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

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ABSTRACT

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 plasmids 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 *fi*$^+$ *S.typhimurium* strains and one *fi*$^-$ strain demonstrated that the *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 μm.

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 μm 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.

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SECTION II. MOLECULAR STUDIES OF NON-TRANSFERRING PLASMIDS

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

HA = 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, 1963a). 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 2×10^{-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 5×10^{-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 Δ into these lines resulted in mobilisation 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 Δ 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 Δ 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 Δ, A and SSu appeared to exist independently in the host cell. Transfer of T resulted from recombination of the tetracycline resistance determinant with Δ forming a single linkage group designated T-Δ (Anderson and Lewis, 1965b).

Transduction by phage P1kc of the Δ-mediated resistance transfer systems (Anderson and Natkin, 1972) supported these postulates, which were originally based on conjugation studies. The R factor T-Δ 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 Δ, the resistance determinant was transduced independently of Δ to recipient cells. After transduction A (or SSu) was not transferable but introduction of Δ 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-Δ 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,Δ and SSu,Δ 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 (ColV-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 Fukasawa, 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.*, 1964). R factors were therefore divided into two types on the basis of their effect on F fertility. Those that inhibited F fertility were termed i^+ or fi^+; R factors without any effect on F fertility were designated 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 β-galactosidase synthesis in K12 when the z^+ gene of the *lac* operon is transferred to a z^-i^- 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 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 transfer the plasmid at high frequency, are agglutinated by sex-fimbrial-specific antiserum, and are visibly lysed by F-specific phages. If the fertility of these *fi*$^+$ 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 *fi*$^+$ 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 *fi*$^+$ R factors required not only a product of the R factor but also a product encoded by F itself. Cells carrying the *fi*$^+$ R factor R100, and newly infected with F*lac*, could retransfer F*lac* at high frequency. However when these cells carried both R100 and a second F factor (F*his*), retransfer of F*lac* 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 fi$^+$ R factors, R100 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 fi$^+$ R factor 334 (= 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, Qβ, 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 fi$^+$ 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 fi$^-$ 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 *fi*$^+$ plasmids were normally under the control of a cytoplasmic repressor which could also act on the F factor. Cells carrying derepressed mutants of *fi*$^+$ R factors showed a high degree of sex fimbriation; these included lines carrying R100-1 (Nishimura *et al.*, 1967) and R1*dr* (Meynell 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 *fi*$^+$ R factors (Meynell 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 Meynell, 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 *fi*$^-$ R factors are self-repressed but do not inhibit F fertility. HFT cultures can be produced, as had been first demonstrated with the *fi*$^-$ ColIb factor (Stocker *et al.*, 1963). Derepressed mutants of *fi*$^-$ 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 R64*dr* and R144*dr*, which are derepressed mutants of *fi*$^-$ 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 *fi*$^+$ plasmids determine the synthesis of F-like fimbriae, while many *fi*$^-$ 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 *fi*$^+$ 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 (Cuzin 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 ColVBtrp 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 ColIbdrd 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 Δ-mediated transfer systems demonstrated that Δ and its derived R factor T-Δ 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-Δdrp1. 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).

Mechanismsof 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 fi^+ R factors were indistinguishable but the chloramphenicol acetyltransferase determined by the fi^- R factor R387 showed several differences from the enzyme coded by the fi^+ 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 10×10^6 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, Clowes, 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-caesium 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, Δ 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 20×10^6 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 26×10^6 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 Δ transfer systems confirmed the postulates based on the genetic evidence. The resistance determinants A and SSu and the transfer factor Δ exist as independent covalently-closed circular DNA molecules in K12. The molecular weights of A, SSu and Δ 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-Δ 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-Δ 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 Δ (or T-Δ)

per chromosome (Humphreys et al., 1972; Smith et al., 1974).

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 x 10^6, 54 x 10^6 and 12 x 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 x 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 x 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) (CSSuT) 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 (H_1), TP116 (H_2) and TP125 (B) dissociate in this *S.typhimurium* host but usually form a single molecular species in K12 (Humphreys, Willshaw and Anderson, 1974).

Reassociation and heteroduplex studies

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 *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 I_1 and I_2. 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 *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 *fi*$^+$ F-like R factors and the *fi*$^-$ 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 *Flac* 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 Flac 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.

SECTION I. GENETIC STUDIES

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 μ2 (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 sterilised 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:

μ2	K12-983	Hfr Broda 10	10^{11} pfu/ml
fd	K12-983	Hfr Broda 10	10^{12} pfu/ml
If1	24R468	S.typhimurium 36 T-Δdrp1	10^{9} pfu/ml
φ2	1R713	K12 F^-	10^{10} pfu/ml
01		S.typhi 01	10^{12} pfu/ml

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 SO_4 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:

$Na_2 HPO_4 2H_2O$	35 g
$KH_2 PO_4$	30 g
NaCl	5 g
$(NH_4)_2 SO_4$	10 g
Fe 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:

Name	Source	Concentration in plates (µg/ml)
Ampicillin (Penbritin)	Glaxo Laboratories	100
Benzylpenicillin B.P. (Crystapen)	Glaxo Laboratories	100
Chloramphenicol B.P. (Chloromycetin)	Parke, Davis & Co.	20
Kanamycin sulphate B.P.C. (Kannasyn)	Bayer Products Co.	20
Nalidixic acid (Negram)	Winthrop Laboratories	40
Paromomycin sulphate	Parke, Davis & Co.	60
Spectinomycin dihydrochloride pentahydrate	Upjohn Ltd.	100
Streptomycin sulphate B.P.	Glaxo Laboratories	20, 40 or 500
Sulphathiazole B.P.C.	May & Baker Ltd.	100
Tetracycline hydrochloride (Tetracyn)	Pfizer Ltd.	5 or 10

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

ERL No.	Original No.	Description	Source
1R713	K12-703	Escherichia coli K12 F^- prototrophic	Prof. W. Hayes
14R525	"	1R713 Nal^r	Dr. M. J. Lewis
38R666	"	1R713 Str^r	"
14R483	"	14R525 $ColE^r$	"
24R357	"	14R525 $ColI^r$	"
1R716	K12-712	K12 F^- pro$^-$ lac$^-$ trp$^-$ his$^-$ Str^r	Prof. W. Hayes
14R519	K12-711	K12 F^- pro$^-$ lac$^-$ trp$^-$ his$^-$ Nal^r	"
22R80	C1142	K12-ROW. Colicin indicator strain	Prof. P. Fredericq
44R610	"	E.coli C	"
42R366 [a,b]	"	Salmonella typhimurium phage type 36 plasmid free	"
42R500 [a]	"	S.typhimurium phage type 36 plasmid free	"
34R99 [b]	"	S.typhimurium phage type 36 Nal^r plasmid free	"
18R613 [b]	"	S.typhimurium phage type 36 Str^r. Carries the fi^+ plasmid $MP10_{36}$.	"

[a] 42R366 and 42R500 are derivatives of two independently isolated strains of S.typhimurium phage type 36. These strains do not carry the fi^+ plasmid $MP10_{36}$, present in the wild strains.

[b] 42R366, 34R99 and 18R613 are derived from the same parent strain of S.typhimurium phage type 36, RT576.

Table 2. Standard plasmid-carrying strains

ERL No.	Original No.	Description	Plasmid-determined antibiotic resistance	Source
21R306	K12-501	K12 F^+ met^- lac^+	-	Prof. W. Hayes
16R399	W1655(Flac)	K12 Flac	-	Prof. W. Hayes
30R893	.	14R519 carrying FlacT [a]	T	.
32R1000	.	K12 Hfr Hayes prototrophic Nal^r	-	Prof. W. Hayes
18R312	.	1R713 , Δ	-	Anderson & Lewis (1965b)
26R844	.	1R713 , T-Δ	T	" " "
20R770	.	1R713 , T-Δdrp1	T	N. D. F. Grindley
22R969	.	1R713 , A-Δdrp1	A	" "
30R587	.	1R713 , R144-3 [b] (KColIb)	K	Meynell & Cooke (1969)
28R781	.	1R713 , 334drp1 [c]	ACSSu	.
18R951	.	1R716 carrying the fi$^+$ F-like transfer factor X	-	Anderson, Pitton & Mayhew (1968)
34R621	.	1R713, TP110 (KColIb)	K	Anderson & Smith (1972b)
20R675	C1 136	K12-ROW , ColE1 Colicin indicator strain	-	Prof. P. Fredericq
20R676	C1 137	K12-ROW , ColE2 " " "	-	" "
44R160	.	14R525 , ColE3 " " "	-	.
22R82	C1 223	K12-ROW , ColIa-CA53 " " "	-	Prof. P. Fredericq
22R83	C1 232	K12-ROW , ColIb-P9 " " "		" "

[a] FlacT is a recombinant between Flac and the tetracycline resistance marker of the I-like factor T-Δ (Anderson & Smith, 1972b).

[b] R144-3 is a derepressed mutant of the I-like R factor R144.

[c] 334drp1 is a derepressed mutant of the F-like R factor 334 isolated from S.paratyphi B 7268 (also known as R1).

Table 3. Reference plasmids of defined compatibility groups [a]

Plasmid No.	Compatibility group	Resistances and other markers	Origin	Reference
R1-19K$^-$	F_{II}	ACSSu	S.paratyphi B England, 1964	Meynell & Cooke (1969)
240	F_{II}	T, *fi*$^+$	S.typhimurium England, 1963	Grindley *et al.* (1971)
TP129	F_{IV}	T, *fi*$^+$	S.typhimurium England, 1963	Hedges & Datta (1972)
F_0-*lac*	F_V	Lac$^+$	S.typhi .	Falkow & Baron (1962); Datta (1975)
TP102	I_1	K, *fi*$^+$	S.typhimurium England, 1968	Grindley & Anderson (1971)
TP114	I_2	K	E.coli Scotland, 1967	Grindley *et al.* (1972)
TP118	N	AS	S.enteritidis England, 1969	Anderson & Threlfall (1970); Grindley *et al.* (1972)
TP125	B	CSSuT	Shigella dysenteriae Central America, 1969	Grindley *et al.* (1972)
TP117	H_1	T	S.typhimurium England, 1961	Grindley *et al.* (1972)
TP116	H_2	CSSu	S.typhi Spain, 1969	Anderson & Smith (1972a); Grindley *et al.* (1972)
S-a	W	CKSSu	Shigella sp. Japan	Watanabe *et al.* (1968)

[a] All these plasmids are auto-transferring.

Table 4. Plasmids investigated

Plasmid No. or designation	Antibiotic resistance or colicinogeny	Species of original host strain	Phage type of original host strain	Year and place of origin	Source or ERL No.
NTP1	A	Salmonella typhimurium	29	England, 1964	RT1
NTP2 [a]	SSu	Salmonella typhimurium	29	England, 1964	RT1
NTP3	ASu	Salmonella typhimurium	29	ERL, 1967	9R314
NTP4	ASSu	-	-	ERL, 1968	13R135
NTP5	T	Salmonella typhimurium	49	Scotland, 1969	9M3779
NTP6	A	Salmonella typhimurium	168	England, 1972	12M521
NTP7	ASSu	Salmonella typhimurium	157	Argentina, 1972	12M3614
NTP8	Colicin E1	Escherichia coli	-	-	K30 Prof. P. Fredericq
NTP9	Colicin E2	Shigella sonnei	-	-	P9 "
NTP10	Colicin E3	Escherichia coli	-	-	CA38 "
NTP11	K	Salmonella virchow	-	England, 1974	44R376
K (= fi⁻K)	K	Salmonella typhimurium	29	England, 1965	5M4136
fi⁺K	K	Salmonella typhimurium	29	England, 1965	"
$MP10_{36}$	-	Salmonella typhimurium	36	England, 1964	RT576 (4M3543)
$MP10_{LT2}$	-	Salmonella typhimurium	4	.	LT2[b] (= 42R93)
$MP10_{8}$	-	Salmonella typhimurium	8	England, 1956	21R341
$MP10_{14}$	-	Salmonella typhimurium	14	Wales, 1948	21R337
$MP10_{74}$	-	Salmonella typhimurium	74	Kenya, 1960	21R340

[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 [a]

ERL No.	Species of host strain. Phage type of S.typhimurium or Salmonella O group	Origin
8M3993	S.typhimurium 32	Human, Scotland, 1968
	" 1	Human, England, 1968
	" 44	Human, Scotland, 1969
	" 6	Animal, England, 1969
	" 12a	Human, England, 1970
	" 12a	Animal, England, 1971
	" 56	Human, England, 1971
	" 95	Animal, England, 1971
	" Untypable	Animal, Singapore, 1973
	" 104	Human, Spain, 1973
	" 156	Human, New Zealand, 1973
	S.agona B	Human, England, 1970
	S.brandenburg B	Human, Wales, 1970
42R652	S.bredeney B	Human, England, 1970
	S.derby B	Human, England, 1970
	S.montevideo C	Human, England, 1970
42R653	S.newport C	Human, England, 1970
42R654	S.panama D	Human, England, 1970
	S.stanley B	Human, England, 1970
	S.chester B	Human, England, 1970
	S.indiana B	Human, England, 1970
EC4240	E.coli	Human, England, 1970
EC4316	E.coli	Human, England, 1970
	E.coli	Animal, England, 1973
3EC635	E.coli	Animal, England, 1973
	E.coli	Animal, Ireland, 1970

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

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	S.brandenburg B	Human, Wales, 1970
42R652	S.bredeney B	Human, England, 1970
	S.derby B	Human, England, 1970
	S.montevideo C	Human, England, 1970
42R653	S.newport C	Human, England, 1970
42R654	S.panama D	Human, England, 1970
	S.stanley B	Human, England, 1970
	S.chester B	Human, England, 1970
	S.indiana B	Human, England, 1970
EC4240	E.coli	Human, England, 1970
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	E.coli	Animal, England, 1973
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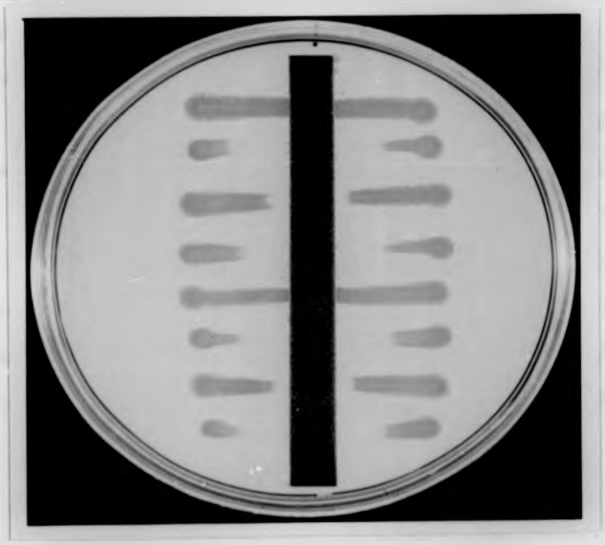
[a] All these strains are sensitive to spectinomycin, that is, the streptomycin resistance is probably caused by a phosphorylating enzyme (Ozanne et al., 1969).

