^{-}\); no transfer of SSu was detected.

However, lines of 8M3993 carrying ASu and T-A transferred both ampicillin and streptomycin resistance to K12. Selection on ampicillin resulted in transfer of ASu without SSu, but all progeny selected on streptomycin were resistant to ampicillin, streptomycin and sulphonamides. A further cross to \textit{S. typhimurium} 36 demonstrated that a recombinant ASSu plasmid had been formed. This determinant was transferred by T-A at a frequency of 3 \times 10^{-3} from K12 to \textit{S. typhimurium} 36 in an overnight cross. This frequency was the same as that of the transfer of the original ASu determinant by T-A. It appeared that the SSu of 8M3993 could be mobilised only after recombination with the ASu determinant.

Studies with the other six strains showing ASu/SSu stability

The strains were \textit{S. bredeny} (42R652), \textit{S. newport} (42R653), \textit{S. panama} (42R654) and three \textit{E. coli} strains ED4240, ED4316 and 3EC635. Streptomycin-sulphonamide resistance was not transferable from the three salmonella strains and could not be mobilised after introduction of \(\Delta\), X or 240 into the wild strains (see Table 14). However, in the strain of \textit{S. panama}, 42R654, SSu was mobilised at low frequency.
after introduction of FlacT.

The *E. coli* strain 3EC635 did not transfer SSu, and the resistances were not mobilised by Δ, 240 or the F-like transfer factor X. The remaining *E. coli* strains EC4240 and EC4316 directly transferred SSu to *S. typhimurium* 36 (34R99). Unselected *S. typhimurium* progeny from both crosses were examined for transfer factors but none were detected in these tests. The resistant *S. typhimurium* 36 progeny were tested for transfer and mobilisation of SSu; the results are shown in Table 15.

Table 15. The SSu plasmids of EC4240 and EC4316

<table>
<thead>
<tr>
<th>Progeny</th>
<th>No. of colonies tested</th>
<th>Transfer of SSu by Δ or X to K12F-</th>
<th>Mobilisation of SSu by Δ or X to K12F-</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. typhimurium</em> 36, SSu (ex EC4240)</td>
<td>25</td>
<td>0/25</td>
<td>2/25</td>
</tr>
<tr>
<td><em>S. typhimurium</em> 36, SSu (ex EC4316)</td>
<td>20</td>
<td>1/20</td>
<td>1/19</td>
</tr>
</tbody>
</table>

The *S. typhimurium* 36 progeny carrying SSu from EC4240 and EC4316 were also used for compatibility experiments with ASu. ASu and SSu were compatible in both strains as shown in Table 16.
Table 16. Compatibility tests with SSu plasmids from E. coli EC4240 and EC4316

<table>
<thead>
<tr>
<th>Plasmid in donor strain</th>
<th>Plasmid in recipient strain</th>
<th>Selection</th>
<th>Analysis of progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASu (NTP3)</td>
<td>SSu (ex EC4240)</td>
<td>A</td>
<td>ASu, SSu 0/1136</td>
</tr>
<tr>
<td>ASu (NTP3)</td>
<td>SSu (ex EC4316)</td>
<td>A</td>
<td>ASu, SSu OA-1S-/809</td>
</tr>
</tbody>
</table>

It appears from these results that the streptomycin-sulphonamide resistances of EC4240 and EC4316 are coded by plasmids unrelated to the SSu determinant NTP2. Although the resistances were readily transferable from the wild E. coli strains to S. typhimurium, SSu was then only rarely transferable or mobilizable to K12F-. The reason for these findings is at present unknown.

Transfer of resistance determinants by different transfer factors

Studies on Class 2 transfer systems have shown that determinants seem to be mobilized by many different transfer factors (Anderson, 1966; 1968). However some relationships between determinants and transfer factors appear to be specific. Three resistance determinants have been examined to determine the frequency of transfer of both the determinant and the transfer factor.
1. Transfer of A (NTP1) and SSu (NTP2) in K12. The transfer of these two determinants was compared using two derepressed transfer systems. The transferable plasmids were T-δdrp1 and FlacT; transfer from K12 to K12. As shown in Table 17 the A determinant was transferred very efficiently (up to 30% in 1 h) by T-δdrp1 but only at a frequency of $10^{-4}$ by FlacT. SSu was also mobilised at higher frequency by T-δdrp1 than by FlacT, but the transfer frequencies were lower than those found with A.

Table 17. Transfer of A (NTP1) and SSu (NTP2) in derepressed transfer systems in K12 a.

<table>
<thead>
<tr>
<th>Plasmids in donor strain</th>
<th>Selection</th>
<th>Frequency of transfer in 1 h crosses</th>
<th>R-type of progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (NTP1) + T-δdrp1</td>
<td>A</td>
<td>$3 \times 10^{-1}$</td>
<td>0.3% A; 99.7% AT</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>$6 \times 10^{-1}$</td>
<td>60% T; 40% AT</td>
</tr>
<tr>
<td>A (NTP1) + FlacT</td>
<td>A</td>
<td>$1 \times 10^{-4}$</td>
<td>100% AT</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>$10^{0}$</td>
<td>100% T</td>
</tr>
<tr>
<td>SSu (NTP2) + T-δdrp1</td>
<td>Su b</td>
<td>$4 \times 10^{-3}$</td>
<td>0.2% SSu; 99.8% SSuT</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>$8 \times 10^{-1}$</td>
<td>98.4% T; 1.6% SSuT</td>
</tr>
<tr>
<td>SSu (NTP2) + FlacT</td>
<td>Su</td>
<td>$4 \times 10^{-6}$</td>
<td>0.7% SSu; 99.9% SSuT</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>$6 \times 10^{-1}$</td>
<td>100% T</td>
</tr>
</tbody>
</table>

a Donor to recipient ratio of 1 : 10.

b All colonies selected on sulphonamides were also resistant to streptomycin.
The frequency of transfer of the A determinant by T-Adrp1 is higher in a 1 h cross than that of A by repressed T-A in overnight crosses (usually about $2 \times 10^{-2}$). There is spread of the transfer factor alone in the recipient population in overnight crosses, whereas in a derepressed system the transfer reaches a maximum in less than 1 h. Therefore derepressed transfer systems provide a good method of studying the efficiency of transfer of determinants by transfer factors.

2. Transfer of A (NTP1) and SSu (NTP2) from K12 to *S. typhimurium* type 36. The transfer of A and SSu by T-Adrp1 from K12 to *S. typhimurium* 36 was measured in 1 h crosses (Table 17a). In both matings the frequency of transfer of the transfer factor was lower than in the crosses from K12 to K12.

*S. typhimurium* 36 clearly acts as a poorer recipient in short matings with K12 donors. Analysis of the progeny from the two crosses revealed that transfer of the determinant alone was obtained at very high frequency. This is a significant difference to the results with two K12 strains in which the frequency of transfer of the determinant alone was less than 0.3%. The reason for this difference between *S. typhimurium* 36 and K12 as recipients is unknown.

3. Transfer of the T determinant NTP5 in K12. The transfer of NTP5 was also compared in two derepressed transfer systems; the results are shown in Table 18.
Table 17a. Transfer of A (NTP1) and SSu (NTP2) from K12 to *S*. *typhimurium*.

<table>
<thead>
<tr>
<th>Plasmids in donor strain</th>
<th>Selection</th>
<th>Frequency of transfer in 1 h crosses</th>
<th>R-type of progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (NTP1) + T-Adr1</td>
<td>A</td>
<td>4 x 10^{-4}</td>
<td>81.6% A; 18.4% AT</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>1 x 10^{-4}</td>
<td>61.6% T; 38.4% AT</td>
</tr>
<tr>
<td>SSu (NTP2) + T-Adr1</td>
<td>Su a</td>
<td>4 x 10^{-3}</td>
<td>99% SSu; 5% SSuT</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>5 x 10^{-4}</td>
<td>63% T; 37% SSuT</td>
</tr>
</tbody>
</table>

a Colonies selected on sulphonamides were also resistant to streptomycin.

Table 18. Transfer of the T determinant NTP5 a

<table>
<thead>
<tr>
<th>Plasmids in donor strain</th>
<th>Selection</th>
<th>Frequency of transfer in 1 h crosses</th>
<th>R-type of progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTP5 + R1-19 K− (ACSSu)</td>
<td>T</td>
<td>3 x 10^{-4}</td>
<td>0.3% T; 99.7% ACSSuT</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>4 x 10^{-1}</td>
<td>100% ACSSu</td>
</tr>
<tr>
<td>NTP5 + A-Adr1 b</td>
<td>T</td>
<td>2 x 10^{-2}</td>
<td>1.3% T; 98.7% AT</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>2 x 10^{-1}</td>
<td>95% A; 7% AT</td>
</tr>
</tbody>
</table>

a Donor to recipient ratio of 1 : 10.

b A-Adr1 is a derepressed mutant of the I-like R factor A-Δ isolated in this laboratory by N. D. F. Grindley.
The transfer of the T determinant NTP5 by the I-like R factor A-Adrp1 was more efficient than mobilisation by the F-like R factor R1-19 K⁻. This is a similar specificity to that shown by A and SSu.

Properties of the kanamycin resistance determinant (K).

A determinant for resistance to neomycin and kanamycin (K) was originally isolated from a strain of S.typhimurium phage type 29 (Anderson, Pitton and Mayhew, 1968; Anderson, Mayhew and Grindley, 1969). From its original host strain, 5M^136, the K determinant was transferred by an fi⁺ transfer factor which was designated X (Anderson, Pitton and Mayhew, 1968). After mating 5M^136 with K12 F⁻, lines carrying K alone were isolated and K was easily mobilised by the F factor and formed a Class 2 transfer system. The K determinant was originally fi⁺ (= fi⁺K), but transfer by F gave rise to K12 F⁺K lines, which were derepressed and lysed by the F-specific phage u2. Spontaneous mutation of fi⁺K probably yielded fi⁻K by loss of the fi⁺ and possibly other regions. K is non auto-transferring in both its fi⁺ and fi⁻ states.

For most experiments described in this thesis the fi⁻ form of the K determinant was used and it will be referred to as K. Strains carrying K were resistant to neomycin, kanamycin and paromomycin; this suggests that the resistances are caused by phosphorylation of the antibiotic. To study the properties of K transfer by F in K12 and S.typhimurium, K was mobilised by Flac using the triparental cross, the final recipient being S.typhimurium 36 (RT576). Lines of
both K12 and *S. typhimurium* 36 carrying Flac and K were lysed in surface spot tests by phage μ2. The transfer of K by Flac from *S. typhimurium* 36 to K12 and to *S. typhimurium* 36 is shown in Table 19.

Table 19. Transfer of K by Flac in *S. typhimurium* 36 and K12

<table>
<thead>
<tr>
<th>Cross</th>
<th>Donor</th>
<th>Recipient</th>
<th>Time</th>
<th>Frequency of K transfer</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. typhimurium</em> 36(K,Flac)</td>
<td>K12</td>
<td>30 min</td>
<td>$3 \times 10^{-5}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 h</td>
<td>$7 \times 10^{-4}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>18 h</td>
<td>$2 \times 10^{-4}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>S. typhimurium</em> 36</td>
<td>30 min</td>
<td>$7 \times 10^{-5}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 h</td>
<td>$2 \times 10^{-4}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>18 h</td>
<td>$7 \times 10^{-5}$</td>
<td></td>
</tr>
</tbody>
</table>

K was transferred by Flac to K12 at a higher frequency than to an *S. typhimurium* 36 in overnight cross, although there was no difference in transfer in the short crosses. Selected and unselected progeny from both crosses were tested with phage μ2. All K12 progeny carrying Flac and K, or Flac alone, were sensitive to the phage. One hundred and forty-eight *S. typhimurium* 36(K,Flac) lines were lysed by μ2 but
all of 337 S. *typhimurium* 36 clones that had received Flac alone were resistant to μ2. Thus, it appeared that Flac, which was derepressed in K12, was repressed in *S. typhimurium* 36 and its derepression in this system seemed to depend on the presence of the K determinant.

The repression of F in *S. typhimurium* 36 was confirmed by measuring Flac transfer from *S. typhimurium* 36(K,Flac) and *S. typhimurium* 36(Flac). As shown in Table 20, Flac transfer was reduced approximately 500 fold in 2 h crosses from *S. typhimurium* 36(Flac) compared with transfer from *S. typhimurium* 36(K,Flac) as donor. The degree of F fimbriation of the two donor strains was determined by electron microscopy. The cultures were grown in peptone water and phage μ2 was added at a multiplicity of 100 phage per bacterium. Preparations were negatively stained with sodium silicotungstate, and the degree of F fimbriation was examined in the electron microscope. Of 28 *S. typhimurium* 36(K,Flac) cells observed, 18 carried sex fimbriae, while *S. typhimurium* 36 carrying Flac alone showed only one F-fimbriated cell out of 36 examined. It was suggested that *S. typhimurium* 36 produces a repressor which inhibits the fertility of F and of repressor-minus mutants of F-like R factors, and that the repression was reversed by the product of a locus, designated *der*, associated with the K determinant (Smith *et al.*, 1970; Gridley *et al.*, 1971).

The derepression of the F factor by K in *S. typhimurium* was further investigated by studying a selection of different phage types. FlacT was introduced into the *S. typhimurium* strains by overnight crosses. FlacT is a recombinant of Flac and the tetracycline resistance marker
Table 20. Transfer of Flac from *S. typhimurium* 36(K,Flac) and *S. typhimurium* 36(Flac).

<table>
<thead>
<tr>
<th>Cross</th>
<th>Time</th>
<th>Frequency of Flac transfer</th>
<th>Sensitivity of donor to phage p2</th>
<th>Proportion of donor cells carrying F fimbriae (electron microscopy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor</td>
<td>Recipient</td>
<td>2 h</td>
<td>18 h</td>
<td>24 h</td>
</tr>
<tr>
<td><em>S. typhimurium</em> 36(K,Flac)</td>
<td>K12</td>
<td>2 h</td>
<td>6 x 10^{-2}</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18 h</td>
<td>6 x 10^{-1}</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>S. typhimurium</em> 36</td>
<td>2 h</td>
<td>4 x 10^{-2}</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>18 h</td>
<td>3 x 10^{-1}</td>
<td></td>
</tr>
<tr>
<td><em>S. typhimurium</em> 36(Flac)</td>
<td>K12</td>
<td>2 h</td>
<td>2 x 10^{-5}</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18 h</td>
<td>5 x 10^{-1}</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>S. typhimurium</em> 36</td>
<td>2 h</td>
<td>2 x 10^{-5}</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>18 h</td>
<td>3 x 10^{-2}</td>
<td></td>
</tr>
</tbody>
</table>

+ = Visible lysis with phage p2.
- = No visible lysis with phage p2.
of T-Å. It is indistinguishable from the original Flac except for the drug resistance marker, and the T resistance facilitates selection of recipient cells into which FlacT has been introduced (Anderson and Smith, 1972b). The strains were drug-sensitive and no transfer factors could be detected in any of them when they were tested with the triparental cross for determinant mobilisation. Seventeen of 22 S. typhimurium strains carrying FlacT were insensitive to μ2. The K determinant was then introduced into these strains and the resulting lines, which carried both FlacT and K, were tested with μ2 (Table 21). At least five colonies carrying K and FlacT were examined in each experiment. FlacT was lost at high frequency from three strains, 21R342, 21R343 and 21R344, and the results were therefore omitted. All the remaining 14 strains tested, which had received K, had become sensitive to μ2, although there was some variation in the degree of visible lysis. The fi+ character is present in most of the S. typhimurium strains examined, and the effect of introducing K suggests that the inhibition may be similar in nature to that found in S. typhimurium 36. However, the experiments do not indicate whether the fi+ property is determined by an independent plasmid or the bacterial chromosome. If the fi+ region were on a plasmid, derepression of F by K in S. typhimurium could be caused by incompatibility between K and that plasmid, resulting in elimination of the latter, in which case the derepression would be simply the result of loss of the fi+ plasmid.
Table 21. Reactions of *S*. *typhimurium* strains with F-specific phage μ2

<table>
<thead>
<tr>
<th>ERL No.</th>
<th>Phage type of <em>S</em>. <em>typhimurium</em> strain</th>
<th>Sensitivity to μ2 of <em>S</em>. <em>typhimurium</em> strains carrying FlacT</th>
<th>Sensitivity to μ2 of <em>S</em>. <em>typhimurium</em> strains carrying FlacT and K</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT576</td>
<td>36*</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>21R339</td>
<td>1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>21R336</td>
<td>4</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>1R213</td>
<td>4 (= LT2)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>21R341</td>
<td>8</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>21R343</td>
<td>12a</td>
<td>-**</td>
<td>*</td>
</tr>
<tr>
<td>21R337</td>
<td>14</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>21R342</td>
<td>32</td>
<td>-**</td>
<td>*</td>
</tr>
<tr>
<td>19R688***</td>
<td>36</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>21R340</td>
<td>74</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>21R344</td>
<td>104</td>
<td>-**</td>
<td>*</td>
</tr>
<tr>
<td>21R338</td>
<td>160</td>
<td>+</td>
<td>*</td>
</tr>
<tr>
<td>21R325</td>
<td>168</td>
<td>+</td>
<td>*</td>
</tr>
<tr>
<td>21R326</td>
<td>169</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>21R327</td>
<td>170</td>
<td>+</td>
<td>*</td>
</tr>
<tr>
<td>21R328</td>
<td>173</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>21R329</td>
<td>180</td>
<td>+</td>
<td>*</td>
</tr>
<tr>
<td>21R330</td>
<td>181</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>21R331</td>
<td>182</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>21R332</td>
<td>184</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>21R333</td>
<td>185</td>
<td>-</td>
<td>✓</td>
</tr>
<tr>
<td>21R334</td>
<td>186</td>
<td>+</td>
<td>*</td>
</tr>
<tr>
<td>21R335</td>
<td>187</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

* = Clear lysis with μ2.
ι = Turbid lysis with μ2.
- = No visible lysis with μ2.
control strain.
** FlacT was very unstable in these *S*. *typhimurium* strains.
*** Type 36 independent of RT576.
Compatibility experiments with the K determinant

Experiments were performed to determine whether the K determinant was compatible with transferable plasmids of known compatibility groups. Each standard plasmid was transferred to K12F⁻lac⁻Str⁻ carrying K selecting for a property coded by the incoming plasmid. Progeny were examined for the presence of both plasmids and tested for stability as described in Materials and Methods. The results of these experiments are shown in Table 22. K coexisted stably with plasmids representing groups F_I, F_II, F_IV, F_V, I, B, N, H_1, H_2 and W.

The K determinant was also tested in compatibility experiments with non-transferring plasmids (Table 23). K was compatible with A (NTP1), SSu (NTP2), T (NTP5), A (NTP6) and the three colicin factors. Since NTP11 also codes for resistance to kanamycin alone it has not been tested with the K determinant.

It is clear from these results that the K determinant represents a distinct compatibility type from groups of both transferable and non-transferring plasmids studied in this laboratory.
Table 22. Compatibility experiments with the K determinant and standard transferable plasmids

<table>
<thead>
<tr>
<th>Plasmid in donor strain</th>
<th>Compatibility group</th>
<th>Selection</th>
<th>Analysis of progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>R-type</td>
</tr>
<tr>
<td>FlacT</td>
<td>F_I</td>
<td>T</td>
<td>K, T</td>
</tr>
<tr>
<td>R1-19K^-</td>
<td>F_II</td>
<td>C</td>
<td>K, ACSu</td>
</tr>
<tr>
<td>TP129</td>
<td>F_IV</td>
<td>T</td>
<td>K, T</td>
</tr>
<tr>
<td>F_0-lac</td>
<td>F_V</td>
<td>-</td>
<td>K, lac^+</td>
</tr>
<tr>
<td>T-4</td>
<td>L_4</td>
<td>T</td>
<td>K, T</td>
</tr>
<tr>
<td>TP118</td>
<td>N</td>
<td>A</td>
<td>K, A</td>
</tr>
<tr>
<td>TP125</td>
<td>B</td>
<td>C</td>
<td>K, CSuT</td>
</tr>
<tr>
<td>TP117</td>
<td>H_1</td>
<td>T</td>
<td>K, T</td>
</tr>
<tr>
<td>TP116</td>
<td>H_2</td>
<td>C</td>
<td>K, CSu</td>
</tr>
<tr>
<td>S-a</td>
<td>W</td>
<td>C</td>
<td>K, CSu</td>
</tr>
</tbody>
</table>

a The recipient strain in each case was K12^F-lac^-Str^- carrying the K determinant.

b F_0-lac is compatible with all other F-like plasmids examined so far (Datta, 1975; unpublished results of this laboratory).
Table 23. Compatibility experiments with the K determinant and other non-transferring plasmids.

<table>
<thead>
<tr>
<th>Plasmid in donor strain</th>
<th>Plasmid in recipient strain</th>
<th>Selection</th>
<th>Analysis of progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>-</td>
<td>K</td>
<td>0/385</td>
</tr>
<tr>
<td>A (NTP1)</td>
<td>K</td>
<td>K, A</td>
<td>0/272</td>
</tr>
<tr>
<td>SSu (NTP2)</td>
<td>K</td>
<td>K, SSu</td>
<td>2K&lt;sup&gt;-&lt;/sup&gt; 0S&lt;sup&gt;-&lt;/sup&gt;/242</td>
</tr>
<tr>
<td>T (NTP5)</td>
<td>K</td>
<td>K, T</td>
<td>0/323</td>
</tr>
<tr>
<td>A (NTP6)</td>
<td>K</td>
<td>K, A</td>
<td>0/434</td>
</tr>
<tr>
<td>ColE1</td>
<td>K</td>
<td>K, ColE1</td>
<td>1K&lt;sup&gt;-&lt;/sup&gt; 0Col&lt;sup&gt;-&lt;/sup&gt;/498</td>
</tr>
<tr>
<td>ColE2</td>
<td>K</td>
<td>K, ColE2</td>
<td>0/230</td>
</tr>
<tr>
<td>ColE3</td>
<td>K</td>
<td>K, ColE3</td>
<td>1K&lt;sup&gt;-&lt;/sup&gt; 0Col&lt;sup&gt;-&lt;/sup&gt;/250</td>
</tr>
</tbody>
</table>

<sup>a</sup> The K determinant was mobilised by F or FlacT in these compatibility experiments.
Recombination between the \( \text{fi}^+ \) property of \textit{S. typhimurium} 36 and \text{KColIb}

It was suggested that the \( \text{fi}^+ \) character of \textit{S. typhimurium} 36 and other \textit{S. typhimurium} strains was determined by a plasmid (page 90). In order to test this hypothesis experiments were performed in an attempt to transfer the \( \text{fi}^+ \) property into \text{K12}.

A line of \textit{S. typhimurium} 36 into which a \text{KColIb} R factor TP110 had been introduced two years earlier was examined for mobilisation of the \( \text{fi}^+ \) property. The strain was mated overnight with \text{K12 HfrH Nal}^r with selection on kanamycin and nalidixic acid. Of fifty progeny tested with phage \( \mu_2 \), one was resistant to the phage, while the rest were visibly lysed like the HfrH control strain. This single line of HfrH (KColIb) was then crossed with \text{K12(Flac)} to determine whether the \( \text{fi}^+ \) character could be transferred by KColIb to another strain. All K12 lines carrying Flac and KColIb were resistant to \( \mu_2 \), indicating that the \( \text{fi}^+ \) property was transferred by the R factor.

A compatibility experiment was performed to examine whether the \( \text{fi}^+ \) region was covalently linked to the KColIb factor. The I-like R factor T-\( \Delta \) was mated with the \( \mu_2 \)-resistant line of HfrH (KColIb) and progeny were selected on tetracycline. All lines were fully sensitive to \( \mu_2 \) and had lost KColIb. It was concluded that the \( \text{fi}^+ \) character of \textit{S. typhimurium} 36 had recombined with KColIb; the recombinant plasmid was designated KColIb \( \text{fi}^+ \).

These observations support the hypothesis that the \( \text{fi}^+ \) property of \textit{S. typhimurium} is plasmid-born, but the results could also be explained by recombination between KColIb and a chromosomal \( \text{fi}^+ \).
marker. However there is no evidence for association of I-like plasmids with the chromosome (Edwards and Meynell, 1969; N. D. F. Grindley and E. S. Anderson, unpublished observations).

The properties of KColIb fi+ were investigated further by measuring the effect of the plasmid on F fertility in HfrH. The frequency of pro transfer to a K12F- recipient was measured in 1 h crosses. The results including the controls are shown in Table 24.

Table 24. The effect of KColIb fi+ on K12 HfrH

<table>
<thead>
<tr>
<th>Strain</th>
<th>Reaction with phage μ2</th>
<th>Frequency of pro transfer in 1 h crosses</th>
</tr>
</thead>
<tbody>
<tr>
<td>K12 HfrH</td>
<td>+</td>
<td>2.9 x 10^-2</td>
</tr>
<tr>
<td>K12 HfrH (KColIb)</td>
<td>+</td>
<td>1.9 x 10^-2</td>
</tr>
<tr>
<td>K12 HfrH (KColIb fi+)</td>
<td>-</td>
<td>3.0 x 10^-5</td>
</tr>
</tbody>
</table>

+ = visible lysis with μ2 in spot tests.
- = no visible lysis with μ2.

The presence of KColIb fi+ reduced the frequency of pro transfer from HfrH approximately a thousandfold.
Recombination between the \( f_i^+ \) property of \( S.\text{typhimurium} \) 36 and the ASu determinant NTP3.

Lines of \( S.\text{typhimurium} \) 36 (RT576) into which the ASu determinant NTP3 had been introduced five years earlier, were examined for loss of resistance markers. Of 651 colonies examined in one experiment, 436 had lost both ampicillin and sulphonamide resistance and 7 colonies had lost sulphonamide resistance only. Crosses with these seven ampicillin-resistant lines demonstrated that A was transferable to K12 F\(^-\) at a frequency of \( 10^{-7} \). This was a surprising result because ASu is a non-transferring plasmid and no transfer factor activity could be demonstrated with the host strain, \( S.\text{typhimurium} \) 36, when it was tested in triparental crosses for mobilisation of resistance determinants such as SSu. Further experiments confirmed that ampicillin resistance was transferable from K12 to K12 at the same low frequency (about \( 10^{-7} \)). The \( f_i \) character of the new plasmid was examined by transferring Flac\( T \) to K12 lines carrying the R factor. All 20 colonies tested were resistant to the F-specific phage \( \mu 2 \) in surface spot tests. The new R factor was \( f_i^+ \); it was provisionally designated A* (Smith et al., 1973b).

The A* plasmid was tested for compatibility with the K determinant. K was transferred by Flac to \( S.\text{typhimurium} \) 36 carrying A* with selection by kanamycin. After a 7 h mating all ten progeny lines examined were resistant to ampicillin and kanamycin. These clones were grown in drug-free nutrient broth
for 5 h and examined for segregation by replica plating. Of 338 colonies tested, two had lost kanamycin resistance and 266 had lost ampicillin resistance. There was no loss of resistance markers from the donor and recipient strains carrying K and A* respectively. Examination of progeny selected with kanamycin after an overnight cross revealed that eight of ten lines were sensitive to ampicillin. Loss of ampicillin resistance was accompanied by loss of the fi+ character: lines carrying K and the F factor were visibly lysed by phage μ2, while lines still resistant to ampicillin were fi+ character. These results suggest that the A region of the ASu determinant has recombined with the plasmid present in S.typhimurium 36 which determines the fi+ character. This recombinant R factor A* is incompatible with the K determinant. The introduction of K into several S.typhimurium strains reversed the inhibition of F fertility in all cases (Table 21). Therefore the fi+ character of these S.typhimurium strains is probably like that of S.typhimurium 36, and is determined by a plasmid.
SECTION II. MOLECULAR STUDIES OF NON-TRANSFERRING PLASMIDS

Materials and Methods

Reagents

The reagents were obtained as follows:

Brij 58
Sarkosyl NL35 (sodium dodecyl sarcosinate)
Sodium lauryl sulphate (SLS)
Sodium deoxycholate
Lysozyme (three times crystallized)
Ribonuclease (RNase)
Pronase (protease Type VI)
Cytochrome c (Type III)
Ammonium acetate. Analar grade
Formaldehyde. Analar grade
Ethidium bromide
Caesium chloride
2' deoxyadenosine
Trizma Base and Trizma HCl (Tris)
Hydroxyapatite

Other chemicals were analytical reagent grade from B.D.H. Chemicals Ltd. or Hopkin and Williams Ltd.
The radiochemicals and reagents for scintillation counting were:

Thymidine - (methyl-³H) specific activity >15 Ci/m mole. (TRK120) Radiochemical Centre, Amersham.

2,5'-diphenyloxazole (PPO) Packard Instrument Co. Inc.
1,4'-bis-[2-(4-methyl-5-phenyloxazolyl)] benzene (dimethyl POPOP) Packard Instrument Co. Inc.

Sulphur-free toluene B.D.H. Chemicals Ltd.

Scintillation fluid contained 4g of PPO, 0.2g of dimethyl POPOP per litre of sulphur-free toluene.

Buffers and solutions

M9-glucose minimal liquid medium

This was prepared as follows:

M9 salts (10 x concentrated) 10 ml
20% glucose 1 ml
0.1 M Mg SO₄ 1 ml
0.01 M CaCl₂ 1 ml
0.2% Thiamine 1 ml
Trace elements 0.1 ml
Distilled water 86 ml

The M9 salts solution (10 x concentrated) contains per litre:

Na₂HPO₄ 60 g
KH₂PO₄ 30 g
NaCl 5 g
NH₄Cl 10 g
Constituents of trace elements per litre:

- Na₂₃ B₄ O₇ • 10H₂O  
  88 mg
- CuSO₄ • 5H₂O  
  393 mg
- Fe₃(SO₄)₂ • 9H₂O  
  0.91 mg
- MnCl₂ • 4H₂O  
  72 mg
- (NH₄)₆Mo₇O₂₄ • 4H₂O  
  36.8 mg
- ZnSO₄ • 7H₂O  
  8.81 mg

Saline - EDTA = 0.15M NaCl, 0.1M EDTA pH 8.0.

Standard saline citrate (SSC) = 0.15M NaCl, 0.015M trisodium citrate, pH 7.0.

Phosphate buffer (PB) was an equimolar mixture of Na₂HPO₄ and NaH₂PO₄.

It was prepared at 0.4M in 5 litre volumes:

\[
\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O} \quad 178 \text{ g} \\
\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O} \quad 156 \text{ g} \\
\text{EDTA} \quad 9.3 \text{ g}
\]

This solution was diluted to 0.14M.

Sucrose solutions (15% and 50% W/W) for sucrose gradients contained 0.01M EDTA, 0.06M KCl and 0.02M Tris, pH 7.3.

Solutions for lysis procedures

a) For Brij lysis method

25% sucrose also contained 0.05M Tris and was adjusted to pH 8.0.

Lysozyme solution contained 5 mg/ml in 0.25M Tris, pH 8.0.

Brij lysis mixture consisted of 1% Brij 58; 0.4% sodium deoxycholate; 0.0625M EDTA and 0.05M Tris, pH 8.0.

(Ethidium bromide stock solution contained 700 µg/ml in TES buffer).
b) Sarkosyl lysis method

TES buffer = 0.05M Tris, 0.005M EDTA, 0.05M NaCl, pH 8.0.

Spheroplast forming mixture contained 1 mg/ml lysozyme, 
0.5 mg/ml RNase and 100 mg/ml sucrose in TES buffer.

Growth of strains for isolation of plasmid DNA

In most experiments the host strains for the plasmids were 
1R713 or 14R525. Strains were grown overnight in M9-glucose medium 
and the following morning 1 ml was added to 20 ml of fresh medium 
in a 125 ml flask with a side arm. The flask was shaken in an 
Aquatherm G86 water bath shaker (New Brunswick Scientific Co. Inc.) 
at 37° C, the platform rotating at 200 r.p.m. Growth was monitored 
with a Klett Summerson photo-electric colorimeter (A. Thomas Co.). 
1 ml of deoxyadenosine (5 mg/ml) was added in early exponential 
phase followed by 0.1 mCi/ml of ³H-thymidine. The cells were then 
grown to late exponential phase and harvested.

Isolation of plasmid DNA

a) Preparation of cleared lysates. The cells were harvested by 
centrifugation from M9 medium and washed with cold phosphate 
buffer (0.1M, pH 7.0). The lysis procedure was that of Clewell 
and Helinski (1969). The conditions given were used for 20 ml 
cultures and were altered for other volumes. The cells were 
resuspended in 0.66 ml of cold 25% sucrose in a 10 ml polycarbonate 
tube (M.S.E.), and were converted to spheroplasts by treatment
with 0.13 ml of lysozyme solution followed after 5 min at 0°C by 0.27 ml of EDTA (0.25M pH 8.0). After another 5 min at 0°C with occasional mixing, the spheroplasts were lysed by adding 1.1 ml of Brij lysis mixture. The suspension became viscous and cleared after 2 to 5 min. The lysate (volume = 2.16 ml) was centrifuged in a fixed angle-rotor (10 x 10 ml) at 26,000 r.p.m. at 4°C for 25 min in an M.S.E. Superspeed 65 (about 48,000 g at average radius). More than 99.5% of the chromosomal DNA is usually pelleted by this centrifugation leaving the supernatant, termed the "cleared lysate". The cleared lysate was made up to 3.8 g with dist. H₂O and 1.6 ml of ethidium bromide (700 μg/ml) was added. 5.2 g of caesium chloride was dissolved in this solution, which was centrifuged (96,000 g) at 36,000 r.p.m. in the 10 x 10 ml fixed angle rotor for approximately 60 h at 18°C.