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

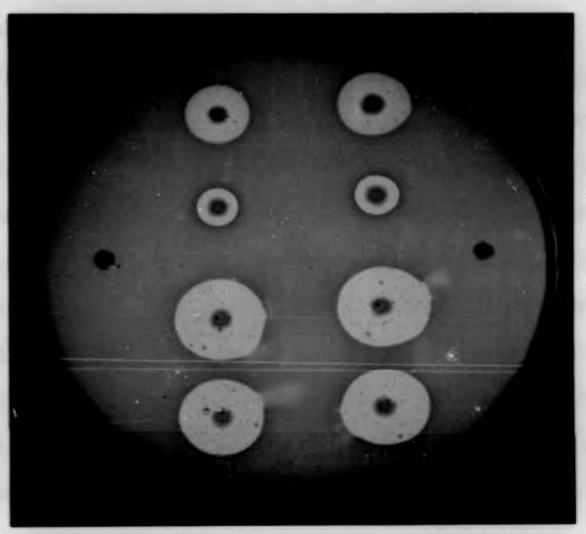
Sulphonamide resistance testing. Broth cultures were diluted 10^{-4} in saline and spotted on laked-blood agar plates with and without sulphathiazole. Resistant and sensitive control strains were included on each plate. All cultures grow on the control plate lacking sulphathiazole, whereas only strains 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° 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~~).

Plate 2. Colicinogeny testing of bacterial strains

A



---- Colicin Ia

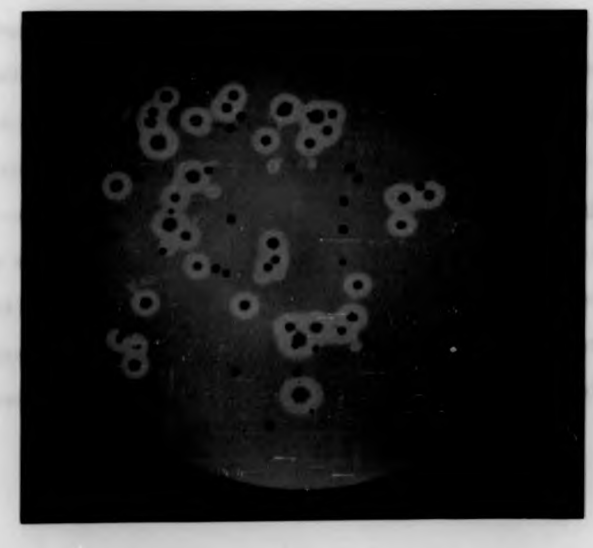
---- Colicin E1

---- Colicin E2

---- Colicin E3

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.

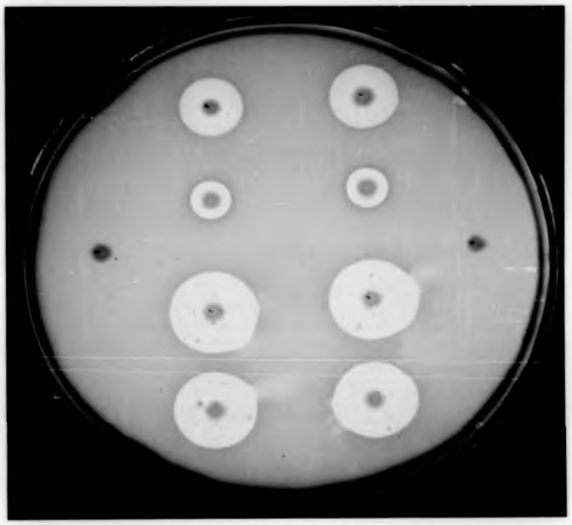
B



Detection of colicin-producing colonies in a recipient population after a mating experiment.

Plate 2. Colicinogeny testing of bacterial strains

A



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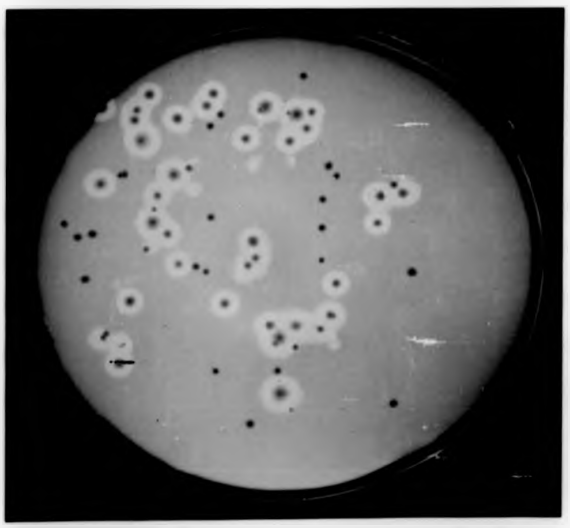
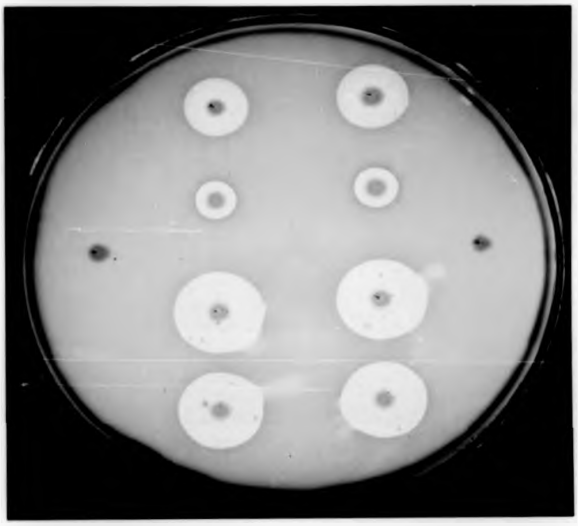


Detection of colicin-producing colonies in a recipient population after a mating experiment.

Plate 2. Colicinogeny testing of bacterial strains

A



---- Colicin Ia

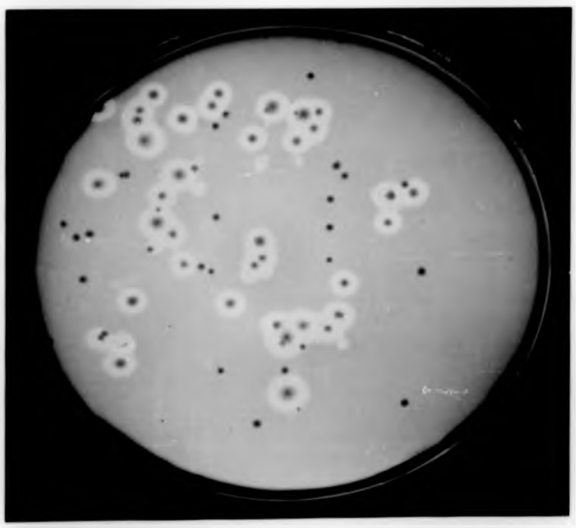
---- Colicin E1

---- Colicin E2

---- Colicin E3

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.

B



Detection of colicin-producing colonies in a recipient population after a mating experiment.

Transferability of drug resistance and colicinogeny

Broth cultures of donor and recipient strains were grown on a Luckham Rotatest shaker at 37° C to exponential phase and contained approximately 2 x 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

tested for colicin production.

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 μ2 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 μ2, 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 µg/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.

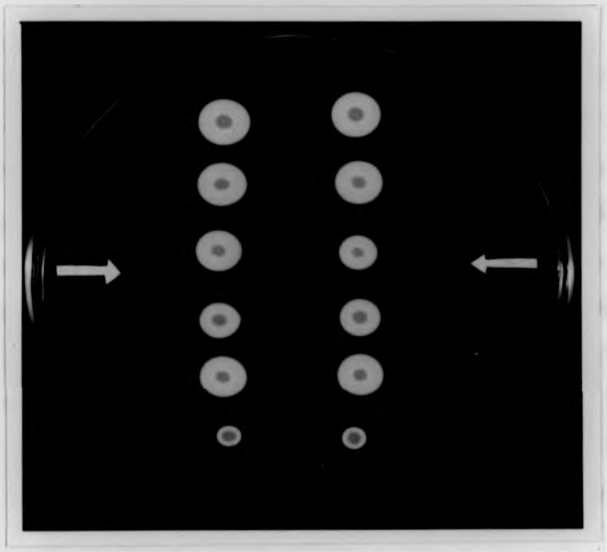
Phage multiplication experiments

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 µ2, fd or If1 (titre 5×10^5 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° 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 µ2 and fd) or K12 T-Δ*drp*1 (for If1). This derepressed mutant of the R factor T-Δ 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

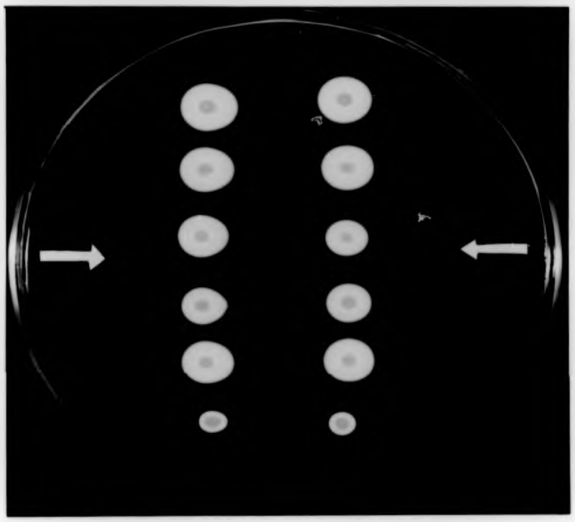


The strains were all derivatives of K12F⁻ (1R713). The cultures were spotted in duplicate.

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.

Plate 3. Detection of β-lactamase production



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1	K12 (NTP1)
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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 K12F$^-$ 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 K12F$^-$Nal^r 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

Donor	X	Recipient	Selection	Time of cross	Frequency of transfer	Analysis of progeny
S.typhimurium (9M3779)		K12F⁻ (24R357)	Tetracycline	2 h	4×10^{-5}	$^{20}/_{20}$ T, ColIa
9M3779		24R357	Tetracycline	18 h	5×10^{-3}	$^{20}/_{20}$ T, ColIa
9M3779		24R357	Unselected	18 h	.	$^{9}/_{20}$ ColIa; $^{11}/_{20}$ T⁻ ColIa⁻
K12(NTP5, ColIa)		S.typhimurium 36 (42R366)	Tetracycline	2 h	2×10^{-6}	$^{5}/_{20}$ T; $^{15}/_{20}$ T, ColIa
K12(NTP5, ColIa)		42R366	Tetracycline	18 h	1×10^{-4}	$^{20}/_{20}$ T, ColIa
K12(NTP5, ColIa)		42R366	Unselected	18 h	.	$^{8}/_{20}$ ColIa; $^{12}/_{20}$ T⁻ColIa⁻

Strains carrying the ColIa 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_1 R factor A-Δdrp1 to K12 carrying NTP5 and ColIa. All 20 colonies selected on ampicillin, or ampicillin and tetracycline, lost the ability to produce colicin but retained NTP5. This confirmed that NTP5 and ColIa are independent plasmids, of which ColIa is a member of group I_1.

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-Δdrp1 in a triparental cross with K12 F^- as the final recipient. The ampicillin resistance determinant, designated NTP6, was easily mobilised by T-Δdrp1. A K12 line carrying NTP6 and T-Δdrp1 was mated with S.typhimurium 36 for 2 h. Eight of 10 lines selected on penicillin did not carry T-Δdrp1 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-Δdrp1) and 1R713.

The S.typhimurium strain 12M3614, 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 to S.typhimurium 36 was performed; 18 of 20 resistant progeny did not transfer ASSu to K12. This ASSu determinant NTP7 was then mobilised by T-Δ 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-Δ.