The tubes were pierced with an M.S.E. tube piercer and 8-drop fractions were collected. 10 μl samples were spotted on to Whatman 3MM filter paper discs and these were washed in 5% trichloroacetic acid (TCA) for 5 min. The discs were transferred to 95% ethanol and finally to ether before drying. The dried discs were placed in vials with 5 ml scintillation fluid and counted in a Nuclear Enterprises liquid scintillation spectrometer. Fractions containing DNA were identified by the presence of ³H label and pooled. Ethidium bromide was removed by shaking three times with an equal volume of isopropanol followed by a final extraction with ether. The DNA solution
was dialysed against distilled water for 4 h at 4°C and then
dialysed against 0.15M ammonium acetate or against 0.14M PB for
DNA reassociation studies.

b) Dye-buoyant density gradient centrifugation of Sarkosyl lysates.

Plasmid DNA was isolated by the method of Bazaral and Helinski
(1968). The plasmid-carrying strains were grown in M9-glucose
medium and treated as follows from 5 ml volumes. The cells
were harvested by centrifugation, washed in TES buffer at 0°C
and resuspended in 0.4 ml of spheroplast-forming mixture. The
suspension was incubated at 37°C for 10 min, then chilled in
ice for 5 min. 0.2 ml of 2% Sarkosyl solution was added, and
the suspension was mixed well before adding 0.4 ml of TES buffer.
DNA shearing was effected by passing the lysate several times
through a narrow-tipped pipette (1 ml). 0.8 ml of the sheared
lysate was mixed with 3 ml of distilled water and 1.6 ml of
ethidium bromide (700 µg/ml in TES) before dissolving 5.2 g of
cesium chloride in the mixture. The solution was centrifuged
in a 10 x 10 ml fixed angle rotor at 36,000 r.p.m. for 60 h at
18°C. The tube was pierced as before and the gradients were
dripped slowly to avoid sucking the upper chromosomal band into
the lower plasmid band. In a cesium chloride-ethidium bromide
density gradient the covalently-closed plasmid DNA has a higher
density than the chromosomal DNA, and bands below it in the density
gradient. The gradient was fractionated and sampled as described
previously. Fractions containing plasmid DNA were pooled, ethidium
bromide was removed and the solution was dialysed against 0.15 M
ammonium acetate or against 0.14M PB.
c) **Sucrose gradients.** In some experiments plasmid DNA was purified by sedimentation through a sucrose gradient. 15-50% linear neutral sucrose gradients (25 ml) were prepared and 1 ml of the cleared lysate was layered on the top of a gradient. The tubes were centrifuged at 26,000 r.p.m. in a 3 x 23 ml swing-out rotor for 15 h at 4°C. The tubes were pierced as above and 20-drop fractions were collected in small tubes. Samples of 50 μl were spotted on to filter paper discs and treated as described previously. Fractions containing plasmid DNA, identified by the presence of 3H-label, were pooled and concentrated by further centrifugation in a cesium chloride gradient. 6.6 g of cesium chloride was dissolved in 5.4 g of the solution containing the plasmid DNA and the tubes were centrifuged at 36,000 r.p.m. in the 10 x 10 ml angle rotor for approximately 60 h at 18°C. The plasmid DNA was then prepared for electron microscopy or reassociation experiments.

**Electron microscopy**

Carbon coated grids were prepared for electron microscopy by the micro-version of the spontaneous adsorption method of Lang and Mitani (1970). The DNA solution was diluted (20 to 100 fold) in 0.15M ammonium acetate - 0.07M formaldehyde. 1 μl of cytochrome c (1 mg/ml) was added to 0.8 ml of the DNA solution and 50 μl drops of the mixture were transferred to a Teflon slab. A monolayer of denatured protein forms at the surface of the drops and the DNA spreads through a liquid in a manner similar to a film.
adsorbs to this layer. The drops were left to stand for approximately 30 - 60 min before transferring the surface films to the grids. These were washed by touching a surface of absolute alcohol for 10 sec.

The grids were rotary-shadowed with platinum at an angle of 7° to 10° in an Edwards E12E3 vacuum-coating unit. Platinum wire (about 1 in of 0.005 in diam.) was wound on a piece of tungsten wire 0.05 in in diameter which was located between the terminals of the unit. The grids were placed on a holder which had been lightly smeared with Araldite CY212 epoxy resin. The platinum was evaporated at 5 x 10^{-6} torr by passing a current of 5 amps through the tungsten wire.

The grids were examined and micrographs taken with an AEI EM6B electron microscope. Micrographs of open circular molecules were enlarged eight times, the molecules were traced on to paper and measured with a map measurer. The magnification of the microscope was calibrated with a carbon replica of a diffraction grating (2160 lines per mm). The contour lengths were calculated and the molecular weights derived from these data, assuming 1 μm = 2.07 x 10^{6} daltons (Lang, 1970).

Preparation of total unlabelled DNA

Total unlabelled DNA was prepared by a modification (Grindley et al., 1973b) of the method of Marmur (1961). Between 0.5 and 1 ml of a broth culture of the strain (about 5 x 10^{8} organisms/ml) was spread on each of eight 6 in diameter nutrient agar plates, supplemented
with antibiotics when required. The plates were incubated overnight at 37°C, the cells were harvested, washed in 25 ml saline - EDTA before resuspending in 100 ml of the same solution. 4 ml of 2% SLS and 2 ml of pronase (5 mg/ml) were added to the suspension which was incubated at 37°C until a clear lysate was obtained. Lysis was usually complete in 2 - 3 h, but occasionally it was necessary to leave the suspension overnight. 25 ml of 5M sodium perchlorate was added, followed by 100 ml of 24:1 (vol/vol) chloroform-isooamyl alcohol. This mixture was well shaken for 30 min and the phases were separated by centrifugation at 5,000 r.p.m. for 10 min. The upper aqueous layer was collected at 0°C and the DNA was precipitated by adding two volumes of cold 95% ethanol. The DNA was spooled on to a glass rod by stirring gently and then redissolved in 45 ml of 0.1 x SSC. The salt concentration was adjusted to 1 x SSC by addition of 5 ml of 10 x SSC. Ribonuclease (1.3 ml of a 2 mg/ml solution) was added and the solution was incubated at 60°C for 1 h. The ribonuclease was extracted with 1 volume of phenol and the mixture was left for about 18 h. One volume of chloroform-isooamyl alcohol was added and the mixture was agitated for 30 min. Protein separated at the interface between the DNA suspension and the chloroform-isooamyl alcohol. This was removed together with the protein. Three or four extractions were made in this way until no interphase material was visible. The DNA was precipitated with cold ethanol as described above and resuspended in SSC. This was repeated twice and the DNA was finally resuspended in 9 ml of 0.1 x SSC and adjusted to 1 x SSC. The DNA was then precipitated twice with 2.5 volumes of 2-ethoxyethanol
before resuspending in 9 ml of 0.01 SSC in 0.025M EDTA. The concentration of DNA was kept above 0.5 mg/ml at this stage because it is poorly precipitated from dilute solutions by ethoxyethanol. A sample (25 μl) in 1.25 ml distilled water was examined in a spectrophotometer at 230, 260 and 280 nm. It was assumed that an OD_260 of 1 was equivalent to 50 μg/ml (Mandel and Marmur, 1967). The DNA concentration of each preparation was adjusted to 0.6 mg/ml before use in the reassociation experiments.

**DNA reassociation experiments**

The techniques were based on the methods of Brenner et al. (1969a) and Guerry and Falkow (1971) as described by Grindley et al. (1973b). Labelled and unlabelled DNA preparations were treated in an MSE 100 watt ultrasonic disintegrator at an amplitude of about 8 microns (peak to peak) for 2 min. This reduced the molecular weight of the DNA to about 4 x 10^5 daltons (Grindley, 1974). The DNA solutions, labelled and unlabelled, were then boiled for 15 min to denature the DNA. The 3H-labelled plasmid DNA was mixed with 5 ml of hydroxyapatite (HA) which had been washed to equilibrium with 0.14M PB and the mixture was incubated at 75° C for 5 min. The HA was removed by centrifugation and samples of the supernatant were added to the reassociation mixtures. The amount of labelled DNA which binds to HA immediately after denaturation is reduced by this precaution (Brenner et al., 1969a). Approximately 2 x 10^-3 μg of denatured 3H-labelled plasmid DNA (200-400 cpm) was used for each experiment. This amount was added in a volume of between 25 and 100 μl.
Each reassociation mixture contained 0.5 ml unlabelled DNA (0.6 mg/ml), 0.35 ml 0.4M PB, the labelled plasmid DNA and the required volume of distilled water to make a total volume of 1 ml. The labelled plasmid DNA is present with a 2000 to 7000-fold excess of unlabelled plasmid DNA. The control contained 0.5 ml distilled water instead of the unlabelled DNA. The mixtures were incubated for 16 h at 75°C. Reassociation occurs in free solution so that in the time employed more than 80% of the unlabelled fragments had reassociated while reassociation of labelled DNA strands with each other was less than 7%. The mixture in each tube was added to a settled bed of HA (10 ml) previously equilibrated with 0.14M PB by washing three times with the buffer. 15 ml of 0.14M PB was added and the mixtures incubated at 75°C for 5 min. The HA was sedimented by centrifugation at 3,500 r.p.m. for 4 min in a MSE Minor centrifuge placed in an incubator at 75°C. This centrifuge batch procedure (Brenner et al., 1969b) allows eight mixtures to be processed simultaneously. The supernatant was collected and kept at 0°C while the HA was washed twice more with 15 ml volumes of 0.14M PB. This washing with 0.14M PB elutes the single-stranded DNA, while the reassociated duplex DNA remains bound to HA at this molarity of PB. The 0.14M PB washings from each tube were pooled, 250 µg of calf thymus DNA added as carrier and trichloroacetic acid added to a concentration of >9%. The flasks were kept at 0°C for 2 - 3 h to facilitate precipitation of the DNA. 15 ml of 0.4M PB was added to the HA and the tubes
boiled for 15 min. The washing with 0.4M PB was repeated three times and the washings were treated as described above.

The mixtures were filtered through Whatman GF/B 2.5 cm glass fibre filter discs (previously soaked in 2% TCA). The discs were washed with 1% or 2% TCA and 2% acetic acid, dried and counted in 5 ml of scintillation fluid. Each sample was counted for 100 min and the degree of reassociation calculated as shown in the Results section.
Isolation of plasmid DNA

The non-transferring plasmids NTP1 to NTP11 listed in Table 4 (page 48) were all isolated from cleared lysates of plasmid-carrying strains. The host strains were 1R713 or its nalidixic acid-resistant derivative 14R525. With this method most of the chromosomal DNA is pelleted by centrifugation, leaving the cleared lysate containing most of the plasmid DNA. Samples were taken from each lysate before and after the clearing spin, and the acid-precipitable $^3$H-label recovered in the cleared lysate was compared with that in the whole lysate. The proportion of DNA recovered in the cleared lysates was calculated as shown in Table 25. A disadvantage of this method is that some of the plasmid DNA may be included in the cell wall-membrane debris which is sedimented with the chromosomal DNA in the clearing spin.

The cleared lysates were subjected to caesium chloride-ethidium bromide density gradient centrifugation. The results of fractionating two such gradients are shown in Fig. 1. As described earlier certain plasmids can be isolated as "relaxation complexes". These consist of protein specifically bound to the supercoiled plasmid DNA and on treatment with agents including pronase and ethidium bromide these complexes relax to give the plasmid DNA in open circular form (Clewell and Helinski, 1969). It appears from the results in Fig. 1 that the A determinant NTP1 is isolated predominantly as supercoiled DNA. In contrast, ColE2 is present mainly in the open circular form,
Table 25. % $^3$H-label recovered in cleared lysates of plasmid-carrying strains

<table>
<thead>
<tr>
<th>No.</th>
<th>Plasmid</th>
<th>R-type or colicinogeny</th>
<th>% $^3$H-label in cleared lysate ( \text{(mean)} )</th>
<th>Number of experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTP1</td>
<td>A</td>
<td></td>
<td>4.0</td>
<td>7</td>
</tr>
<tr>
<td>NTP2</td>
<td>SSu</td>
<td></td>
<td>1.5</td>
<td>2</td>
</tr>
<tr>
<td>NTP3</td>
<td>ASu</td>
<td></td>
<td>2.2</td>
<td>2</td>
</tr>
<tr>
<td>NTP4</td>
<td>ASSu</td>
<td></td>
<td>2.2</td>
<td>2</td>
</tr>
<tr>
<td>NTP5</td>
<td>T</td>
<td></td>
<td>1.5</td>
<td>3</td>
</tr>
<tr>
<td>NTP6</td>
<td>A</td>
<td></td>
<td>2.3</td>
<td>2</td>
</tr>
<tr>
<td>NTP7</td>
<td>ASSu</td>
<td></td>
<td>2.0</td>
<td>2</td>
</tr>
<tr>
<td>NTP8</td>
<td>Colicin E1</td>
<td></td>
<td>1.5</td>
<td>2</td>
</tr>
<tr>
<td>NTP9</td>
<td>Colicin E2</td>
<td></td>
<td>1.8</td>
<td>2</td>
</tr>
<tr>
<td>NTP10</td>
<td>Colicin E3</td>
<td></td>
<td>1.9</td>
<td>2</td>
</tr>
<tr>
<td>NTP11</td>
<td>K</td>
<td></td>
<td>3.1</td>
<td>2</td>
</tr>
</tbody>
</table>

\( ^b \) - Control experiments with the plasmid-free host strain 1K713.

\( ^a \) As a % of the $^3$H-label present in the total lysate before the clearing spin.

a As a % of the $^3$H-label present in the total lysate before the clearing spin.

b Control experiments with the plasmid-free host strain 1K713.
FIGURE 1  Dye buoyant density gradient centrifugation of cleared lysates

A (NTP1)

covalently closed

open circular

COL E2

bottom  FRACTION NO.  top

CPM x 10^-3

CPM x 10^-1
indicating that it is isolated as a relaxation complex (Helinski and Clewell, 1971; Humphreys, et al., 1972). Fractions containing plasmid DNA were identified as described in Materials and Methods. Samples of the plasmid DNA were then prepared for electron microscopy.

Estimation of contour lengths

Carbon-coated grids carrying the plasmid DNA were prepared and examined in the electron microscope. Open circular molecules were photographed and the micrographs were enlarged (x 8) and the molecules were traced onto paper and measured. Details of contour length measurements are given in Table 26. The molecular weights were calculated from these lengths assuming that the mass per unit length of DNA observed by electron microscopy is 2.07 x 10^6 daltons per µm (Lang, 1970). This observation requires that the DNAs contain no modified bases. The DNA of bacteriophage ϕX174 was used as the standard for contour length measurements. Grids of the replicative form of ϕX174 were prepared as described previously and the mean contour length (MCL) was calculated from measurements of open circular molecules (Table 26).

The two plasmids coding for resistance to ampicillin only, NTP1 and NTP6, can be distinguished by their mean contour lengths: 2.70 µm and 3.10 µm respectively. A grid carrying both plasmid DNAs was prepared and 57 open circular molecules were measured. The values were bimodal in distribution as shown in Fig. 2. The lengths of NTP1 and NTP6 were compared with that of the replicative
Table 26. Contour lengths and molecular weights of the non-transferring plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>R-type or colicinogeny</th>
<th>No. of molecules measured</th>
<th>Contour length (μm)</th>
<th>Molecular weight (x 10^-6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTP1</td>
<td>A</td>
<td>40</td>
<td>2.45-2.86</td>
<td>2.70</td>
</tr>
<tr>
<td>NTP2</td>
<td>SSu</td>
<td>33</td>
<td>2.60-2.97</td>
<td>2.74</td>
</tr>
<tr>
<td>NTP3</td>
<td>ASu</td>
<td>37</td>
<td>3.67-4.18</td>
<td>3.91</td>
</tr>
<tr>
<td>NTP4</td>
<td>ASSu</td>
<td>30</td>
<td>3.87-4.42</td>
<td>4.24</td>
</tr>
<tr>
<td>NTP5</td>
<td>T</td>
<td>18</td>
<td>3.00-3.33</td>
<td>3.16</td>
</tr>
<tr>
<td>NTP6</td>
<td>A</td>
<td>17</td>
<td>2.98-3.19</td>
<td>3.10</td>
</tr>
<tr>
<td>NTP7</td>
<td>ASSu</td>
<td>25</td>
<td>4.29-4.80</td>
<td>4.53</td>
</tr>
<tr>
<td>NTP8</td>
<td>Colicin E1</td>
<td>18</td>
<td>2.15-2.43</td>
<td>2.28</td>
</tr>
<tr>
<td>NTP9</td>
<td>Colicin E2</td>
<td>22</td>
<td>2.08-2.37</td>
<td>2.22</td>
</tr>
<tr>
<td>NTP10</td>
<td>Colicin E3</td>
<td>22</td>
<td>2.27-2.42</td>
<td>2.34</td>
</tr>
<tr>
<td>NTP11</td>
<td>K</td>
<td>14</td>
<td>2.61-2.96</td>
<td>2.84</td>
</tr>
<tr>
<td>Phage ϕX174 a</td>
<td></td>
<td>58</td>
<td>1.70-1.87</td>
<td>1.79</td>
</tr>
</tbody>
</table>

a The MCL of 1.79 μm is in good agreement with that of 1.83 μm obtained by Gordon (1973).
FIGURE 2 Contour length measurements of NTP 1 and NTP 6 prepared on the same grid

NTP 1
Mean 2.70 μm

NTP 6
Mean 3.10 μm
form DNA of phage φX174 which was included in the same preparation as an internal standard. The lengths of NTP1 and NTP6 relative to φX174 were 1.48 and 1.68 respectively (Table 27).

Table 27. Contour lengths of NTP1 and NTP6

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Plasmid</th>
<th>Contour length (µm)</th>
<th>Number of φX174 lengths</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. of molecules measured</td>
<td>Range</td>
</tr>
<tr>
<td>NTP1</td>
<td>NTP1</td>
<td>22</td>
<td>2.56-2.81</td>
</tr>
<tr>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTP6</td>
<td>NTP6</td>
<td>14</td>
<td>2.95-3.09</td>
</tr>
<tr>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>φX174</td>
<td>φX174</td>
<td>31</td>
<td>1.74-1.86</td>
</tr>
</tbody>
</table>

The contour lengths of the resistance determinants of the A transfer systems provide information on their interrelationships. The A and SSu determinants, originally present in S. typhimurium type 29 (RT1) have very similar contour lengths: 2.70 and 2.74 µm respectively. ASu was produced by ultraviolet irradiation of RT1 and has a MCL of 3.91 µm, 1.17 µm longer than SSu. This means that a considerable portion of the A determinant must therefore be present in ASu. Recombination between SSu (2.74 µm) and ASu (3.91 µm) yielded ASSu (NTP4) which is 4.24 µm in length. The wild ASSu determinant NTP7 with an MCL of 4.53 µm is slightly longer than the laboratory-made ASSu. The measurements for the three colicin factors show that they are the shortest plasmids studied in
this investigation and there is little difference between their contour lengths. Electron micrographs of some of the plasmids are shown in Plates 4 and 5.
Plate 4. Electron micrographs of
(A) NTP2, length 2.74 μm
(B) NTP5, length 3.16 μm

at a magnification of 120,000.
Plate 5. Electron micrograph of NTP1, length 2.70 μm, at a magnification of 85,000, showing open circular and supercoiled DNA.
Estimation of copy numbers of the non-transferring plasmids

The approximate number of copies of a plasmid per chromosome is estimated from the relative amounts of plasmid and chromosomal DNA, as measured by tritiated thymidine uptake over several generations of growth. The copy number can then be calculated for plasmids of known molecular weight, assuming the molecular weight of the E.coli chromosome to be $2.5 \times 10^9$ (Cooper and Helmstetter, 1968). These estimates are likely to be low, because of the limitations of the methods used to isolate plasmid DNA. Two different isolation procedures have been employed here.

1. Preparation of "cleared lysates" (see Materials and Methods).
   This method does not depend on a particular form of the plasmid DNA but relies on differential centrifugation: most of the chromosomal DNA is pelleted with the cell debris, leaving the majority of plasmid DNA molecules in the supernatant. The results shown in Table 25 indicate that 99.9% of the chromosomal DNA was removed in this way. The percentage of the $^3$H-label recovered in the cleared lysates of plasmid-carrying strains was given in Table 25. The number of copies of each plasmid per chromosome has been calculated from these values with the known molecular weights for the plasmids (Table 26) and the E.coli chromosome. The copy numbers are listed in Table 28.

2. Sarkosyl lysates. The disadvantage of Sarkosyl lysis followed by caesium chloride-ethidium bromide centrifugation is that certain plasmids, such as ColE1, "relax" to an open circular form in the
presence of ethidium bromide. This form bands in the same position as linear chromosomal DNA in the density gradient (Clewell and Helinski, 1969). The determination of copy number by this method may therefore be low for plasmids that exist as "relaxation complexes" and are thereby converted to open circular form. The relative amounts of plasmid and chromosomal DNA were estimated in each preparation. The result of fractionation of a caesium chloride-ethidium bromide gradient of a Sarkosyl lysate of K12 carrying NTP6 is shown in Fig. 3. Estimates of the number of copies per chromosome of the plasmids have been made from these values and are also given in Table 28.

All eleven non-transferring plasmids exist as multiple copies per chromosome. In general, there was good agreement between the values obtained from the two methods used for estimation of plasmid copy number. The A determinant NTP1, present in about 18 copies per chromosome, was found in greater numbers than any other plasmid, including the second A determinant NTP6.
Table 28. Estimation of the number of copies of the non-transferring plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>R-type or colicinogeny</th>
<th>1) Cleared lysates</th>
<th>2) Sarkosyl lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. of copies per chromosome</td>
<td>% $^3$H-label in plasmid peak</td>
</tr>
<tr>
<td>NTP1</td>
<td>A</td>
<td>17.7</td>
<td>4.2</td>
</tr>
<tr>
<td>NTP2</td>
<td>SSu</td>
<td>6.6</td>
<td>1.8</td>
</tr>
<tr>
<td>NTP3</td>
<td>ASu</td>
<td>6.7</td>
<td>2.9</td>
</tr>
<tr>
<td>NTP4</td>
<td>ASSu</td>
<td>6.3</td>
<td>2.7</td>
</tr>
<tr>
<td>NTP5</td>
<td>T</td>
<td>5.7 b</td>
<td>0.6</td>
</tr>
<tr>
<td>NTP6</td>
<td>A</td>
<td>9.0</td>
<td>3.6</td>
</tr>
<tr>
<td>NTP7</td>
<td>ASSu</td>
<td>5.3</td>
<td>2.2</td>
</tr>
<tr>
<td>NTP8</td>
<td>Colicin E1</td>
<td>7.8</td>
<td>0.9</td>
</tr>
<tr>
<td>NTP9</td>
<td>Colicin E2</td>
<td>9.6</td>
<td>1.7</td>
</tr>
<tr>
<td>NTP10</td>
<td>Colicin E3</td>
<td>9.7</td>
<td>1.8</td>
</tr>
<tr>
<td>NTP11</td>
<td>K</td>
<td>13.1</td>
<td>3.6</td>
</tr>
</tbody>
</table>

a Calculated from the % $^3$H-label recovered in each cleared lysate as shown in Table 25.

b The values obtained by caesium chloride-ethidium bromide centrifugation of Sarkosyl lysates were consistently lower (range of copy numbers in six experiments: 1.5-3.9) than those obtained by the cleared lysate method (3 experiments: 4.2-7.3 copies).
FIGURE 3  Caesium chloride - ethidium bromide gradient of a Sarkosyl lysate of *E. coli* K12 carrying NTP 6
(Note the change of scale)
Molecular studies of the kanamycin resistance determinant K and the 
$fi^+$ plasmid from *S.typhimurium*.

The kanamycin resistance determinant identified in *S.typhimurium*
type 29 ($54\,136$) was originally $fi^+ (= fi^+K)$ but spontaneous mutation
of this plasmid yielded $fi^-K$ referred to as K in this thesis. Both
forms of the K determinant were isolated from K12 strains by caesium
chloride-ethidium bromide density gradient centrifugation of cleared
lysates. The contour lengths were determined by electron microscopy
and the details are given in Table 29. The MCL of the $fi^-$ form of
K is $18.1 \mu m$, whereas $fi^+K$ has an MCL of $30.3 \mu m$ (Plate 6).

The *S.typhimurium* 36 strains RT576 and 34R99 were examined for
plasmid DNA. RT576 showed the $fi^+$ property, whereas 34R99 is a
mutant of *S.typhimurium* 36 that no longer inhibits F fertility.
Lysis of *S.typhimurium* was unsatisfactory in some cases and it was
necessary to increase the time of incubation with the Brij-
deoxycholate mixture. The cleared lysates were centrifuged in
cesium chloride-ethidium bromide density gradients. The DNA profile
of RT576 showed two bands but no satellite (plasmid) band was
detected with 34R99. Plasmid DNA from RT576, and a sample from the
single band obtained with 34R99, were examined by electron microscopy.
The contour length of the plasmid from RT576 is $27.3 \mu m$, corresponding
to a molecular weight of $56.5 \times 10^6$ (Table 29). No open circular
or covalently-closed supercoiled DNA molecules were found in the
preparation of 34R99. These results support the hypothesis that the
$fi^+$ property of *S.typhimurium* 36 is plasmid-borne.
The K determinant was transferred from K12HfrH K to RT576. A resulting line, 42R79, which had received K but not the F factor, was examined further. When FlacT was transferred to 42R79, progeny carrying FlacT and K were visibly lysed by phage χ2 in surface spot tests. The fi+ property of RT576 had thus been reversed by K, as observed in previous experiments. Plasmid DNA was isolated from 42R79 and examined by electron microscopy. There was a single molecular species with a MCL of 17.3 μm (Table 29) which is in good agreement with the length of K determined after isolation from the K12 host strain. No DNA molecules of 27 - 28 μm, corresponding in length to the fi+ plasmid of RT576, were detected in this preparation.

These results support the suggestion that the derepression of the F factor by K in S.typhimurium is caused by incompatibility between K and the fi+ plasmid. Selection for K would result in loss of the fi+ plasmid as observed in this experiment.

The S.typhimurium strains LT2 (= 42R93), 21R337, 21R340 and 21R341 were also examined for plasmid DNA. In the case of LT2 a cleared lysate was prepared and subjected to caesium chloride-ethidium bromide dye-buoyant density gradient centrifugation. Cleared lysates of strains 21R337, 21R340 and 21R341 were centrifuged in 15-50% sucrose gradients. Fractions containing labelled plasmid DNA were concentrated in caesium chloride gradients. The DNA from all four preparations was prepared for electron microscopy and the details of contour length measurements are shown in Table 29. The lengths of the plasmids from these four S.typhimurium strains of independent origin fall between 27.8 and 29.3 μm. Thus the fi+ plasmids of these strains appear to be very closely related to each other if not identical, and have been given the collective
designations MP10 (= *S. typhimurium* plasmid 10). Those isolated from type 36 and LT2 can thus be designated $\text{MP}^{10}_{36}$ and $\text{MP}^{10}_{\text{LT2}}$ respectively (Smith et al., 1973b).
Plate 6. Electron micrograph of the $f_{1-h} K$ determinant, length 18.1 μm, at a magnification of 60,000.
Table 29. Contour lengths and molecular weights of K and the \( \text{fi}^+ \) plasmid of \textit{S.typhimurium}

<table>
<thead>
<tr>
<th>Strain No.</th>
<th>Description</th>
<th>Contour length (( \mu \text{m} ))</th>
<th>No. of molecules measured</th>
<th>Range</th>
<th>Mean</th>
<th>Standard deviation</th>
<th>Molecular weight ((\times 10^{-6}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>42R79</td>
<td>\textit{S.typhimurium} 36 carrying ( \text{fi}^{-} \text{K} )</td>
<td>10</td>
<td>16.6-17.8</td>
<td>17.3</td>
<td>0.4</td>
<td>35.8</td>
<td></td>
</tr>
<tr>
<td>38R960</td>
<td>K12 carrying ( \text{fi}^{-} \text{K} )</td>
<td>32</td>
<td>17.6-18.7</td>
<td>18.1</td>
<td>0.3</td>
<td>37.5</td>
<td></td>
</tr>
<tr>
<td>42R339</td>
<td>K12 carrying ( \text{fi}^{+} \text{K} )</td>
<td>10</td>
<td>29.8-32.1</td>
<td>30.3</td>
<td>0.9</td>
<td>62.7</td>
<td></td>
</tr>
<tr>
<td>RT576</td>
<td>\textit{S.typhimurium} 36</td>
<td>12</td>
<td>26.5-28.5</td>
<td>27.3</td>
<td>0.6</td>
<td>56.5</td>
<td></td>
</tr>
<tr>
<td>42R93</td>
<td>\textit{S.typhimurium} 4 (= LT2)</td>
<td>11</td>
<td>27.2-28.8</td>
<td>27.8</td>
<td>0.5</td>
<td>57.6</td>
<td></td>
</tr>
<tr>
<td>21R337</td>
<td>\textit{S.typhimurium} 14</td>
<td>7</td>
<td>27.6-29.8</td>
<td>28.6</td>
<td>0.9</td>
<td>59.2</td>
<td></td>
</tr>
<tr>
<td>21R340</td>
<td>\textit{S.typhimurium} 74</td>
<td>14</td>
<td>28.7-30.1</td>
<td>29.3</td>
<td>0.4</td>
<td>60.7</td>
<td></td>
</tr>
<tr>
<td>21R341</td>
<td>\textit{S.typhimurium} 8</td>
<td>10</td>
<td>28.4-30.0</td>
<td>29.3</td>
<td>0.6</td>
<td>60.7</td>
<td></td>
</tr>
</tbody>
</table>
Number of plasmid copies per chromosome

For plasmids of known molecular weight the number of copies of the plasmid per chromosome can be calculated from the percentage of total labelled DNA that is recovered as plasmid DNA, assuming that the E. coli or S. typhimurium chromosome has a molecular weight of $2.5 \times 10^9$ daltons.

The percentage of $^3$H-label recovered in cleared lysates of K12 strains carrying $^f^i^{-}$ K was 2.7. The molecular weight of K is $37.5 \times 10^6$, so the number of K copies per chromosome is 1.8.

S. typhimurium 36 carrying MP10$_{36}$ gave 2.2% recovery of $^3$H-label in the cleared lysate. MP10$_{36}$ has a molecular weight of $56.5 \times 10^6$; the copy number per chromosome is therefore 1.0. Most transferable plasmids have a molecular weight greater than $20 \times 10^6$ daltons and are present as one to two copies per chromosome (Clowes, 1972; Grindley et al., 1973b).

DNA reassociation experiments with $^f^i^{-}$ K and MP10$_{36}$

DNA reassociation experiments were performed at 75º C as described in Materials and Methods. A tube containing the labelled plasmid DNA and unlabelled chromosomal DNA from the host strain was included in each set of experiments. This allows the determination of any homology between the plasmid and the chromosome. A reaction mixture containing only the $^3$H-labelled plasmid DNA (called the Control) was included to correct for the labelled DNA, which binds
to hydroxyapatite immediately after denaturation ("zero-time" binding), and also for reassociation between labelled DNA strands.

**Calculation of the degree of reassociation**

The results of the reassociation of $^3$H-labelled DNA of MP10$_{36}$ and MP10$_{LT2}$ with unlabelled DNA from RT576, 34R99 (*S.*typhimurium $36 \text{fi}^-$), 42R79 (*S.*typhimurium 36 K) and 1R713 (K12F$^-$) are shown in Table 30. The counts per minute (cpm) recovered in the 0.14M PB and 0.4M PB washes are listed in columns A and B. The background counts have been subtracted in each case. The total cpm (column C) were lower for the reaction with unlabelled DNA from RT576, which is the homologous reaction. It has been shown that the loss of counts is caused by the unlabelled DNA in the reassociation mixture. Most of the unlabelled DNA is recovered in the 0.4M PB washes and the presence of this DNA probably causes increased quenching of the low energy tritium radiation (Grindley et al., 1973b; Grindley, 1974).

The results have been corrected for loss of counts recovered by the 0.4M PB washes. An overall correction factor ($k$) can be calculated as follows:

$$\text{Total cpm in 0.14M PB} + k \times \text{Total cpm in 0.4M PB} = n \times \text{Total cpm in control where } n = \text{number of reaction mixtures in which quenching occurs.}$$

For $^3$H-MP10$_{36}$

$$786.8 + k \times 289.9 = 4 \times 304.2$$

$$k = 1.48$$
The counts in the 0.4M PB washes were then corrected, using k (Column D), and the new corrected totals are shown in Column E. In the Control reaction for MP10 \textsubscript{36}, 6.4\% (19.4/304.2) of the counts were recovered in the 0.4M PB washes. Since this does not represent reassociation with unlabelled DNA, 6.4\% of the total counts have been calculated in each reaction (Column F). These values have been subtracted from the corrected 0.4M PB figures in Column D and also from the totals in Column E. The new corrected values are listed in Columns G and H. The degree of reassociation can then be calculated (Column I).

The results can also be expressed relative to the reassociation with DNA of the same plasmid (= 100) and with that of the chromosome (= 0). Relative degree of reassociation:

\[
\text{Relative degree of reassociation} = \frac{\text{Absolute } \% \text{ reassociation} - \% \text{ reassociation with chromosomal DNA}}{100}
\]

\[
\text{Relative degree of reassociation} = \frac{\% \text{ reassociation of "homologous" reaction} - \% \text{ reassociation with chromosomal DNA}}{100}
\]

The results given in Table 30 have been calculated in this way so that inter-plasmid homologies can be examined.

Results of DNA reassociation experiments

1. Between plasmids of \textit{S. typhimurium} and the \textit{fi\textsuperscript{x}} form of the K determinant.

\textsuperscript{H}-labelled plasmid DNA was isolated from RT576 and LT2. DNA reassociation reactions were examined at 75\° C with unlabelled DNA from RT576, 42R79, 34R99 and 1R713. The detailed results are
Table 30. Detailed results of reassociation reactions between plasmids of *S. typhimurium* and the K determinant

<table>
<thead>
<tr>
<th>Strains used for preparing unlabelled DNA</th>
<th>Recovered in washes</th>
<th>Counts/min</th>
<th>Correction for reassociation in the control</th>
<th>Reassociation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1M PB</td>
<td>0.4M PB</td>
<td>Total A+B</td>
<td>B x k a</td>
</tr>
<tr>
<td>MP10&lt;sub&gt;36&lt;/sub&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expt I</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT576</td>
<td>11.7</td>
<td>187.0</td>
<td>198.7</td>
<td>276.8</td>
</tr>
<tr>
<td>42R79</td>
<td>192.8</td>
<td>64.8</td>
<td>257.6</td>
<td>95.9</td>
</tr>
<tr>
<td>34R99</td>
<td>275.4</td>
<td>18.7</td>
<td>294.1</td>
<td>27.7</td>
</tr>
<tr>
<td>1R713</td>
<td>306.9</td>
<td>19.4</td>
<td>326.3</td>
<td>28.7</td>
</tr>
<tr>
<td>CONTROL b</td>
<td>284.8</td>
<td>19.4</td>
<td>304.2</td>
<td></td>
</tr>
<tr>
<td>MP10&lt;sub&gt;122&lt;/sub&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT576</td>
<td>19.6</td>
<td>199.9</td>
<td>219.5</td>
<td>269.9</td>
</tr>
<tr>
<td>42R79</td>
<td>254.4</td>
<td>77.3</td>
<td>331.7</td>
<td>104.4</td>
</tr>
<tr>
<td>34R99</td>
<td>295.3</td>
<td>24.6</td>
<td>319.9</td>
<td>33.2</td>
</tr>
<tr>
<td>1R713</td>
<td>320.8</td>
<td>22.1</td>
<td>342.9</td>
<td>29.8</td>
</tr>
<tr>
<td>CONTROL</td>
<td>306.1</td>
<td>25.6</td>
<td>331.7</td>
<td></td>
</tr>
</tbody>
</table>

The values for the quench correction factor (k) have been calculated as follows:

For MP10<sub>36</sub>: 786.8 + 289.9 x k = 1216.8  
  k = 1.48

For MP10<sub>122</sub>: 890.1 + 323.9 x k = 1326.8  
  k = 1.35

The proportion of counts per minute (%) in the Control recovered in the 0.4M PB washes were as follows: MP10<sub>36</sub>, 6.4%; MP10<sub>122</sub>, 7.7%.

Key of strains

RT576  *S. typhimurium* 36 carrying MP10<sub>36</sub>

42R79  *S. typhimurium* 36 K

34R99  *S. typhimurium* 36 fi

1R713  *E. coli* K12 F−
given in Table 30 and are summarised in Table 31. There was no homology (<3%) between the _S_. _typhimurium_ plasmid DNA and the DNA from 34R99 or 1R713. The result with 34R99 suggests that this _fi^-_ mutant of _S_. _typhimurium_ 36 does not carry a plasmid. It is also clear that there is little or no homology between MP1O_LT2 and chromosomal DNA from _E_. _coli_ K12 or _S_. _typhimurium_ 36. The findings with labelled plasmid DNA from RT576 and LT2 indicate that the plasmids in these hosts are very closely related. This conclusion can probably be extended to this group of _fi^+_ plasmids generally in _S_. _typhimurium_.

Between 23 and 30% of the MP1O plasmid is homologous with the K determinant. In the case of MP1O_LT2 (MCL, 27.3 μm) and K (MCL, 17.3 μm) there is approximately 8 μm of homologous DNA.

---

**Table 31. Reassociation between plasmids of _S_. _typhimurium_ and the K determinant**

<table>
<thead>
<tr>
<th>Strains used for preparing unlabelled DNA</th>
<th><em>S</em>. <em>typhimurium</em> strains used for isolation of 3H-labelled plasmid DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT576 ( <em>S</em>. <em>typhimurium</em> 36 )</td>
<td>RT576 (MP1O_LT2) 91</td>
</tr>
<tr>
<td>42R79 ( <em>S</em>. <em>typhimurium</em> 36 K )</td>
<td>30</td>
</tr>
<tr>
<td>34R99 ( <em>S</em>. <em>typhimurium</em> 36 <em>fi^-</em> )</td>
<td>3</td>
</tr>
<tr>
<td>1R713 (K12^--_)</td>
<td>2</td>
</tr>
</tbody>
</table>

*The values given are the means of the results of three separate experiments.*
2. DNA homology between MP10, K and plasmids of different compatibility groups

Unlabelled DNA was prepared from strains bearing plasmids which represent Groups F, F', I, I', N, B and H. Reassociation experiments were then performed with ³H-labelled plasmid DNA of MP10 and K (Table 32).

Between 14 and 21% of MP10 reassociated with the F-like plasmids of Groups F and F'. The R factor 240, like MP10, is fi⁺, while F and R1-19K are both fi⁻ and derepressed F-like plasmids. No homology was observed between MP10 and the members of the other plasmid groups. The experiments with ³H-labelled K confirmed the homology between K and the S.typhimurium fi⁺ plasmid. 48% of K reassociated with MP10 which is equivalent to 8.5 µm of homologous DNA.

The other results with K show that it has less than 10% homology with plasmids belonging to any of the groups studied in this investigation. These DNA reassociation experiments with ³H-labelled MP10 and K are shown as histograms in Figure 4.
Table 32. Reassociation between MP^{10}_{36}K and plasmids of different compatibility groups a

<table>
<thead>
<tr>
<th>Total unlabelled DNA from strains bearing plasmids</th>
<th>Compatibility group</th>
<th>MP^{10}_{36}</th>
<th>No. of expts.</th>
<th>K</th>
<th>No. of expts.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP^{10}_{36}</td>
<td>.</td>
<td>100</td>
<td>3</td>
<td>48</td>
<td>3</td>
</tr>
<tr>
<td>K</td>
<td>.</td>
<td>31</td>
<td>3</td>
<td>100</td>
<td>3</td>
</tr>
<tr>
<td>F</td>
<td>F_{I}</td>
<td>16</td>
<td>2</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>240</td>
<td>F_{II}</td>
<td>21</td>
<td>2</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>R1-19K^-</td>
<td>F_{II}</td>
<td>14</td>
<td>3</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>TP102</td>
<td>I_{1}</td>
<td>2</td>
<td>1</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>TP114</td>
<td>I_{2}</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>TP120</td>
<td>N</td>
<td>0</td>
<td>1</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>TP113</td>
<td>B</td>
<td>0</td>
<td>1</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>TP117</td>
<td>H_{1}</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

a The values indicate the degree of reassociation at 75°C of \(^{3}H\)-labelled plasmid DNA with unlabelled plasmids, relative to the reassociation both with DNA of the same plasmid ( = 100) and with that of the K12 chromosome ( = 0).
FIGURE 4  Reassociation of $^3$H-labelled plasmid DNA of MP10$_{3b}$ and K with unlabelled DNA from plasmid-bearing strains.

$^3$H-MP10$_{3b}$

$^3$H-K

GROUP

MP10$_{3b}$ K  F  240 R1-19K$^-$ TP102 TP114 TP120 TP113 TP117
DISCUSSION

Genetic properties of the non-transferring plasmids

Previous studies have established that there are two classes of genetic transfer systems in enterobacteria. In Class 1 the resistance or other determinant(s) and the transfer factor form a covalently-bonded complex which is transferred as a single linkage group. In contrast, in Class 2 transfer systems, the determinant(s) and the transfer factor are independent plasmids.

The eleven non-transferring plasmids NTP1 to NTP11, described in Section I, all form Class 2 transfer systems. Except for the A determinant NTP6, the plasmids were originally present in strains carrying transfer factors. The transfer factor and determinant were transferred to standard strains, the transfer factor usually being transmitted at a higher frequency than the determinant. Separation of the two plasmids was most easily demonstrated after interrupted crosses with transfer from K12 to S. typhimurium. The resistance or colicin determinants were then non-auto-transferring but could be mobilised by transferable plasmids. Mobilisation of a determinant by a transfer factor in order to introduce it into a strain carrying another plasmid was used to examine compatibility relationships of these non-transferring plasmids.

The results of compatibility experiments indicated that the eleven plasmids appeared to fall into at least six groups. The A determinant NTP1 was compatible with all the other determinants tested. Since NTP1 and NTP6 code for resistance to ampicillin only,
it has not been possible to test for compatibility between them.

Four plasmids belong to the second group of which SSu (NTP2) is the prototype. Previous studies demonstrated incompatibility between SSu and ASu (Anderson et al., 1968). The two ASSu determinants NTP4 and NTP7 are other members of this group. NTP4 was formed by recombination between ASu and SSu (Anderson, 1969), while NTP7 was identified in a strain of S. typhimurium isolated in South America. The T determinant NTP5 and the colicin factor CoLE1 were compatible with all the other plasmids and also with each other; they were tentatively classified as groups 3 and 4. The colicin determinants CoLE2 and CoLE3 showed a low degree of incompatibility with each other, but coexisted stably with the other determinants. Since CoLE2 and CoLE3 have about 80% of DNA homology in reassociation and heteroduplex experiments (Inselburg, 1973) they would be expected to belong to the same compatibility group. Limited incompatibility was observed between CoLE2 and CoLE3 (Inselburg, 1974). The K determinant NTP11 was tested with representatives of the groups described above and was compatible with each of the plasmids. NTP11 presumably belongs to another group of non-transferring plasmids, provisionally designated group 6.

The occurrence of SSu resistance determinants was also explored in wild strains resistant to streptomycin and sulphonamides. Nineteen of 26 wild strains examined carried SSu plasmids that were incompatible with ASu. This suggests these determinants are probably identical with, or closely related to, the prototype SSu of S. typhimurium type 29 (Anderson and Lewis, 1965a,b). The SSu
determinants were identified in different phage types of *S. typhimurium*, several salmonella serotypes and five independent *E. coli* strains. The cultures, of both animal and human origin, were isolated in different parts of the world over a period of five years. The identification of homologous plasmids in cultures from both animal and human sources supports the idea of a common pool of R factors and enterobacteria which act as sources of transferable drug resistance in both types of host (Anderson, 1968, 1975b; Anderson et al., 1973). Recent studies in this laboratory have demonstrated a high degree of DNA homology between R factors belonging to the same compatibility group identified in strains of both animal and human origin (Anderson, Humphreys and Willehaye, J. Gen. Microbiol., in press).

Although incompatibility was clearly shown with most of the strains resistant to streptomycin and sulphonamides, ASu coexisted stably with the putative SSu determinants in seven strains. Streptomycin-sulphonamide resistance was not directly transferable from five of these strains, and mobilisation with a transfer factor was shown in only one case. In contrast, the SSu determinants incompatible with ASu were easily mobilisable by A and the F-like transfer factor X. These results suggest that certain non-transferring plasmids may be highly specific in their ability to be mobilised by transfer factors. The *S. typhimurium* type 32 strain (8M3993) carried an SSu plasmid which was not directly transferable or mobilisable. Lines of 8M3993 carrying ASu and T-A could transfer ampicillin, streptomycin, sulphonamide and tetracycline resistances. Transfer
of ASu alone was demonstrated but all progeny selected on streptomycin carried a recombinant ASSu plasmid. It appeared that the recombination with ASu in the wild strain was necessary for transfer of the SSu plasmid of 8M3993. Another possibility is that the S resistance region of this strain recombined with ASu. The SSu of 8M3993 may thus be related to the prototype SSu determinant but lacks the functions potentiating its mobilisation by transferable plasmids. Studies on the molecular nature of the SSu plasmid of 8M3993 are required to test this possibility. ASu was also compatible with the SSu plasmids of three E.coli strains. SSu was directly transferable from two of these wild strains to S.typhimurium 36 and further experiments indicated that the respective E.coli strains carried Class 1 resistance transfer systems.

The wild strains were chosen because they were resistant to streptomycin and sulphonamides but sensitive to spectinomycin. It has been established that in strains carrying resistance plasmids coding for streptomycin and spectinomycin resistance, the antibiotics are inactivated by an adenylylating enzyme, whereas in strains resistant to streptomycin but sensitive to spectinomycin inactivation of streptomycin is by phosphorylation (Ozanne et al., 1969; Benveniste et al., 1970). It has also been observed that strains carrying most I-like plasmids coding for streptomycin resistance inactivate the antibiotic by phosphorylation (Hedges, 1972). SSu and other determinants may sometimes recombine with I-like transfer factors, to form Class 1 transfer systems.
The incidence of non-transferring plasmids in wild strains of S.typhimurium was investigated by Anderson (1965a, 1968). The plasmids originally identified were SSu determinants. It was also observed that the same non-auto-transferring resistance determinant could be mobilised by different transfer factors, and the same transfer factor could mobilise different determinants (Anderson and Lewis, 1965b; Anderson, 1966, 1968). Smith and Linggood (1970) have more recently explored wild E.coli in the same way. They have found SSu plasmids, T determinants, a ColE1 factor, and a determinant for α-haemolysin production, and have also demonstrated the capacity of different transfer factors to mobilise the same determinant and of the same transfer factor to mobilise different determinants (Smith and Heller, 1973). In most cases the transfer systems belonged to Class 2, but probable covalent bonding between a T determinant and certain transfer factors was also found.

At present there is little information on the mechanism of transfer of a determinant by a transfer factor in Class 2 systems, where the respective plasmids are independent of each other in the host cell. It has been suggested that there is an association between the two plasmids during transfer, but once established in the recipient cell the two plasmids resume their independence. One possibility is that there is hydrogen bonding between a limited number of complementary base pairs of the transfer factor and the determinant (Anderson et al., 1969). Alternatively, the determinant may pass through the conjugation tube without any specific linkage or association with the transfer factor. Reeves and Willette (1974)
examined the ability of several F-like plasmids and the I-like R factor R64-11 to transfer ColE1. Two F-like R factors, R100 and R136, transferred ColE1 at low frequency, whereas F, ColV2 and the other plasmids transferred ColE1 very efficiently. Further experiments demonstrated that all 10 tra gene products required for formation of F fimbriae were necessary for high frequency transfer of ColE1. The products of traD and traI, unnecessary for fimbrial synthesis, were not required for efficient transfer of ColE1. It was suggested that a product of either ColE1 itself or of the chromosome is required for initiation of transfer of ColE1 DNA.

As described in Section I there is a significant difference between K12 and S. typhimurium as recipients in crosses with Class 2 transfer systems. In K12 x K12 crosses with SSu and T-Adr+, progeny selected on streptomycin or sulphonamides had almost always acquired both SSu and T-Adr+. In contrast, with S. typhimurium as recipient and with the same donor strain and selection, 99% of the progeny carried SSu alone. The frequency of T-Adr+ transfer was much reduced in comparison with the K12 system. This result with S. typhimurium suggests that if there is an association between the determinant and the transfer factor in such crosses, the determinant enters the recipient first and in the majority of cases the transfer factor is either not transferred intact or is not transferred at all. There is no evidence of covalent linkage between transfer factor and determinant during transfer in Class 2 systems.
The molecular characteristics of the non-transferring plasmids

The molecular studies in Section II established that the non-transferring plasmids NTP1 to NTP11 are between 2.22 and 4.53 µm in length. Previous studies demonstrated that A (NTP1), SSu and the three colicin factors belong to the group of plasmids with molecular weights less than $10 \times 10^6$ daltons and are present as multiple copies per chromosome (Bazaral and Helinski, 1968; Humphreys et al., 1972; review Clowes, 1972). The contour length measurements distinguished between two plasmids, NTP1 and NTP6, which conferred resistance to ampicillin alone. NTP1 has a mean contour length of 2.70 µm, whereas the length of NTP6 is 3.10 µm. These results also showed that the laboratory-made ASSu was similar in size, 4.24 µm, to the wild ASSu determinant NTP7 (4.53 µm).

The eleven non-transferring plasmids are all present in multiple copies per chromosome, although there is a considerable range of copy numbers (Table 28). Unfortunately, the methods used do not provide a reliable estimate of the number of copies per chromosome, as has been stated in the Results section. Certain plasmids, such as CoLE1 and CoLE2, relax to open circular form in the presence of ethidium bromide, and this form bands with the chromosomal DNA in the density gradient. A (NTP1) and SSu exist mainly in a non-complexed form with approximately 89% of the plasmid DNA isolated as covalently-closed circular molecules which band below the chromosomal DNA in density gradients (Humphreys et al., 1972).
The other resistance determinants studied in this thesis do not appear to exist as relaxation complexes to a significant extent. There was a lower recovery of the T determinant NTP5 from caesium chloride-ethidium bromide centrifugation of Sarkosyl lysates compared with that from cleared lysates. However, experiments in which pronase was added to the cleared lysate did not indicate a conversion to open circular form as observed with plasmids such as ColE1. Estimates of the number of copies per chromosome also demonstrated that A (NTP1) is present in greater numbers than any other non-transferring plasmid, including the second A determinant NTP6. The differences in copy number presumably reflect differences in the control of replication of the plasmids. The four plasmids belonging to the SSu group have values for the number of copies per chromosome between 5.0 and 8.9 and those for ColE2 and ColE3 were between 8.1 and 11.2 (Table 28). The inference that there is a genuine difference in copy numbers of determinants of different types is supported by enzyme assay of K12 strains carrying the plasmids. Extracts of strains carrying NTP1 show approximately twice the \( \beta \)-lactamase activity of those carrying ASu (NTP3) or the second A determinant NTP6 (Humphreys and Anderson, unpublished observations). The same gene codes for \( \beta \)-lactamase in the case of NTP1 and NTP3, and therefore the difference in \( \beta \)-lactamase activity is probably due to the higher number of A gene copies in K12(NTP1) compared with K12 (NTP3). The TEM-type of \( \beta \)-lactamase appears to be coded for by these plasmids.

A number of other workers have also described the molecular
properties of some non-transferring resistance plasmids. A tetracycline resistance determinant from a strain of *Salmonella* (Guineé and Willems, 1967) had a molecular weight of about $6 \times 10^6$ daltons and was present in 16 - 29 copies per chromosome (van Embden and Cohen, 1973; Guerry, van Embden and Falkow, 1974). This plasmid could be mobilised by a large variety of transfer factors including those carrying regions coding for enterotoxin production, haemolysin synthesis or the K88 antigen. After transfer to minicells, five discrete polypeptide species were found in plasmid-carrying cells but the functions of these polypeptides have not yet been elucidated. Barth and Grinter (1974) examined a number of SSu plasmids isolated from a wide range of bacterial species. Nine of the 12 plasmids had a molecular weight of about $5.7 \times 10^6$ daltons, two others were $6.3 \times 10^6$ and $9.3 \times 10^6$ and the remaining plasmid consisted of three species, of molecular weight $7.4 \times 10^6$, $14.7 \times 10^6$ and $21.4 \times 10^6$. DNA reassociation experiments demonstrated 80 to 93% homology between 10 of the plasmids, indicating a common evolutionary origin.

The ampicillin resistance determinant RSP1030 has a molecular weight of $5.5 \times 10^6$ daltons and exists in about 30 copies per chromosome equivalent (Crosa, Luttrell and Falkow, 1975; Heffron et al., 1975). In the original host strain this plasmid is part of a Class 2 transfer system in which the transfer factor is I-like (unpublished observations of this laboratory). It was originally described as a single transferable plasmid designated R111 and defined as *com* 8 (Chabbert et al., 1972).
The K determinant and the $\phi^+$ plasmid of *S. typhimurium*

The preliminary genetic experiments on the non-transferring K determinant from a type 29 strain of *S. typhimurium* $5M^{136}$, indicated that it formed part of a Class 2 transfer system (Anderson, Pitton and Mayhew, 1968; Anderson, Mayhew and Grindley, 1969). Introduction of the $\phi^-$ form of K into *S. typhimurium* 36 carrying Flac resulted in the derepression of the F factor. This led to an investigation of F fertility in *S. typhimurium* in general and its relationship with the K determinant. Inhibition of F in *S. typhimurium* was observed in at least 14 of 22 further *S. typhimurium* strains of independent origin and belonging to 13 different phage types. It was also established that these wild strains were drug-sensitive, and no transfer factors could be detected using the triparental cross for determinant mobilisation. The $\phi^-$ K determinant reversed the inhibition of F fertility in all cases. It was originally suggested that the derepression was associated with a locus (termed der) on the K determinant (Smith et al., 1970). However an alternative explanation was that incompatibility existed between K and a plasmid present in *S. typhimurium* which coded for the $\phi^+$ property (Anderson and Smith, 1972b). Selection for K would result in loss of the resident $\phi^+$ plasmid and derepression of F. Molecular studies confirmed that this latter hypothesis was correct (Smith et al., 1973b).

The $\phi^+$ plasmid carried by *S. typhimurium* 36 has a molecular weight of $56.5 \times 10^6$ daltons. It has been designated $MP^{10}_{36}$ (Smith et al., 1973b). No plasmid DNA could be detected in a
mutant line of S. typhimurium which no longer inhibited F fertility. Examination of four other 
$fi^+$ S. typhimurium strains, including the standard LT2, revealed the presence of a plasmid in each strain, 
with molecular weights between 57.6 and $60.7 \times 10^6$ daltons. Dowman and Meynell (1970) had previously demonstrated the presence of 
plasmid DNA in LT2 by density gradient centrifugation but no property was assigned to the so-called "cryptic" plasmid. It is clear from the genetic and molecular studies that the plasmid-determined $fi^+$ property is widely distributed in strains of 
S. typhimurium. However, apart from phage restriction, other functions of this MP10 plasmid remain obscure. Incompatibility of the $fi^+$ plasmid with the resistance determinant K was demonstrated by introduction of the $fi^- K$ into S. typhimurium 36 (strain RT576). K has a molecular weight of $37.5 \times 10^6$ daltons in K12, and examination of the plasmid DNA after introduction of K into RT576 revealed a homogeneous monomolecular species with a molecular weight of $35.8 \times 10^6$ daltons. MP10$^-_{36}$ had evidently been displaced by the $fi^- K$, this resulted in loss of the $fi^+$ property.

This K determinant was isolated in amounts corresponding to approximately one copy of the plasmid per chromosome. This is in contrast to the number of copies for the other non-transferring plasmids NTP1 to NTP11, which all exist as multiple copies per chromosome. The molecular weight of K ($37.5 \times 10^6$ daltons) is of the same order as those of certain groups of transferable plasmids (Glowes, 1972; Grindley et al., 1973b) whereas most non-transferring plasmids have molecular weights less than $10 \times 10^6$ daltons.
DNA reassociation experiments confirmed that the \( \text{fi}^+ \) plasmids of \( S.\text{typhimurium} \) 36 and LT2 (\( = \text{MP}^{10}_{\text{LT}2} \)) are very closely related if not identical. There was little or no homology between \( \text{MP}^{10}_{\text{36}} \) and chromosomal DNA of \( E.\text{coli} \) K12 or \( S.\text{typhimurium} \). These results also demonstrated that almost half of K is homologous with \( \text{MP}^{10}_{\text{36}} \). As K was first identified in a strain of \( S.\text{typhimurium} \) type 29, it seems probable that it was formed by recombination between an acquired kanamycin resistance determinant and the \( \text{fi}^+ \) (MP10) plasmid of \( S.\text{typhimurium} \) 29. The K determinant originally isolated from \( S.\text{typhimurium} \) 29 was \( \text{fi}^+ \), and is designated \( \text{fi}^+ \) K. Spontaneous mutation of this plasmid gave rise to \( \text{fi}^- \) K. Reassociation experiments with labelled \( \text{MP}^{10}_{\text{36}} \) indicated some homology (up to 20%) with F-like R factors and the F factor, but not with plasmids of the other compatibility groups examined. The \( \text{fi}^- \) K had less than 10% homology with plasmids representing groups F_I, F_{II}, I_1, I_2, B, N and H_4. If the above suggestion on the origin of K is correct, the region of MP10 which is homologous with F-like plasmids must have been lost in the formation of the \( \text{fi}^- \) form of K.

The MP10 plasmid of \( S.\text{typhimurium} \) has been shown to restrict certain non-donor specific phages to which \( S.\text{typhimurium} \) lacking this plasmid is sensitive (Maureen de Saxe, personal communication). The \( \text{fi}^+ \) K determinant causes the same phage restriction as \( \text{MP}^{10}_{\text{36}} \) in \( S.\text{typhimurium} \), while \( \text{fi}^- \) K does not block these phages. Thus the \( \text{fi}^+ \) and respective phage-restricting region(s) of \( \text{fi}^+ \) K were probably lost together, although it is not yet known whether they
are identical or closely linked. Conjugation experiments indicated that no transfer activity could be identified in MP10\textsubscript{36} or related plasmids, and the K determinant was also non-transferring in both its $f_{1}^{+}$ and $f_{1}^{-}$ states. However, the recombinant between the A of ASu and MP10\textsubscript{36} termed A-MP10\textsubscript{36} (or A*) transferred to K12 at low frequency. This new R factor was incompatible with K, and like MP10\textsubscript{36} it was $f_{1}^{+}$. These observations suggest that MP10 may be a transfer factor with very low intrinsic transfer activity: the conditions under which its actual transfer can be observed may be satisfied only in recombinants such as A-MP10\textsubscript{36}. Alternatively, MP10\textsubscript{36} may be a defective transfer factor, the transfer defect of which has been at least partially repaired by insertion of A. It has not yet been possible to establish which explanation is correct, or why MP10 is so widely distributed in \textit{S. typhimurium}.

Further discussion

Previous studies on incompatibility have usually involved transferable plasmids which are present as a single copy per chromosome. However, the non-transferring plasmids NTP\textsubscript{1} to NTP\textsubscript{11} exist in multiple copies per chromosome, and any hypothesis of incompatibility must be considered in relation to these observations. It has been suggested that plasmid incompatibility involves competition for specific cellular attachment sites that govern replication and uniform segregation into daughter cells at cell division (Jacob \textit{et al.}, 1963; Anderson, 1966; Anderson \textit{et al.}, 1968).
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Further discussion

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Competition between two plasmids for the same site would lead to establishment of only one plasmid. Alternatively, incompatibility may result from inhibition of replication by a plasmid-specified cytoplasmic inhibitor (Pritchard, Barth and Collins, 1969).

The replication of the multiple-copy ColE1 factor has been followed in a density shift experiment (Bazaral and Helinski, 1970). Some copies appear to replicate once per generation, others twice, and some molecules not at all in this time. It was concluded that copies were selected at random for replication from a pool of ColE1 molecules. Similar experiments with the plasmid from E.coli 15, molecular weight $1.5 \times 10^6$ daltons (Cozzarelli, Kelly and Kornberg, 1968) also indicated that this plasmid has a mode of replication similar to that of ColE1 (Goebel and Schrempf, 1972). 

Studies on segregation kinetics of ColE1 in a K12\mutant strain which is temperature-sensitive for the production of DNA polymerase I, suggested that the inheritance of ColE1 was random (Durkacz and Sherratt, 1973). At present there is no information on the nature of the replication mechanism for the resistance determinants studied in this thesis. SSu and ASu are both present in multiple copies and are incompatible with each other. This incompatibility cannot be explained on the basis of random replication of these two plasmids, because a pool of SSu and ASu molecules would be expected to be formed with the appearance of stable hybrids. 

For plasmids such as SSu and ASu there is probably a master copy governing both replication and segregation. Incompatibility would then result from competition between the two master copies for a
single attachment site. Alternatively, incompatibility may result from the requirement of membrane attachment for uniform segregation of the plasmid into daughter cells. The selected copy would act as a master copy in terms of genetic continuity and the other copies in the daughter cells would have to be diluted out or become inactive. A random mechanism for replication could be reconciled with this second hypothesis.

The results with ColE2 and ColE3 indicated that there was a low degree of incompatibility after short crosses, with loss of the incoming plasmid to a greater extent. These observations can be reconciled with a random replication and segregation mechanism for these colicin factors. If one or very few ColE2 molecules are transferred to a recipient carrying ColE3 after a short mating, a random mechanism for the segregation into daughter cells may well result in a number of cells not receiving the ColE2 factor. It would be expected on this hypothesis that established lines carrying ColE2 and ColE3 would have a pool of both types of molecule, and there would be selection at random for replication from this pool. The experiments with such lines demonstrated a very low rate of loss of either ColE2 or ColE3, as would be predicted on the above hypothesis. However, recombination may occur readily between these two plasmids, since they share about 80% of DNA homology (Inselburg, 1973), which would yield similar results. A detailed analysis of incompatibility with non-transferring plasmids clearly requires studies of the replication mechanisms in each case. Only one transferable plasmid, R6K, has been shown to
exist in multiple copies per chromosome in K12 (Kontomichalou, Mitani and Clowes, 1970). R6K is the prototype of compatibility group X, and other R factors have been allotted to this group (Hedges et al., 1973). It was concluded that their incompatibility could not be interpreted as operating through a mechanism which imposes "stringent" control on plasmid replication.

Molecular studies of incompatibility have demonstrated that there is a reduction in the rate of replication of the superinfecting DNA (Falkow et al., 1971; LeBlanc and Falkow, 1973). This may be the result of the unique membrane attachment site of a given plasmid being already occupied so that a membrane site for normal replication is unavailable for the superinfecting plasmid. Alternatively, it has been suggested that the presence of an incompatible plasmid in the recipient resulted in the production of a specific repressor of plasmid replication. Plasmid establishment and incompatibility have also been investigated with staphylococcal penicillinase plasmids by Novick and Brodsky (1972). Their results suggested that entry of the plasmid into a recipient was a gradual process with at least two distinct components. These were the initiation of replication and heritable stability. Incompatibility in this system appeared to involve a temporary inhibition of replication and a longer inhibition of stabilisation. However, these observations were made in staphylococci, and plasmid transfer in these organisms is effected by transduction and not by conjugation. It is not known to what degree the control of plasmid replication and genetic continuity is similar in enterobacteria to that in staphylococci.
In this thesis the studies of Class 2 transfer systems demonstrate that the resistance determinant and the transfer factor are independent plasmids. Class 1 systems may arise from recombination between the components of Class 2 systems, and the formation of A-Δ (Class 1) by recombination between ASu and A, which have ordinarily a Class 2 relationship, may be an example of this process. Alternatively, Class 1 factors may be formed by recombination between transfer factors and the respective regions of the bacterial chromosome. This is discussed later.

The ASu determinant NTP3 was formed by ultraviolet irradiation of S.typhimurium type 29 carrying A and SSu (Anderson et al., 1968). The A region of ASu can recombine with auto-transferring and non auto-transferring plasmids. In addition to the formation of A-Δ, translocation of the A region of ASu yields ASSu (NTP4) from recombination with SSu (Anderson, 1969; 1975a). Similarly, as described earlier in this thesis, the A region of ASu recombined with the fli+ plasmid of S.typhimurium MP1036 to form A-MP1036 or A+. Other resistance genes can also be translocated from one plasmid to another. For example, FlacT is a recombinant between Flac and the tetracycline resistance marker of T-Δ (Anderson and Smith, 1972b; Anderson, 1975a). There appears to be little or no DNA homology between some of these recombining units. Thus, plasmids in which most of the DNA is unrelated in base sequence can nevertheless recombine with each other (Anderson, 1975a).
Transposition of ampicillin resistance has recently been described by other workers (Hedges and Jacob, 1974; Heffron et al., 1975). Examination of a number of plasmids coding for ampicillin resistance established that there is a common sequence of DNA, about $3 \times 10^6$ daltons, on each of the plasmids. This sequence or "transposon A", which includes the TEM ß-lactamase gene, is also present on the non-transferring A determinant RSF1030 and on A (NTP1) and ASu (NTP3). It was suggested that R factors specifying this ß-lactamase arose as a result of the transposition of this sequence of DNA from plasmid to plasmid. Although the transposition of genes has been clearly demonstrated, the genetic mechanisms concerned are unclear. In the case of the A transposon the DNA sequence may be similar to, or analogous with, the insertion sequences IS1 and IS2 described by Starlinger and Saedler (1972) (Heffron et al., 1975). Such sequences were first found as insertions in certain spontaneous, strongly polar mutations.