One of the lines of S.typhimurium 36 which transferred ASSu, without introduction of T-Δ, 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-Δdrp1 (I_1) and TP114 (I_2) were transferred to this strain and to $K12F^-$ (Table 7). There was no surface exclusion of T-Δdrp1 or TP114, and the resulting progeny were stable for ASSu and either T-Δdrp1 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

Plasmids in donor strain	Plasmids in recipient strain	Time of cross	Selection	Frequency of transfer
T-Δdrp1	NTP7, TP151	1 h	T	9×10^{-1}
I_1	-	1 h	T	3×10^{-1}
TP114	NTP7, TP151	1 h	K	1×10^{-3}
I_2	-	1 h	K	5×10^{-4}
NTP7, TP151	T-Δ	18 h	A [a]	2×10^{-1}
	TP114	18 h	A	3×10^{-3}
	-	18 h	A	1×10^{-1}

[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-transferring 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 plasmids 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 fi^- R factor T-Δ. 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^{-1} in a 1 h cross. NTP1 to NTP11 are therefore fi^- 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 (NTP4) 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 14R525, 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).

Non-transferring plasmid in donor strain	Non-transferring plasmid in recipient strain	Selection	Analysis of progeny	
			R-type or colicinogeny	Segregation of clones (pooled data)
A	-	A	A	$^{0}/_{792}$
(NTP1)	SSu (NTP2)	A	A, SSu	$^{0}/_{343}$
	T (NTP5)	A	A, T	$^{0}/_{354}$
	ColE1	A	A, ColE1	$^{1A^-\ 0ColE1^-}/_{519}$
	ColE2	A	A, ColE2	$^{0}/_{469}$
	ColE3	A	A, ColE3	$^{0}/_{432}$
	K (NTP11)	A	A, K	$^{0A^-\ 1K^-}/_{768}$
SSu	-	S	SSu	$^{0}/_{435}$
(NTP2)	T (NTP5)	S	SSu, T	$^{0}/_{428}$
	A (NTP6)	S	SSu, A	$^{0}/_{803}$
	ColE1	S	SSu, ColE1	$^{0}/_{620}$
	ColE2	S	SSu, ColE2	$^{0}/_{319}$
	ColE3	S	SSu, ColE3	$^{0}/_{783}$
	K (NTP11)	S	SSu, K	$^{0}/_{544}$
ASu	-	A	ASu	$^{0}/_{214}$
(NTP3)	SSu (NTP2)	A	ASu/SSu	$^{50A^-\ 318S^-}/_{614}$
	"	AS	ASu/SSu	$^{1125A^-\ 1058^-}/_{2269}$

Table 9. Compatibility experiments with T (NTP5), A (NTP6) and K (NTP11)

Non-transferring plasmid in donor strain	Non-transferring plasmid in recipient strain	Selection	Analysis of progeny	
			R-type or colicinogeny	Segregation of clones (pooled data)
T	-	T	T	0/648
(NTP5)	A (NTP6)	T	T, A	0/628
	ColE1	T	T, ColE1	0/456
	ColE2	T	T, ColE2	0/402
	ColE3	T	T, ColE3	0/746
	K (NTP11)	T	T, K	0/1002
A	-	A	A	0/436
(NTP6)	ColE1	A	A, ColE1	0/375
	ColE2	A	A, ColE2	0/343
	ColE3	A	A, ColE3	0/496
	K (NTP11)	A	A, K	0/329
K	-	K	K	0/317
(NTP11)	ColE1	K	K, ColE1	0/600
	ColE2	K	K, ColE2	0/396
	ColE3	K	K, ColE3	0/273

Table 10. Compatibility test with ASSu (NTP7)

Non-transferring plasmid in donor strain	Non-transferring plasmid in recipient strain	Selection	Analysis of progeny	
			Designation [a]	Loss of ampicillin resistance
ASSu	-	A	-	$^{0}/_{674}$
(NTP7)	SSu (NTP2)	A	Colony 1	$^{34}/_{170}$
			" 2	$^{0}/_{147}$
			" 3	$^{4}/_{122}$
			" 4	$^{51}/_{99}$
			" 5	$^{0}/_{168}$
			" 6	$^{150}/_{191}$
			" 7	$^{0}/_{125}$
			" 8	$^{13}/_{176}$
			" 9	$^{0}/_{101}$
			" 10	$^{34}/_{182}$

[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-Δ 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.

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Table 11. Compatibility experiments with ColE1, ColE2 and ColE3.

Colicin factor in donor strain	Colicin factor in recipient strain	Selection [a]	Duration of cross	Analysis of progeny	
				Colicinogeny	Segregation of clones (pooled data)
ColE1	-	T	18 h	$^{29}/_{105}$ ColE1	^{1}ColE1^{-}/330
	ColE2	T	2 h	$^{19}/_{20}$ ColE1, ColE2	$^{0}/_{245}$
	ColE2	T	18 h	$^{5}/_{30}$ ColE1, ColE2	$^{0}/_{394}$
	ColE3	T	2 h	$^{9}/_{10}$ ColE1, ColE3	^{0}ColE1^{-} ^{1}ColE3^{-}/832
ColE2	ColE1	T	18 h	$^{2}/_{20}$ ColE1, ColE2	$^{0}/_{190}$
ColE3	-	T	2 h	$^{10}/_{10}$ ColE3	$^{0}/_{290}$
	ColE1	T	2 h	$^{37}/_{40}$ ColE1, ColE3	^{0}ColE1^{-} ^{1}ColE3^{-}/768
	ColE1	T	18 h	$^{9}/_{10}$ ColE1, ColE3	$^{0}/_{253}$

[a] Colonies were selected on tetracycline and examined for colicin production.

Table 12. Compatibility experiments with ColE2 and ColE3.

Colicin factor in donor strain	Colicin factor in recipient strain	Selection [a]	Duration of cross	Analysis of progeny	
				Colicinogeny	Segregation of clones (pooled data)
ColE2	-	T	2 h	$^{6}/_{10}$ ColE2	$^{0}/_{183}$
	-	T	18 h	$^{6}/_{10}$ ColE2	$^{0}/_{608}$
	ColE3	T	2 h	$^{9}/_{10}$ ColE2, ColE3	^{8}ColE2^{-} ^{4}ColE3^{-}/1610
	ColE3	T	18 h	$^{2}/_{10}$ ColE2, ColE3	^{0}ColE2^{-} ^{4}ColE3^{-}/281
ColE3	-	T	2 h	$^{40}/_{40}$ ColE3	$^{0}/_{616}$
	-	T	18 h	$^{23}/_{35}$ ColE3	$^{0}/_{1752}$
	ColE2	T	2 h	$^{31}/_{40}$ ColE2, ColE3	^{5}ColE2^{-} ^{242}ColE3^{-}/2366
	ColE2	T	18 h	$^{24}/_{70}$ ColE2, ColE3	^{1}ColE2^{-} ^{12}ColE3^{-}/1706

[a] Colonies were selected on tetracycline and examined for colicin production.

Table 13. Compatibility groups of the non-transferring plasmids

Group	1	2	3	4	5	6	Not yet grouped
Plasmids	A (NTP1)	SSu (NTP2) ASu (NTP3) ASSu (NTP4) ASSu (NTP7)	T (NTP5)	ColE1	ColE2 ColE3	K (NTP11)	A (NTP6)

Compatibility tests with wild strains resistant to streptomycin and sulphonamides

The wild enterobacterial strains carrying streptomycin-sulphonamide resistance listed in Table 5 (page 49) and in Table 14 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: EC4240, 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 Δ 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 (EC4240 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

Wild strain	Triparental crosses for determinant mobilisation		Segregation of clones after transfer of ASu into wild SSu strains			Conclusion
	by Δ	by transfer factor X	No. of clones tested	Loss of ASu	Loss of SSu	
S.typhimurium 32 8M3993	-	-	1741	1	0	Compatible
S.typhimurium 1	+	+	156	84	5	Incompatible
S.typhimurium 44	+	+	502	182	249	"
S.typhimurium 6 *	+	+	439	105	317	"
S.typhimurium 12a (Human)	+	+	235	122	83	Incompatible
S.typhimurium 12a (Animal)	+	+	171	130	6	"
S.typhimurium 56	+	+	268	168	16	"
S.typhimurium 95	+	+	147	94	39	"
S.typhimurium Untypable	+	+	98	59	20	"
S.typhimurium 104	+	+	713	278	292	"
S.typhimurium 156	+	+	317	109	132	"
S.agona	+	+	428	146	183	Incompatible
S.brandenburg	+	+	752	199	4	Incompatible
S.bredeney 42R652	-	-	1117	3	5	Compatible
S.derby	+	+	580	73	260	Incompatible
S.montevideo	+	+	650	248	236	Incompatible
S.newport 42R653	-	-	812	0	1	Compatible
S.panama 42R654	-	-	677	3	1	Compatible
S.stanley	+	+	459	189	169	Incompatible
S.chester *	+	+	580	228	225	Incompatible
S.indiana	+	+	558	217	230	Incompatible
E.coli EC4240 *	+	+	211	0	0	Compatible
E.coli EC4316 *	+	+	338	0	2	Compatible
E.coli 3EC451	+	+	353	19	203	Incompatible
E.coli 3EC635	-	-	1622	0	3	Compatible
E.coli EC4201	+	+	318	25	170	Incompatible

* These wild strains carry a transfer factor.

Further investigation of 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 S.typhimurium. T-Δ 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-Δ 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 S.typhimurium 36 demonstrated that a recombinant ASSu plasmid had been formed. This determinant was transferred by T-Δ at a frequency of 3×10^{-3} from K12 to S.typhimurium 36 in an overnight cross. This frequency was the same as that of the transfer of the original ASu determinant by T-Δ. 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 S.bredeney (42R652), S.newport (42R653), S.panama (42R654) and three E.coli strains EC4240, EC4316 and 3EC635. Streptomycin-sulphonamide resistance was not transferable from the three salmonella strains and could not be mobilised after introduction of Δ, X or 240 into the wild strains (see Table 14). However, in the strain of 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

Progeny	No. of colonies tested	Transfer of SSu to K12F⁻	Mobilisation of SSu by Δ or X to K12F⁻
S.typhimurium 36, SSu (ex EC4240)	25	$^{0}/_{25}$	$^{2}/_{25}$
S.typhimurium 36, SSu (ex EC4316)	20	$^{1}/_{20}$	$^{1}/_{19}$

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 EC4240 and EC4316

Plasmid in donor strain	Plasmid in recipient strain	Selection	Analysis of progeny	
			R-type	Segregation of clones
ASu (NTP3)	SSu (ex EC4240)	A	ASu, SSu	$^{0}/1136$
ASu (NTP3)	SSu (ex EC4316)	A	ASu, SSu	$OA^{-}\ 1S^{-}/809$

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 mobilisable 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 mobilised 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 was 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].

Plasmids in donor strain	Selection	Frequency of transfer in 1 h crosses	R-type of progeny
A (NTP1) + T-Δdrp1	A	3×10^{-1}	0.3% A; 99.7% AT
	T	6×10^{-1}	60% T; 40% AT
A (NTP1) + FlacT	A	1×10^{-4}	100% AT
	T	10^{0}	100% T
SSu (NTP2) + T-Δdrp1	Su [b]	4×10^{-3}	0.2% SSu; 99.8% SSuT
	T	8×10^{-1}	98.4% T; 1.6% SSuT
SSu (NTP2) + FlacT	Su	4×10^{-6}	0.1% SSu; 99.9% SSuT
	T	6×10^{-1}	100% T

[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-Δdrp1 is higher in a 1 h cross than that of A by repressed T-Δ in overnight crosses (usually about 2 x 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-Δdrp1 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 0.5%. 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.

Plasmids in donor strain	Selection	Frequency of transfer in 1 h crosses	R-type of progeny
A (NTP1) + T-Δdrp1	A	4×10^{-4}	81.6% A; 18.4% AT
	T	1×10^{-4}	61.6% T; 38.4% AT
SSu (NTP2) + T-Δdrp1	Su [a]	4×10^{-3}	95% SSu; 5% SSuT
	T	5×10^{-4}	63% T; 37% SSuT

[a] Colonies selected on sulphonamides were also resistant to streptomycin.

Table 18. Transfer of the T determinant NTP5 [a]

Plasmids in donor strain	Selection	Frequency of transfer in 1 h crosses	R-type of progeny
NTP5 + R1-19 K^-	T	3×10^{-4}	0.3% T; 99.7% ACSSuT
(ACSSu)	C	4×10^{-1}	100% ACSSu
NTP5 + A-Δdrp1 [b]	T	2×10^{-2}	1.3% T; 98.7% AT
	A	2×10^{-1}	93% A; 7% AT

[a] Donor to recipient ratio of 1 : 10.

[b] A-Δdrp1 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-Δdrp1 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, 5M4136, the K determinant was transferred by an fi^+ transfer factor which was designated X (Anderson, Pitton and Mayhew, 1968). After mating 5M4136 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 μ2. 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

Cross		Time	Frequency of K transfer
Donor	Recipient		
S.typhimurium 36(K,Flac)	K12	30 min	3×10^{-5}
		2 h	7×10^{-4}
		18 h	2×10^{-1}
	S.typhimurium 36	30 min	7×10^{-5}
		2 h	2×10^{-4}
		18 h	7×10^{-3}

K was transferred by Flac to K12 at a higher frequency than to *S.typhimurium* 36 in an 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; Grindley 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).

Cross		Time	Frequency of Flac transfer	Sensitivity of donor to phage μ2	Proportion of donor cells carrying F fimbriae (electron microscopy)
Donor	Recipient				
S.typhimurium 36(K,Flac)	K12	2 h	1×10^{-2}	+	$^{18}/_{28} = 64\%$
		18 h	6×10^{-1}		
	S.typhimurium 36	2 h	4×10^{-2}		
		18 h	3×10^{-1}		
S.typhimurium 36(Flac)	K12	2 h	2×10^{-5}	-	$^{1}/_{31} = 2.8\%$
		18 h	5×10^{-1}		
	S.typhimurium 36	2 h	2×10^{-5}		
		18 h	3×10^{-2}		

+ = Visible lysis with phage μ2.