The role of insertion sequences in the formation of some F-like R factors has recently been identified. The sequence IS1 occurs as a direct tandem duplication located at both junctions of the transfer factor and resistance determinant components of R1, R6 and R100 (Hu et al., 1975; Ptashne and Cohen, 1975). These R factors, which usually exist as a single plasmid in K12, are found as three size classes of circular DNA molecules in certain other hosts such as Proteus mirabilis (see Introduction). It has been postulated that reciprocal recombination occurs at the ends of the two IS1 sequences leading to dissociation (or association) of the transfer factor and R determinant components. This reversible process is dependent on
the presence of antibiotics as well as on the host strain. Recombination between IS1 regions could also produce molecules with several copies of the \( R \) determinant component, as suggested by Rownd and coworkers (Rownd and Mickel, 1971; Rownd \textit{et al.}, 1975).

Although the genetic and molecular characteristics of a large number of plasmids have been described in recent years, the origin of the resistance genes carried on these plasmids remains obscure. This applies to auto-transferring as well as non auto-transferring resistance plasmids. It has been suggested that the resistance markers are chromosomal in origin. However, in most cases the mechanism of chromosomal resistance to a given antibiotic is different from that of plasmid-mediated resistance. A model in which chromosomal genes coding for an antibiotic-inactivating enzyme may be picked up by plasmids has been proposed by Shaw (1971). Some strains of \textit{E.coli}, \textit{Serratia marcescens} and \textit{Proteus mirabilis} have low activities of the chloramphenicol acetylating enzyme but are sensitive to the antibiotic (Shaw, 1967). Chloramphenicol-resistant mutants were made from some of the \textit{P.mirabilis} strains and extracts from these strains showed increased levels of enzyme activity. The enzymes produced by the sensitive strains and the resistant mutants show a high degree of homology with the chloramphenicol acetyltransferase encoded by \( R \) factors. The differences lie in the affinity of the enzymes for chloramphenicol. It was suggested that enzymes which inactivate antibiotics arise through an increased affinity for the antibiotic as substrate. The genes responsible for these enzymes may be picked up from the chromosome by transfer factors to form \( R \) factors.
The origin of plasmid-borne enterobacterial genes coding for resistance to antibiotics may be the antibiotic-producing actinomycetes (Benveniste and Davies, 1973b). Some of the actinomycetes that synthesize antibiotics possess inactivating enzymes analogous with those encoded by enterobacterial plasmids. It has been demonstrated recently that a Streptomyces plasmid codes for the synthesis of, and resistance to, the same antibiotic (Kirby, Wright and Hopwood, 1975). The relationship of such a plasmid to those found in the enterobacteria has not yet been established, but further investigations of this type are clearly necessary. In addition, further studies of translocation of genetic segments between plasmids, transferring and non-transferring, can be expected to yield more detailed information about insertion sequences and other recombination regions of DNA. This may clarify the phylogeny of resistance factors.
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Other publications


Fertility Inhibition in Strains of Salmonella typhimurium

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Summary. The \( f^+ \) property recently described in a strain of Salmonella typhimurium phage type 30, which was reversed by the introduction of the neomycin-kanamycin resistance determinant K (Smith et al., 1970), has been observed in at least 14 of 22 further S. typhimurium strains of independent origin. The 14 strains represented 13 different phage types of S. typhimurium. The \( f^+ \) property was reversed in all 14 strains by the introduction of K. The derepressing effect of K originally described may be caused by displacement of a plasmid coding for the \( f^+ \) character.

The \( f^+ \) character of the original strain of S. typhimurium 30, and of a further but unrelated strain of the same phage type, has apparently recombined with KColIb factors in lines stored for at least 2 years. The KColIb/\( f^+ \) plasmids so formed are stable in K12, and are displaced as a single linkage group in compatibility experiments with another F-like plasmid.

Strains of Escherichia coli K12 (= K12) carrying the F factor are subject to inhibition of fertility by other plasmids, which are described as “fertility inhibition” + (\( f^+ \)) (Egawa and Hirota, 1962; Watanabe and Fukasawa, 1962). The \( f^+ \) character is usually demonstrated by the transfer of the respective plasmid to F+ and Hfr strains of K12, and examination of the F fertility of the resulting strains. It is most easily monitored by testing the sensitivity of these strains to a male-specific phage (Watanabe, Fukasawa, and Takano, 1962).

When F is transferred to certain salmonellae, the recipient strains show a low degree of F fertility (Mäkelä, Lederberg, and Lederberg, 1962; Easterling et al., 1969). Transfer of the F-lac factor into S. typhimurium phage type 30 (= S. typhimurium 30) also yielded a strain in which F fertility was repressed; this was reversed by the introduction of a determinant for neomycin-kanamycin resistance (K) (Smith et al., 1970). S. typhimurium 30 F-lac strains were resistant to phage \( \mu 2 \) in surface spot tests, whereas S. typhimurium 30 F-lac K strains were sensitive to the phage. Strains of S. typhimurium 30 carrying repressor-minus mutants to F-like R factors also showed repression of fertility which was reversed by introducing K. It was suggested that S. typhimurium 30 produces a repressor which inhibits the fertility of F and of repressor-minus mutants of F-like R factors, and that the repression is reversed by the product of a locus, designated der, associated with the K determinant (Grindley et al., 1971).

A selection of different phage types has been studied in order to investigate the incidence of the \( f^+ \) character in S. typhimurium in general.
Materials and Methods

Apart from the *S. typhimurium* strains examined, which are listed in Table 1, we used K12HfrH (= HfrH); 30R893, a strain of K12lac~ carrying F-lacT, a recombinant of F::lac and the tetracycline (T) resistance marker of the I-like R factor T::I (Anderson and Lewis, 1965; Anderson, 1968; Anderson and Smith, in preparation); and 38R93 which is K12lac~ carrying both F::lac and K. The F-lacT plasmid is indistinguishable from the original F::lac except for the drug resistance marker, and the T resistance facilitates selection of recipient cells into which F-lacT has been introduced.

No transfer factors could be detected in any of the *S. typhimurium* strains examined, when they were tested with the triparental cross for determinant mobilisation (Anderson, 1965).

F-facT was introduced into the *S. typhimurium* strains by overnight crosses at 37°C between 30R893 and the respective recipients, using a donor:recipient ratio of 1:1. K was similarly introduced from 38R93 into the resulting strains, using selection with tetracycline and kanamycin to ensure that the progeny contained both F-lacT and K.

An R factor used in these investigations was KCollb, which also codes for kanamycin resistance. We have no present indication that the K moiety of this complex is related to the K determinant studied in these experiments. Transfer of the KCollb R factor to HfrH was carried out by the same method as that used for F-lacT transfer to *S. typhimurium*.

Croses involving K12HfrH as a donor were performed with a donor:recipient ratio of 1:10 and were of 1 h duration.

The male-specific phage μ2 was routinely used for testing the repressive effect of *S. typhimurium* strains on the F-lacT factor. Strain 30R893 (= K12 F-lacT) is fully sensitive to this phage. These tests were carried out by the surface spot method (Grindley, Grindley, and Anderson, 1970).

Results and Discussion

The results are summarised in Table 1. Seventeen of 22 *S. typhimurium* strains carrying F-lacT were insensitive to μ2. This may indicate that these strains have the same f+ character as *S. typhimurium* 36, the results with which are given in the table as a control. Those *S. typhimurium* strains which appeared to have this character were investigated further by transferring to them the K determinant from a suitable donor in overnight crosses (Anderson, Mayhew, and Grindley, 1969). The descendant lines, which carried both F-lacT and K, were tested with μ2, with the results shown in Table 1. F-lacT was lost at high frequency from three strains, 21R342, 21R343 and 21R344. Although the introduction of K produced μ2 sensitivity in each of these strains in the presence of F-lacT, the rate of loss of the latter plasmid was too high to yield reproducible results. They are therefore omitted from further discussion. All the remaining 14 strains tested which had received K had become sensitive to μ2, although there was some variation in the degree of visible lysis. Thus, the f+ character is present in most of the *S. typhimurium* strains examined, and the effect of introducing K suggests that the inhibition may be similar in nature to that originally described in *S. typhimurium* 36. However, the experiments do not indicate whether the f+ property is determined by an independent plasmid or the bacterial chromosome.

As we have mentioned above, it was previously suggested that the derepression by K could be caused by the product of a "derepressor" locus associated with the resistance determinant, that is, that it is positive in nature. If the f+ region...
Table 1. Reactions of *S. typhimurium* strains with male-specific phage μ2

<table>
<thead>
<tr>
<th>ERL No.</th>
<th>Phage type of <em>S. typhimurium</em> strain</th>
<th>Sensitivity to μ2 of <em>S. typhimurium</em> strains carrying F-lac<em>T</em></th>
<th>Sensitivity to μ2 of <em>S. typhimurium</em> strains carrying F-lac<em>T</em> and K</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT576</td>
<td>36*</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>21R339</td>
<td>1</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>21R336</td>
<td>4</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>1R213</td>
<td>4 (=LT2)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>21R341</td>
<td>8</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>21R343</td>
<td>12a</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>21R337</td>
<td>14</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>21R342</td>
<td>32</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>1R686</td>
<td>36***</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>21R340</td>
<td>74</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>21R344</td>
<td>104</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>21R338</td>
<td>109</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>21R325</td>
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<td>+</td>
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<td>—</td>
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<tr>
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<td>+</td>
<td>—</td>
</tr>
<tr>
<td>21R328</td>
<td>173</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>21R329</td>
<td>180</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>21R330</td>
<td>181</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>21R331</td>
<td>182</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>21R332</td>
<td>184</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>21R334</td>
<td>186</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>21R335</td>
<td>187</td>
<td>+</td>
<td>—</td>
</tr>
</tbody>
</table>

+ = Clear lysis with μ2. ± = Turbid lysis with μ2. — = No visible lysis with μ2. V = Variable results (see text). * Control strain (see text). ** F-lac*T was very unstable in these *S. typhimurium* strains. *** Independent of RT576.

were on a plasmid, however, derepression of F by K in *S. typhimurium* could be caused by incompatibility between K and that plasmid, resulting in elimination of the latter, in which case the derepression would be simply the result of loss of the f+i+ plasmid. Incompatibility between homologous plasmids has been shown for transfer factors (Scaife and Gross, 1962; Anderson, 1968) and for resistance determinants (Anderson et al., 1968). It may be explained by competition for a unique attachment site which is necessary for plasmid replication and segregation.

Further investigation of *S. typhimurium* 36 has shown that the f+i+ property can be mobilised with an I-like f-i+ R factor, KCol1b, which codes for kanamycin resistance and colicinogeny. This R factor was isolated from a strain of *S. typhimurium* phage type 104 in the Enteric Reference Laboratory. A line of *S. typhimurium* 36 into which the KCol1b factor had been introduced two years earlier, was crossed with HfrH. Of 50 HfrH(KCol1b) lines tested with phage μ2, one was resistant to μ2 while 49 were fully sensitive to the phage. In order to determine...
Table 2. The effect of the \( \text{fi}^+ \) character of \( S. \text{typhimurium} \) 36 on K12HfrH

<table>
<thead>
<tr>
<th>Strain</th>
<th>Reaction with phage ( \mu_2 ) in spot tests</th>
<th>Frequency of ( \text{pro} ) transfer in 1 h crosses</th>
</tr>
</thead>
<tbody>
<tr>
<td>K12HfrH</td>
<td>+</td>
<td>2.9 ( \times ) 10(^{-3} )</td>
</tr>
<tr>
<td>K12HfrH(KColIb)</td>
<td>+</td>
<td>1.9 ( \times ) 10(^{-4} )</td>
</tr>
<tr>
<td>K12HfrH(KColIb/( \text{fi}^+ ))(^* )</td>
<td>-</td>
<td>3 ( \times ) 10(^{-5} )</td>
</tr>
</tbody>
</table>

+ = Visible lysis with \( \mu_2 \). — = No visible lysis with \( \mu_2 \). * Denotes the presence of the \( \text{fi}^+ \) character acquired by KColIb from \( S. \text{typhimurium} \) 36.

whether the \( \mu_2 \) insensitivity of this single line was caused by acquisition of the \( \text{fi}^+ \) character by KColIb, the plasmid was introduced into K12 F-lac, and a number of resulting colonies were tested for sensitivity to \( \mu_2 \). All colonies tested were resistant to the phage. The \( \text{fi}^+ \) property of \( S. \text{typhimurium} \) 36 thus appears to have been mobilised by KColIb.

The effect of this \( \text{fi}^+ \) character on F fertility was examined by measuring the frequency of \( \text{pro} \) transfer to a suitable K12F- recipient from HfrH carrying the recombinant plasmid, in a 1 h cross. The results of this experiment, together with the appropriate controls, are shown in Table 2.

As Table 2 shows, the frequency of \( \text{pro} \) transfer from HfrH is reduced approximately a thousandfold by the presence of the \( \text{fi}^+ \) character, now associated with KColIb.

If this \( \text{fi}^+ \) region is linked to KColIb, displacement of KColIb in the \( \mu_2 \)-resistant HfrH strain by an incompatible K12F- recipient from HfrH carrying the recombinant plasmid, in a 1 h cross. This supports the hypothesis that the \( \text{fi}^+ \) character has recombined with the KColIb factor. This recombinant plasmid has been designated KColIb/\( \text{fi}^+ \), and we have concluded that the \( \text{fi}^+ \) region concerned is that of \( S. \text{typhimurium} \) 36.

We have also mobilised the \( \text{fi}^+ \) character of 19R688, a further strain of \( S. \text{typhimurium} \) 36, apparently of independent origin from RT576 (see Table 1), using another KColIb R factor.

These observations support the possibility that the \( \text{fi}^+ \) property of \( S. \text{typhimurium} \) is plasmid-borne, but the results could also be explained by recombination between the KColIb \( \text{R} \) factors and a chromosomal marker. However, we have no other evidence of recombination between KColIb and the chromosome, and the hypothesis that the \( \text{fi}^+ \) character of \( S. \text{typhimurium} \) is carried by a plasmid seems more plausible at present.

The majority of transferable \( \text{fi}^+ \) plasmids found in wild strains are F-like. It has been suggested that they synthesise a repressor which is responsible for their normally repressed state and which also inhibits F fertility (Meynell and Datta, 1963). Recently, however, Grindley and Anderson (1971) have described a group of \( \text{R} \) factors which are both \( \text{fi}^+ \) and I-like and these showed some variation...
in the degree of inhibition of F fertility. Experiments with a series of "repressor-insensitive" mutants of F-lac (Grindley, Grindley, Smith, and Anderson, in preparation) indicate that the f/ marker mobilised by KCol1b has similarities with that of the F-like R factor R100 (Egawa and Hirota, 1962), and is distinguishable from that of the f/* I-like factors described by Grindley and Anderson (1971).

This survey establishes that the f/* character is widely distributed in S. typhimurium, and probably in other enterobacteria. It raises questions about the origin of the f/* markers of the R factors in general.

References

Watanabe, T., Fukasawa, T., Takano, T.: Conversion of male bacteria of *Escherichia coli* K12 to resistance to f phages by infection with the episome "resistance transfer factor". Virology 17, 218–219 (1962).

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Molecular Studies of an $fi^+$ Plasmid from Strains of *Salmonella typhimurium*

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Received July 19, 1973

Summary. Plasmid DNA has been isolated from five $fi^+$ strains of *Salmonella typhimurium* of independent origin, including type 36 and LT2. The mean contour length of the plasmid was between 27.3 and 29.3 μm. A variant line of *S. typhimurium* type 36 which was $fi^-$ yielded no plasmid DNA. These results support the hypothesis that the $fi^+$ property of *S. typhimurium* is coded by a plasmid. In *S. typhimurium* 36 this plasmid, designated MP10, also appears to code for restriction of non-donor-specific phages. Molecular studies indicate that superinfection of *S. typhimurium* 36 with the kanamycin resistance determinant K, which results in loss of the $fi^+$ property, is correlated with loss of MP10. Reassociation experiments demonstrate a high degree of homology between the DNA of all five *S. typhimurium* plasmids, and between MP10 and K. MP10 has some homology with F and F-like R factors, but not with plasmids of other compatibility groups. A recombinant between an ampicillin resistance determinant and MP10 is autotransferable at low frequency. The significance of these findings is discussed.

The "fertility inhibition +" ($fi^+$) character was first described in relation to R factors that inhibited the fertility of strains of *Escherichia coli* K 12 (= K 12) carrying the F factor (Nakaya, Nakamura and Murata, 1960; Watanabe et al., 1964). Later studies established that most $fi^+$ plasmids were F-like, since they coded for the synthesis of F-fimbriae (Meynell, Meynell and Datta, 1968). However, the $fi^+$ character is not limited to F-like plasmids; for example, it also occurs in some I-like R factors (Grindley and Anderson, 1974) and in at least one member of the N compatibility group (Grindley, Grindley and Anderson, 1972).

It has been demonstrated that F fertility is inhibited in *Salmonella typhimurium*. When F lac was transferred to *S. typhimurium* phage type 36 (= *S. typhimurium* 36), the resulting progeny showed low donor ability, absence of visible lysis with F-specific phage μ2, and reduction of sex fimbriation (Smith et al., 1970). The $fi^+$ property was later observed in at least 14 of 22 further *S. typhimurium* strains of independent origin and belonging to 13 different phage types (Anderson and Smith, 1972). All the *S. typhimurium* strains examined were drug-sensitive, and no transfer factors could be detected in these strains when they were tested with the triparental cross for determinant mobilisation (Anderson, 1965). The introduction of a kanamycin resistance determinant (K) into these *S. typhimurium* strains reversed the inhibition of F fertility in all cases. It was suggested that the $fi^+$ character of *S. typhimurium* was carried by a plasmid, and that the derepression of F by K could be caused by incompatibility between K and that plasmid.

*Requests for reprints to E. S. Anderson.*
Selection for K would result in loss of the \(fi^+\) plasmid so that the \(S.\ typhimurium\) strains would no longer inhibit F fertility (Anderson and Smith, 1972).

In this paper we describe molecular studies demonstrating the presence of a plasmid in several \(fi^+\) \(S.\ typhimurium\) strains. We have isolated plasmid DNA from \(S.\ typhimurium\) 36 both before and after superinfection with the K determinant. The loss of the \(fi^+\) character is correlated with displacement of the resident plasmid by the K determinant. DNA reassociation experiments involving the \(S.\ typhimurium\) plasmids, K and a number of plasmids belonging to various compatibility groups are also presented.

**Materials and Methods**

**Bacterial Strains, Plasmids and Phages.** The strains of *Escherichia coli* K12 and *Salmonella typhimurium* are listed in Table 1. The plasmids of various compatibility groups used in DNA reassociation experiments are shown in Table 2. Visible lysis of strains carrying the F factor was detected in surface spot tests with F-specific phage \(\mu_2\) (Dettori et al., 1961).

<table>
<thead>
<tr>
<th>Enteric Reference</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laboratory No.</td>
<td></td>
</tr>
<tr>
<td>1R713</td>
<td><em>Escherichia coli</em> K12 F' prototrophic</td>
</tr>
<tr>
<td>38R960</td>
<td>1R713 carrying K(^a)</td>
</tr>
<tr>
<td>RT676</td>
<td><em>S. typhimurium</em> phage type 36</td>
</tr>
<tr>
<td>34R99</td>
<td><em>S. typhimurium</em> 36 (fi^-) (^b)</td>
</tr>
<tr>
<td>42R79</td>
<td><em>S. typhimurium</em> 36 carrying K</td>
</tr>
<tr>
<td>42R93</td>
<td><em>S. typhimurium</em> phage type 4 ((=LT2))</td>
</tr>
<tr>
<td>21R337</td>
<td><em>S. typhimurium</em> phage type 14</td>
</tr>
<tr>
<td>21R340</td>
<td><em>S. typhimurium</em> phage type 74</td>
</tr>
<tr>
<td>21R341</td>
<td><em>S. typhimurium</em> phage type 8</td>
</tr>
</tbody>
</table>

\(^a\) The kanamycin resistance determinant of Anderson et al. (1969).

\(^b\) When the F factor is transferred to 34R90, the resulting progeny are visibly lysed by the F-specific phage \(\mu_2\). This line is thus designated *S. typhimurium* 36 \(fi^-\).

**Media.** Bacterial strains for conjugation experiments were grown in nutrient broth. Crosses were plated on MacConkey agar containing suitable concentrations of the appropriate antibiotics. Counter selection against the K12 donor strain was exercised with colicin E2 when *S. typhimurium* was used as the recipient strain (Anderson and Lewis, 1965). Strains used for the preparation of \(^3\)H-labelled plasmid DNA were grown in M9 liquid medium with glucose as the sole carbon source. For the preparation of total unlabelled DNA, strains were grown on Difco nutrient agar plates containing antibiotics as required.

**Mating Conditions.** Cultures of donor and recipient strains containing approximately \(2 \times 10^8\) organisms/ml were mixed in a ratio of 1:1 and incubated overnight at 37°C.

**Isolation of Plasmid DNA.** The bacteria were grown in M9 liquid medium and the DNA was labelled with \(^3\)H-thymidine added early in exponential growth. The cells were lysed by the method of Clewell and Holinski (1969). The "cleared lysates" so obtained were subjected to cesium chloride-ethidium bromide dye-buoyant density gradient centrifugation. Alternatively, cleared lysates of the strains were centrifuged on 25 ml linear 15–50% sucrose gradients. Fractions containing labelled plasmid DNA were concentrated in cesium chloride gradients. Plasmid DNA recovered from the gradients was prepared for electron microscopy or DNA reassociation experiments (see Grindley et al., 1973).
Table 2. Plasmids of various compatibility groups

<table>
<thead>
<tr>
<th>Plasmid designation</th>
<th>Compatibility group</th>
<th>Resistance markers</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>F_1</td>
<td>T</td>
<td>Hayes (1952)</td>
</tr>
<tr>
<td>240</td>
<td>F_1</td>
<td>—</td>
<td>Grindley et al. (1971)</td>
</tr>
<tr>
<td>R1-19K^-c</td>
<td>F_1</td>
<td>ACSSu</td>
<td>Meynell and Cooke (1969)</td>
</tr>
<tr>
<td>TP102</td>
<td>I_1</td>
<td>K</td>
<td>Grindley and Anderson (1971)</td>
</tr>
<tr>
<td>TP114</td>
<td>I_1</td>
<td>K</td>
<td>Grindley et al. (1972)</td>
</tr>
<tr>
<td>TP120^-c</td>
<td>N</td>
<td>ASSuT</td>
<td>Grindley et al. (1972)</td>
</tr>
<tr>
<td>TP113</td>
<td>R</td>
<td>K</td>
<td>Grindley et al. (1972)</td>
</tr>
<tr>
<td>TP117</td>
<td>H</td>
<td>T</td>
<td>Grindley et al. (1972)</td>
</tr>
</tbody>
</table>

Symbols for drug resistances: A, ampicillin; C, chloramphenicol; K, kanamycin; S, streptomycin; Su, sulphonamides; T, tetracyclines.

* All K factors were isolated from wild enterobacterial strains defined in the Enteric Reference Laboratory.
* 240 was isolated from S. typhimurium 3M4400 and is probably identical with R136.
* Spontaneous segregant of R1-19 no longer coding for kanamycin resistance.
* TP120 was isolated from S. typhimurium 2M1818 and is probably identical with R46, R Brighton and R1818.

Preparation of Total Unlabelled DNA and DNA Reassociation Experiments. Total unlabelled DNA was prepared by a modification (Grindley et al., 1973) of the method of Marmur (1961). Reassociation experiments with denatured DNA were carried out as described previously (Grindley et al., 1973); the techniques are based on the methods of Brenner et al. (1961).

Electron Microscopy. Grids carrying the plasmid DNAs were prepared by the method of Lang and Mitani (1970), rotary shadowed with platinum, and examined with an A.E.I. EM6B electron microscope. Micrographs of open circular molecules were enlarged, traced and measured (see Grindley et al., 1973).

Results

Isolation of Plasmid DNA from Strains of S. typhimurium. The S. typhimurium strains RT570, 34R99, LT2 (= 421R9), 21R337, 21R340 and 21R341 were examined for plasmid DNA as described in Materials and Methods. All those strains showed the fi+ property except 34R99, a mutant of S. typhimurium ST no longer inhibits F fertility (see Table 1).

Lysis of S. typhimurium was unsatisfactory in some experiments and it was necessary in these cases to increase the time of incubation with the detergent mixture. Cleared lysates of RT570, 34R99 and LT2 were centrifuged in caesium chloride-ethidium bromide density gradients. The DNA profiles of RT570 and LT2 showed two bands. The denser band was assumed to be covalently closed circular plasmid DNA which had been separated from the lighter chromosomal open circular plasmid DNA. No satellite (plasmid) band was detected with 34R99. Plasmid DNA from RT570 and LT2 and a sample from the single band obtained with 34R99 were examined by electron microscopy.

Cleared lysates of strains 21R337, 21R340 and 21R341 were centrifuged in 15-50% sucrose gradients. Fractions containing plasmid DNA were pooled, concentrated by banding in caesium chloride density gradients, and prepared for electron microscopy.
Table 3. Contour lengths and molecular weights of the plasmids of *S. typhimurium*

<table>
<thead>
<tr>
<th>Strain No.</th>
<th>Description and phage type</th>
<th>Contour length (μm)</th>
<th>Molecular weighta (daltons × 10⁴)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. of molecules measured</td>
<td>Range</td>
</tr>
<tr>
<td>RT576</td>
<td><em>S. typhimurium</em> 36</td>
<td>12</td>
<td>26.5-28.5</td>
</tr>
<tr>
<td>LT2 (= 42R93)</td>
<td><em>S. typhimurium</em> 4</td>
<td>11</td>
<td>27.2-28.8</td>
</tr>
<tr>
<td>21R337</td>
<td><em>S. typhimurium</em> 14</td>
<td>7</td>
<td>27.6-28.9</td>
</tr>
<tr>
<td>21R340</td>
<td><em>S. typhimurium</em> 74</td>
<td>14</td>
<td>28.7-30.1</td>
</tr>
<tr>
<td>21R341</td>
<td><em>S. typhimurium</em> 8</td>
<td>10</td>
<td>28.4-30.0</td>
</tr>
<tr>
<td>42R79</td>
<td><em>S. typhimurium</em> 36 carrying K</td>
<td>10</td>
<td>16.6-17.8</td>
</tr>
<tr>
<td>38R900</td>
<td>K12 carrying K</td>
<td>32</td>
<td>17.6-18.7</td>
</tr>
</tbody>
</table>

* Calculated on the assumption that 1 μm = 2.07 × 10⁶ daltons (Lang, 1970).

Open circular and covalently closed supercoiled DNA molecules were found in all preparations except that of 34R99. Micrographs of open circular molecules were enlarged, traced and measured. The details of contour length measurements and molecular weights are shown in Table 3. The lengths of plasmids from five *S. typhimurium* strains of independent origin fell between 27.3 and 29.3 μm.

*K 12 and *S. typhimurium 36 Strains Carrying the K Determinant.* The K determinant was isolated from K12K (38R900) by caesium chloride-ethidium bromide density gradient centrifugation of a cleared lysate. The mean contour length of K is 18.1 μm (Table 3).

K was transferred from K12HfrH K to *S. typhimurium* 36 (RT570) (Anderson, Mayhew and Grindley, 1969). A resulting line, 42R79, which had received K but not the F factor, was examined further. When the F factor was transferred to 42R79, progeny carrying F and K were visibly lysed by the F-specific phage ϕ2 in surface spot tests. The φ⁺ property of RT570 had thus been reversed by K, as observed in previous experiments (Smith et al., 1970; Anderson and Smith, 1972). Plasmid DNA was isolated from 42R79 by caesium chloride-ethidium bromide dye-buoyant density gradient centrifugation of a cleared lysate. Examination of DNA from the plasmid band by electron microscopy revealed a single molecular species of 17.3 μm mean contour length (Table 3). This measurement is in good agreement with the length of K determined after isolation from the K12 host strain (38R900). No DNA molecules of 27 μm, corresponding in length to the φ⁻ plasmid of RT570, were detected in preparations of 42R79.

**DNA Reassociation Experiments.** Reassociation experiments between ³²P-labelled plasmid DNA isolated from RT570 and LT2, and unlabelled DNA from RT570, 34R99 and 42R79 (*S. typhimurium* 36 carrying K) yielded the results shown in Table 4. The lack of homology of the *S. typhimurium* plasmids with DNA from 34R99 suggests that this mutant of *S. typhimurium* 36 does not carry a plasmid, which confirms the findings described above. The similarity of the results with labelled plasmid DNA from RT570 and LT2 indicates that the two plasmids are very closely related. Preliminary experiments with three further
Table 4. Reassociation between plasmids of S. typhimurium and the K determinant

<table>
<thead>
<tr>
<th>Strains used for preparing unlabelled DNA</th>
<th>S. typhimurium strains used for isolation of 3H-labelled plasmid DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT576 (S. typhimurium 36)</td>
<td>91</td>
</tr>
<tr>
<td>42R79 (S. typhimurium 36 carrying K)</td>
<td>30</td>
</tr>
<tr>
<td>34R99 (S. typhimurium 36 fi-)</td>
<td>3</td>
</tr>
</tbody>
</table>

* The values show the reassociation (%) at 75°C of 3H-labelled plasmid DNA with total unlabelled DNA of the strains in the left hand column.

Table 5. Homologies between MP10<sub>m</sub>, K and plasmids of various compatibility groups

<table>
<thead>
<tr>
<th>Total unlabelled DNA from strains bearing plasmids</th>
<th>Compatibility group</th>
<th>Strains used for the isolation of 3H-labelled plasmid DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP10&lt;sub&gt;m&lt;/sub&gt;</td>
<td>.</td>
<td>100</td>
</tr>
<tr>
<td>K</td>
<td>.</td>
<td>31</td>
</tr>
<tr>
<td>F</td>
<td>F&lt;sub&gt;H&lt;/sub&gt;</td>
<td>14</td>
</tr>
<tr>
<td>246</td>
<td>F&lt;sub&gt;H&lt;/sub&gt;</td>
<td>2</td>
</tr>
<tr>
<td>K1-19K&lt;sup&gt;-&lt;/sup&gt;</td>
<td>F&lt;sub&gt;H&lt;/sub&gt;</td>
<td>14</td>
</tr>
<tr>
<td>TP102</td>
<td>I&lt;sub&lt;s&lt;/sub&gt;</td>
<td>0</td>
</tr>
<tr>
<td>TP114</td>
<td>I&lt;sub&gt;s&lt;/sub&gt;</td>
<td>0</td>
</tr>
<tr>
<td>TP120</td>
<td>N</td>
<td>0</td>
</tr>
<tr>
<td>TP113</td>
<td>B</td>
<td>0</td>
</tr>
<tr>
<td>TP117</td>
<td>H</td>
<td>0</td>
</tr>
</tbody>
</table>

* The values indicate the degree of reassociation at 75°C of 3H-labelled plasmid DNA with unlabelled plasmids, relative to the reassociation both with DNA of the same plasmid (= 100) and with that of the K12 chromosome (= 0). Both 3H-labelled plasmids gave a reassociation value of 3-4% with chromosomal DNA from either E. coli K12 or S. typhimurium.

S. typhimurium strains (21R337, 340 and 341) suggest that there is a high degree of homology between the plasmids in these strains and those in S. typhimurium 36 and LT2. As we believe this to apply to this group of fi<sup>+</sup> plasmids generally in S. typhimurium, we propose giving them the collective designation MP10 (= S. typhimurium plasmid 10): those isolated from type 36 and LT2 can thus be designated MP10<sub>m</sub> and MP10<sub>LTS</sub> respectively. Approximately 30% of MP10<sub>m</sub> is homologous with the K determinant. This result, together with the degree of reassociation of labelled DNA of K with unlabelled DNA from RT576, indicates that MP10<sub>m</sub> and K have approximately 8 µm of homologous DNA.

Reassociation experiments between 3H-labelled plasmid DNA from RT576 and unlabelled DNA from members of various compatibility groups indicated some homology between MP10<sub>m</sub>, the F-like R factors and the F factor (see Table 5).
However, little or no homology was observed with members of the other plasmid groups. The results with the labelled DNA of K show that it has very little homology with plasmids belonging to any of the compatibility groups studied in our earlier experiments (Grindley et al., 1973).