- = No visible lysis with phage μ2.

of T-Δ. 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

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

\+ = Clear lysis with μ2.

± = 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^r 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_1, 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 [a]

Plasmid in donor strain	Compatibility group	Selection	Analysis of progeny	
			R-type	Segregation of clones
F*lac*T	F_I	T	K, T	$^0/_{365}$
R1-19K$^-$	F_{II}	C	K, ACSu	$^0/_{252}$
TP129	F_{IV}	T	K, T	$^0/_{271}$
F_o-*lac* [b]	F_V	-	K, Lac$^+$	$^0/_{273}$
T-Δ	I_1	T	K, T	$^{0}T^{-}1K^{-}/_{279}$
TP118	N	A	K, A	$^0/_{348}$
TP125	B	C	K, CSuT	$^{0}C^{-}4K^{-}/_{534}$
TP117	H_1	T	K, T	$^0/_{295}$
TP116	H_2	C	K, CSu	$^0/_{291}$
S-a	W	C	K, CSu	$^0/_{435}$

[a] The recipient strain in each case was K12F$^-$*lac*$^-$Str^r carrying the K determinant.

[b] F_o-*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

Plasmid in donor strain	Plasmid in recipient strain	Selection	Analysis of progeny	
			R-type or colicinogeny	Segregation
K [a]	-	K	K	$^{0}/385$
	A (NTP1)	K	K, A	$^{0}/272$
	SSu (NTP2)	K	K, SSu	$2K^{-}\ 0S^{-}/242$
	T (NTP5)	K	K, T	$^{0}/323$
	A (NTP6)	K	K, A	$^{0}/434$
	ColE1	K	K, ColE1	$1K^{-}\ 0Col^{-}/498$
	ColE2	K	K, ColE2	$^{0}/230$
	ColE3	K	K, ColE3	$1K^{-}\ 0Col^{-}/250$

[a] The K determinant was mobilised by F or FlacT in these compatibility experiments.

Recombination between the *fi*$^+$ property of *S.typhimurium* 36 and KColIb

It was suggested that the *fi*$^+$ character of *S.typhimurium* 36 and other *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 *fi*$^+$ property into K12.

A line of *S.typhimurium* 36 into which a KColIb R factor TP110 had been introduced two years earlier was examined for mobilisation of the *fi*$^+$ property. The strain was mated overnight with K12 HfrH Nal^r with selection on kanamycin and nalidixic acid. Of fifty progeny tested with phage μ2, 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 K12(*Flac*) to determine whether the *fi*$^+$ character could be transferred by KColIb to another strain. All K12 lines carrying *Flac* and KColIb were resistant to μ2, indicating that the *fi*$^+$ property was transferred by the R factor.

A compatibility experiment was performed to examine whether the *fi*$^+$ region was covalently linked to the KColIb factor. The I-like R factor T-Δ was mated with the μ2-resistant line of HfrH (KColIb) and progeny were selected on tetracycline. All lines were fully sensitive to μ2 and had lost KColIb. It was concluded that the *fi*$^+$ character of *S.typhimurium* 36 had recombined with KColIb; The recombinant plasmid was designated KColIb *fi*$^+$.

These observations support the hypothesis that the *fi*$^+$ property of *S.typhimurium* is plasmid-borne, but the results could also be explained by recombination between KColIb and a chromosomal *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

Strain	Reaction with phage μ2	Frequency of *pro* transfer in 1 h crosses
K12 HfrH	+	2.9×10^{-2}
K12 HfrH (KColIb)	+	1.9×10^{-2}
K12 HfrH (KColIb *fi*$^+$)	-	3.0×10^{-5}

+ = 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 *fi*$^+$ property of *S.typhimurium* 36 and the ASu determinant NTP3.

Lines of *S.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.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 *fi* character of the new plasmid was examined by transferring F*lac*T to K12 lines carrying the R factor. All 20 colonies tested were resistant to the F-specific phage μ2 in surface spot tests. The new R factor was *fi*$^+$; it was provisionally designated A* (Smith *et al.*, 1973b).

The A* plasmid was tested for compatibility with the K determinant. K was transferred by F*lac* to *S.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 $\underline{fi}^+$ character: lines carrying K and the F factor were visibly lysed by phage μ2, while lines still resistant to ampicillin were $\underline{fi}^+$. These results suggest that the A region of the ASu determinant has recombined with the plasmid present in S.typhimurium 36 which determines the $\underline{fi}^+$ 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 $\underline{fi}^+$ 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	Atlas Chemical Corp.
Sarkosyl NL35 (sodium dodecyl sarcosinate)	Geigy (U.K.) Ltd.
Sodium lauryl sulphate (SLS)	B.D.H. Chemicals Ltd.
Sodium deoxycholate	Allen and Hanburys Ltd.
Lysozyme (three times crystallized)	B.D.H. Chemicals Ltd.
Ribonuclease (RNase)	Cambrian Chemicals Ltd.
Pronase (protease Type VI)	Sigma Chemical Co. Ltd.
Cytochrome c (Type III)	Sigma Chemical Co. Ltd.
Ammonium acetate. Analar grade	B.D.H. Chemicals Ltd.
Formaldehyde. Analar grade	B.D.H. Chemicals Ltd.
Ethidium bromide	Calbiochem. Ltd.
Caesium chloride	Serva Feinbiochemica.
2' deoxyadenosine	Sigma Chemical Co. Ltd.
Trizma Base and Trizma HC1 (Tris)	Sigma Chemical Co. Ltd.
Hydroxyapatite	Bio-Rad Laboratories Ltd.

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-^{3}H) specific activity >15 Ci/mmole. (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 $MgSO_4$	1 ml
0.01 M $CaCl_2$	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:

Na_2HPO_4	60 g
KH_2PO_4	30 g
NaCl	5 g
NH_4Cl	10 g

Constituents of trace elements per litre:

$Na_2 B_4 O_7 \cdot 10H_2O$	88 mg
$Cu SO_4 \cdot 5H_2O$	393 mg
$Fe_2(SO_4)_3 \cdot 9H_2O$	0.91 mg
$Mn Cl_2 \cdot 4H_2O$	72 mg
$(N H_4)_6 Mo_7O_{24} \cdot 4H_2O$	36.8 mg
$Zn SO_4 \cdot 7H_2O$	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 $Na_2 HPO_4$ and NaH_2PO_4.

It was prepared at 0.4M in 5 litre volumes:

$Na_2 HPO_4 \cdot 2H_2O$	178 g
$Na H_2PO_4 \cdot 2H_2O$	156 g
EDTA	9.3 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 ^{3}H-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. H_2O 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 at 36,000 r.p.m. (96,000 g) 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 caesium 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 caesium 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 ^{3}H-label, were pooled and concentrated by further centrifugation in a caesium chloride gradient. 6.6 g of caesium 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 Langand 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

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^{o} to 10^{o} 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 5×10^{-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 = 2.07×10^{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 5×10^{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 25% 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-isoamyl 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-isoamyl alcohol was added and the mixture was agitated for 30 min. Protein separated at the interface between the DNA suspension and the chloroform-isoamyl 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 4×10^5 daltons (Grindley, 1974). The DNA solutions, labelled and unlabelled, were then boiled for 15 min to denature the DNA. The ^{3}H-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 2×10^{-3} µg of denatured ^{3}H-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 >5%. 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 5% TCA). The discs were washed with 1% or 5% 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.

SECTION II. RESULTS

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,

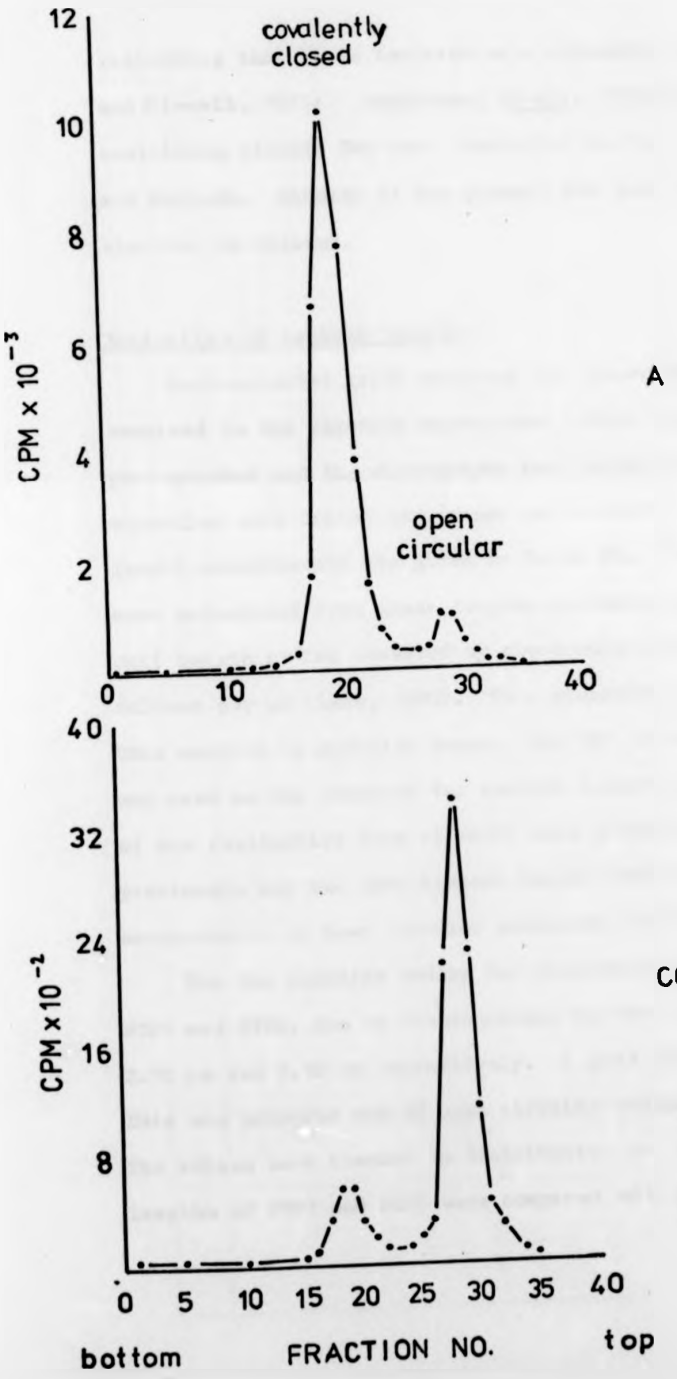
Table 25. % ^{3}H-label recovered in cleared lysates of plasmid-carrying strains

Plasmid		% ^{3}H-label in cleared lysate [a] (mean)	Number of experiments
No.	R-type or colicinogeny		
NTP1	A	4.0	7
NTP2	SSu	1.5	2
NTP3	ASu	2.2	2
NTP4	ASSu	2.2	2
NTP5	T	1.5	3
NTP6	A	2.3	2
NTP7	ASSu	2.0	2
NTP8	Colicin E1	1.5	2
NTP9	Colicin E2	1.8	2
NTP10	Colicin E3	1.9	2
NTP11	K	3.1	2
- [b]	-	0.54	12

[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 1R713.

FIGURE 1 Dye buoyant density gradient centrifugation of cleared lysates



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 on to 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 2.07×10^6 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).

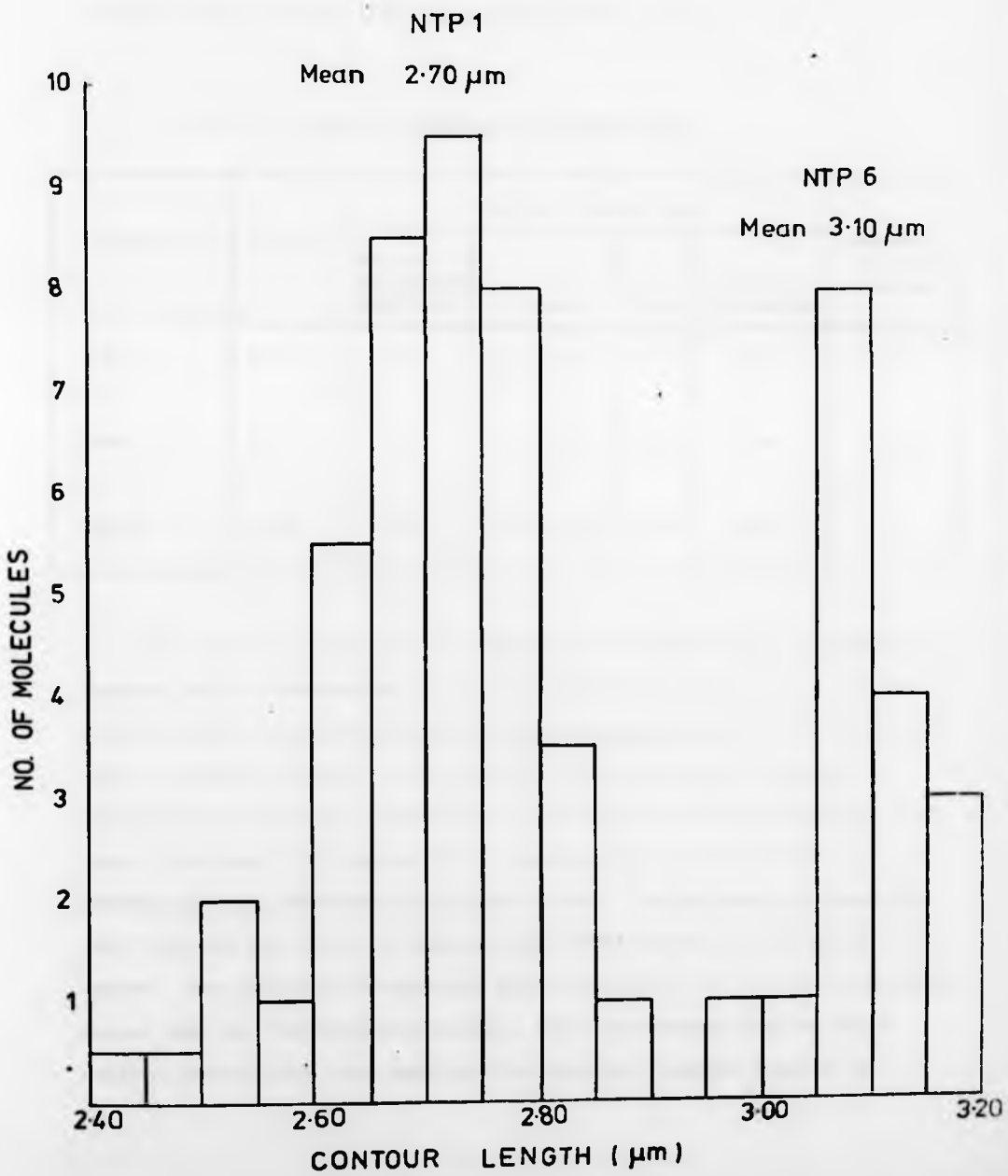
The two plasmids coding for resistance to ampicillin only, NTP1 and NTP6, can be distinguished by their mean contour lengths: 2.70 μm and 3.10 μm respectively. A grid carrying both plasmid DNAs was prepared and 57 open circular molecules were measured. The values were bimodal in distribution as shown 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

Plasmid		Contour length (μm)				Molecular weight ($\times 10^{-6}$)
No.	R-type or colicinogeny	No. of molecules measured	Range	Mean	Standard deviation	
NTP1	A	40	2.45-2.86	2.70	0.09	5.6
NTP2	SSu	33	2.60-2.97	2.74	0.09	5.7
NTP3	ASu	37	3.67-4.18	3.91	0.13	8.1
NTP4	ASSu	30	3.87-4.42	4.24	0.13	8.8
NTP5	T	18	3.00-3.33	3.16	0.09	6.5
NTP6	A	17	2.98-3.19	3.10	0.05	6.4
NTP7	ASSu	25	4.29-4.80	4.53	0.16	9.4
NTP8	Colicin E1	18	2.15-2.43	2.28	0.08	4.7
NTP9	Colicin E2	22	2.08-2.37	2.22	0.07	4.6
NTP10	Colicin E3	22	2.27-2.42	2.34	0.04	4.8
NTP11	K	14	2.61-2.96	2.84	0.10	5.9
Phage φX174 [a]		58	1.70-1.87	1.79	0.04	3.7

[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



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).

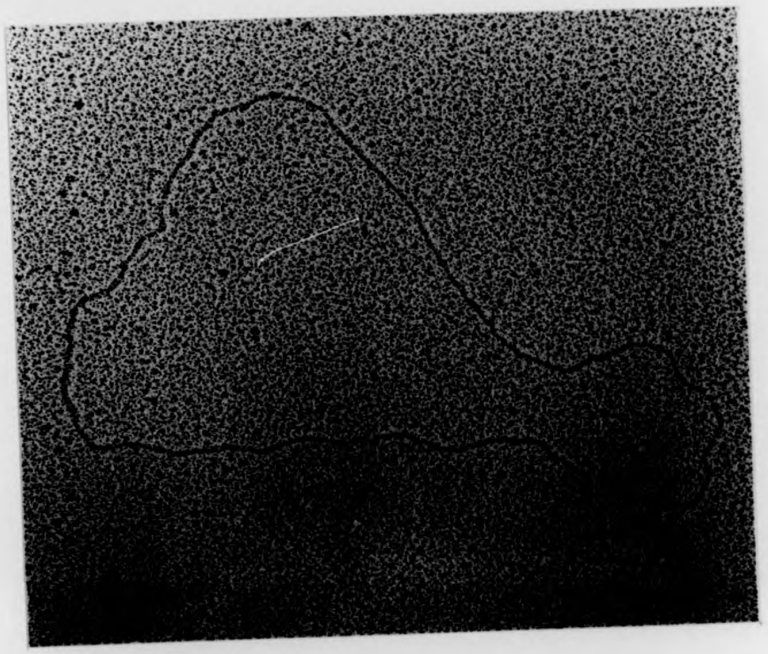
Table 27. Contour lengths of NTP1 and NTP6

Preparation	Plasmid	Contour length (μm)				Number of φX174 lengths
		No. of molecules measured	Range	Mean	Standard deviation	
NTP1	NTP1	22	2.56-2.81	2.67	0.05	1.48
+						
NTP6	NTP6	14	2.95-3.09	3.03	0.04	1.68
+						
φX174	φX174	31	1.74-1.86	1.80	0.04	.

The contour lengths of the resistance determinants of the Δ 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.

A



B

Plate 4. Electron micrographs of (A) NTP2, length 2.74 µm
(B) NTP5, length 3.16 µm

at a magnification of 120,000.

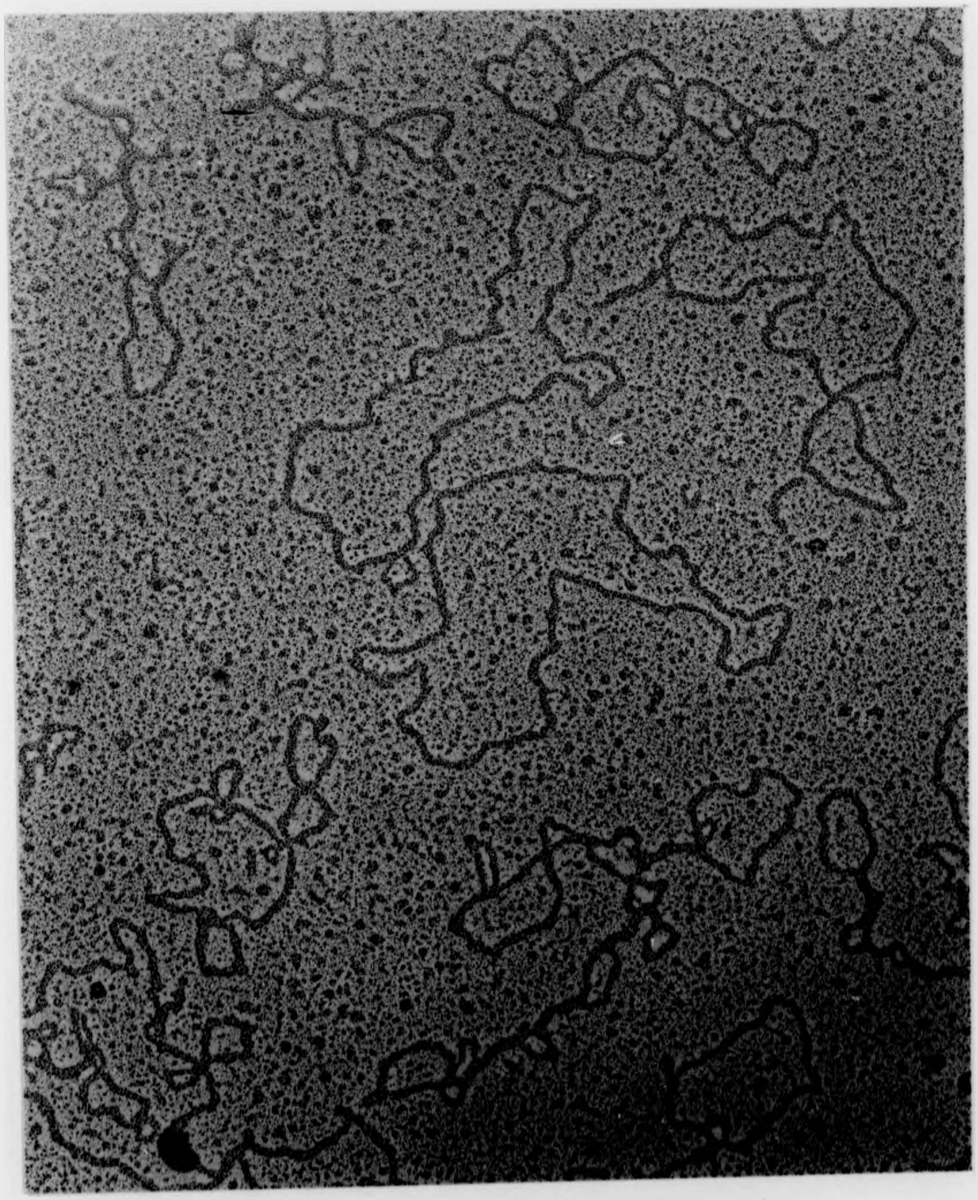


Plate 5. Electron micrograph of NTP1, length 2.70 μm, at a magnification of 85,000, showing open circular and supercoiled DNA.

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 2.5×10^9 (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 99.5% 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.

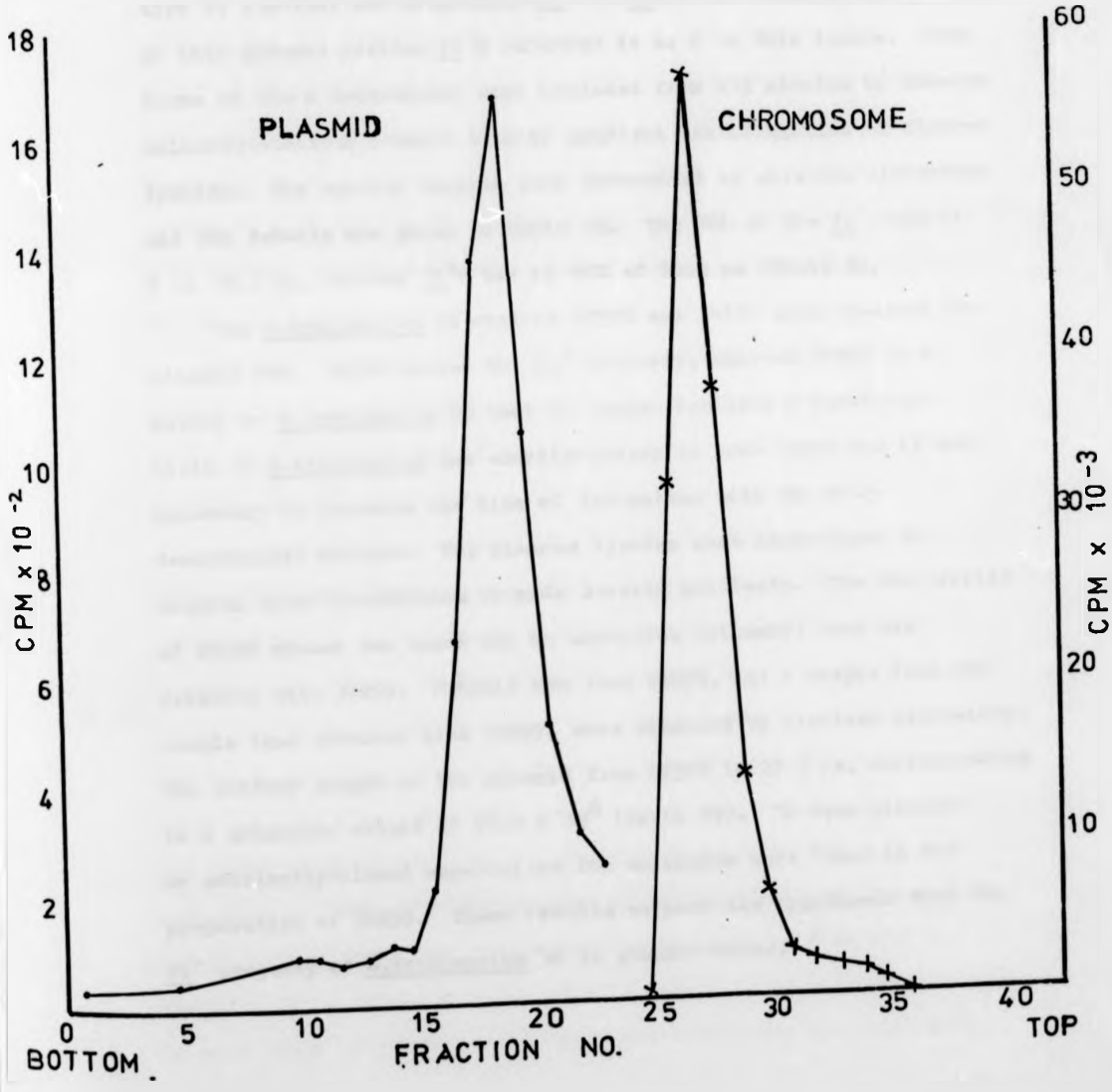
Table 28. Estimation of the number of copies of the non-transferring plasmids

Plasmid		1) Cleared lysates	2) Sarkosyl lysis	
No.	R-type or colicinogeny	No. of copies per chromosome [a]	% ^{3}H-label in plasmid peak	No. of copies per chromosome
NTP1	A	17.7	4.2	18.5
NTP2	SSu	6.6	1.8	8.0
NTP3	ASu	6.7	2.9	8.9
NTP4	ASSu	6.3	2.7	7.6
NTP5	T	5.7 [b]	0.6	2.4 [b]
NTP6	A	9.0	3.6	14.1
NTP7	ASSu	5.3	2.2	5.7
NTP8	Colicin E1	7.8	0.9	5.0
NTP9	Colicin E2	9.6	1.7	9.0
NTP10	Colicin E3	9.7	1.8	9.6
NTP11	K	13.1	3.6	15.1

[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 (5M4136) 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 µm, whereas *fi*$^+$K has an MCL of 30.3 µm (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 caesium 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 µm, corresponding to a molecular weight of 56.5×10^6 (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 μ2 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

designation MP10 (= *S.typhimurium* plasmid 10). Those isolated from type 36 and LT2 can thus be designated $MP10_{36}$ and $MP10_{LT2}$ respectively (Smith *et al.*, 1973b).