**Discussion**

Plasmid DNA in the form of covalently closed circular molecules was isolated from five *S. typhimurium* strains with the $f^+$ character. In contrast, no such molecules were detected in the mutant line of *S. typhimurium* 36 (34R99) which no longer inhibits F fertility. These results support the hypothesis that the $f^+$ property of *S. typhimurium* is plasmid-borne (Anderson and Smith, 1972). The mean contour lengths of these plasmids fall within a small range, 27.3 to 29.3 μm. The lengths of the plasmids are of the same order as those of certain groups of transferable plasmids (Grindley et al., 1973; review: Clowes, 1972). However, we have been unable to mobilise resistance determinants in triparental crosses with these *S. typhimurium* $f^+$ plasmids (Anderson and Smith, 1972). Recently, a recombinant between MP10m and an ampicillin (A) resistance determinant has been identified. This plasmid, designated A*, transfers to K12F at low frequency, about $10^{-7}$ in overnight crosses. This suggests that MP10m may be a transfer factor with very low intrinsic transfer activity; the conditions under which its actual transfer can be observed may be satisfied only in recombinants such as A*. Alternatively, MP10m may be a defective transfer factor, the transfer defect of which has been at least partially repaired by integration of A. This study will be presented in detail in a later paper.

Studies by other workers have demonstrated a "silent" plasmid in a strain of *S. typhimurium* LT2, but no function could be attributed to the plasmid (Dowman and Meynell, 1970; Spratt, Rowbury and Meynell, 1973). Our results show that LT2 carries a plasmid that determines the $f^+$ property. Its molecular weight is $57 \times 10^6$ daltons (Table 3), which agrees well with the estimate of $60 \times 10^6$ daltons for the size of the LT2 plasmid calculated from its sedimentation behaviour in alkaline sucrose gradients (Spratt et al., 1973).

A "mutant" plasmid, designated FlacS, has been identified in a strain of *S. typhimurium* LT2 which carried the temperature-sensitive $F_{\text{tai}}$ plasmid (Macrina and Balbinder, 1973). K12 strains carrying FlacS were not visibly lysed by F-specific phages, whereas K12$F_{\text{tai}}$ strains were lysed by these phages. Sedimentation in alkaline sucrose gradients showed that FlacS was considerably larger than $F_{\text{tai}}$. Macrina and Balbinder suggested that FlacS resulted from a nonreciprocal exchange between $F_{\text{tai}}$ and some segment of *S. typhimurium* DNA which may be derived from the host chromosome or a "cryptic" plasmid.

Our results suggest that FlacS may be a recombinant of $F_{\text{tai}}$ with an $f^+$ plasmid present in the *S. typhimurium* strain. We have isolated recombinants between MP10m and the I-like R factor KCollh (Anderson and Smith, 1972).

The $f^+$ property of *S. typhimurium* strains is reversed by the introduction of the kanamycin resistance determinant, K. The isolation of plasmid DNA from *S. typhimurium* 36 carrying K yielded a single molecular species of 17.3 μm and...
Plasmid of *S. typhimurium*

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no molecules of 27 \(\mu\)m. The reversal of the \(fi^+\) character by the K determinant is therefore correlated with loss of the 27 \(\mu\)m *S. typhimurium* 36 plasmid. These results support the suggestion by Anderson and Smith (1972) that the derepression of the F factor by K in *S. typhimurium* is caused by incompatibility between K and the \(fi^+\) plasmid. Selection for K would result in loss of the \(fi^+\) plasmid as observed in these studies.

Compatibility experiments with K show that it coexists stably with plasmids representing Groups F\(_{\text{I}}\), F\(_{\text{II}}\), F\(_{\text{Ia}}\), B, N, H and W, and with the F\(_0\)-lac plasmid of Falkow and Baron (1962). As K was first identified in a strain of *S. typhimurium* type 29 (Anderson, Pitton, and Mayhew, 1968), it may have been formed by recombination between an acquired kanamycin resistance determinant and the \(fi^+\) (MP10) plasmid of *S. typhimurium* 29. This plasmid thus represents a distinct compatibility group.

Estimation of the percentage of \(^3\)H-labelled DNA present in a cleared lysate compared with that in the whole lysate before clearing, suggests that the K determinant is present in K12 as approximately one copy per chromosome. In contrast, the A and SSu resistance determinants of the A transfer system exist as multiple copies per chromosome (Smith, Anderson, and Clowes, 1970; Humphreys Grindley and Anderson, 1972). There are also marked differences in size; A and SSu have molecular weights of \(5 \times 10^9\) and \(5.4 \times 10^9\) respectively, whereas K has a molecular weight of \(37.5 \times 10^9\). This K determinant therefore differs fundamentally from resistance determinants such as A and SSu.

The DNA reassociation experiments confirm that the \(fi^+\) plasmids of several *S. typhimurium* strains are very closely related to each other if not identical. The results also demonstrate that there is approximately 8 \(\mu\)m of DNA in common between MP10\(_{34}\) and the K determinant. In the case of MP10\(_{34}\), which is 27.3 \(\mu\)m in length, the shared proportion amounts to 30%, while in that of the K determinant (17.3 \(\mu\)m) it amounts to 48%. The incompatibility observed between these two plasmids is therefore correlated with a high degree of DNA homology. Studies with several R factor compatibility groups showed, in general, a correlation between incompatibility and close DNA homology, an indication of phylogenetic relationship (Grindley et al., 1973). Reassociation experiments with plasmids of various compatibility groups establish that there is homology between the *S. typhimurium* \(fi^+\) plasmids and F-like R factors and the F factor. In contrast, little or no homology was detected between K and plasmids representing Groups F\(_{\text{I}}\), F\(_{\text{II}}\), F\(_{\text{Ia}}\), B, N and H.

The molecular studies presented here therefore confirm the genetic findings on fertility inhibition and its reversal by K in strains of *S. typhimurium* (Anderson and Smith, 1972). MP10\(_{34}\) has recently been shown to restrict certain non-donor-specific phages to which *S. typhimurium* lacking this plasmid is sensitive (Maureen de Saxe, personal communication). The K determinant is \(fi^-\) and does not restrict these phages. The K determinant originally isolated from *S. typhimurium* type 29 was \(fi^+\) (= \(fi^+\)K) and restricted a typing phage in *S. paratyphi* B (Anderson et al., 1968). This plasmid apparently causes the same phage restriction as MP10\(_{34}\) in *S. typhimurium*. Spontaneous mutation of \(fi^+\)K probably gave rise to \(fi^-\)K, perhaps by excision of the \(fi^+\) and phage restricting region(s) of \(fi^+\)K.
The "silent" or "cryptic" plasmid present in many strains of *S. typhimurium*, including LT2, is evidently responsible for the widespread ft+ character of the serotype. The significance of the wide distribution of such a plasmid in *S. typhimurium*, and the possibility of the presence of analogous elements in other salmonellae, are under investigation.

References


Falkow, S., Baron, L. S.: Episomic element in a strain of *Salmonella typhi*. J. Bact. 84, 581-589 (1962)


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Genetic and Molecular Characterisation of Some Non-transferring Plasmids

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Summary. Compatibility and molecular studies were performed on a number of non-auto-transferring plasmids for drug resistance and colicinogeny. The ampicillin (A) and streptomycin-sulphonamide (SSu) resistance determinants of Salmonella typhimurium type 29 are compatible with each other, and thus represent different compatibility groups. The laboratory-made resistance determinant ASu is incompatible with SSu and was used for compatibility studies with other determinants. Nineteen of 20 wild streptomycin-sulphonamide resistant strains of salmonellae and Escherichia coli carried non-transferring SSu determinants incompatible with ASu, and therefore probably phylogenetically related to the SSu determinant of type 29. A wild tetracycline resistance determinant (T) and the non-transferring colicinogeny determinants E1, E2 and E3 were compatible with each other and with A and SSu. A tentative classification can thus be suggested for these non-transferring plasmids which places A, SSu and its homologues, T, ColE1, ColE2 and ColE3 in separate compatibility groups. Molecular studies of ten of the plasmids showed that they consisted of covalently-closed circular DNA molecules with mean contour lengths between 2.22 and 4.53 μm. All were present in multiple copies per chromosome in E. coli K12.

Studies on transferable drug resistance led to the definition of two classes of resistance transfer systems (Anderson, 1968; Anderson, 1969; Anderson and Threlfall, 1970; Anderson and Natkin, 1972). In Class 1 the resistance determinant(s) and the transfer factor form a covalently-bonded complex which is transferred as a single linkage group. The complex has a unique membrane attachment site, which is that of the transfer factor (Anderson et al., 1968). The R factors discovered in Japan were the first examples of this class (review: Watanabe, 1963). The tetracycline R factor T-A of the A-mediated resistance transfer systems also belongs to Class 1 (Anderson and Lewis, 1965b).

In Class 2 transfer systems the resistance determinant(s) and the transfer factor are discrete plasmids, independent of each other in the host cell, and each occupies its own attachment site. The transfer factor and resistance determinant can be transferred separately or together, the transfer factor usually being transmitted at a substantially higher frequency than the determinant. In interrupted crosses, especially when the transfer is from Escherichia coli K12 to Salmonella typhimurium, transfer of the resistance determinant alone can be easily demonstrated (Anderson and Lewis, 1965b; Anderson, 1968). The resistance determinant is then non-auto-transferring but can be mobilised by a transfer factor in a triparental cross (Anderson, 1965). Examples of this second class were originally identified in the A-mediated transfer systems of S. typhimurium phage type 29. Ampicillin resistance (A), and the linked streptomycin-sulphonamide resistances (SSu) are coded by two plasmids which are independent of each other.
and of the \( \Lambda \) transfer factor (Anderson and Lewis, 1965a, b; Anderson, 1968). These Class 2 systems have been designated \( A, \Lambda \) and SSu, \( \Lambda \) respectively (Anderson and Natkin, 1972).

Molecular studies on the plasmids of the \( \Lambda \) transfer systems confirmed the postulates based on the genetic evidence. The resistance determinants \( A \) and SSu and the transfer factor \( \Lambda \) exist as independent covalently-closed circular DNA molecules in K12. The molecular weights of \( A \), SSu and \( \Lambda \) are approximately \( 5.6 \), \( 5.7 \) and \( 59 \times 10^6 \) daltons respectively. \( A \) and SSu are present as multiple copies per chromosome, whereas there is approximately only one copy of \( \Lambda \) per chromosome (Smith, Anderson and Clowes, 1970; Humphreys, Grindley and Anderson, 1972; Milliken, Anderson and Clowes, unpublished). Several other plasmids, such as ColEl, are also small, non-transferring, and exist as multiple copies per chromosome (Bazarak and Helinski, 1968; Cozzarelli, Kelly and Kornberg, 1968; Hardy et al., 1973). These plasmids can also form Class 2 transfer systems; an example is the transfer of ColEl by the F factor (Fredericq, 1954; Clowes, 1964).

The property of compatibility is now widely used for classifying bacterial plasmids, and several "compatibility groups" of auto-transferable plasmids have been defined. Members of each group are generally compatible with those of other groups, but are incompatible with each other. Anderson et al. (1968) demonstrated incompatibility between two homologous resistance determinants, SSu and ASu. The ASu determinant was produced by ultraviolet irradiation of a strain of \( S. \) typhimurium carrying the \( A \) and SSu resistance determinants. ASu apparently resulted from insertion of at least part of the \( A \) determinant into SSu, with loss or inactivation of the streptomycin resistance gene (Anderson et al., 1968; Anderson, 1969).

In this paper we describe a number of non-transferring plasmids which form Class 2 transfer systems. We have studied the compatibility of these plasmids. A selection of wild enterobacterial strains carrying streptomycin-sulphonamide resistance have also been examined in order to investigate the incidence of SSu resistance determinants homologous with the prototype SSu of Anderson and Lewis (1965a, b). The molecular characters of ten non-transferring plasmids have been determined with \( E. \) coli K12 as the host strain.

**Materials and Methods**

**Bacterial Strains and Plasmids.** The standard strains of \( E. \) coli K12 and \( S. \) typhimurium are listed in Table 1. We have designated resistance (or colicin) determinants which form Class 2 transfer systems as "non-transferring plasmids" (NTP). The origin of these plasmids is described in Table 2. Wild enterobacterial strains resistant to streptomycin and sulphonamides are listed in Table 3.

**Media.** Strains for conjugation experiments were grown in nutrient broth (Anderson and Lewis, 1965a) and crosses were plated on MacConkey or nutrient agar containing suitable concentrations of the appropriate antibiotics. Counter-selection against the K12 donor strains was exercised with colicin E2 (Anderson and Lewis, 1965a, b) or nalidixic acid (40 \( \mu \)g/ml). Salmonella 01 phage (Felix and Callow, 1943) was used to eliminate \( S. \) typhimurium donor strains. For the preparation of \( ^{3}H \)-labelled plasmid DNA, strains were grown in M9 liquid medium containing tritiated thymidine, with glucose as the sole carbon source.

**Conjugation Experiments.** Exponential-phase broth cultures of donor and recipient strains, containing about \( 2 \times 10^8 \) organisms/ml, were mixed in a ratio of 1:10 for interrupted crosses, and in equal quantities for overnight crosses. Interrupted crosses were terminated after 30-60 min. The duration of overnight crosses was usually about 18 h.
Table 1. Standard bacterial strains

<table>
<thead>
<tr>
<th>Enteric Reference Laboratory No.</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1R713</td>
<td><em>Escherichia coli</em> K12 F&lt;sup&gt;+&lt;/sup&gt; prototrophic</td>
</tr>
<tr>
<td>14R525</td>
<td>1R713 N&lt;sub&gt;a&lt;/sub&gt;</td>
</tr>
<tr>
<td>22R80</td>
<td>K12-ROW, Colicin indicator strain.</td>
</tr>
<tr>
<td>20R675</td>
<td>K12 carrying ColE1 = K12(ColE1). Sensitive to colicin E2 and E3 but resistant to E1</td>
</tr>
<tr>
<td>20R676</td>
<td>K12 carrying ColE2 = K12(ColE2). Sensitive to colicin E1 and E3 but resistant to E2</td>
</tr>
<tr>
<td>RT576</td>
<td><em>Salmonella typhimurium</em> phage type 36</td>
</tr>
</tbody>
</table>

<sup>a</sup> N<sub>a</sub>r, nalidixic acid-resistant mutant.

Table 2. Non-transferring plasmids

<table>
<thead>
<tr>
<th>Plasmid number</th>
<th>Antibiotic resistance or colicinogen</th>
<th>Species of original host strain</th>
<th>Phage type of original host strain</th>
<th>Year and place of origin</th>
<th>Source or Enteric Reference Laboratory No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTP1</td>
<td>A&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>Salmonella typhimurium</em></td>
<td>29</td>
<td>England 1964</td>
<td>RT1, Anderson and Lewis (1965a, b)</td>
</tr>
<tr>
<td>NTP2</td>
<td>SSu&lt;sup&gt;b&lt;/sup&gt;</td>
<td><em>Salmonella typhimurium</em></td>
<td>29</td>
<td>England 1964</td>
<td>RT1, Anderson and Lewis (1965a, b)</td>
</tr>
<tr>
<td>NTP3</td>
<td>ASu</td>
<td><em>Salmonella typhimurium</em></td>
<td>29</td>
<td>Enteric Reference Laboratory</td>
<td>9R314, Anderson et al. (1968)</td>
</tr>
<tr>
<td>NTP4</td>
<td>ASSu</td>
<td>–</td>
<td>–</td>
<td>Enteric Reference Laboratory</td>
<td>13R135, Anderson (1969)</td>
</tr>
<tr>
<td>NTP5</td>
<td>T</td>
<td><em>Salmonella typhimurium</em></td>
<td>49</td>
<td>Scotland 1969</td>
<td>9M3779</td>
</tr>
<tr>
<td>NTP6</td>
<td>A</td>
<td><em>Salmonella typhimurium</em></td>
<td>168</td>
<td>England 1972</td>
<td>12M521</td>
</tr>
<tr>
<td>NTP7</td>
<td>ASSu</td>
<td><em>Salmonella typhimurium</em></td>
<td>157</td>
<td>Argentina 1972</td>
<td>12M3014</td>
</tr>
<tr>
<td>NTP8</td>
<td>colicin E1</td>
<td><em>Escherichia coli</em> K30</td>
<td>–</td>
<td>–</td>
<td>Prof. P. Fredericq</td>
</tr>
<tr>
<td>NTP9</td>
<td>colicin E2</td>
<td><em>Shigella sonnei</em> P9</td>
<td>–</td>
<td>–</td>
<td>Prof. P. Fredericq</td>
</tr>
<tr>
<td>NTP10</td>
<td>colicin E3</td>
<td><em>Escherichia coli</em> CA38</td>
<td>–</td>
<td>–</td>
<td>Prof. P. Fredericq</td>
</tr>
</tbody>
</table>

Symbols for antibiotic resistances: A = ampicillin, S = streptomycin, Su = sulphonamides, T = tetracyclines.

<sup>a</sup> Strains conferring ampicillin resistance are also resistant to carbenicillin.

<sup>b</sup> Strains carrying SSu, ASSu (NTP4) or ASSu (NTP7) are resistant to streptomycin but not to spectinomycin. This suggests that these plasmids code for a streptomycin phospho transferase (Danne et al., 1969).
Table 3. Wild strains resistant to streptomycin and sulphonamides

<table>
<thead>
<tr>
<th>ERL No.</th>
<th>Species of host strain and phage type of <em>S. typhimurium</em></th>
<th>Origin</th>
<th>Compatibility of SSu resistance with ASu</th>
</tr>
</thead>
<tbody>
<tr>
<td>8M3993</td>
<td><em>S. typhimurium</em> 32</td>
<td>Human, Scotland 1968</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td><em>S. typhimurium</em> 1</td>
<td>Human, England 1968</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td><em>S. typhimurium</em> 44</td>
<td>Human, Scotland 1969</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td><em>S. typhimurium</em> 6</td>
<td>Animal, England 1969</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td><em>S. typhimurium</em> 12a</td>
<td>Human, England 1970</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td><em>S. typhimurium</em> 12a</td>
<td>Animal, England 1971</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td><em>S. typhimurium</em> 55</td>
<td>Human, England 1971</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td><em>S. typhimurium</em> 85</td>
<td>Animal, England 1971</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td><em>S. typhimurium</em> Untypable</td>
<td>Animal, Singapore 1973</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td><em>S. typhimurium</em> 104</td>
<td>Human, Spain 1973</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td><em>S. typhimurium</em> 156</td>
<td>Human, New Zealand 1973</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td><em>S. agona</em> (B)*</td>
<td>Human, England 1970</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td><em>S. brandenburg</em> (B)</td>
<td>Human, Wales 1970</td>
<td>—</td>
</tr>
<tr>
<td>42R652</td>
<td><em>S. bredeney</em> (B)</td>
<td>Human, England 1970</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td><em>S. derby</em> (B)</td>
<td>Human, England 1970</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td><em>S. montevideo</em> (B)</td>
<td>Human, England 1970</td>
<td>—</td>
</tr>
<tr>
<td>42R653</td>
<td><em>S. newport</em> (B)</td>
<td>Human, England 1970</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td><em>S. Panama</em> (B)</td>
<td>Human, England 1970</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td><em>S. stanley</em> (B)</td>
<td>Human, England 1970</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td><em>S. chester</em> (B)</td>
<td>Human, England 1970</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td><em>S. Indiana</em> (B)</td>
<td>Human, England 1970</td>
<td>—</td>
</tr>
<tr>
<td>EC4240</td>
<td><em>E. coli</em></td>
<td>Human, England 1970</td>
<td>+</td>
</tr>
<tr>
<td>EC4316</td>
<td><em>E. coli</em></td>
<td>Human, England 1970</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td><em>E. coli</em></td>
<td>Animal, England 1973</td>
<td>—</td>
</tr>
<tr>
<td>3EC835</td>
<td><em>E. coli</em></td>
<td>Animal, England 1973</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td><em>E. coli</em></td>
<td>Animal, Ireland 1970</td>
<td>—</td>
</tr>
</tbody>
</table>

+ = Compatible, — = Incompatible.

These strains are all sensitive to spectinomycin (see Table 2).

Salmonella O groups are shown in parenthesis after serotype designations.

Colicinogeny. Strains were tested for colicin production by the method of Fredericq (1957). The K12 strains listed in Table 1 were used as differential indicators for colicins E1, E2 and E3.

Detection of β-lactamase Production. Release of β-lactamase was examined by the technique of Anderson and Lewis (1965a). Individual colonies were grown on starch-nutrient agar plates, and indole-penicillin developer was applied after about 18 h incubation at 37°C.

Compatibility Experiments. Compatibility between two non-transferring plasmids was tested by introducing one into a strain carrying the other, and examining the progeny for the presence of the resident plasmid. When both plasmids were present, segregation was studied by growing picks of the respective clones for 5 h in broth at 37°C, followed by plating on nutrient agar plates. These master plates were replicated on to nutrient agar containing the relevant antibiotics. In experiments with the colicin factors, master plates were replicated on to nutrient agar and the colonies tested for colicin production. Three to six hybrid clones were plated in each experiment, and usually at least 100 colonies of each were replicated.

Isolation of Plasmid DNA for Electron Microscopy. Cells were grown in M9 liquid medium and lysed with a mixture of lysozyme, EDTA, Brj 38 and sodium deoxycholate (Clewes and Helliwell, 1970). The resulting "clear lysates" were subjected to caesium chloride-ethidium bromide density gradient centrifugation. Fractions containing plasmid DNA were
Characterisation of Non-transferring Plasmids

Identification and preparation for electron microscopy. Full details of these techniques have been published previously (Grindley, Humphreys and Anderson, 1973).

Estimation of Copy Numbers of the Plasmids. The approximate number of copies of a plasmid per chromosome is estimated from the relative amounts of plasmid and chromosomal DNA, as measured by tritiated thymidine uptake over several generations of growth. The copy number can then be calculated for plasmids of known molecular weight, assuming the molecular weight of the chromosome to be $2.5 \times 10^8$ (Cooper and Helmstetter, 1968). Two different isolation procedures have been employed.

1. Preparation of "Cleared Lysates" (see above). More than 99.5% of the chromosomal DNA is pelleted, leaving the supernatant ("cleared lysate") containing the plasmid DNA. The proportion of acid-precipitable $^3H$-label recovered in the cleared lysate was compared with that in the whole lysate before the clearing spin.

2. Dye-buoyant Density Gradient Centrifugation of Sarkosyl Lysates. Plasmid DNA was isolated by the method of Bazaral and Helinski (1968). In caesium chloride-ethidium bromide density gradient centrifugation, the covalently-closed plasmid DNA has a higher density than the chromosomal DNA, and bands below it in the density gradient. The relative amounts of plasmid and chromosomal DNA were estimated in each preparation.

Results

Strains Carrying Non-transferring Plasmids (see Table 2). The resistance determinants of the $\Delta$-mediated transfer systems have been described previously (Anderson and Lewis, 1965a, b; Anderson et al., 1968; Anderson, 1969). These plasmids, A (NTP1), SSu, ASu and ASSu (NTP4), were transferred to K12 by either $\Delta$ or its derived R factor, T-A (Anderson and Lewis, 1965b). The colicin determinants ColEl, ColE2 and ColE3 were transferred to K12 from the wild colicinogenic strains. The T determinant NTP5 was transferred to K12 by the I-like transfer factor present in the original host strain. Lines carrying T alone were detected after a 30 min mating between K12 strains. However, it was easier to demonstrate transfer of T alone by a 30 min cross from K12 to S. typhimurium type 36. The second A determinant, NTP6, was identified in an S. typhimurium strain which carried non-transferring ampicillin resistance. It was mobilised by T-A and transferred to K12. ASSu (NTP7) was initially transferred to K12 from the wild host strain 12M3614, which carries an $\eta$-I-like transfer factor of compatibility group I$_8$ (Anderson and Threlfall, unpublished observations). The resistance determinant NTP7 was then separated from the transfer factor by an interrupted cross from the K12 host strain into S. typhimurium type 36, after which NTP7 was mobilised by T-A and introduced into K12 for DNA studies.

Compatibility Experiments. Pairs of non-transferring plasmids were tested for compatibility by transferring one plasmid into a strain carrying the other. Previous studies by Anderson et al. (1968) demonstrated that the homologous resistance determinants SSu and ASu were incompatible. ASSu (NTP4) arose by recombination between these two plasmids (Anderson, 1969). Thus, SSu, ASu and ASSu all belong to the same compatibility group, of which SSu is the prototype. The remaining non-transferring plasmids were tested for compatibility with SSu and with each other where possible. The A determinant NTP1 was identified in the same S. typhimurium type 29 strain, RT1, as SSu, and these two determinants coexist stably in the same cell (Anderson and Lewis, 1965a, b). NTP1 is also compatible with T, ColE1, ColE2 and ColE3. The second A determinant,
XTP6, also coexists stably with SSu, T and the three colicin factors. Since XTP1 and XTP6 code for resistance to ampicillin only, we have so far been unable to test for compatibility between them. The T determinant XTP3 was tested with A (XTP1), SSu, A (XTP6) and the three colicin factors; it was compatible with all these plasmids.

As the ASSu determinant XTP7 codes for resistance to ampicillin, streptomycin and sulphonamides, it was tested for compatibility with SSu by transfer to *S. typhimurium* type 36 already carrying SSu, selection being exercised for ampicillin resistance. The progeny were examined for segregation by replica plating. Up to 78% loss of ampicillin resistance was detected in some clones. Lines that were stable for A, S and Su were mated with K12, selecting for streptomycin resistance only. If both ASSu and SSu were present in the stable donor strain there should be independent transfer of the plasmids, so that a proportion of colonies selected on streptomycin should be sensitive to ampicillin. However, all the progeny selected on streptomycin carried ASSu only. The original cross had evidently resulted in the displacement of SSu by ASSu. We can thus conclude that ASSu (XTP7) belongs to the SSu compatibility group.

The three colicin factors shown to be compatible with the resistance determinants were tested against each other. *S. typhimurium* type 36 was employed as the host in these experiments, because it is immune to all three colicins. ColE1, ColE2 and ColE3 are compatible with each other. The results of the compatibility experiments are summarised in Table 4.

### Table 4. Compatibility groups of the non-transferring plasmids

<table>
<thead>
<tr>
<th>Groups</th>
<th>A (XTP1) group</th>
<th>SSu group</th>
<th>Compatible plasmids not yet grouped</th>
</tr>
</thead>
<tbody>
<tr>
<td>Examples</td>
<td>A (XTP1)</td>
<td>SSu</td>
<td>A (XTP6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ASu</td>
<td>T</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ASSu (XTP4)</td>
<td>ColE1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ASSu (XTP7)</td>
<td>ColE2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ColE3</td>
</tr>
</tbody>
</table>

* Many wild enterobacterial strains resistant to streptomycin and sulphonamides carry plasmids belonging to the SSu group (see Table 3).

**Compatibility Tests with Wild Strains Resistant to Streptomycin and Sulphonamides.** The ASSu determinant was transferred to a number of wild enterobacterial strains resistant to streptomycin and sulphonamides. The resistant progeny were examined for segregation after growth in drug-free nutrient broth. The results of these compatibility tests are shown in Table 3.

Incompatibility was detected in 19 of the 26 strains, which comprised ten different phage types of *S. typhimurium*, ten salmonella aerotypes and five independent *E. coli* strains. As shown in Table 3, the strains are of both human and animal origin and include cultures isolated in several different countries. The nineteen strains in which incompatibility was found probably carry SSu resistance determinants which are identical with, or closely related to, the
Characterisation of Non-transferring Plasmids

original SSu determinant of Anderson and Lewis (1965a, b). The SSu determinants in these 19 strains are easily mobilisable by A and an F-like transfer factor in triparental crosses.

The ASu determinant was compatible with the SSu resistance in seven of the strains tested: one S. typhimurium (8M3993); and three other salmonella serotypes, S. bredeney (42R652), S. newport (42R653) and S. panama (42R654). Three E. coli strains showed ASu/SSu stability: EC4240, EC4316 and 3EC635. Streptomycin-sulphonamide resistance was not transferable, nor was it mobilisable by A, from five of these strains (8M3993, 42R652, 42R653, 42R654, 3EC635). In one of them, a strain of S. panama, 42R654, SSu was mobilised by the F factor of K12. We have so far been unsuccessful in mobilising the SSu resistances of the four remaining strains with any transfer factors.

The line of S. typhimurium type 32 (8M3993), into which ASu had been introduced with T-A, was investigated further. Both ampicillin and streptomycin resistance were now transferable from this strain to K12. Selection on ampicillin resulted in transfer of ASu without SSu, but all progeny selected on streptomycin were resistant to ampicillin, streptomycin and sulphonamides. Further crosses to S. typhimurium type 36 demonstrated that such progeny carried a non-transferring recombinant ASSu plasmid. This new ASSu was transferred by T-A at the same frequency as the original ASu determinant. Studies on the nature of the SSu resistance of 8M3993 are being continued.

The two E. coli strains EC4240 and EC4316 directly transferred their streptomycin-sulphonamide resistances to S. typhimurium type 36, that is, they already possessed transfer factors. The properties of these plasmids are being investigated.

Contour Length Measurements. The non-transferring plasmids listed in Table 2 were all isolated from cleared lysates of plasmid-carrying strains. Plasmid DNA was prepared for electron microscopy, and micrographs of open circular molecules were enlarged, traced and measured. Details of contour length measurements and molecular weights are shown in Table 5.

The two A determinants NTPhwml NTP8 can be distinguished by their mean contour lengths on electron microscopy: 2.70 pm and 3.10 pm respectively. A grid carrying both plasmid DNAs was prepared and 57 open circular molecules were measured. The values were bimodal in distribution, with means corresponding to the measurements of NTP1 and NTP6 prepared on separate grids. The lengths of NTP1 and NTP6 were compared with that of the replicative form DNA of phage pX174, which was included in the same preparation as an internal standard. The lengths of NTP1 and NTP6 relative to pX174 were 1.48 and 1.68 respectively. This confirmed that NTP1 was shorter than NTP6, as our direct measurements indicated.

The contour lengths of the resistance determinants of the A transfer systems provide information on their interrelationships. ASu was produced by ultraviolet irradiation of S. typhimurium strain RT1 carrying A (NTP1) and SSu (Anderson et al., 1968); the mean contour length of A is 2.70 pm and that of SSu, 2.74 pm. The contour length of ASu is 3.91 pm, 1.17 pm longer than SSu. A considerable portion of the A determinant must therefore be present in ASu, but possibly not the region responsible for attachment of A to the membrane, since A and ASu
Table 5. Contour lengths and molecular weights of the plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Contour length (μm)</th>
<th>Molecular weight × 10^6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>Mean</td>
</tr>
<tr>
<td>A (NTP1)</td>
<td>40</td>
<td>2.45-2.86</td>
</tr>
<tr>
<td>SSu</td>
<td>33</td>
<td>2.60-2.97</td>
</tr>
<tr>
<td>ASu</td>
<td>37</td>
<td>3.07-4.18</td>
</tr>
<tr>
<td>ASSu (NTP4)</td>
<td>30</td>
<td>3.87-4.42</td>
</tr>
<tr>
<td>T</td>
<td>18</td>
<td>3.00-3.33</td>
</tr>
<tr>
<td>A (NTP9)</td>
<td>17</td>
<td>2.98-3.19</td>
</tr>
<tr>
<td>ASSu (NTP7)</td>
<td>25</td>
<td>4.29-4.80</td>
</tr>
<tr>
<td>ColE1</td>
<td>18</td>
<td>2.15-2.43</td>
</tr>
<tr>
<td>ColE2</td>
<td>22</td>
<td>2.08-2.37</td>
</tr>
<tr>
<td>ColE3</td>
<td>22</td>
<td>2.27-2.42</td>
</tr>
</tbody>
</table>

* Calculated on the assumption that 1 μm = 2.07 × 10^6 daltons (Lang, 1970).

The DNA of bacteriophage φX174 was used as the standard for contour length measurements. The mean contour length of the replicative form of φX174, calculated from measurement of 58 circular molecules, was 1.79 μm. This result is in good agreement with that of 1.83 μm obtained by Gordon (1973).

are compatible. Recombination between SSu (2.74 μm) and ASu (3.91 μm) yielded ASSu, which is 4.24 μm in length.

Our contour length measurements for the three colicin factors are in reasonable agreement with previously published values (Roth and Helinski, 1967; Inselburg and Fuke, 1970; Inselburg, 1973).

Estimation of Copy Numbers of the Non-transferring Plasmids. The number of copies of the resistance and colicin determinants per chromosome was calculated as described in Materials and Methods. At present no single method gives a wholly reliable estimate of copy number, so we have employed two different plasmid DNA isolation procedures. Preliminary centrifugation in the preparation of cleared lysates pellets most of the chromosomal DNA and cell wall-membrane debris. Some plasmid DNA may be included in this sedimenting material; this may result in a low estimate of copy number. The disadvantage of Sarkosyl lysis followed by cesium chloride-ethidium bromide centrifugation is that certain plasmids, such as ColE1, “relax” to open circular form in the presence of ethidium bromide. This form bands in the same position as linear chromosomal DNA in the density gradient (Clewell and Helinski, 1969). The determination of copy number by this method may therefore be low for plasmids that exist as “relaxation complexes” and are thereby converted to open circular form.

The results presented in Table 6 demonstrate that all ten non-transferring plasmids exist as multiple copies per chromosome.

In general, there is good agreement between the values obtained from the two methods used for estimation of plasmid copy number. We do not yet know why there is a low recovery of the T determinant NTP5 from cesium chloride-ethidium bromide centrifugation of Sarkosyl lysates compared with that from
Table 6. Estimation of copy numbers of the plasmids

<table>
<thead>
<tr>
<th>Non-transferring plasmid</th>
<th>% $^3$H-label in the cleared lysate relative to the total lysate*</th>
<th>% $^3$H-label in the plasmid band relative to the chromosomal DNAb</th>
<th>Number of plasmid copies per chromosomec</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (NTP1)</td>
<td>4.0</td>
<td>4.2</td>
<td>10.7–18.7</td>
</tr>
<tr>
<td>SSu</td>
<td>1.5</td>
<td>1.8</td>
<td>5.8–8.9</td>
</tr>
<tr>
<td>ASu</td>
<td>2.2</td>
<td>2.9</td>
<td>5.8–8.9</td>
</tr>
<tr>
<td>ASSu (NTP4)</td>
<td>2.2</td>
<td>2.7</td>
<td>5.3–7.6</td>
</tr>
<tr>
<td>T</td>
<td>1.5</td>
<td>0.6</td>
<td>4.2–7.3; 1.5–3.9d</td>
</tr>
<tr>
<td>A (NTP8)</td>
<td>2.3</td>
<td>3.1</td>
<td>8.8–14.1</td>
</tr>
<tr>
<td>ASSu (NTP7)</td>
<td>2.0</td>
<td>2.2</td>
<td>5.0–5.7</td>
</tr>
<tr>
<td>ColE1</td>
<td>1.5</td>
<td>0.9</td>
<td>5.0–9.5</td>
</tr>
<tr>
<td>ColE2</td>
<td>1.8</td>
<td>1.7</td>
<td>8.2–10.9</td>
</tr>
<tr>
<td>ColE3</td>
<td>1.9</td>
<td>1.8</td>
<td>8.1–11.2</td>
</tr>
</tbody>
</table>

* In 12 experiments, a mean of 0.54% chromosomal DNA was recovered in cleared lysates of the K12F+ strain (17).6
b Caesium chloride-ethidium bromide density gradient centrifugation of Sarkosyl lysates.

The range of copy numbers has been calculated from the values of experiments with both cleared lysates and Sarkosyl lysis.

d The values obtained by caesium chloride-ethidium bromide centrifugation of Sarkosyl lysates were consistently lower (range of copy numbers in six experiments: 1.5–3.9) than those obtained by the cleared lysate method (3 experiments: 4.2–7.3 copies).

cleared lysates. However, we believe that this difference is genuine, and it will be further investigated.

The prototype A determinant NTP1, present in about 18 copies per chromosome, was consistently found in greater numbers than any other plasmid, including the second A determinant NTP6. The inference that there is a genuine difference in copy number of these two A determinants is supported by $\beta$-lactamase assay of K12 strains carrying them. Extracts of strains carrying NTP1 show approximately twice the $\beta$-lactamase activity of those carrying NTP6 (Humphreys and Anderson, unpublished observations). Similarly, the diameter of zones of $\beta$-lactamase diffusion on starch-nutrient agar from colonies of K12 (NTP1) are greater than those of K12 (NTP6). If the two $\beta$-lactamases are identical in nature, as our activity profile determinations suggest, and are synthesised at the same rate by each A gene copy, the difference in $\beta$-lactamase activity may be due to the higher number of A gene copies in K12 (NTP1).

Discussion

The ten non-transferring plasmids described all form Class 2 transfer systems, in which resistance (or colicin) determinants and transfer factors are independent replicons. Incompatibility has previously been demonstrated between two homologous resistance determinants, SSu and ASu (Anderson et al., 1968). Two ASu determinants, NTP4 and NTP7, also belong to this compatibility group, of which SSu is the prototype. NTP4 was formed by recombination between ASu...
and SSu (Anderson, 1969); it is similar in size 4.24 μm, to the wild ASSu determinant NTP7 (4.53 μm).

Two A determinants, NTP1 and NTP6, are compatible with all the other determinants, but compatibility between NTP1 and NTP6 could not be tested because of identity of the resistance markers. The T resistance determinant and the three colicin factors are compatible with SSu, with both A determinants, and also with each other. The compatibility of ColE2 with ColE3 is interesting, because these plasmids show about 80% of DNA homology (Inselburg, 1973), and might thus be expected to belong to the same compatibility group.

We have examined a number of wild enterobacterial strains resistant to streptomycin and sulphonamides in compatibility experiments with ASu. The presence of SSu plasmids was established by the demonstration of incompatibility with ASu in 19 of 26 strains of independent origin and diverse nature. These SSu plasmids are thus homologous with the prototype SSu identified in *S. typhimurium* type 29 (Anderson and Lewis, 1965a, b). The identification of homologous plasmids in cultures of both animal and human origin supports the idea of a common pool of R factors and enterobacteria, which act as sources of transferable drug resistance in both types of host (Anderson, 1968; Anderson et al., 1973). The wide geographical distribution of similar SSu plasmids establishes their phylogenetic relationship. Since it is unlikely that they are descendants of a single ancestral SSu plasmid, they probably had a similar genetic origin in enterobacteria in different parts of the world.

The incidence of non-transferring plasmids in wild strains of *E. coli* has also been investigated by Smith and Linggood (1970). They isolated a number of determinants which were mobilisable by the triparental cross of Anderson (1965). The plasmids they identified were SSu determinants, T determinants, a ColE1 factor and a determinant for α-haemolysin production. The transfer of determinants by several different transfer factors was also studied (Smith and Heller, 1973). Their findings are in accordance with the observations of Anderson (1965, 1968), that the same determinant can be mobilised by different transfer factors, and that the same transfer factor can mobilise different determinants. Class 2 transfer systems were demonstrated by Smith and his co-workers in most cases, but probable covalent bonding between a determinant and certain transfer factors was also found.

Molecular studies demonstrate that the ten non-transferring plasmids described here are between 2.22 and 4.53 μm in length. The contour length measurements distinguish between two plasmids, NTP1 and NTP6, which confer resistance to ampicillin. NTP1 has a mean contour length of 2.70 μm, whereas that of NTP6 is 3.10 μm. Preliminary DNA reassociation experiments indicate some homology between these two A determinants. The ten plasmids are all present as multiple copies per chromosome. Previous studies established that A (NTP1), SSu and the three colicin factors exist as multiple copies per chromosome (Bazaral and Helinski, 1968; Humphreys, Grindley and Anderson, 1972; Milliken, Anderson and Clowes, unpublished). The G+C content of plasmid DNA can be determined by analytical ultracentrifugation in cesium chloride. The A determinant NTP1 has a G+C content of 40%, whereas those of SSu and ASu are 61% and 58% respectively (Grindley and Anderson, unpublished observations). From these
data we have calculated that about 95% of SSu and 50% of A are present in the ASu determinant. This calculation is based on the assumption that the genomes of A and SSu are each homogeneous in G+C content.

We have described another type of non-transferring plasmid, the kanamycin resistance determinant K. The K determinant originally isolated from *S. typhimurium* type 29 is $\textit{fi}^+$, and non-transferring but mobilisable in a triparental cross (Anderson, Pitton and Mayhew, 1968). The $\textit{fi}^+$K gave rise to $\textit{fi}^-$K, which forms a Class 2 transfer system with the F factor (Anderson, Mayhew and Grindley, 1969). Molecular studies of $\textit{fi}^-$K demonstrate that it has a mean contour length of 18.1 μm, and is present as approximately one copy per chromosome (Smith et al., 1973). This $\textit{fi}^-$K is incompatible with the recently defined $\textit{fi}^+$ plasmid widely distributed in strains of *S. typhimurium* (Anderson and Smith, 1972). In view of this incompatibility and the DNA homology between K and the $\textit{fi}^+$ plasmid, we have suggested that the $\textit{fi}^+$K determinant was formed by recombination between a kanamycin resistance marker and the $\textit{fi}^+$ plasmid of *S*. *typhimurium* type 29 (Smith et al., 1973). The K determinant therefore differs fundamentally from resistance determinants such as A and SSu.

It has been suggested that plasmid incompatibility involves competition for specific cellular attachment sites that govern replication and uniform segregation into daughter cells at cell division. Competition between two plasmids for the same site leads to establishment of only one plasmid. The resistance determinants SSu and ASu exist in multiple copies but are incompatible with each other. The simple membrane attachment site hypothesis must therefore be considered in relation to these results. Bazaral and Helinski (1970) followed the replication of the multiple-copy ColE1 determinant in a density-shift experiment, and concluded that copies are selected at random for replication from a pool of ColE1 molecules. Studies on the segregation kinetics of ColE1 led Durkacz and Sherratt (1973) also to suggest that the inheritance of ColE1 may be random. If a random selection mechanism for replication exists in certain systems, membrane attachment of the replicating copy may still be required. We have no information on the nature of the replication mechanism for the incompatible determinants SSu and ASu. There may be a master copy governing both replication and segregation. Incompatibility would then result from competition between the two master copies for the single membrane attachment site. Alternatively, incompatibility may result from the requirement of membrane attachment of a plasmid for uniform segregation into daughter cells. Only one plasmid, chosen at random from the pool, could occupy the site at the time of segregation during the cell cycle. This copy would effectively act as a master copy in terms of genetic continuity of the respective plasmid. The inference of this would be that the remaining cytoplasmic plasmid copies in the daughter cells would be diluted out or would become inactive and degrade, that is, that they would no longer constitute viable replicons. A random mechanism for replication could be reconciled with this hypothesis.

Previous studies on incompatibility have usually involved transferable plasmids which exist as a single copy per chromosome. However, the R factor R6K is transferable but is present in multiple copies (Kontomichalou, Mitani and Clowes, 1970). Recently, two R factors, R485 and R487, isolated from *Proteus* 17*
morganii, have been found to be incompatible with R6K (Hedges et al., 1973). This appears to be the first example of incompatibility involving a transferable plasmid which exists in multiple copies.

Our studies on non-transferring plasmids demonstrate that this type of plasmid can be distinguished by a number of criteria, including compatibility. Since compatibility is widely used for categorisation of bacterial plasmids, it is important to determine whether the transfer systems studied belong to Class 1 or Class 2 (see Introduction). If this is not investigated, plasmids which constitute in fact Class 2 transfer systems may be assigned to spurious new compatibility groups because of incompatibility between determinant plasmids which are independent of transfer factors and vice versa. Genetic studies demonstrate that resistance determinants and transfer factors of Class 2 systems can be separated and characterised individually. As yet, incompatibility has not been demonstrated between a small non-transferring determinant, existing in multiple copies, and a transferable plasmid usually present as a single copy per chromosome.

These studies underline the importance of the subdivision of bacterial transfer systems into Classes 1 and 2. They also indicate that, if the hypothesis that all plasmids, whether or not they are autotransferring, have membrane attachment sites, the number of such sites must be high.

Acknowledgements. We thank Dr. R. L. Sinsheimer for phage q-X174, Mrs. L. Ward for providing strains of ten S8u-resistant salmonella serotypes, and Dr. E. J. Threlfall for the strain carrying the ASSu (NTP7) determinant.

References
Characterisation of Non-transferring Plasmids


*Note Added in Proof.* Recent experiments suggest that there is a low degree of incompatibility between ColE2 and ColE3. This incompatibility was manifest in 2 h crosses but the crosses described above, which were overnight, yielded apparently stable hybrids. These studies will be published in detail at a later date.

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Characterisation of Derepressed Mutants of an F-like R Factor

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Summary. Inhibition of transfer of an F-like plasmid probably requires at least two components, one of which appears to be the same for most, perhaps all, F-like plasmids, while the other, the P product, is relatively plasmid-specific. Our investigations suggest that the plasmid-specific component of the transfer inhibitor is the same for the R factors R100 and 240, but different for 334. Using this finding a series of derepressed mutants of 240 have been shown to fall into three main groups: the first two contain mutants which are defective in one or other of the two components of the transfer inhibitor, while the third group comprises those mutants which are insensitive to the inhibitor. Our results also suggest that R100 and 240 code for the same plasmid-specific proteins necessary for transfer.

The group of R factors described as \(i^+\) (Egawa and Hirota, 1962) or \(f^+\) (Watanabe et al., 1964) inhibit the fertility of the sex factor F. Most of these R factors code for sex fimbriae similar to those produced by F\(^+\) strains of Escherichia coli K12, and have therefore been called F-like R factors (Meynell and Datta, 1966). It has been postulated that such \(f^+\) R factors code for a cytoplasmic inhibitor, which acts on the R factor itself to maintain the wild-type (repressed) state (Egawa and Hirota, 1962), and on the F factor in \(R^+F^+\) strains (Meynell and Datta, 1965). Since the F factor itself is naturally derepressed and sensitive to repression by \(f^+\) R factors, it was postulated to lack this inhibitor.

Finnegan and Willetts (1971) showed that cells carrying a wild-type R factor, R100, and newly infected with Flac, could retransfer Flac at high frequency. However, when these cells carried both R100 and a second F factor (Fhis), retransfer of Flac was inhibited. This suggested that inhibition of F transfer by an R factor required not only a product of the R factor, but also a product encoded by F, which was either slowly synthesized or slow to act, since the incoming F factor failed to provide it. Finnegan and Willetts (1971) therefore concluded that inhibition of F fertility by F-like R factors required both a component specified by an R factor locus designated \(\text{fin}\), and a plasmid-specific product, encoded by the F factor itself, which they called \(P_F\).

Mutants of Flac were isolated which were derepressed in the presence of an \(f^+\) R factor in lines in which both plasmids were established; these mutants were called \(\text{tra}P\) (Finnegan and Willetts, 1971). When such an Flac \(\text{tra}P\) mutant was transferred into cells carrying both an \(f^+\) R factor and Fhis, the immediate retransfer of Flac \(\text{tra}P\) was inhibited, indicating that this mutant was sensitive to the complete inhibitor, that is, the \(\text{fin}\) product of the R factor and the \(P\) product of Fhis. It was suggested that the \(\text{tra}P\) gene coded for the \(P\) product, although this could not be proved (Finnegan and Willetts, 1971).

Finnegan and Willetts also suggested that, by analogy with the inhibition of F transfer, both a \(\text{fin}\) product and an R factor-specified, plasmid-specific product \(P_R\), were necessary for inhibition of R factor transfer. Recently, evidence
supporting this suggestion was obtained with several F-like plasmids (Finnegan and Willetts, 1972). It thus appears that at least two components are necessary for inhibition of F-like plasmids: the fin product, which appears to be the same or similar for most, perhaps all, F-like plasmids; and the P product which shows some plasmid specificity. It is not known how these products interact to cause the inhibition of plasmid transfer.

Lawn and Meynell (1970) examined the ability of antibody produced against F-like fimbriae specified by one plasmid to bind to those produced by strains carrying other plasmids, and subdivided F-like plasmids into four groups. Two R factors which specified serologically similar sex fimbriae were R100 and R136, while R1 was shown to specify fimbriae of a different serotype. The plasmids F and ColV-K94 also coded for fimbriae that were serologically indistinguishable.

In this paper we demonstrate that the P_R products of the two F-like R factors R100 and 240 (= R136) are the same or similar, and are distinguishable from the P_R product of a third F-like R factor, 334 (= R1). Using this finding, we characterise a series of derepressed transfer mutants of 240.

Materials and Methods

Bacterial Strains. The Escherichia coli K12 strains used are shown in Table 1. Only the markers relevant to our investigations are shown. The donors in all crosses were derivatives of JC6255 carrying R factors. The “intermediates” in the experiments described below were JC5455 and derivatives of it carrying R factors. JC3051 was the final recipient in all crosses.

<table>
<thead>
<tr>
<th>Table 1. Bacterial strains</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strain No.</strong></td>
</tr>
<tr>
<td>JC6255^a</td>
</tr>
<tr>
<td>JC5455^a</td>
</tr>
<tr>
<td>JC3051^a</td>
</tr>
<tr>
<td>38R223</td>
</tr>
<tr>
<td>38R444</td>
</tr>
<tr>
<td>38R447</td>
</tr>
<tr>
<td>38R020</td>
</tr>
</tbody>
</table>

^a JC3051, 5455 and 6255 are derivatives of JC8500 (Achtman, Willetts, and Clark, 1971), and were obtained from D. J. Finnegan.
^b Details of these plasmids are shown in Table 2.
T6^f = resistance to phage T6. Str^f = chromosomal resistance to streptomycin.

Transferable Plasmids. These are shown in Table 2. The tetracycline-sensitive segregants, R100T— and R100-1T— were isolated in this laboratory. The derepressed mutants of the F-like R factor 240 (= R136) and their isolation were described by Grindley et al. (1971). Those mutants of 240 which remained fi^+ and were not repressed by the fi^+ 1-like R factor 782 were called “operator constitutive” (oc) mutants (Grindley et al., 1971). As there is no evidence that inhibition of transfer occurs through an operator at the level of transcription, we have renamed these mutants 240drl—8; they were previously called 240 d^a1—8.

Media. Bacterial strains were grown in L broth (Lennox, 1955) from single colonies, and crosses were plated on nutrient agar. Appropriate antibiotics were incorporated in the plates to select for R factor-carrying strains. Streptomycin (2000 μg/ml) was used to select for recipient strains.

Mating Conditions. As the F-like R factors studied here could not coexist stably in the same cell, the effect of one such R factor on derepressed mutants of the same or another plasmid was investigated by examining a transient population of cells carrying both R factors.
Characterisation of Derepressed Mutants of an F-like R Factor

Table 2. Transferable plasmids

<table>
<thead>
<tr>
<th>Designation</th>
<th>Description</th>
<th>Resistances carried</th>
</tr>
</thead>
<tbody>
<tr>
<td>R100T⁻</td>
<td>T⁻ segregant of the F⁻ F-like R factor, R100</td>
<td>CSSu</td>
</tr>
<tr>
<td>R100-1</td>
<td>mutant of R100, derepressed in transfer and no longer F⁻</td>
<td>TCSSu</td>
</tr>
<tr>
<td>R100-1T⁻</td>
<td>T⁻ segregant of R100-1</td>
<td>CSSu</td>
</tr>
<tr>
<td>240</td>
<td>F-like R factor isolated from S. typhimurium 4406 (also known as R136)</td>
<td>T</td>
</tr>
<tr>
<td>240T⁻</td>
<td>segregant of 240, sensitive to tetracyclines</td>
<td>—</td>
</tr>
<tr>
<td>240d⁻1</td>
<td>mutant of 240, derepressed in transfer and no longer F⁻</td>
<td>T</td>
</tr>
<tr>
<td>240d⁻1,2,4 to 8 and R136draHb</td>
<td>mutants of 240, derepressed in transfer and still F⁻</td>
<td>T</td>
</tr>
<tr>
<td>334</td>
<td>F-like R factor isolated from S. paratyphi B 7268 (also known as R1)</td>
<td>ACSSu</td>
</tr>
</tbody>
</table>

Symbols for plasmid-borne antibiotic resistances: A=ampicillin; C=chloramphenicol; S=streptomycin; Su=sulphonamides; T=tetracyclines.

For this we used the method devised by Finnegan and Willetts (1971) to characterise their mutants of Falc. An early exponential phase culture of JC6255 (0.6 ml) carrying a mutant of one factor was crossed with 1.4 ml of a stationary phase culture of JC5455 carrying the wild-type R factor. After 45 min, mating was interrupted by incubating for 15 min with T6 phage to which the donor strain JC6255 is sensitive. The number of cells of JC5455 carrying both plasmids, which act as donors in the final cross, was then measured by plating on nutrient agar containing the appropriate antibiotics. The efficiency of retransfer from this intermediate donor strain was immediately tested by mixing 0.2 ml of the culture with 1.8 ml of an exponential phase culture of the final recipient JC3051. Suitable dilutions were plated after 30 min to determine frequencies of transfer of the R factors from the intermediate strain JC5455.

Male-Specific Phage Sensitivity of Strains Carrying Two Incompatible R Factors. An exponential phase culture of JC6255, carrying a derepressed mutant of 240 or R100-1, was crossed with an exponential phase culture of JC5455 carrying 334. After about 4 h the mating mixture was spread on nutrient plates containing antibiotics selecting for both R factors, and spotted with the F-specific phage μ2.

Results

Plasmid Specificity of Components of the Transfer Inhibitor. As we have already mentioned, it has been suggested that at least two components are required to form an active inhibitor of the transfer of an F-like plasmid. The first of these is the product of the fin gene. This appears to be the same for most, if not all, F-like plasmids. The second component, called P, is slowly synthesized (or slow to act) in newly-infected cells, and shows some plasmid specificity.

If two F-like R factors code for the same P product, this will result in inhibition of retransfer of a mutant of one factor, which lacks one or other of the components
of the transfer inhibitor (that is, either \( \text{fin}^- \) or \( \text{P}^- \)) from cells also carrying the other R factor in the wild repressed state, because the strain carrying the repressed R factor will contain both the \( \text{fin} \) product and \( \text{P} \). We examined the immediate retransfer of 240\(i^-1 \), from intermediate lines, newly infected with this derepressed R factor, and carrying either of the wild-type R factors, 334 or R100T\(^- \) (see Methods). The results are shown in Table 3, lines 1 and 2.

<table>
<thead>
<tr>
<th>R factor in donor strain</th>
<th>Intermediate strain</th>
<th>Frequency of transfer of donor R factor from newly-infected intermediate cells (as % of retransfer from the R(^- ) intermediate JC5455)</th>
</tr>
</thead>
<tbody>
<tr>
<td>240(i^-1 )</td>
<td>38R444</td>
<td>334</td>
</tr>
<tr>
<td>240(i^-1 )</td>
<td>38R223</td>
<td>R100T(- )</td>
</tr>
<tr>
<td>R100(-1 )</td>
<td>38R444</td>
<td>334</td>
</tr>
<tr>
<td>R100(-1 )</td>
<td>38R447</td>
<td>240</td>
</tr>
</tbody>
</table>

The derepressed R factors retransfer from the R\(^- \) intermediate under the same conditions at a frequency of about \( 10^{-1} \) per intermediate donor.

When 334 was present in the intermediate strain, the retransfer of 240\(i^-1 \) was not inhibited. Assuming that 240\(i^-1 \) is sensitive to the 334-specified \( \text{fin} \) product, this result indicates that a slowly synthesised, or slowly acting 240-specific product is required for inhibition of the transfer of 240\(i^-1 \) by 334. This therefore confirms, for 240, the finding of Finnegan and Willetts (1972) that F-like plasmids require a plasmid-specific product \( \text{P} \) for inhibition of their transfer.

The assumption that 334 codes for a \( \text{fin} \) product which can act on 240\(i^-1 \) (and R100\(-1 \)) was confirmed by examining the effect of 334 on the sensitivity to the F-specific phage \( \mu^2 \) of strains carrying derepressed mutants of 240 or of R100 (see Methods). Strains carrying both 334 and either of the \( \text{fin}^- \) mutants, 240\(i^-1 \) or R100\(-1 \), were not visibly lysed by \( \mu^2 \) in surface spot tests (see Table 4). Strains carrying either 240\(i^-1 \) or R100\(-1 \) alone were sensitive to the phage. The R factors 240 and R100 are therefore sensitive to the \( \text{fin} \) product specified by 334.

The immediate retransfer of 240\(i^-1 \) from the intermediate strain carrying R100T\(- \) was reduced 500-fold (Table 3, line 2). This shows that 240\(i^-1 \) is sensitive to the complete transfer inhibitor specified by R100T\(- \), and therefore that R100T\(- \) codes for a \( \text{P} \) product which can also act on 240.

The frequencies of retransfer of R100\(-1 \) from newly-infected intermediate lines carrying either 334 or 240 are also shown in Table 3 (lines 3 and 4). From these
results, by analogy with the conclusions drawn from the results obtained with
240i-1, we can infer that R100-1 also requires a P_R product for transfer inhibition,
and that such a P product is specified by 240 but not by 334.

Characterisation of Derepressed Mutants of 240. We examined retransfer of a
series of derepressed mutants of 240 from cells carrying R100T-
and newly infected
with the mutant of 240. The results were similar to those of analogous experiments
using as the intermediate strain 38R626, which carried a tetracycline-sensitive
segregant of 240 (see Table 5).

On the basis of those two sets of results it appears that the derepressed
mutants of 240 can be divided into three groups. The first group comprises the
four mutants R130drdH8, 240drp5, 240drp8 and 240dr/>8. Transfer of these four

Table 5. Retransfer of derepressed mutants of 240 from newly-infected R+ strains

<table>
<thead>
<tr>
<th>Derepressed mutant</th>
<th>Frequency of transfer* of the donor plasmid from the newly-infected intermediate strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>38R223 (R100T-)</td>
<td>38R626 (240T-)</td>
</tr>
<tr>
<td>R130drdH8</td>
<td>40</td>
</tr>
<tr>
<td>240drp5</td>
<td>50</td>
</tr>
<tr>
<td>6</td>
<td>33</td>
</tr>
<tr>
<td>8</td>
<td>28</td>
</tr>
<tr>
<td>1</td>
<td>5.9</td>
</tr>
<tr>
<td>4</td>
<td>0.7</td>
</tr>
<tr>
<td>2</td>
<td>0.2</td>
</tr>
<tr>
<td>7</td>
<td>0.0</td>
</tr>
<tr>
<td>240i-1</td>
<td>0.2</td>
</tr>
</tbody>
</table>

a Expressed as a percentage of the retransfer of the donor plasmid from the R- inter­
mEDIATE strain JC5455.
b The R100T- plasmid transferred from cells carrying both it and a derepressed mutant of
240, at about half the frequency of the derepressed mutant itself. Transfer of R100T- from
cells carrying it alone, was not detectable in a thirty minute cross.

mutants was little changed by the presence of either R100T- or 240T- in
the intermediate strain. The second group contains the mutants 240drp1 and 240drp4. Both
these mutations were partially repressed by R100T- (15- to 17-fold) or 240T-
(23- to 44-fold), but were at least ten times less repressed than the mutant 240i-1,
which no longer codes for an active fin product). The third group of mutants,
240drp2 and 240drp7, was repressed by both R100T- (170- to 500-fold) and 240T-
(300- to 500-fold), so that the frequency of transfer of these mutants from the
intermediate donor cells was similar to that of 240i-1.

The intermediate strains 38R223 and 38R626 must produce all the components
necessary for complete repression of transfer of 240. Mutants of 240 which remain
derepressed when newly transferred to these strains may have a mutation in the
site of action of the transfer inhibitor, similar to that suggested for the traO
mutants of Flac (Finnegan and Willetts, 1971). The first group of mutants of 240:
R130drdH8, 240drp5, 6, and 8, fall into this class. Derepressed mutants of 240,
whose transfer from newly-infected intermediate cells of 38R223 or 38R626 was
fully inhibited, fall into two groups: those which no longer repress F-mediated
fertility and are therefore fin- (240i-1) (Grindley et al., 1971); and those which
still repress F and are therefore \( \text{fin}^+ \) (240drp2 and 7). This second group, being sensitive to the complete wild-type inhibitor encoded by the resident plasmid in the intermediate strain, must be deficient in a second component of this inhibitor. The second component must be relatively plasmid-specific since the mutants of this group are not repressed by 334 in established lines (see Table 4). The mutants 240drp2 and 240drp7, therefore, have the properties expected of 240 carrying a mutation in the \( \text{traP} \) gene.

The two mutants 240drp1 and 240drp4 do not appear to fall into any of these categories. When these mutants of 240 were alone in established lines they were fully derepressed. However, they were partially repressed in the presence of an active inhibitor of fertility in newly-infected cells. If these mutants carried lesions in the site of action of the inhibitor synthesised by themselves, making them partially sensitive to this inhibitor, they would be only partially derepressed in established lines. However, their efficiency of transfer was indistinguishable from that of the other derepressed mutants of 240, all of which transferred at a frequency of \( 4-8 \times 10^{-3} \) in 30 min. If mutants 240drp1 and 4 coded for an altered \( \text{fin} \) product, they should no longer inhibit F transfer, while if they specified an altered P product, their transfer from the intermediate strains 38R223 and 38R626 which carry R100T\(^-\) and 240T\(^-\) respectively, would be fully rather than partially repressed.

If we can assume that the results with 240drp1 and 4 are not caused by a quantitative difference in either inhibitor production or sensitivity of one of the mutant types \( \text{traP} \) or \( \text{traO} \), their behaviour could be explained on the following hypothesis: if a third product, rapidly synthesised in newly-infected cells, were necessary for transfer inhibition, 240drp1 and 4 could code for a defective form of this product, retaining the ability to interact with the site of action of the normal product, but unable to contribute to transfer inhibition. If such a mutant were newly transferred into a cell carrying R100T\(^-\) (or 240T\(^-\)) the altered product would compete with the normal equivalent already present in the intermediate strain, resulting in partial inhibition of retransfer of the 240drp mutant. Any mutant which failed to produce this postulated third component of the transfer inhibitor would be classified as a \( \text{traP} \) mutant if retransfer were examined from newly-infected cells also carrying the wild-type plasmid. Mutants classified as \( \text{traP} \) may therefore include mutations in more than one gene.

In the experiments described above, in which we examined retransfer of various derepressed mutants from newly-infected cells carrying wild-type R factors, we also examined the transfer of the wild-type R factor present in the intermediate strain. The R factor R100T\(^-\) is transfer-defective, its transfer not normally being detectable in a 30 min cross. However, in the experiments summarised in Table 3, the R factor R100T\(^-\), in the intermediate strain, transferred in all cases at about 50% of the frequency of retransfer of the derepressed mutant of 240. The incoming mutant of 240, therefore codes for transfer proteins that can be used by R100. Moreover, when these proteins are produced at a derepressed rate (by the strains carrying the mutants R136drpH8, 240drp5, 240drp6 and 240drp9) R100T\(^-\) is also transferred at a derepressed frequency.

The mutant 240\(^+\)1 retransferred at a derepressed rate from intermediate cells also carrying 334 (Table 3). In this experiment, however, the R factor 334 transferred from the intermediate strain at its usual repressed rate (ca. \( 2 \times 10^{-3} \)).
Characterisation of Derepressed Mutants of an F-like R Factor

A similar result was obtained with the derepressed plasmid R100-1 and the wild-type 334. It was therefore concluded that the repressed F-like R factor 334 was unable to use the transfer proteins produced at a derepressed level by 240 and R100. There is thus at least one component of the proteins, necessary for transfer, which is the same for R100 and 240 but different for 334. Willetts (1971) has shown that the traJ and traI products, both necessary for transfer of the F factor, are plasmid-specific, since complementation does not occur between R100-1 and Flac carrying mutations in either of these genes. Our findings could be explained by the presence of R factor analogues of one or both of the traJ and traI products. These would be the same for R100 and 240, but different for 334 and for F.

Discussion

Finnegan and Willetts (1971) proposed a model for inhibition of transfer of the F factor by an f' F-like R factor, suggesting that at least two components were required to form an active transfer inhibitor. The first component is the product of the fin gene of the R factor. The second component, called P, is specified by a gene carried by the F factor itself, and is slowly synthesised or slow to act in newly-infected cells. Recent work has shown that this model can be extended to include the inhibition of transfer of F-like plasmids (Finnegan and Willetts, 1972).

Lawn and Meynell (1970) subdivided the sex fimbriae encoded by several F-like plasmids into four serotypes. Strains carrying the R factors R100 and R130 (240) produced fimbriae of the same serotype, which suggested that there might be other similarities between R100 and 240. Our results, given above, show that R100 and 240 code for interchangeable P products, distinguishable from that of 334 (which codes for fimbriae of a different serotype). Finnegan and Willetts (1972) have independently obtained the same results, and have also shown that the P products of F and ColV-K84, which code for fimbriae of the same serotype, are indistinguishable.

Willetts (1971) observed that traJ or traI mutants of Flac were not complemented by R100-1, which suggested that the products of these genes were plasmid-specific. Our investigations suggest that at least one component of the transfer proteins specified by 240 is relatively plasmid-specific, since it promotes the transfer of R100 but not of 334. This specificity may indicate that the protein concerned is coded by a traJ or traI gene of the R factor. Thus, not only do R100 and 240 code for serologically indistinguishable fimbriae (Lawn and Meynell, 1970) but their P products and the plasmid-specific component(s) of their transfer proteins are interchangeable.

We have characterised a series of derepressed mutants of 240 using the fact that R100 specifies the same fin and P products as 240. These mutants fall into three main groups.

1. Those with a mutation in the fin gene (240f-1).
2. Those with a mutation in a gene which codes for a plasmid-specific component of the transfer inhibitor, presumed to be the P product (240g/p2 and 7). These are analogous to the traP mutants of Flac.
3. Those which are insensitive to the complete transfer inhibitor produced by R100 and are therefore presumed to have a mutation in the site of action of the inhibitor (R130g/rf118, 240g/p5, 6, and 8). These are analogous to the tra0 mutants of Flac.