Plate 6. Electron micrograph of the fi⁻ K determinant, length 18.1 µm, at a magnification of 60,000.

Table 29. Contour lengths and molecular weights of K and the *fi*$^+$ plasmid of *S.typhimurium*

Strain No.	Description	Contour length (μm)				
		No. of molecules measured	Range	Mean	Standard deviation	Molecular weight$_{-6}$ (x 10^{-6})
42R79	*S.typhimurium* 36 carrying *fi*$^-$K	10	16.6-17.8	17.3	0.4	35.8
38R960	K12 carrying *fi*$^-$K	32	17.6-18.7	18.1	0.3	37.5
42R339	K12 carrying *fi*$^-$K	10	29.8-32.1	30.3	0.9	62.7
RT576	*S.typhimurium* 36	12	26.5-28.5	27.3	0.6	56.5
42R93	*S.typhimurium* 4 (= LT2)	11	27.2-28.8	27.8	0.5	57.6
21R337	*S.typhimurium* 14	7	27.6-29.8	28.6	0.9	59.2
21R340	*S.typhimurium* 74	14	28.7-30.1	29.3	0.4	60.7
21R341	*S.typhimurium* 8	10	28.4-30.0	29.3	0.6	60.7

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 2.5×10^9 daltons.

The percentage of ^{3}H-label recovered in cleared lysates of K12 strains carrying fi$^-$ K was 2.7. The molecular weight of K is 37.5×10^6, 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 56.5×10^6; the copy number per chromosome is therefore 1.0. Most transferable plasmids have a molecular weight greater than 20×10^6 daltons and are present as one to two copies per chromosome (Clowes, 1972; Grindley et al., 1973b).

DNA reassociation experiments with fi$^-$ K and $MP10_{36}$

DNA reassociation experiments were performed at 75° 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 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:

Total cpm in 0.14M PB + k x Total cpm in 0.4M PB = n x Total cpm in control where n = 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 $MP10_{36}$, 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:

$$= \frac{\text{Absolute \% reassociation - \% reassociation with chromosomal DNA} \times 100}{\text{\% reassociation of "homologous" reaction - \% reassociation with chromosomal DNA.}}$$

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 S.typhimurium and the fi$^-$ form of the K determinant.

^{3}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

^{3}H-labelled plasmid DNA	Strains used for preparing unlabelled DNA	Counts/min								Reassociation	
		Recovered in washes			Corrected for Preferential quenching		Correction for reassociation in the control				
		A 0.14M PB	B 0.4M PB	C Total A+B	D B x k [a]	E New total A+D	F x% of E	G D - F	H E - F	I G/H	J
$MP10_{36}$ Expt I	RT576	11.7	187.0	198.7	276.8	288.5	18.5	258.3	270.0	95.7	100
	42R79	192.8	64.8	257.6	95.9	288.7	18.5	77.4	270.2	28.6	27.7
	34R99	275.4	18.7	294.1	27.7	303.1	19.4	8.3	283.7	2.9	0
	1R713	306.9	19.4	326.3	28.7	335.6	21.5	7.2	314.1	2.3	0
	CONTROL [b]	284.8	19.4	304.2							
$MP10_{LT2}$	RT576	19.6	199.9	219.5	269.9	289.5	22.3	247.6	267.2	92.7	100
	42R79	254.4	77.3	331.7	104.4	358.8	27.6	76.8	331.2	23.2	22.9
	34R99	295.3	24.6	319.9	33.2	328.5	25.3	7.9	303.2	2.6	0
	1R713	320.8	22.1	342.9	29.8	350.6	27.0	2.8	323.6	0.9	0
	CONTROL	306.1	25.6	331.7							

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

For $MP10_{36}$: 786.8 + 289.9 x k = 1216.8 k = 1.48

For $MP10_{LT2}$: 890.1 + 323.9 x k = 1326.8 k = 1.35

[b] The proportion of counts per minute (x%) in the Control recovered in the 0.4M PB washes were as follows: $MP10_{36}$, 6.4%; $MP10_{LT2}$, 7.7%.

Key of strains

RT576	S.typhimurium 36 carrying $MP10_{36}$
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 $MP10_{36}$ and $MP10_{LT2}$ 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 MP10 plasmid is homologous with the K determinant. In the case of $MP10_{36}$ (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

Strains used for preparing unlabelled DNA	S.typhimurium strains used for isolation of ^{3}H-labelled plasmid DNA	
	RT576 ($MP10_{36}$) [a]	LT2 ($MP10_{LT2}$)
RT576 (S.typhimurium 36)	91	93
42R79 (S.typhimurium 36 K)	30	23
34R99 (S.typhimurium 36 fi⁻)	3	3
1R713 (K12F⁻)	2	1

[a] The values given are the means of the results of three separate experiments.

2. DNA homology between $MP10_{36}$, K and plasmids of different compatibility groups

Unlabelled DNA was prepared from strains bearing plasmids which represent Groups F_I, F_{II}, I_1, I_2, N, B and H_1. Reassociation experiments were then performed with ^3H-labelled plasmid DNA of $MP10_{36}$ and K (Table 32).

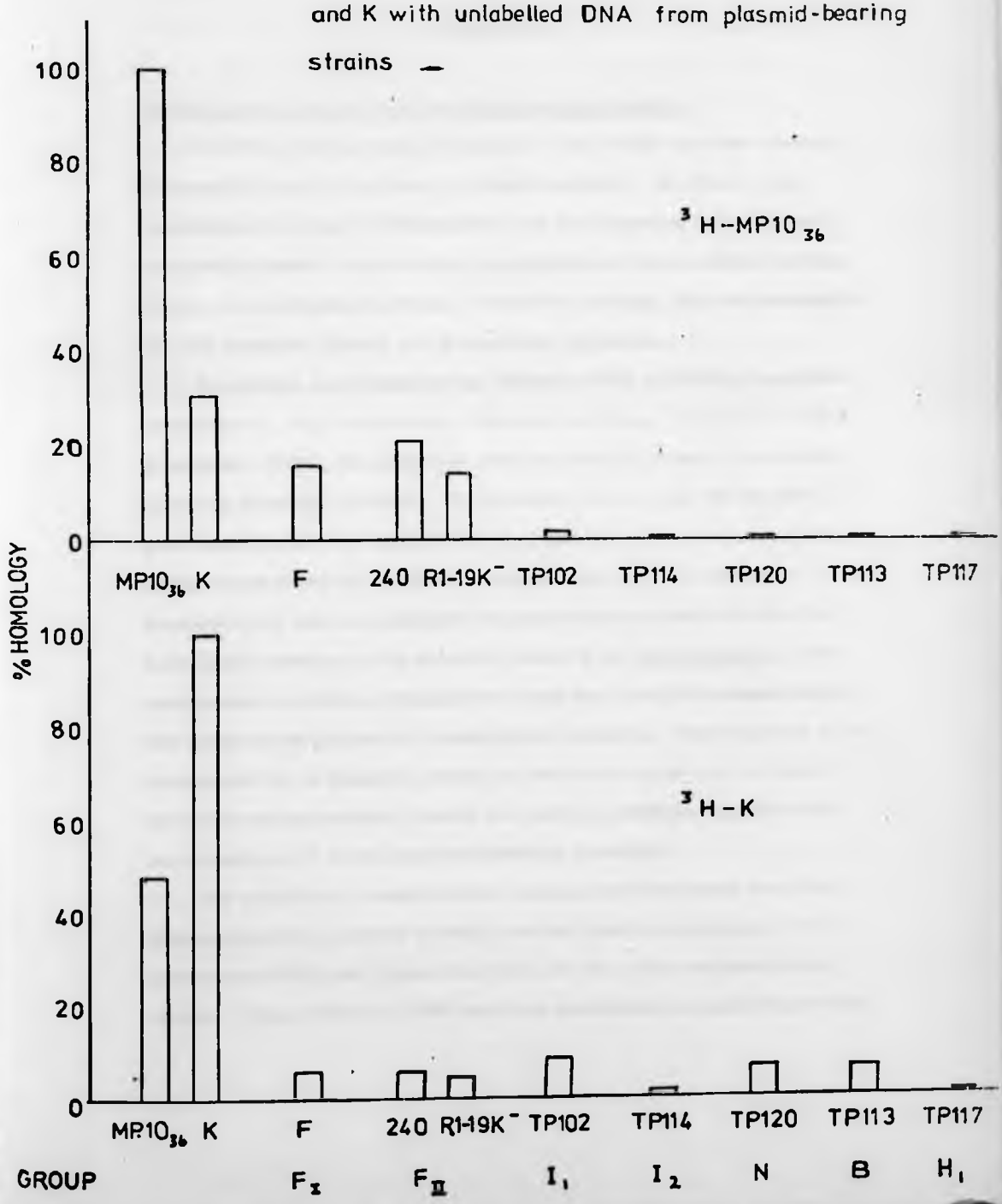
Between 14 and 21% of $MP10_{36}$ reassociated with the F-like plasmids of Groups F_I and F_{II}. The R factor 240, like $MP10_{36}$, is *fi*$^+$, while F and R1-19K$^-$ are both *fi*$^-$ and derepressed F-like plasmids. No homology was observed between $MP10_{36}$ and the members of the other plasmid groups. The experiments with ^3H-labelled K confirmed the homology between K and the *S.typhimurium* *fi*$^+$ plasmid. 48% of K reassociated with $MP10_{36}$ which is equivalent to 8.3 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 $MP10_{36}$ and K are shown as histograms in Figure 4.

Table 32. Reassociation between $MP10_{36}$ fi$^-$ K and plasmids of different compatibility groups [a]

Total unlabelled DNA from strains bearing plasmids	Compatibility group	^{3}H-labelled plasmid DNA			
		$MP10_{36}$	No. of expts.	K	No. of expts.
$MP10_{36}$	•	100	3	48	3
K	•	31	3	100	3
F	F_I	16	2	6	2
240	F_{II}	21	2	6	2
R1-19K$^-$	F_{II}	14	3	5	2
TP102	I_1	2	1	9	2
TP114	I_2	0	1	2	2
TP120	N	0	1	7	2
TP113	B	0	1	7	2
TP117	H_1	0	1	1	1

[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 $MP10_{36}$ and K with unlabelled DNA from plasmid-bearing strains —



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 Willshaw, 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 Δ 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-Δ 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 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 Willetts (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-Δdrp1, progeny selected on streptomycin or sulphonamides had almost always acquired both SSu and T-Δdrp1. In contrast, with S.typhimurium as recipient and with the same donor strain and selection, 95% of the progeny carried SSu alone. The frequency of T-Δdrp1 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 10×10^6 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 85% 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 β-lactamase activity of those carrying ASu (NTP3) or the second A determinant NTP6 (Humphreys and Anderson, unpublished observations). The same gene codes for β-lactamase in the case of NTP1 and NTP3, and therefore the difference in β-lactamase activity is probably due to the higher number of A gene copies in K12(NTP1) compared with K12 (NTP3). The TEM-type of β-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 S.panama (Guineé and Willems, 1967) had a molecular weight of about 6 x 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 x 10^6 daltons, two others were 6.3 and 9.3 x 10^6 and the remaining plasmid consisted of three species, of molecular weight 7.4, 14.7 and 21.4 x 10^6. DNA reassociation experiments demonstrated 80 to 93% homology between 10 of the plasmids, indicating a common evolutionary origin.

The ampicillin resistance determinant RSF1030 has a molecular weight of 5.5 x 10^6 daltons and exists in about 30 copies per chromosome equivalent (Crosa, Luttropp 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 *fi*$^+$ 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 *fi*$^-$ 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 *fi*$^-$ 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 *fi*$^+$ property (Anderson and Smith, 1972b). Selection for K would result in loss of the resident *fi*$^+$ plasmid and derepression of F. Molecular studies confirmed that this latter hypothesis was correct (Smith *et al.*, 1973b).

The *fi*$^+$ plasmid carried by *S.typhimurium* 36 has a molecular weight of 56.5×10^6 daltons. It has been designated $MP10_{36}$ (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 x 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 x 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 x 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 x 10^6 daltons) is of the same order as those of certain groups of transferable plasmids (Clowes, 1972; Grindley et al., 1973b) whereas most non-transferring plasmids have molecular weights less than 10 x 10^6 daltons.

DNA reassociation experiments confirmed that the *fi*$^+$ plasmids of *S.typhimurium* 36 and LT2 (= $MP10_{LT2}$) are very closely related if not identical. There was little or no homology between $MP10_{36}$ and chromosomal DNA of *E.coli* K12 or *S.typhimurium*. These results also demonstrated that almost half of K is homologous with $MP10_{36}$. As K was first identified in a strain of *S.typhimurium* type 29, it seems probable that it was formed by recombination between an acquired kanamycin resistance determinant and the *fi*$^+$ (MP10) plasmid of *S.typhimurium* 29. The K determinant originally isolated from *S.typhimurium* 29 was *fi*$^+$, and is designated *fi*$^+$ K. Spontaneous mutation of this plasmid gave rise to *fi*$^-$ K. Reassociation experiments with labelled $MP10_{36}$ 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 *fi*$^-$ K had less than 10% homology with plasmids representing groups F_I, F_{II}, I_1, I_2, B, N and H_1. 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 *fi*$^-$ form of K.