4 Make, gen. Genet. 120
It is difficult to explain the behaviour of two mutants, 240drp1 and 4, within the model for transfer inhibition outlined above. These mutants are partially sensitive to the complete transfer inhibitor in newly-infected cells, although they are fully derepressed in established lines. To explain the properties of these mutants, we suggest that a third component, rapidly synthesised in newly-infected cells, may be implicated in transfer inhibition.

Note Added in Proof. The surface exclusion properties of the R factors we have examined conform with the plasmid specificity of their postulated P products and transfer proteins. Surface exclusion was found (200- to 500-fold reduction in transfer frequency) in matings between strains carrying R100-1 and 240drp2. No exclusion was shown in matings between strains carrying R1drd19 and either R100-1 or 240drp2 (although R1did19 was excluded by a strain carrying R1drd19 itself).

References


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Derepression of F-lac in Salmonella typhimurium by a determinant for kanamycin resistance

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AND E. S. ANDERSON

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(Received 20 July 1970)

SUMMARY

When a determinant for neomycin-kanamycin resistance (K) was transferred by an F-lac factor into Salmonella typhimurium, the resulting KF-lac strain was sensitive to the male-specific phage μ and F-lac was derepressed. However, F-lac alone is repressed in S. typhimurium. When kanamycin resistance is spontaneously lost from S. typhimurium KF-lac an element persists which derepresses F-lac in S. typhimurium. The results are consistent with the hypothesis that a locus der, for derepression of F-lac in S. typhimurium, lies on the K plasmid. The R factor Rldrd19 is derepressed in K12 but is repressed in S. typhimurium. It also is derepressed by der. In contrast to F-lac and R1, another R factor, R136drdH8, is derepressed in both K12 and S. typhimurium, so that the intervention of der is unnecessary for its derepression in the salmonella host.

A determinant for resistance to neomycin and kanamycin (K) was described recently by Anderson, Mayhew & Grindley (1969). This determinant was isolated from a strain of S. typhimurium phage type 29 in which it was associated with an $f^+$ transfer factor. The association between K and the transfer factor is characteristic of the class of R factors first observed in S. typhimurium, in which the transfer factor and the resistance determinant regularly segregate in transfer, and are independent of each other in the host cell (Anderson & Lewis, 1965a, b; Smith, Anderson & Clowes, 1970).

For the experiments described in this paper, the K determinant, without its original transfer factor, was isolated in Escherichia coli K12F- (= K12). It was then mobilized with an F-lac factor, using the triparental cross for determinant mobilization (Anderson, 1965), the final recipient being S. typhimurium, phage type 30 (= S. typhimurium). Except for the presence of lac, which was simply used as a marker for F transfer to S. typhimurium, the resulting R factor, KF-lac, was indistinguishable from the KF resistance factor described previously (Anderson et al. 1969; Grindley, Grindley & Anderson, 1970). The effects of K on F-lac, described below, are thus identical with its effects on the F factor alone.

Transfer of F-lac from S. typhimurium KF-lac and S. typhimurium F-lac to K12 and S. typhimurium is shown in Table 1.

This table shows that S. typhimurium KF-lac transfers F-lac to both K12 and S. typhimurium at a frequency a 100-fold higher than that from S. typhimurium F-lac to the same recipients. All of 148 S. typhimurium KF-lac recombinants tested were sensitive to the male-specific phage μ (Dettori, Maccauro & Piccinin, 1961). By contrast, 337 S. typhimurium clones that had received F-lac alone were resistant to μ, although K12 carrying F-lac only is sensitive to μ. Thus, the F-lac factor, which is derepressed in K12,
is ordinarily repressed in *S. typhimurium*, and its derepression in this system seems to be affected by the K determinant. The reactions of *S. typhimurium* and K12 lines with phage _λ_2 are summarized in Table 2.

### Table 1. Transfer of _F-lac_ from *Salmonella typhimurium* KF- _lac_ and _S. typhimurium_ _F-lac_ in 2 _h_ crosses

<table>
<thead>
<tr>
<th>Cross</th>
<th>Frequency of <em>F-lac</em> transfer</th>
<th>Sensitivity of donor to phage _µ_4</th>
<th>Proportion of donor cells carrying <em>F</em> fimbriae (electron microscopy)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. typhimurium</em> KF- <em>lac</em> x K12</td>
<td>1 × 10⁻⁴</td>
<td>+</td>
<td>18/28 = 64%</td>
</tr>
<tr>
<td><em>S. typhimurium</em> <em>F-lac</em> x K12</td>
<td>2 × 10⁻⁴</td>
<td>−</td>
<td>1/36 = 2.8%</td>
</tr>
<tr>
<td><em>S. typhimurium</em> (K)⁻ <em>F-lac</em>* x K12</td>
<td>1 × 10⁻⁴</td>
<td>+</td>
<td>21/47 = 44.7%</td>
</tr>
</tbody>
</table>

* Resulting from spontaneous loss of K from *S. typhimurium* KF- _lac_.

+ = Visible lysis in spot tests with phage _µ_4 on surface culture (Grindley et al. 1970); − = No visible lysis with _µ_4.

### Table 2. Reactions of _S. typhimurium_ and K12 strains with _male-specific_ phage _µ_4

<table>
<thead>
<tr>
<th>Strains</th>
<th>Reaction with _µ_4</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. typhimurium</em> <em>F-lac</em></td>
<td>−</td>
</tr>
<tr>
<td><em>S. typhimurium</em> KF- <em>lac</em></td>
<td>+</td>
</tr>
<tr>
<td><em>S. typhimurium</em> (K)⁻ <em>F-lac</em>*</td>
<td>+</td>
</tr>
<tr>
<td><em>S. typhimurium</em> (KF- <em>lac</em>)*</td>
<td>+</td>
</tr>
<tr>
<td>K12 <em>F-lac</em></td>
<td>+</td>
</tr>
<tr>
<td><em>S. typhimurium</em></td>
<td>−</td>
</tr>
<tr>
<td>K12 <em>F⁻</em></td>
<td>−</td>
</tr>
</tbody>
</table>

+ = Visible lysis with _µ_4; − = No visible lysis with _µ_4.

* Spontaneous loss of K.
† Spontaneous loss of both K and _F-lac_.

The state of repression of _S. typhimurium_ KF- _lac_ and _S. typhimurium_ _F-lac_ was also investigated by determining the degree of _F_ fimbriation in the electron microscope (see Table 1). Specific adsorption of phage _µ_4, followed by negative staining with sodium silicotungstate, was used for the identification of _sex_ fimbriae. Of 28 _S. typhimurium_ KF- _lac_ cells observed, 18 (64%) carried _sex_ fimbriae, while _S. typhimurium_ _F-lac_ showed only one _sex_ fimbriated cell out of 36 examined (2.8%).

Other workers have also observed the repression of an _F_ factor in _S. typhimurium_ (Mäkelä, Lederberg & Lederberg, 1962; Easterling et al. 1969), although no explanation has been suggested and derepression does not seem to have been described.

Lines of _S. typhimurium_ KF- _lac_ were then examined for spontaneous loss of K, and it was found that when such loss occurred the derepression persisted, as shown in Table 1. This was also confirmed by observation of _sex_ fimbriation by electron microscopy.

Further experiments with _S. typhimurium_ carrying the derepressed _F-lac_ factor without K showed that, although the derepressed state was stable in that host strain, subsequent transfer of this element in which _F-lac_ was retested retained the derepression. In overnight crosses, the frequency of transfer was 8 × 10⁻⁴. This frequency is very similar to that in overnight crosses to _S. typhimurium_.
pressed in *S. typhimurium*, and its derepression in this system seems to be a K determinant. The reactions of *S. typhimurium* and K12 lines with the repression in Table 2.

1. **Transfer of F-lac from Salmonella typhimurium KF-lac and S. typhimurium F-lac in 2 h crosses**

<table>
<thead>
<tr>
<th>Cross</th>
<th>Frequency of F-lac transfer</th>
<th>Sensitivity of donor to phage $\mu_2$</th>
<th>Proportion of donor cells carrying F fimbrili (electron microscopy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{KF-lac} \times \text{K12}$</td>
<td>$1 \times 10^{-2}$</td>
<td>+</td>
<td>$18/28 = 64%$</td>
</tr>
<tr>
<td>$\text{F-lac} \times \text{K12}$</td>
<td>$2 \times 10^{-3}$</td>
<td>+</td>
<td>$1/36 = 2.8%$</td>
</tr>
<tr>
<td>$\text{F-lac} \times \text{K12}$</td>
<td>$2 \times 10^{-3}$</td>
<td>+</td>
<td>$21/47 = 44.7%$</td>
</tr>
</tbody>
</table>

; from spontaneous loss of K from *S. typhimurium* KF-lac.  
; lysis in spot tests with phage $\mu_2$ on surface culture (Grindlly et al. 1970);  
; able lysis with $\mu_4$.

**Table 2. Reactions of S. typhimurium and K12 strains with male-specific phage $\mu_2$**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Reaction with $\mu_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. typhimurium F-lac</em></td>
<td>$+$</td>
</tr>
<tr>
<td><em>S. typhimurium KF-lac</em></td>
<td>$-$</td>
</tr>
<tr>
<td><em>S. typhimurium (K) F-lac</em></td>
<td>$+$</td>
</tr>
<tr>
<td><em>S. typhimurium (KF-lac)</em>$\dagger$</td>
<td>$-$</td>
</tr>
<tr>
<td>K12 F-lac</td>
<td>$+$</td>
</tr>
<tr>
<td>S. typhimurium</td>
<td>$-$</td>
</tr>
<tr>
<td>K12 F-</td>
<td>$-$</td>
</tr>
</tbody>
</table>

$+$ = Visible lysis with $\mu_5$; $-$ = No visible lysis with $\mu_5$.  
$*$ Spontaneous loss of K.  
$\dagger$ Spontaneous loss of both K and F-lac.

Expression of *S. typhimurium* KF-lac and *S. typhimurium* F-lac was also evaluated the degree of F fimbrilation in the electron microscope (see of phage $\mu_2$, followed by negative staining with sodium of the identification of sex fimbrilia. Of 28 *S. typhimurium* KF-lac (K12) carried sex fimbrilia, while *S. typhimurium* F-lac showed the repress state; was stable in that host strain.
subsequent transfer of this F-lac to S. typhimurium produced a majority of recombinants in which F-lac was repressed. However, about 4% of 220 recombinant clones tested retained the derepression. As F-lac in this experiment transferred at $2 \times 10^{-1}$ in an overnight cross, the frequency of transfer of derepression was $4\%$ of $2 \times 10^{-1}$, that is, $8 \times 10^{-4}$. This frequency is very similar to that of K transfer from S. typhimurium KF-lac in overnight crosses to S. typhimurium, about $10^{-3}$.

![Diagram](attachment:image.png)

**Fig. 1.** Proportion of F-lac and K in S. typhimurium. * (+); (−): reactions with male-specific phage μ. ** F-lac was transferred at a frequency of c. $2 \times 10^{-1}$ in overnight crosses in all cases.

It should be noted that whether F-lac is in the repressed or derepressed state in S. typhimurium, it is derepressed in K12.

An S. typhimurium line that had spontaneously lost both K and F-lac (see Table 2) was then investigated. When a (repressed) F-lac factor was transferred from S. typhimurium into this strain, sensitivity to μ was regained. The recipient strain had therefore retained the element for derepression of the F-lac factor, in spite of the loss of K and F-lac. Crosses from the resultant derepressed S. typhimurium F-lac strain into S. typhimurium established that F-lac was transferred at the same frequency as before, that is, $1.8 \times 10^{-4}$, and that it was derepressed in 6.5% of S. typhimurium F-lac recipients. Thus, the frequency of transfer of the derepression of F-lac was unchanged (c. $1 \times 10^{-3}$).

These observations are summarized in Fig. 1.
The results suggest that a derepressor locus, which we designate der, is associated with the K plasmid. This hypothesis is supported by the fact that the transfer frequency of der by S. typhimurium der F- lac is the same as that of the original K plasmid. Spon-
taneous loss of kanamycin resistance may result from mutation in, or deletion of, the resistance locus, and retention of der may indicate that the remainder of the plasmid is intact. This residuum, carrying der, would be transferable by F-lac in the same way as the original K plasmid.

Alternatively, der could be on a separate plasmid from K, in which case the plasmids should be lost independently of each other. However, if this hypothesis is correct the two plasmids must be postulated to be very closely associated during transfer in spite of their independence, as we have been unable to separate the kanamycin resistance from the derepression property by conjugation.

If der is effectively part of the K plasmid, the properties of derepression and kanamycin resistance should be readily co-transduced, whereas if the two markers are on indepen-
dent plasmids, co-transduction should be a very rare event. Bacteriophage P22 was used to derepress property by conjugation.

Two derepressed R factors, R1306 and R1306dH8 (Meynell & Datta, 1967) were investigated. These will be referred to as R1 and R136 hereafter. R1 carries resistance to ampicillin, kanamycin, streptomycin and sulphonamides, and R130 resistance to tetra-
cyclines only. R1 and R136 were originally identified in strains of S. paratyphi B phage type 3a var 4 and S. typhimurium phage type 28 respectively (E. S. Anderson & N. Datta, unpublished).

K12 carrying R1 or R136 is sensitive to µp. K12 R1 was crossed with S. typhimurium and S. typhimurium der, to yield S. typhimurium R1 and S. typhimurium der R1 progeny respectively. Forty-nine of 50 colonies of S. typhimurium R1 gave no visible lysis with µp, while all of 40 S. typhimurium der R1 colonies gave good lysis with the phage. In the colony of S. typhimurium R1 which gave lysis with µp, this lysis was more turbid than that on S. typhimurium der R1.

Transfer of R1 from µp-insensitive S. typhimurium R1 to S. typhimurium occurred at a repressed frequency of 2·5 x 10^-4 in 2 h, and 1·6 x 10^-4 overnight. The µp-sensitive line of S. typhimurium R1, in contrast, transferred R1 at a derepressed frequency of 10^-1 in 2 h and 4·5 x 10^-1 overnight. The derepression was not transferred, however, since all of 100 progeny tested from the overnight cross were insensitive to µp.

S. typhimurium der R1 transferred R1 to S. typhimurium at a frequency of 1·5 x 10^-4 in a 2 h cross, and 3 x 10^-1 overnight. Ten of 100 colonies from the overnight cross were sensitive to µp. Thus, der was transferred by R1 at a frequency of about 10^-3, similar to that of its transfer by F-lac.

When R136 was transferred from K12 to S. typhimurium and S. typhimurium der, all the S. typhimurium R136 and S. typhimurium der R136 progeny tested (20 of each) gave good lysis with µp.

The presence of der in S. typhimurium therefore potentiated derepression of R136dH8, as did F-lac in that host. Since R136dH8 is already derepressed in S. typhimurium, there was no evidence that its state of derepression was affected by der.

It may be significant that F and R136dH8 are i- mutants which do not synthesize repressor, but are sensitive to repressor in K12, while R136dH8 is an o' mutant which is insensitive to repressor in K12 (Front.).

The possibility that S. typhimurium may state of F-lac and R136dH8 without the presence of the plasmid, it is reasonable to suppose that S. typhimurium R136dH8 as it is in S. typhimurium.

On the basis of the above evidence, it is assumed that R136dH8 possesses no transfer factor, carries only the lactose-ferm

These phenomena are under further investigation.

[Note added in proof.] Recent experiments have shown that R136dH8 is insensitive to repressor but not o' mutants of F-lac.

We thank Dr Anne M. Field for culture of S. paratyphi H, Dr Datta for providing derepressed mutants of R136dH8, and Dr E. S. Anderson for providing derepressed mutants of F-lac.

References


H. R. Smith and others

At that a derepressor locus, which we designate der, is associated with a hypothesis is supported by the fact that the transfer frequency of \textit{F} lac is the same as that of the original K plasmid. Sper- nomycin resistance may result from mutation in, or deletion of, the old retention of der may indicate that the remainder of the plasmid is um, carrying der, would be transferable by F-lac in the same way as mid.

or could be on a separate plasmid from K, in which case the plasmids dependently of each other. However, if this hypothesis is correct the be postulated to be very closely associated during transfer in spite asce, as we have been unable to separate the 

by part of the K plasmid, the properties of derepression and kanamycin be readily co-transduced, whereas if the two markers are on indepen- transduction should be a very rare event. Bacteriophage P22 was 

amurium KF-lac. The resulting phage preparation (titre c. \(8 \times 10^{10}\) diluted with toluene, and 1 ml was mixed with an equal quantity of an amurium F-lac (c. \(5 \times 10^6\) organisms/ml). After 30 min at \(37^\circ\) C the 

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mid.

The resulting phage preparation (titre c. \(8 \times 10^{10}\) diluted with toluene, and 1 ml was mixed with an equal quantity of an amurium F-lac (c. \(5 \times 10^6\) organisms/ml). After 30 min at \(37^\circ\) C the 

plasmid is

mid.

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plasmid is

mid.

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plasmid is

mid.
is insensitive to repressor in K12 (Frydman & Meynell, 1969; Meynell & Cooke, 1969). The possibility that S. typhimurium actively represses both F and R1ddy19 must therefore be explored.

Alternatively, S. typhimurium may be (passively) unable to express the derepressed state of F-lac and R1ddy19 without the intervention of an element such as der. In any case, it is reasonable to suppose that the derepressing effect of der is the same in S. typhimurium R1ddy19 as it is in S. typhimurium F-lac.

On the basis of the above evidence we conclude that the K plasmid, which apparently possesses no transfer factor, carries a locus der, which derepresses F-lac and R1ddy19 in S. typhimurium.

These phenomena are under further examination and our findings will be reported later.

[Note added in proof.] Recent experiments support the hypothesis that S. typhimurium represses i~ but not of mutants of F-like plasmids. This work is being prepared for publication.

We thank Dr Anno M. Field for carrying out the electron microscopy, and Dr Naomi Datta for providing derepressed mutants of R1 and R136. Requests for reprints should be addressed to Dr E S. Anderson.

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SHORT PAPER

The effects of Salmonella typhimurium on derepressed mutants of F-like factors

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(Received 3 December 1970)

SUMMARY

Derepressed mutants of F-like transfer factors, isolated by mutagenesis, were characterized as repressor-minus (i−) or operator-constitutive (oc). Mutants of the i− class are derepressed in K12 but repressed in Salmonella typhimurium. They are derepressed in S. typhimurium by a kanamycin resistance determinant carrying a locus der, described previously. Most oc mutants of F-like factors are derepressed in both K12 and S. typhimurium. However, one mutant of F-lac was oc in K12 but was repressed in S. typhimurium. It was derepressed by der. Repression by S. typhimurium is different from that by f+ factors, since der reverses the former but does not affect the latter. Possible interpretations of these findings are discussed.

In a previous article (Smith et al. 1970) we described the effect of S. typhimurium phage type 31 (= S. typhimurium) on an F-lac factor and on the derepressed F-like R factors Rldrd19 and R130drdH8. S. typhimurium carrying either F-lac or Rldrd19 showed poor donor ability and was insensitive to the male-specific phage μ4; that is, the factors were derepressed in the S. typhimurium host. In contrast, R130drdH8 remained derepressed in S. typhimurium. The fact that F and Rldrd19 are repressor-minus (i−) mutants while R130drdH8 is an operator-constitutive (oc) mutant (Meynell & Cooke, 1969) was felt to be possibly significant. Repressor-minus mutants do not produce an active repressor but remain sensitive to the repressor, while oc mutants are insensitive to the repressor although they still code for its synthesis.

An earlier paper described the transfer of a kanamycin resistance determinant (K) by the F factor (Anderson, Mayhew & Grindley, 1969). Later work showed that the K determinant derepressed S. typhimurium strains carrying F-lac and Rldrd19 (Smith et al. 1970). K could also be transferred by either F-lac or Rldrd19 from such strains to S. typhimurium. On the basis of these and other results, we postulated the existence of a locus on the K plasmid responsible for the derepression of F-lac and Rldrd19 in S. typhimurium. This locus was designated der.

In this paper we describe the effect of S. typhimurium on further derepressed mutants of F-like R factors and on oc mutants of F-lac. It should be borne in mind that F-lac is i−, so that oc mutants of F-lac are both i− and oc. Table 1 shows the F-like factors studied and their derivation.

Derepressed mutants of the R factors were obtained by the selection procedure of Edwards & Meynell (1968) after treatment of the R factor in Escherichia coli K12 (= K12) with ethyl methane sulphonate (EMS) or N-methyl-N′-nitro-N-nitroso-
guanidine (NG). EMS was used at 0-2, 0-3 or 0-4 mM in broth, and NG at 50,250 or 500 μg/ml in 0-1 M citrate buffer, pH 5-5. A standard exposure time of 30 min to each mutagen was used. Derepressed colonies were detected by their sensitivity to phage μS. To determine whether the derepressed R factors were i- or o′, they were transferred into a K12 strain carrying the factor 782, which also confers resistance to kanamycin. Factor 782 is incompatible with the i- I-like factor 4 of Anderson & Lewis (1965). Strains carrying factor 782 propagate the I-specific phage If1. However, 782 represses the fertility and male-specific phage sensitivity of strains carrying the F factor; that is, it is i+, in spite of being I-like; it is the prototype of a group of factors shortly to be described (J. N. Grindley and E. S. Anderson, in the press). Since 782 is compatible with both F and F-like R factors, it is useful for testing the repressor sensitivity of derepressed F-like factors. Derepressed mutants which were repressed by factor 782 were designated i-, while those that remained derepressed were designated o′. The results were confirmed by examining the effect of the derepressed mutants on the fertility of HfrH. Although direct selection for derepression after mutagenesis yields mainly o′ mutants, we also obtained i- mutants in this way.

### Table 1. F-like factors and their derepressed mutants

<table>
<thead>
<tr>
<th>Ref. no.</th>
<th>Resistance*</th>
<th>Derepressed mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>334t</td>
<td>ACKSSu</td>
<td>i- 334 o′</td>
</tr>
<tr>
<td>R 1t</td>
<td>ACKSSu</td>
<td>R 1rd 19</td>
</tr>
<tr>
<td>240t</td>
<td>T</td>
<td>240 i- and 2</td>
</tr>
<tr>
<td>F-lac‡</td>
<td></td>
<td>F-lac o′ 1 to 3</td>
</tr>
</tbody>
</table>

* A, Ampicillin; C, chloramphenicol; K, neomycin-kanamycin; S, streptomycin; Su, sulphonamides; T, tetracyclines.
† R factors 334 and R1 were isolated from a strain of S. paratyphi B BB7268. Factor 334 is a kanamycin-sensitive segregant of the original R factor which carried the resistances ACKSSu (see Pitton & Anderson, 1970).
‡ R factor 240 was freshly isolated from its wild host strain of S. typhimurium 3M4466. An earlier isolation (E. S. Anderson and N. Datta, unpublished) was designated R136 (Meynell & Datta, 1966).
§ The F-lac factor of Jacob and Adelberg (1959), supplied by Professor W. Hayes.

The o′ mutants of F-lac were isolated by a modification of the method of Friedmann et al. (1970). K12F-lac carrying 782 was treated with EMS or NG as described above. The treated strain was then incubated overnight in nutrient broth and crossed in a ratio of 20:1, with a K12F-lac-Strr recipient carrying 782. Mating was interrupted at 30 min, when 1 ml samples of the mating mixtures were diluted into 100 ml of broth containing 500 μg/ml of streptomycin. After 3 h incubation at 37 °C, 1 ml quantities were subcultured to 100 ml of M9 minimal medium containing 500 μg streptomycin/ml and with lactose (0-2 %) as the sole carbon source. The mixture was allowed to grow overnight. The resultant cultures were then crossed in a ratio of 20:1 with a K12F-lac-Strr recipient carrying 782. Mating was interrupted at 30 min, when 1 ml samples of the mating mixtures were diluted into 100 ml of broth containing 500 μg/ml of streptomycin. After 3 h incubation at 37 °C, 1 ml quantities were subcultured to 100 ml of M9 minimal medium containing 500 μg streptomycin/ml and with lactose (0-2 %) as the sole carbon source. The mixture was allowed to grow overnight. The resultant cultures were then crossed in a ratio of 20:1 with a K12F-lac-Strr recipient carrying 782. After 30 min, suitable dilutions were plated on MacConkey plates containing 20 μg/ml of nalidixic acid; the plates were incubated overnight at 37 °C. Lactose-fermenting colonies were then purified and tested with male-specific phage μS. The majority of colonies tested were sensitive to phage μS in spite of the presence of the i+ factor 782, and therefore carried F-lac o′ mutants. The three F-lac o′ mutants studied were isolated in independent derepression experiments. They were separated from factor 782 by interrupted crosses into K12F-lac-Strr.

### Table 2. Characteristics of derepressed factors

<table>
<thead>
<tr>
<th>Derepressed factor</th>
<th>334 o′</th>
</tr>
</thead>
<tbody>
<tr>
<td>R 1rd 19</td>
<td></td>
</tr>
<tr>
<td>240 o′ 1 to 8</td>
<td></td>
</tr>
<tr>
<td>240 i- and 2</td>
<td></td>
</tr>
<tr>
<td>F-lac o′ 1 to 3</td>
<td></td>
</tr>
<tr>
<td>F-lac</td>
<td></td>
</tr>
</tbody>
</table>

* Visible lysing.

### Table 3. Sensitivity to phage pf1 of K12F-i+ and F-lac o′

<table>
<thead>
<tr>
<th>Derepressed factor</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>334 o′ 1</td>
<td></td>
</tr>
<tr>
<td>R 1rd 19</td>
<td></td>
</tr>
<tr>
<td>240 o′ 1 to 8</td>
<td></td>
</tr>
<tr>
<td>240 i- and 2</td>
<td></td>
</tr>
<tr>
<td>F-lac o′ 1</td>
<td></td>
</tr>
<tr>
<td>F-lac o′ 2 and 3</td>
<td></td>
</tr>
<tr>
<td>F-lac</td>
<td></td>
</tr>
</tbody>
</table>

NT, Not tested; +, visible lysis.

With the exception of R1rd 19 obtained by these methods. Each factor is carried on a different plasmid.

Table 2 shows the effect of 782 on the derepressed mutants, and that derepressed mutants were selected with EMS and NG.

It is evident from Table 2 that derepression with EMS or NG does not significantly reduce the fertility on K12, or the conferring μS sensitivity on K12, of HfrH, presumably because the derepressed mutants do not significantly reduce the fertility of HfrH. Although selection for derepression after mutagenesis yields mainly o′ mutants, we also obtained i- mutants in this way.
EMS was used at 0-2, 0-3 or 0-4 M in broth, and NG at 50, 250 or 500 µg/ml buffer, pH 5-5. A standard exposure time of 30 min to each mutagen was used. Colonies were detected by their sensitivity to phage λ. To determine repressed R factors were i- or o, they were transferred into a K12 strain F+ of Anderson & Lewis (1965). Strains carrying the I-specific phage If1. However, 782 represses the fertility and age sensitivity of strains carrying the F factor; that is, it is i+, in spite of the fact that 782 is the prototype of a group of factors shortly to be described (J. N. S. Anderson, in the press). Since 782 is compatible with both F and r, it is useful for testing the repressor sensitivity of derepressed F-like mutants which were repressed by factor 782 were designated i+, and further derepressed were designated o. The results were confirmed by the effect of the derepressed mutants on the fertility of HfrH. Although direct repression after mutagenesis yields mainly o mutants, we also obtained derepressed mutants.

Table 1. F-like factors and their derepressed mutants

<table>
<thead>
<tr>
<th>Resistance*</th>
<th>Derepressed mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>i-</td>
</tr>
<tr>
<td>ACSSu</td>
<td>334o+1</td>
</tr>
<tr>
<td>ACKSSu</td>
<td>R1prog19</td>
</tr>
<tr>
<td>T</td>
<td>240o-1 and 2</td>
</tr>
<tr>
<td>F-lac</td>
<td>F-laco+1</td>
</tr>
</tbody>
</table>

*a resistant to 
† resistant to 
‡ resistant to 
§ resistant to 
\* resistant to 
\** resistant to 
\*** resistant to

Table 1. F-like factors and their derepressed mutants
With the exception of Rldrd19, all the derepressed mutants listed in Table 1 were obtained by these methods. Each number refers to an independent isolation.

Table 2 shows the effect of 782 on the phage $\mu_2$ sensitivity of the K12 strains carrying the derepressed mutants, and the effect of these mutants on the fertility of HfrH.

It is evident from Table 2 that the $i^-$ mutants Rldrd19 and 240$^{-}$-1 and 2 confer sensitivity to phage $\mu_2$ on K12$^{-}$, and are repressed by factor 782 in the same host. They do not significantly reduce the fertility of HfrH. The $o^-$ mutants, in contrast, while conferring $\mu_2$ sensitivity on K12, are not repressed by factor 782, but reduce the fertility of HfrH, presumably because they still produce repressor.

### Table 2. Characterization of derepressed mutants

<table>
<thead>
<tr>
<th>Derepressed factor</th>
<th>Sensitivity to $\mu_2$ of K12 carrying the factor</th>
<th>Sensitivity to $\mu_2$ of K12 carrying the factor &amp; 782</th>
<th>Sensitivity to Transfer frequency of pro from HfrH+factor (HfrH = 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>334o+1</td>
<td>+</td>
<td>+</td>
<td>0.001</td>
</tr>
<tr>
<td>Rldrd19</td>
<td>-</td>
<td>-</td>
<td>0.5</td>
</tr>
<tr>
<td>240o+1 to 8</td>
<td>+</td>
<td>+</td>
<td>0.07</td>
</tr>
<tr>
<td>240$^{-}$-1 and 2*</td>
<td>-</td>
<td>-</td>
<td>0.5-1</td>
</tr>
<tr>
<td>F-laco+1 to 3</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>F-lac</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

+ , Visible lysis with $\mu_2$; -, no visible lysis with $\mu_2$.

* The derepressed mutants 240$^{-}$-1 and 2 gave good lysis with $\mu_2$ only when grown without shaking. Consequently all $\mu_2$ phage sensitivity tests of strains carrying these factors were carried out on unshaken cultures.

### Table 3. Sensitivity to phage $\mu_2$ of Salmonella typhimurium and S. typhimurium K der carrying derepressed factors

<table>
<thead>
<tr>
<th>Derepressed factor</th>
<th>Type in K12</th>
<th>S. typhimurium</th>
<th>S. typhimurium K der</th>
</tr>
</thead>
<tbody>
<tr>
<td>334o+1</td>
<td>o+</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td>Rldrd19</td>
<td>i-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>240o+1 to 8</td>
<td>o-</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td>240$^{-}$-1 and 2*</td>
<td>o-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>F-laco+1</td>
<td>o+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>F-laco$^{-}$2 and 3</td>
<td>o-</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td>F-lac</td>
<td>i-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

NT, Not tested; +, visible lysis with $\mu_2$; -, no visible lysis with $\mu_2$.

All the derepressed mutants were transferred into S. typhimurium and the resulting progeny were tested with phage $\mu_2$. The factors repressed in S. typhimurium were also transferred to S. typhimurium K der. Table 3 shows the results of these experiments.

This table shows that all the $i^-$ factors were repressed by S. typhimurium. In contrast, all the $o^-$ R factor mutants and two of the three F-laco$^o$ mutants were derepressed in S. typhimurium, although one mutant of F-lac which was $o^-$ in K12 was repressed in S. typhimurium. All the factors repressed in S. typhimurium alone were derepressed when that host carried der.
When the $f^+$ R factor 334 was transferred to *S. typhimurium* F-lac K+der, the F-lac became repressed, and the strain was no longer sensitive to $\mu_+$. Thus, der has no influence on the repressor activity of an $f^+$ R factor. The repressor activity of *S. typhimurium* on $i^-$ derepressed mutants, which is reversed by der, is therefore different from that of the $f^+$ R factors.

The Jacob & Monod (1961) model of regulation was first applied by Egawa & Hirota (1962) to the control of F fertility and sex fimbrial synthesis. Using the same model, we suggest the following as a plausible explanation of our observations. *S. typhimurium* produces a repressor which binds to the operator of F or F-like transfer factors. This repressor is different from that of the F-lac operon, as shown above. The locus der reverses the repression of *S. typhimurium*, perhaps by producing an antirepressor which inactivates the *S. typhimurium* repressor, or by blocking the synthesis of this repressor.

As the $f^+$ R factor repressors tested were not affected by der, it must be postulated that they cannot be bound by this antirepressor, or alternatively that their synthesis is not blocked by the der product.

We have found that, when F-lac or R1d19 is transferred to *S. typhimurium*, although the majority of recombinant colonies are resistant to $\mu_+$, rare recombinant clones are sensitive to the phage. This spontaneous derepression cannot be transferred and is therefore not due to mutation in the transfer factor. When lines which had lost their transfer factor were isolated from these colonies, reinfection with F-lac or R1d19 gave only derepressed progeny. These clones could be spontaneous repressor-minus mutants of *S. typhimurium*, the existence of which is predictable on the hypothesis that the repression of $i^-$ mutants of F-like factors by *S. typhimurium* is caused by a host-synthesized repressor.