The MP10 plasmid of *S.typhimurium* has been shown to restrict certain non-donor specific phages to which *S.typhimurium* lacking this plasmid is sensitive (Maureen de Saxe, personal communication). The *fi*$^+$ K determinant causes the same phage restriction as $MP10_{36}$ in *S.typhimurium*, while *fi*$^-$ K does not block these phages. Thus the *fi*$^+$ and respective phage-restricting region(s) of *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 $MP10_{36}$ or related plasmids, and the K determinant was also non-transferring in both its *fi*$^+$ and *fi*$^-$ states. However the recombinant between the A of ASu and $MP10_{36}$ termed A-$MP10_{36}$ (or A*) transferred to K12 at low frequency. This new R factor was incompatible with K, and like $MP10_{36}$ it was *fi*$^+$. 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-$MP10_{36}$. Alternatively, $MP10_{36}$ 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 *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 NTP1 to NTP11 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 *et al.*, 1963; Anderson, 1966; Anderson *et al.*, 1968).

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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 1.5×10^6 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 Δ, 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 fi^+ plasmid of S.typhimurium $MP10_{36}$ to form A-$MP10_{36}$ 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 3×10^6 daltons, on each of the plasmids. This sequence or "transposon A", which includes the TEM β-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 β-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 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 E.coli, Serratia marcescens and 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 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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Fertility Inhibition in Strains of *Salmonella typhimurium*

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Summary. The *fi*$^+$ property recently described in a strain of *Salmonella typhimurium* phage type 36, 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 *fi*$^+$ 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 *fi*$^+$ character.

The *fi*$^+$ character of the original strain of *S. typhimurium* 36, 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/*fi*$^+$ plasmids so formed are stable in K12, and are displaced as a single linkage group in compatibility experiments with another I-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"+ (*fi*$^+$) (Egawa and Hirota, 1962; Watanabe and Fukasawa, 1962). The *fi*$^+$ 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 36 (= *S. typhimurium* 36) 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* 36 F-*lac* strains were resistant to phage μ2 in surface spot tests, whereas *S. typhimurium* 36 F-*lac* K strains were sensitive of the phage. Strains of *S. typhimurium* 36 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* 36 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 *fi*$^+$ 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 K12*lac*$^-$ carrying F-*lac*T, a recombinant of F-*lac* and the tetracycline (T) resistance marker of the I-like R factor T-Δ (Anderson and Lewis, 1965; Anderson, 1968; Anderson and Smith, in preparation); and 38R93 which is K12*lac*$^-$ carrying both F-*lac* and K. The F-*lac*T 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-*lac*T 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-*lac*T 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-*lac*T and K.

An R factor used in these investigations was KColIb, 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 KColIb R factor to HfrH was carried out by the same method as that used for F-*lac*T transfer to *S. typhimurium*.

Crosses 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 $\mu 2$ was routinely used for testing the repressive effect of *S. typhimurium* strains on the F-*lac*T factor. Strain 30R893 (= K12 F-*lac*T) 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-*lac*T were insensitive to $\mu 2$. This may indicate that these strains have the same *fi*$^+$ 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 descendent lines, which carried both F-*lac*T and K, were tested with $\mu 2$, with the results shown in Table 1. F-*lac*T was lost at high frequency from three strains, 21R342, 21R343 and 21R344. Although the introduction of K produced $\mu 2$ sensitivity in each of these strains in the presence of F-*lac*T, 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 $\mu 2$, although there was some variation in the degree of visible lysis. Thus, 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 originally described in *S. typhimurium* 36. However, the experiments do not indicate whether the *fi*$^+$ 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 *fi*$^+$ region

Table 1. Reactions of *S. typhimurium* strains with male-specific phage μ2

ERL No.	Phage type of *S. typhimurium* strain	Sensitivity to μ2 of *S. typhimurium* strains carrying F-*lac*T	Sensitivity to μ2 of *S. typhimurium* strains carrying F-*lac*T and K
RT576	36*	−	+
21R339	1	−	+
21R336	4	−	+
1R213	4 (= LT2)	−	±
21R341	8	−	+
21R343	12a	−**	V
21R337	14	−	+
21R342	32	−**	V
19R688	36***	−	±
21R340	74	−	+
21R344	104	−**	V
21R338	160	+	·
21R325	168	+	·
21R326	169	−	+
21R327	170	+	·
21R328	173	−	+
21R329	180	+	·
21R330	181	−	+
21R331	182	−	+
21R332	184	−	+
21R333	185	−	±
21R334	186	+	·
21R335	187	−	+

+ = 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 *fi*$^+$ plasmid. Incompatibility between homologous plasmids has been shown for transfer factors (Scaife and Gross, 1962; Anderson, 1966) 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 *fi*$^+$ property can be mobilised with an I-like *fi*$^-$ R factor, KColIb, 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 KColIb factor had been introduced two years earlier, was crossed with HfrH. Of 50 HfrH(KColIb) 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 *fi*$^+$ character of *S. typhimurium* 36 on K12HfrH

Strain	Reaction with phage μ2 in spot tests	Frequency of *pro* transfer in 1 h crosses
K12HfrH	+	2.9×10^{-3}
K12HfrH(KColIb)	+	1.9×10^{-3}
K12HfrH(KColIb*fi*$^+$)*	−	3×10^{-5}

+ = Visible lysis with μ2. − = No visible lysis with μ2. * Denotes the presence of the *fi*$^+$ character acquired by KColIb from *S. typhimurium* 36.

whether the μ2 insensitivity of this single line was caused by acquisition of the *fi*$^+$ character by KColIb, the plasmid was introduced into K12 F-*lac*, and a number of resulting colonies were tested for sensitivity to μ2. All colonies tested were resistant to the phage. The *fi*$^+$ property of *S. typhimurium* 36 thus appears to have been mobilised by KColIb.

The effect of this *fi*$^+$ character on F fertility was examined by measuring the frequency of *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 *pro* transfer from HfrH is reduced approximately a thousandfold by the presence of the *fi*$^+$ character, now associated with KColIb.

If this *fi*$^+$ region is linked to KColIb, displacement of KColIb in the μ2-resistant HfrH strain by an incompatible plasmid should restore the μ2 sensitivity of the HfrH strain (Grindley and Anderson, 1971). Displacement of this KColIb by introduction of T-*Δ* yielded HfrH(T-*Δ*) lines which were fully sensitive to phage μ2. This supports the hypothesis that the *fi*$^+$ character has recombined with the KColIb factor. This recombinant plasmid has been designated KColIb/*fi*$^+$, and we have concluded that the *fi*$^+$ region concerned is that of *S. typhimurium* 36.

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

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

The majority of transferable *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, 1965). Recently, however, Grindley and Anderson (1971) have described a group of R factors which are both *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 *fi*$^+$ marker mobilised by KColIb has similarities with that of the F-like R factor R100 (Egawa and Hirota, 1962), and is distinguishable from that of the *fi*$^+$ I-like factors described by Grindley and Anderson (1971).

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

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

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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 plasmids 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 $MP10_{36}$, 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 $MP10_{36}$. Reassociation experiments demonstrate a high degree of homology between the DNA of all five *S. typhimurium* plasmids, and between $MP10_{36}$ and K. $MP10_{36}$ 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 $MP10_{36}$ 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, 1971) 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.

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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 μ2 (Dettori *et al.*, 1961).

Table 1. Strains of *Escherichia coli* K12 and *Salmonella typhimurium*

Enteric Reference Laboratory No.	Description
1R713	*Escherichia coli* K12 F$^-$ prototrophic
38R960	1R713 carrying K[a]
RT576	*S. typhimurium* phage type 36
34R99	*S. typhimurium* 36 *fi*$^-$ [b]
42R79	*S. typhimurium* 36 carrying K
42R93	*S. typhimurium* phage type 4 (= LT2)
21R337	*S. typhimurium* phage type 14
21R340	*S. typhimurium* phage type 74
21R341	*S. typhimurium* phage type 8

[a] The kanamycin resistance determinant of Anderson *et al.* (1969).
[b] When the F factor is transferred to 34R99 the resulting progeny are visibly lysed by the F-specific phage μ2. 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 2×10^8 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 Helinski (1969). The "cleared lysates" so obtained were subjected to caesium 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 caesium 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[a]

Plasmid designation	Compatibility group	Resistance markers	Reference
F	F_I	—	Hayes (1952)
240[b]	F_{II}	T	Grindley *et al.* (1971)
R1-19K-[c]	F_{II}	ACSSu	Meynell and Cooke (1969)
TP102	I_1	K	Grindley and Anderson (1971)
TP114	I_2	K	Grindley *et al.* (1972)
TP120[d]	N	ASSuT	Grindley *et al.* (1972)
TP113	B	K	Grindley *et al.* (1972)
TP117	H	T	Grindley *et al.* (1972)

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

[a] All R factors were isolated from wild enterobacterial strains defined in the Enteric Reference Laboratory.

[b] 240 was isolated from *S. typhimurium* 3M4466 and is probably identical with R136.

[c] Spontaneous segregant of R1-19 no longer coding for kanamycin resistance.

[d] 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.* (1969).

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 RT576, 34R99, LT2 (= 42R93), 21R337, 21R340 and 21R341 were examined for plasmid DNA as described in Materials and Methods. All these strains showed the *fi*⁺ property except 34R99, a mutant of *S. typhimurium* 36 that 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 RT576, 34R99 and LT2 were centrifuged in caesium chloride-ethidium bromide density gradients. The DNA profiles of RT576 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 and open circular plasmid DNA. No satellite (plasmid) band was detected with 34R99. Plasmid DNA from RT576 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*

Strain No.	Description and phage type	Contour length (μm)				
		No. of molecules measured	Range	Mean	Standard deviation	Molecular weight[a] (daltons $\times 10^{-6}$)
RT576	*S. typhimurium* 36	12	26.5–28.5	27.3	0.6	56.5
LT2 (= 42R93)	*S. typhimurium* 4	11	27.2–28.8	27.8	0.5	57.6
21R337	*S. typhimurium* 14	7	27.6–29.8	28.6	0.9	59.2
21R340	*S. typhimurium* 74	14	28.7–30.1	29.3	0.4	60.7
21R341	*S. typhimurium* 8	10	28.4–30.0	29.3	0.6	60.7
42R79	*S. typhimurium* 36 carrying K	10	16.6–17.8	17.3	0.4	35.8
38R960	K12 carrying K	32	17.6–18.7	18.1	0.3	37.5

[a] Calculated on the assumption that 1 μm = 2.07×10^8 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 (38R960) 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 (RT576) (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 *fi*$^+$ property of RT576 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 (38R960). No DNA molecules of 27 μm, corresponding in length to the *fi*$^+$ plasmid of RT576, were detected in preparations of 42R79.

DNA Reassociation Experiments. Reassociation experiments between ^{3}H-labelled plasmid DNA isolated from RT576 and LT2, and unlabelled DNA from RT576, 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 RT576 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 [a]

Strains used for preparing unlabelled DNA	*S. typhimurium* strains used for isolation of ^{3}H-labelled plasmid DNA	
	RT576 ($MP10_{36}$)	LT2 ($MP10_{LT2}$)
RT576 (*S. typhimurium* 36)	91	93
42R79 (*S. typhimurium* 36 carrying K)	30	23
34R99 (*S. typhimurium* 36 *fi*−)	3	3

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

Table 5. Homologies between $MP10_{36}$, K and plasmids of various compatibility groups[a]

Total unlabelled DNA from strains bearing plasmids	Compatibility group	Strains used for the isolation of ^{3}H-labelled plasmid DNA	
		RT576 ($MP10_{36}$)	42R79 (K)
$MP10_{36}$	.[b]	100	48
K	.[b]	31	100
F	F_I	16	6
240	F_{II}	21	6
R1-19K−	F_{II}	14	5
TP102	I_1	2	9
TP114	I_2	0	2
TP120	N	0	7
TP113	B	0	7
TP117	H	0	1

[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). Both ^{3}H-labelled plasmids gave a reassociation value of 3–4% with chromosomal DNA from either *E. coli* K12 or *S. typhimurium*.
[b] Not yet designated.

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*+ 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 $MP10_{36}$ and $MP10_{LT2}$ respectively. Approximately 30% of $MP10_{36}$ 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 $MP10_{36}$ and K have approximately 8 μm of homologous DNA.

Reassociation experiments between ^{3}H-labelled plasmid DNA from RT576 and unlabelled DNA from members of various compatibility groups indicated some homology between $MP10_{36}$, 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 *fi*$^+$ 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 *fi*$^+$ 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 fi*$^+$ plasmids (Anderson and Smith, 1972). Recently, a recombinant between $MP10_{36}$ 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 $MP10_{36}$ 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, $MP10_{36}$ 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 *fi*$^+$ property. Its molecular weight is 57×10^6 daltons (Table 3), which agrees well with the estimate of 60×10^6 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 *Flac*S, has been identified in a strain of *S. typhimurium* LT2 which carried the temperature-sensitive F_{ts114}*lac* plasmid (Macrina and Balbinder, 1973). K12 strains carrying *Flac*S were not visibly lysed by F-specific phages, whereas $K12F_{ts114}$*lac* strains were lysed by these phages. Sedimentation in alkaline sucrose gradients showed that *Flac*S was considerably larger than F_{ts114}*lac*. Macrina and Balbinder suggested that FlacS resulted from a nonreciprocal exchange between F_{ts114}*lac* and some segment of *S.typhimurium* DNA which may be derived from the host chromosome or a "cryptic" plasmid.