The F-lac$^o 1$ mutant is repressed by *S. typhimurium*, although it remains derepressed in K12 carrying 334 or 782. On the above hypothesis it could therefore be suggested that F-lac$^o 1$ has a mutation in the operator rendering it insensitive to the $f^+$ repressors of 334 and 782 but not affecting its sensitivity to the *S. typhimurium* repressor. The effect of the *S. typhimurium* repressor on F-lac$^o 1$ is reversed by der, as would be expected. These results suggest that $o^-$ mutants of F, and presumably of F-like transfer factors, can be divided into two classes, depending on whether they are repressed or derepressed in *S. typhimurium*. The $o^-$ property can thus be defined, not only in terms of the transferable plasmid itself, but also in terms of the host organism, because, as we have shown, a mutant which is $o^-$ in one host, may be repressed by another.

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N. D. F. Grindley and others

or 334 was transferred to S. typhimurium F-lac K der, the F-lac
the strain was no longer sensitive to μₗ. Thus, der has no influence
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Interactions of Group H Resistance Factors with the F Factor

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The four R factors described in this paper form a single compatibility group which has been previously designated group H. Recombination was demonstrated between any pair of the plasmids TP117, 123, and 124. In contrast, TP116 did not appear to recombine with any other members of the group. TP117, 123, and 124 usually displaced the F factor from Escherichia coli K-12 F+, while TP116 and F coexisted stably in that strain. Deoxyribonucleic acid reassociation experiments showed minimal homology between F and the four group H plasmids. The results indicate that there are limitations to using incompatibility alone for classification of bacterial plasmids.

The ability of certain pairs of plasmids to coexist stably in the same cell has been used in the classification of R factors and other bacterial plasmids. This phenomenon, termed incompatibility, was first demonstrated with the F factor and F lac (27). Watanabe et al. (29) showed that pairs of "fertility inhibition" */* R factors were incompatible but that fi* and fi plasmids were stably maintained in the same cell. Studies on the fi* -like plasmid, A, and its derived R factor, T-A (4), showed that these two plasmids were incompatible (1). Incompatibility has also been demonstrated for nontransfering plasmids such as the resistance determinants SSu and ASu (3).

The phenomenon of incompatibility is especially useful for classifying plasmids which do not enable their hosts to support multiplication of the F specific phage μ2 and fd or the I specific phage II. Several "compatibility groups" of such R factors have been defined (9, 10, 12, 16, 21, 23). Plasmids belonging to one group cannot coexist stably but are compatible with plasmids of other groups.

In this paper we describe experiments with four fi R factors belonging to compatibility group H (5, 16). Three of the four R factors studied displace the F factor from Escherichia coli K-12 F+. This displacement of F by an H group R factor has been independently observed by Waldvogel and Pitton (personal communication). The results of deoxyribonucleic acid (DNA) reassociation experiments between F and the group H plasmids are also presented. The data in this paper will be discussed in relation to other molecular studies on the H group R factors (17).

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. Standard strains used in these experiments are listed in Table 1. The H group R factors and their origin are shown in Table 2. The phages were: the F specific phage, μ2 (13), the female specific phage, μ2 (11), and the I specific phage, II (26).

Media. Strains were grown in nutrient broth, and mating mixtures were plated on MacConkey agar containing suitable concentrations of the appropriate antibiotics. Counter selection against the donor strains was exercised with nalidixic acid (40 μg/ml) or streptomycin (500 μg/ml).

Conjugation experiments. Both cultures of donor and recipient strains containing about 2 x 10^6 bacteria/ml were mixed in a ratio of 1:10 for 1-h crosses, and in equal quantities for overnight crosses. The frequencies of transfer in interrupted crosses are expressed as the proportion of resistant progeny per donor cell, while those in overnight crosses are expressed as the proportion per recipient cell.

Plasmid interactions. Compatibility experiments were performed by transferring one plasmid into a strain carrying another and selecting for the resistance of the donor plasmid. The progeny were then tested for the presence of both the incoming and resident plasmids. When possible, joint selection was also exercised for the resistances of both R factors. The stability of hybrid lines was examined by growing them in nutrient broth for 5 h at 37 C, followed by plating on nutrient agar plates, which were incubated overnight at 37 C. These master plates were replicated onto nutrient agar containing the relevant antibiotics. Strains which were stable for the resistance of the
two plasmids were tested in crosses with K-12F selecting for each resistance marker separately in the progeny. Examination of these exconjugants showed whether the two plasmids coexisted independently or were recombined in the donor strain.

**Phage sensitivity.** The presence of the F factor in K-12 strains was tested with the F-specific phage, $\phi 2$, and the female-specific phage, $\phi 2 K-12F$ or Hfr strains are fully sensitive to $\phi 2$ but restrict viable lysates by $\phi 2$. Strains that have lost F are like the K-12F control, resistant to $\phi 2$ but fully sensitive to $\phi 2$. The tests were carried out by the surface spot method.

**Phage multiplication experiments.** The ability of strains carrying the group H plasmids to support multiplication of the F-specific phage, $\phi 2$, and the female-specific phage, $\phi 2 K-12F$ or Hfr strains have been described (17). The methods of preparing the unlabeled DNA from plasmid-bearing strains and of measuring reassociation of desaturated DNA from different plasmids have also been described (17).

### RESULTS

The four R factors listed in Table 2 have been ascribed to the H compatibility group (16). These plasmids, TP116, TP117, TP123, and TP124, do not enable their hosts to support multiplication of either the F-specific phage, $\phi 2$, or the L-specific phage, $\phi 1$. The R factors were transferred into HfrH, and the resulting strains were tested for sensitivity to phage $\phi 2$. In each case the strains remained fully sensitive to the phage, showing the incoming plasmids to be $fi$. The hybrid strains resulting from these crosses were stable, indicating that the integrated F factor could coexist with these group H plasmids. The results of phage multiplication and fertility inhibition tests with TP118 and TP117 have already been published (16).

### Table 1. Standard strains of Escherichia coli K-12

<table>
<thead>
<tr>
<th>No.</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>14R525</td>
<td>K-12 $^*$prototrophic Nal$^+$</td>
</tr>
<tr>
<td>44R850</td>
<td>14R525 carrying F$^*$</td>
</tr>
<tr>
<td>34R649</td>
<td>K-12F for Str$^+$</td>
</tr>
<tr>
<td>39R883</td>
<td>K-12Flac$^T$</td>
</tr>
<tr>
<td>32R1000</td>
<td>K-12HfrH Nal$^+$</td>
</tr>
</tbody>
</table>

* Nal resistant acid-resistant mutant; Str$^+$ streptomycin-resistant mutant.

The F factor from strain S8-161 (20).

Flac$^T$ is a recombinant between Flac and the tetracycline (T) resistance marker of the I-like R factor T-2 (4, 6).

### Table 2. H group R factors

<table>
<thead>
<tr>
<th>Plasmid no.</th>
<th>Drug resistance $^*$</th>
<th>Species of original host and place of origin</th>
<th>Phase type of original host strain</th>
<th>KFr reference no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP116</td>
<td>CSSu</td>
<td>Nal.annus (Spanish) 1960</td>
<td>C</td>
<td>TP116</td>
</tr>
<tr>
<td>TP117</td>
<td>T</td>
<td>S. typhimurium (England) 1961</td>
<td>1 var 2</td>
<td>TP117</td>
</tr>
<tr>
<td>TP123</td>
<td>C, Ssu, S+</td>
<td>S. typhimurium (Mexico 1972)</td>
<td>Degraded</td>
<td>TP123</td>
</tr>
<tr>
<td>TP124</td>
<td>C, Ssu, S+</td>
<td>S. typhimurium (India 1972)</td>
<td>DN</td>
<td>TP124</td>
</tr>
</tbody>
</table>

$^*$ Symbols for drug resistances: C. chloramphenicol; S, streptomycin; N, nalidixic acid; Su, sulfonamides; T, tetracycline.

### Compatibility experiments with group H plasmids.**

Each H group R factor was transferred into a K-12 strain carrying another plasmid of the group in overnight crosses (Table 3). It is difficult to measure entry exclusion with these plasmids because they have a low and variable frequency of transfer, even into "virgin" strains. For example, the transfer frequency to K-12F in overnight crosses may be as high as $10^{-2}$ or as low as $10^{-5}$.

The results in Table 3 show that the resident H group R factor is usually displaced by the incoming plasmid. When there is joint selection for the resistances of both plasmids, there are marked differences between the pairs of R factors. TP116 did not stably coexist with TP117, 123, or 124, nor did it recombine with them. Unstable hybrid lines carrying TP116 were selected, and each of the other three plasmids showed that TP116 was usually retained while the other plasmid was lost, as had been suggested earlier in experiments with TP116 and TP117 (16). Joint selection for the resistance markers of TP123 and TP124 yielded a high proportion of stable hybrid lines because of recombination between the resident and incoming plasmids. Recombination also occurred between TP117 and both TP123 and TP124.

The four R factors were transferred to K-12F (40R850) in overnight crosses, selecting for the appropriate resistances. The resulting colonies were tested for sensitivity to the F-specific phage, $\phi 2$, and the female-specific phage, $\phi 2$. The progeny were also tested for the resistance to chloramphenicol, streptomycin, and sulfonamides. The results of these experiments have already been published (16).

### Table 3. Incompatibility of H group R factors

<table>
<thead>
<tr>
<th>Plasmid in donor strain</th>
<th>Plasmid in recipient strain</th>
<th>Selected marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP116</td>
<td>TP117</td>
<td>CSSu (T)</td>
</tr>
<tr>
<td>TP116</td>
<td>TP124</td>
<td>CSSu (T)</td>
</tr>
<tr>
<td>TP123</td>
<td>TP116</td>
<td>CSSu (T)</td>
</tr>
<tr>
<td>TP124</td>
<td>TP117</td>
<td>CSSu (T)</td>
</tr>
</tbody>
</table>

* Unstable, rapidly segregating.

† Appropriate segregants of TP117 used for these experiments. The result of spontaneous loss of resistance.

‡ Stable hybrids.

selected and any unselected

Table 4: Transfer of the plasmids TP123 to K-12F resulted in the F factor in the great majority of the progeny of these crosses in which there was resistance to phage $\phi 2$ but full resistance to phage $\phi 2$ would be expected. In contrast, TP116 into K-12F displacement of F. Lines carrying H and F were stable and transferred K-12F in 1-h crosses.

In a separate experiment a transferred to K-12F, 16 tested still carried F and the all these lines had lost the plasmid resistance marker of resistance to chloramphenicol and sulphonamides. The were F and carried all these of TP124. An exconjugant H T segregant of TP124 was the orange to eliminate the F had been confirmed, the transferred to K-12F. The fully sensitive to phage $\phi 2$, which determined incompatibility thus was lost with the T marker. This T segregant.
SMITH ET AL.
J. BACTERIOL.

Compatibility experiments with group H plasmids. Each group H factor was transferred into a K-12 strain carrying another plasmid of the group in overnight crosses (Table 3). It is difficult to measure entry exclusion with these plasmids because they have a low and variable frequency of transfer, even into "virgin" strains. For example, the transfer frequency to K-12F in overnight crosses may be as high as 10^4 or as low as 10^2.

The results in Table 3 show that the resident H group R factor is usually displaced by the incoming plasmid. When there is joint selection for the resistances of both plasmids, there are marked differences between the pairs of R factors. TP116 did not stably coexist with TP117, 123, or 124, nor did it recombine with them. Unstable hybrid lines carrying TP116 and each of the other three plasmids showed that TP116 was usually retained while the other plasmid was lost, as had been suggested earlier in experiments with TP116 and TP117 (16). Joint selection for the resistance markers of TP123 and TP124 yielded a high proportion of stable hybrid lines because of recombination between the resident and incoming plasmids. Recombination also occurred between TP117 and both TP123 and TP124.

Transfer of the group H resistance factors to K-12F'. The four R factors were transferred to K-12F* (40R850) in overnight crosses, selecting for the appropriate resistances. The resulting colonies were tested for sensitivity to the F-specific phage, m^2, and the female-specific phage, v^2. The progeny were also tested for the

<table>
<thead>
<tr>
<th>Plasmid no.</th>
<th>Drug resistance*</th>
<th>Species of original host and place of origin</th>
<th>Phage type of original host strain</th>
<th>ERI reference no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP116</td>
<td>CSSu*</td>
<td>Salmonella typhimurium Spain 1989</td>
<td>C1</td>
<td>IT-4616</td>
</tr>
<tr>
<td>TP117</td>
<td>T</td>
<td>S. typhimurium England 1981</td>
<td>In var 2</td>
<td>IM-1467</td>
</tr>
<tr>
<td>TP123</td>
<td>CSSuT*</td>
<td>S. typhi Mexico 1972</td>
<td>Degraded Vi</td>
<td>IT-4750</td>
</tr>
<tr>
<td>TP124</td>
<td>CSSuT*</td>
<td>S. typhi India 1972</td>
<td>DH-N</td>
<td>IT-4754</td>
</tr>
</tbody>
</table>

* Symbols for drug resistances: C, chloramphenicol; S, streptomycin; N, naldixic acid; T, tetracycline.

* Strains carrying the H factor TP118, 123, and 124 are also resistant to spectinomycin. This suggests that these plasmids code for an adenylate synthetase which inactivates both streptomycin and spectinomycin (71). In this paper we use the symbol S for resistance to both streptomycin and spectinomycin.

See Anderson and Smith (1).
INTERACTIONS OF \( T \) GROUP H RESISTANCE FACTORS

**Table 3. Incompatibility of group H plasmids**

<table>
<thead>
<tr>
<th>Plasmid in donor strain</th>
<th>Plasmid in recipient strain</th>
<th>Selected marker</th>
<th>Progeny carrying both resistance markers</th>
<th>Progeny carrying only incoming Plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP116 ( (CSSu) )</td>
<td>TP117</td>
<td>C</td>
<td>0/10</td>
<td><strong>30/30</strong></td>
</tr>
<tr>
<td>TP116 ( (CSSu) )</td>
<td>TP124</td>
<td>T</td>
<td>0/45</td>
<td></td>
</tr>
<tr>
<td>TP123 ( (T) )</td>
<td>TP117</td>
<td>C</td>
<td>0/10</td>
<td><strong>30/30</strong></td>
</tr>
<tr>
<td>TP124 ( (CSSu) )</td>
<td>TP117</td>
<td>C</td>
<td>0/10</td>
<td><strong>30/30</strong></td>
</tr>
</tbody>
</table>

* Unstable, rapidly segregating hybrids.
* Appropriate segregants of TP123 and TP124 were used for these experiments. They were isolated as a result of spontaneous loss of resistance markers.
* Stable hybrids.

TP116 \( (CSSu) \) and TP124 to K-12F resulted in displacement of the F factor in the great majority of cases. The progeny of these crosses in which F was present were resistant to phage \( \mu \) but fully sensitive to chloramphenicol, streptomycin, and sulphonamides. The remaining 1-114 lines were F and carried all the resistance markers of TP124 but transferred resistance to chloramphenicol, streptomycin, and sulphonamides. The remaining 11-114 lines were F and carried all the resistance markers of TP124. An exconjugant line carrying TP117 was tested still carried F and the R factor. However, all these lines had lost the unselected tetracycline resistance marker of TP124 but retained resistance to chloramphenicol, streptomycin, and sulphonamides. The remaining 1-114 lines were F and carried all the resistance markers of TP124. An exconjugant line carrying TP124 was tested still carried F and the R factor. After 11-114 lines of F had been confirmed, the CSSu R factor was transferred to K-12F. The progeny were fully sensitive to phage \( \mu \), indicating that there was no displacement of F. The region of TP118 which determines incompatibility with \( \mu \) factor was thus lost with the tetracycline resistance marker. This T segregant of TP124 was never transferable and was still incompatible with other H group R factors.

Transfer of the F factor to strains carrying group H plasmids. The F factor was transferred to strains carrying TP116 or TP124 or TP124 in 1-h crosses, FlacT (see Materials and Methods) was used in the donor strain because selection for T simultaneously selects for F. Spontaneous T segregants of TP123 and TP124 were used as recipients. In contrast with the T segregants of TP124 which were compatible with F as described above, these spontaneous T segregants of TP124 and TP124 displaced F when they were transferred to K-12F.

There was no surface exclusion of FlacT by strains carrying TP116, TP123 or TP124. The frequency of transfer of FlacT to these strains was approximately \( \times 10^{-4} \) in 1-h crosses. Progeny from each of these crosses were examined for the presence of both plasmids.

There was no displacement of the R factors in any of the experiments. Lines carrying TP116 and FlacT were stable and showed independent transfer of FlacT to K-12F. When strains carrying TP123 and FlacT were superinfected with FlacT, the resulting progeny carried both plasmids. Segregation tests on these lines, however, showed no loss of TP123T. But FlacT was lost from most of the lines, although a minority (about 10%) were stable and transferrable independently into new recipients. No spontaneous loss of FlacT was detected in the control experiment in which this plasmid was transferred to K-12F.

The findings with TP124T and FlacT were identical with those described for TP123T and FlacT.

**DNA reassociation experiments.** The results of DNA reassociation experiments between the F factor and group H plasmids are shown in Table 5. Minimal homology was found between F and any of the group H plasmids.
Table 5. Homology between the F factor and H group plasmids

<table>
<thead>
<tr>
<th>Unlabeled DNA from strains bearing plasmids</th>
<th>Labeled plasmid DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
</tr>
<tr>
<td>F</td>
<td>100</td>
</tr>
<tr>
<td>TP116</td>
<td>1</td>
</tr>
<tr>
<td>TP117</td>
<td>1.5</td>
</tr>
<tr>
<td>TP123</td>
<td>0</td>
</tr>
<tr>
<td>TP124</td>
<td>3.7</td>
</tr>
</tbody>
</table>

*The values indicate the degree of reassociation of 75 Ci of H-labeled plasmid DNA with unlabeled plasmids, relative to the reassociation both with DNA of the same plasmid (× 100) and with E. coli chromosomal DNA (× 0). Figures showing the degrees of reassociation between the H group plasmids are from a previous paper (17). The mean contour length of the F factor used in these studies, calculated from measurements of 27 circular molecules from electron micrographs, was 28.4 ± 2.0 nm with a standard deviation of 1.0. This is equivalent to a mol wt of 59 ± 10<sup>6</sup> assuming that 1 nm = 2.07 × 10<sup>6</sup> daltons (24). Molecular weights of the group H plasmids, calculated by the same method, are as follows: TIM 16, 144 × 10<sup>6</sup>; TP117, 112 × 10<sup>6</sup>; TP123, 121 × 10<sup>6</sup>; TP124, 120 × 10<sup>6</sup> (17).

These values are results obtained with unlabeled DNA from a strain carrying a CSSu segregant of TP123.

plasmids. The figures given in Table 5 indicate that on the basis of DNA homology the plasmids studied fall into three groups: F; TIM 16; and TP 117, 123, 124. This suggests that these groups are basically unrelated to each other.

In the DNA reassociation experiments, about 45% of H-labeled F factor DNA reassociated with labeled DNA from the K-12F host strain. While the possibility that the preparation of F DNA contains some chromosomal DNA cannot be excluded, our experience suggests that such contamination is unlikely to be above 10%. This result is in accordance with the conclusion of Falkow and Citarella (14) that almost half of the F factor DNA possesses nucleotide sequences homologous with those of the E. coli chromosome, and supports the suggestion that the portion of F (about 50%) which is not homologous with the DNA of F-like R factors (28) may have been derived from continual recombination with the chromosome (14, 28).

DISCUSSION

The results presented here indicate that the four R factors TP116, 117, 123, and 124 form a single compatibility group, designated group H (16). Hybrid lines carrying TP116 and any of the remaining H group factors were unstable, and it was interesting that in each case the segregation usually resulted in retention of TP116 only. However, TP116 did not recombine with the other three plasmids, although recombination was demonstrated between any pair of the H factors TP117, 123, and 124.

DNA reassociation experiments have been used to study the relationship between plasmids of the same and different groups (17, 18). Reassociation between the H group plasmids showed a high degree of homology between three members (TP117, 123, and 124), but these showed only minimal homology with TP116 (17). There was little or no homology between the H group R factors and members of other compatibility groups. These results with TP116 and the other H group plasmids show, therefore, that incompatibility is not always associated with a high degree of DNA homology between plasmids.

Another difference between TP116 and the other group H plasmids is shown after their transfer to K-12F. TP117, 123, and 124 usually displace the F factor from K-12F, while TP116 and F coexist stably in that strain. In one experiment with TP124 and F, there was loss of the (unselected) tetracycline resistance marker without loss of the complete R factor, and the hybrids so formed were stable (see above). It seemed that the region of TP124 responsible for incompatibility with F was close to the T marker. However, similar experiments with spontaneous T segregants of TP123 and 124 yielded progeny which had all acquired the group H factor but had lost F. When FlacT was introduced into lines carrying the spontaneous T segregants of TP123 and 124, 90% of the progeny subsequently lost FlacT. About 10% of the progeny of these crosses, however, were stable FlacT/TP123 (or 124) lines. The T group H plasmids in such hybrids were nevertheless shown by further crosses to have retained the property of displacing the F factor. This phenomenon is being further explored.

The one-sided incompatibility between certain H group plasmids and F has been observed independently by Waldvogel and Pitton (pers. comm.), who showed that a CSSuT resistance factor caused displacement of F. This H factor is almost certainly identical with TP123, since both plasmids were isolated from a strain of Salmonella typhi which caused an extensive outbreak of typhoid fever in Mexico in 1972 (8) and infected American, British, and Swiss visitors to Mexico (5). In contrast to
single compatibility group, designated group H (16). Hybrid lines carrying TP116 and any of the remaining H group factors were unstable, and it was interesting that in each case the segregation usually resulted in retention of TP116 only. However, TP116 did not recombine with the other three plasmids, although recombination was demonstrated between any pair of the R factors TP117, 123, and 124.

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the effect of introducing TP117, TP123, and TP124 into K-12F*, which resulted in loss of the F factor, superinfection of Hfr strains with these H group factors yielded stable hybrids. Presumably the chromosomal integration of F in Hfr lines protects it from "displacement" by the group H plasmids.

The displacement of F by certain plasmids may result in misinterpretation of fertility inhibition tests where K-12F* is used. The R factor R27 (25) was isolated from the same strain of S. typhimurium (1M1407) as TP117. R27 was originally described as fii+, but it did not enable its host to support multiplication of F- or I-specific phages. Since the fii character was apparently determined by transferring R27 into K-12F* and testing the resulting progeny with F-specific phage, it seems probable, in view of our results with TP117, that R27 had displaced F rather than inhibited its fertility. Experiments on the inhibition of transfer by antisera led Harden and Meynell (19) to suggest that strains carrying R27 had F-like fimbiae of the R100 serotype although they were unable to demonstrate F-specific phage propagation by strains carrying R27. Our results with TP117 indicate that it is an fii* H group R factor having minimal homology with F or with an F-like R factor (17). This suggests that the conclusions drawn from serological studies by Harden and Meynell may be erroneous.

DNA reassocation experiments have shown that there is very little homology between F and the four group H plasmids. The ability of three group H factors to displace F cannot, therefore, be explained in terms of genetic similarity. The displacement of a plasmid of one group by an R factor belonging to another group has also been reported by Coetsee et al. (10). The T group plasmid, R394, isolated from Proteus rettgeri, dislodged resident R factors of the N group, but further experiments showed that R394 and N plasmids could stably coexist and be transferred independently. When R394 was transferred to strains carrying N group plasmids, loss of resistance markers from both classes of plasmids was also observed.

It has been suggested that incompatibility involves competition for specific cellular maintenance sites which govern replication and uniform segregation into daughter cells at cell division (2, 3, 22, 29). Competition between two plasmids for the same site could lead to establishment of only one plasmid with loss of the other. In general, closely related plasmids belong to the same compatibility group and coexist stably with members of other groups. However, the three H group R factors that displace the F factor appear to have no significant homology with F. This unilateral incompatibility may involve a different mechanism from that responsible for the incompatibility of homologous plasmids.

The results presented in this paper, together with the molecular studies of Grindley et al. (17), show that there can be important differences between plasmids belonging to a single compatibility group.

The displacement of F by certain group H plasmids indicates that plasmids belonging to one group do not necessarily coexist stably with plasmids of all other groups. The use of incompatibility alone can therefore be misleading in the classification of bacterial plasmids.

LITERATURE CITED


Chloramphenicol Resistance in the Typhoid Bacillus

E. S. ANDERSON, H. R. SMITH

Summary

Chloramphenicol resistance has been reported in individual strains of Salmonella typhi since 1950, but there had been no accounts of epidemics caused by resistant strains of the organism until 1972, when one occurred in Mexico. Two British patients have been infected in that country, and the organism isolated from them corresponds in all respects with the description of the Mexican epidemic strain of S. typhi: It is a degraded VI strain resistant to chloramphenicol, streptomycin, sulphonamides, and tetracyclines. It owes its resistance to an R factor which can be transferred to Escherichia coli and thence to drug-sensitive S. typhi. Although there is a negligible risk of dissemination of this strain in Britain the incident is a reminder of the need to restrict the use of drugs such as chloramphenicol to the serious diseases for which they are virtually specific.

Introduction

Chloramphenicol resistance in Salmonella typhi was apparently first reported in England (Colquhoun and Weetch, 1950). It was subsequently observed in India (Murti et al., 1962), West Africa (Njoku-Obi and Njoku-Obi, 1963), and Greece and Israel (Kontomichalou, 1967; Smpolinsky et al., 1967). The resistance in the last two instances was caused by a transferable extra-chromosomal element known as a resistance factor or R factor.

Studies in the Enteric Reference Laboratory

The minimal inhibitory concentration (M.I.C.) of chloramphenicol for drug-sensitive S. typhi is between 1 and 3 \( \mu \text{g/ml} \) with the Enteric Reference Laboratory. We have examined seven chloramphenicol-resistant strains of S. typhi received between 1966 and 1969. All were foreign in origin. Two were sent from Chile in 1966. One of these belonged to VI—phage type 46 and had a chloramphenicol M.I.C. of 150 \( \mu \text{g/ml} \). The other Chilean culture belonged to VI—phage type A, and its chloramphenicol M.I.C. was 12.5 \( \mu \text{g/ml} \). A strain of S. typhi was isolated in 1967 in Norwich from a patient infected in Aden. This was a degraded VI strain with a chloramphenicol M.I.C. of about 6.0 \( \mu \text{g/ml} \).

These three strains were resistant to chloramphenicol only, and we were unable either to transfer the resistance to recipient strains or to mobilize it with a transfer factor—the agent responsible for the transferability of R factors (Watanabe, 1963; Anderson, 1965a, 1965b; Anderson, 1966).

Three further chloramphenicol-resistant S. typhi strains, two belonging to VI—phage type A and one to VI—phage type EI, were received in 1967 from Kuwait. All were resistant to ampicillin (A), chloramphenicol (C), and tetracyclines (T). The M.I.C.s of the respective drugs for the parent strains were: ampicillin 250 \( \mu \text{g/ml} \), chloramphenicol 250 \( \mu \text{g/ml} \), and tetracyclines 62.5 \( \mu \text{g/ml} \). The resistances were transferred en bloc to Escherichia coli K12 at high frequency (greater than 50\%\) in overnight crosses. The resulting lines transferred the ACT resistance factor at a frequency of about \( 3 \times 10^{-8} \) to the type strains of VI—types A and EI, of S. typhi, which are used as standard recipients in the study of R factors (Anderson, 1966, 1968).

The ACT factor in the Kuwait strains appeared to be uniform throughout. This was interesting, because each patient was suspected to have been infected in a different place—one in Aden, one in Cairo, and one in Pakistan. Two of the strains were isolated by blood culture and one from stools. It can be concluded either that this ACT resistance factor is widely distributed in the Middle East and Pakistan or that the infecting S. typhi strains acquired it in Kuwait. The latter explanation seems the more probable, since we have no evidence from strains of S. typhi sent from the areas other than Kuwait that the ACT factor is common elsewhere.

According to current classification of R factors the ACT factor is categorized as fertility inhibition + (fi+) and F-like (Meynell et al., 1968). It probably originated in a non-pathogenic intestinal commensal such as E. coli, and may well have been common in the Kuwait area during the period in which the three resistant S. typhi strains were encountered. The occurrence of the ACT resistance factor in S. typhi may thus be a reflection of the epidemiology of the R factor itself, as distinct from that of its pathogenic host.

In September 1969 we received a strain of type Cl of S. typhi isolated in London by blood culture from a young man probably infected in Spain. This strain was resistant to chloramphenicol, streptomycin (S), and sulphonamides (Su). The resistances were transferred en bloc at low frequency (about \( 10^{-8} \)) into E. coli K12, and thence at only about \( 10^{-4} \) into VI—types A and Cl of S. typhi in overnight crosses. Despite its low transfer frequency the CSSu resistance factor is very stable, both in its original S. typhi host and in the type strains of A and Cl to which it was transferred. This R factor is of special interest because it is \( fi^+ \) and belongs to a new group of enterobacterial plasmids which we have designated “group H” (N. D. F. Grindley, J. N. Grindley, and E. S. Anderson, in preparation).

The chloramphenicol M.I.C. of the original host strain is about 150 \( \mu \text{g/ml} \).

Chloramphenicol-resistant S. typhi from Mexico

The incidents described above appeared to be discrete, and hitherto we have no indication that they represented the acquisition of chloramphenicol resistance by S. typhi with sufficient frequency for epidemiologically important resistant strains of the pathogen to emerge. Recently, however, the Center for Disease Control (C.D.C.) in Atlanta, Georgia, U.S.A., reported a widespread and protracted outbreak of typhoid fever in Mexico, which started early in 1972 (C.D.C. Weekly Report, 1972a). The strain of S. typhi concerned carries a transferable factor with the resistance pattern CSSuT. Two American tourists have been infected with this strain (C.D.C. Weekly Report, 1972b).

Two British men who recently visited Mexico independently have developed typhoid fever. The cultures of S. typhi isolated in England from these patients correspond precisely with the C.D.C. description of the Mexican strain; they belong to a degraded VI strain, and they carry a CSSuT resistance factor. The M.I.C. of chloramphenicol for these Mexican strains is about 150 \( \mu \text{g/ml} \).
This R factor transfers from the resistant \textit{S. typhi} to \textit{E. coli} K12 at a frequency of about \(10^{-4}\), and from \textit{E. coli} K12 to \textit{Vi}—type A of \textit{S. typhi} at about \(10^{-3}\) in overnight crosses. Like the CSSu factor described above, which came from Spain, it is \(\beta\)-lactamase and belongs to group H. However, apart from the presence of tetracycline resistance it can be distinguished from the Spanish R factor by phage restriction techniques (Anderson, 1966, 1968).

**Discussion**

It can be assumed that chloramphenicol resistance will appear in \textit{S. typhi} from time to time as the result of either mutation or the acquisition of R factors, but the Mexican outbreak seems to be the first example of its appearance in an epidemic strain. This is the type of event most feared by those who are studying the ecology of R factors, because chloramphenicol is the drug of choice in the treatment of typhoid fever. It can be accepted that the R factor in this strain of \textit{S. typhi} originated in one of the non-pathogenic enterobacteria.

The ultimate appearance of epidemic strains of \textit{S. typhi} carrying R factors coding for chloramphenicol resistance is most likely in countries where two conditions are satisfied. The first is that typhoid fever must be common, so that the organism is frequently present in the human intestine. The second condition is that chloramphenicol should be used indiscriminately, so that its widespread selective pressure will promote the emergence of stable R factors coding for the respective resistance. Both these conditions are satisfied in Mexico: It is a country with a relatively high incidence of typhoid fever; and not only is chloramphenicol used on a large scale by doctors but it can be bought by the general public.

The C.D.C. commented that "although detailed studies have not been completed, it appears that many of the patients infected with resistant strains responded poorly to therapy with chloramphenicol, necessitating the use of other antibiotics, principally ampicillin." (C.D.C. Weekly Report, 1972a).

The poor response to chloramphenicol is hardly surprising, but ampicillin, to which the Mexican strain is sensitive in vitro, has proved a disappointment in general for the treatment of typhoid fever, and it would be an error to regard it as anything better than a second-line drug in this respect.

The appearance of this strain of \textit{S. typhi} in British patients is yet another demonstration that, with the extent and speed of modern travel, infection can be rapidly conveyed between widely separated countries. If the causal organisms have additional unwelcome features such as chloramphenicol resistance in \textit{S. typhi}, they present the patients concerned with an augmented risk, because the most effective therapy cannot be used.

Although the importation of chloramphenicol-resistant \textit{S. typhi} is undesirable it need not arouse alarm in Britain, because opportunities for the spread of typhoid in this country are negligible. Nevertheless, if epidemics caused by chloramphenicol-resistant \textit{S. typhi} occur on a sufficiently large scale in countries of high typhoid incidence, such organisms may spread to other countries often enough to present an irksome problem, and the possibility of residual carriers of these imported strains cannot be discounted. The British cases of typhoid infected in Mexico, and the epidemic which caused them, are a warning of this, and are a reminder that if antibiotics such as chloramphenicol are to retain their efficacy for important diseases, their use should be largely if not entirely restricted to those diseases throughout the world.

**References**