Our results suggest that *Flac*S may be a recombinant of F_{ts114}*lac* with an *fi*$^+$ plasmid present in the S. *typhimurium* strain. We have isolated recombinants between $MP10_{36}$ and the I-like R factor KColIb (Anderson and Smith, 1972).

The *fi*$^+$ 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

no molecules of 27 μm. The reversal of the *fi*+ character by the K determinant is therefore correlated with loss of the 27 μm *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_I, F_{II}, I_1, 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 5×10^6 and 5.4×10^6 respectively, whereas K has a molecular weight of 37.5×10^6. 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 μm of DNA in common between $MP10_{36}$ and the K determinant. In the case of $MP10_{36}$, which is 27.3 μm in length, the shared proportion amounts to 30%, while in that of the K determinant (17.3 μm) 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_I, F_{II}, I_1, I_2, 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). $MP10_{36}$ 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 $MP10_{36}$ 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 $f5^+$ 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.

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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 26 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 *Δ* transfer factor (Anderson and Lewis, 1965a, b; Anderson, 1968). These Class 2 systems have been designated A, *Δ* and SSu, *Δ* respectively (Anderson and Natkin, 1972).

Molecular studies on the plasmids of the *Δ* transfer systems confirmed the postulates based on the genetic evidence. The resistance determinants A and SSu and the transfer factor *Δ* exist as independent covalently-closed circular DNA molecules in K12. The molecular weights of A, SSu and *Δ* are approximately 5.6, 5.7 and 59×10^6 daltons respectively. A and SSu are present as multiple copies per chromosome, whereas there is approximately only one copy of *Δ* per chromosome (Smith, Anderson and Clowes, 1970; Humphreys, Grindley and Anderson, 1972; Milliken, Anderson and Clowes, unpublished). Several other plasmids, such as ColE1, are also small, non-transferring, and exist as multiple copies per chromosome (Bazaral 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 ColE1 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* K12F$^-$ 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 μg/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 2×10^8 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

Enteric Reference Laboratory No.	Description
1R713	*Escherichia coli* K12F⁻ prototrophic
14R525	1R713 Nal$^{r a}$
22R80	K12-ROW. Colicin indicator strain.
20R675	K12 carrying ColE1 = K12(ColE1). Sensitive to colicin E2 and E3 but resistant to E1
20R676	K12 carrying ColE2 = K12(ColE2). Sensitive to colicin E1 and E3 but resistant to E2
RT576	*Salmonella typhimurium* phage type 36

[a] Nal^r, nalidixic acid-resistant mutant.

Table 2. Non-transferring plasmids

Plasmid number	Antibiotic resistance or colicinogeny	Species of original host strain	Phage type of original host strain	Year and place of origin	Source or Enteric Reference Laboratory No.
NTP1	A[a]	*Salmonella typhimurium*	29	England 1964	RT1, Anderson and Lewis (1965a, b)
NTP2	S Su[b]	*Salmonella typhimurium*	29	England 1964	RT1, Anderson and Lewis (1965a, b)
NTP3	A Su	*Salmonella typhimurium*	29	Enteric Reference Laboratory	9R314, Anderson *et al.* (1968)
NTP4	A S Su	–	–	Enteric Reference Laboratory	13R135, Anderson (1969)
NTP5	T	*Salmonella typhimurium*	49	Scotland 1969	9M3779
NTP6	A	*Salmonella typhimurium*	168	England 1972	12M521
NTP7	A S Su	*Salmonella typhimurium*	157	Argentina 1972	12M3614
NTP8	colicin E1	*Escherichia coli* K30	–	–	Prof. P. Fredericq
NTP9	colicin E2	*Shigella sonnei* P9	–	–	Prof. P. Fredericq
NTP10	colicin E3	*Escherichia coli* CA38	–	–	Prof. P. Fredericq

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

[a] Strains conferring ampicillin resistance are also resistant to carbenicillin.
[b] Strains carrying S Su, A S Su (NTP4) or A S Su (NTP7) are resistant to streptomycin but not to spectinomycin. This suggests that these plasmids code for a streptomycin phosphotransferase (Ozanne *et al.*, 1969).

Table 3. Wild strains resistant to streptomycin and sulphonamides[a]

ERL No.	Species of host strain and phage type of *S. typhimurium*	Origin	Compatibility of SSu resistance with ASu
8M3993	*S. typhimurium* 32	Human, Scotland 1968	+
	S. typhimurium 1	Human, England 1968	−
	S. typhimurium 44	Human, Scotland 1969	−
	S. typhimurium 6	Animal, England 1969	−
	S. typhimurium 12a	Human, England 1970	−
	S. typhimurium 12a	Animal, England 1971	−
	S. typhimurium 56	Human, England 1971	−
	S. typhimurium 95	Animal, England 1971	−
	S. typhimurium Untypable	Animal, Singapore 1973	−
	S. typhimurium 104	Human, Spain 1973	−
	S. typhimurium 156	Human, New Zealand 1973	−
	S. agona (B)[b]	Human, England 1970	−
	S. brandenburg (B)	Human, Wales 1970	−
42R652	*S. bredeney* (B)	Human, England 1970	+
	S. derby (B)	Human, England 1970	−
	S. montevideo (C)	Human, England 1970	−
42R653	*S. newport* (C)	Human, England 1970	+
42R654	*S. panama* (D)	Human, England 1970	+
	S. stanley (B)	Human, England 1970	−
	S. chester (B)	Human, England 1970	−
	S. indiana (B)	Human, England 1970	−
EC4240	*E. coli*	Human, England 1970	+
EC4316	*E. coli*	Human, England 1970	+
	E. coli	Animal, England 1973	−
3EC635	*E. coli*	Animal, England 1973	+
	E. coli	Animal, Ireland 1970	−

+ = Compatible, − = Incompatible.

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

[b] Salmonella O groups are shown in parenthesis after serotype designations.

Colicinogeny. Strains were tested for colicin production by the method of Frederieq (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 iodine-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, Brij 58 and sodium deoxycholate (Clewell and Helinski, 1969). The resulting "cleared lysates" were subjected to caesium chloride-ethidium bromide density gradient centrifugation. Fractions containing plasmid DNA were

identified and prepared 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 2.5×10^9 (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 ^{3}H-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 *Δ*-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 *Δ* or its derived R factor, T-*Δ* (Anderson and Lewis, 1965b). The colicin determinants ColE1, 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-*Δ* and transferred to K12. ASSu (NTP7) was initially transferred to K12 from the wild host strain 12M3614, which carries an *fi*$^-$ I-like transfer factor of compatibility group I_g (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-*Δ* 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,

NTP6, also coexists stably with SSu, T and the three colicin factors. Since NTP1 and NTP6 code for resistance to ampicillin only, we have so far been unable to test for compatibility between them. The T determinant NTP5 was tested with A (NTP1), SSu, A (NTP6) and the three colicin factors; it was compatible with all these plasmids.

As the ASSu determinant NTP7 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 (NTP7) 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

Groups	A (NTP1) group	SSu group[a]	Compatible plasmids not yet grouped
Examples	A (NTP1)	SSu	A (NTP6)
		ASu	T
		ASSu (NTP4)	ColE1
		ASSu (NTP7)	ColE2
			ColE3

[a] 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 ASu 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 serotypes 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

original SSu determinant of Anderson and Lewis (1965a, b). The SSu determinants in these 19 strains are easily mobilisable by *Δ* 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 *Δ*, 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-*Δ*, 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-*Δ* 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 NTP1 and NTP6 can be distinguished by their mean contour lengths on electron microscopy: 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, 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 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. This confirmed that NTP1 was shorter than NTP6, as our direct measurements indicated.

The contour lengths of the resistance determinants of the *Δ* 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 μm and that of SSu, 2.74 μm. The contour length of ASu is 3.91 μm, 1.17 μm 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

Plasmid	Contour length (μm)				Molecular weight[a] ($\times 10^{-6}$)
	No. of molecules measured	Range	Mean	Standard deviation	
A (NTP1)	40	2.45–2.86	2.70	0.09	5.6
SSu	33	2.60–2.97	2.74	0.09	5.7
ASu	37	3.67–4.18	3.91	0.13	8.1
ASSu (NTP4)	30	3.87–4.42	4.24	0.13	8.8
T	18	3.00–3.33	3.16	0.09	6.5
A (NTP6)	17	2.98–3.19	3.10	0.05	6.4
ASSu (NTP7)	25	4.29–4.80	4.53	0.16	9.4
ColE1	18	2.15–2.43	2.28	0.08	4.7
ColE2	22	2.08–2.37	2.22	0.07	4.6
ColE3	22	2.27–2.42	2.34	0.04	4.8

[a] 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 caesium 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 caesium chloride-ethidium bromide centrifugation of Sarkosyl lysates compared with that from

Table 6. Estimation of copy numbers of the plasmids

Non-transferring plasmid	% ^{3}H-label in the cleared lysate relative to the total lysate[a]	% ^{3}H-label in the plasmid band relative to the chromosomal DNA[b]	Number of plasmid copies per chromosome[c]
A (NTP1)	4.0	4.2	16.7–18.7
SSu	1.5	1.8	5.8–8.0
ASu	2.2	2.9	5.8–8.9
ASSu (NTP4)	2.2	2.7	5.3–7.6
T	1.5	0.6	4.2–7.3; 1.5–3.9[d]
A (NTP6)	2.3	3.6	8.8–14.1
ASSu (NTP7)	2.0	2.2	5.0–5.7
ColE1	1.5	0.9	5.0–9.5
ColE2	1.8	1.7	8.2–10.9
ColE3	1.9	1.8	8.1–11.2

[a] In 12 experiments, a mean of 0.54% chromosomal DNA was recovered in cleared lysates of the K12F$^-$ strain (1R713).
[b] Caesium chloride-ethidium bromide density gradient centrifugation of Sarkosyl lysates.
[c] 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 β-lactamase assay of K12 strains carrying them. Extracts of strains carrying NTP1 show approximately twice the β-lactamase activity of those carrying NTP6 (Humphreys and Anderson, unpublished observations). Similarly the diameter of zones of β-lactamase diffusion on starch-nutrient agar from colonies of K12 (NTP1) are greater than those of K12 (NTP6). If the two β-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 β-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 ASSu 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 (1966, 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 caesium chloride. The A determinant NTP1 has a G+C content of 40%, whereas those of SSu and ASu are 61% and 56% 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 *fi*$^+$, and non-transferring but mobilisable in a triparental cross (Anderson, Pitton and Mayhew, 1968). The *fi*$^+$K gave rise to *fi*$^-$K, which forms a Class 2 transfer system with the F factor (Anderson, Mayhew and Grindley, 1969). Molecular studies of *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 *fi*$^-$K is incompatible with the recently defined *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 *fi*$^+$ plasmid, we have suggested that the *fi*$^+$K determinant was formed by recombination between a kanamycin resistance marker and the *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*

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.

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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 fi^+ (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 fi^+ 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 fi^+ 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 synthesised 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 *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 fi^+ R factor in lines in which both plasmids were established; these mutants were called *traI'* (Finnegan and Willetts, 1971). When such an *Flac traI'* mutant was transferred into cells carrying both an fi^+ R factor and *Fhis*, the immediate retransfer of *Flac traI'* was inhibited, indicating that this mutant was sensitive to the complete inhibitor, that is, the *fin* product of the R factor and the P product of *Fhis*. It was suggested that the *traI'* 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 *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 1. Bacterial strains

Strain No.	Description
JC6255[a]	K12F$^-$
JC5455[a]	K12F$^-$ T6^r
JC3051[a]	K12F$^-$ T6^r Str^r
38R223	JC5455 carrying R100T$^-$[b]
38R444	JC5455 carrying 334[b]
38R447	JC5455 carrying 240[b]
38R626	JC5455 carrying 240T$^-$[b]

[a] JC3051, 5455 and 6255 are derivatives of JC6589 (Achtman, Willetts, and Clark, 1971), and were obtained from D. J. Finnegan.
[b] Details of these plasmids are shown in Table 2.
T6^r = resistance to phage T6. Str^r = 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*$^+$ I-like R factor 782 were called "operator constitutive" (o^c) 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 240*drp*1–8; they were previously called 240 o^c1–8.

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.

Table 2. Transferable plasmids

Designation	Description	Resistances carried
R100T^{-}[a]	T^{-} segregant of the *fi*$^{+}$F-like R factor, R100	CSSu
R100-1	mutant of R100, derepressed in transfer and no longer *fi*$^{+}$	TCSSu
R100-1T^{-}	T^{-} segregant of R100-1	CSSu
240	*fi*$^{+}$ F-like R factor isolated from *S. typhimurium* 4466 (also known as R136)	T
240T^{-}	segregant of 240, sensitive to tetracyclines	—
240*i*$^{-}$1	mutant of 240, derepressed in transfer and no longer *fi*$^{+}$	T
240*drp*1,2,4 to 8 and R136*drd*H8[b]	mutants of 240, derepressed in transfer and still *fi*$^{+}$	T
334	*fi*$^{+}$ F-like R factor isolated from *S. paratyphi B* 7268 (also known as R1)	ACSSu

Symbols for plasmid-borne antibiotic resistances: A=ampicillin; C=chloramphenicol; S=streptomycin; Su=sulphonamides; T=tetracyclines.
The R factors R100 and its derepressed mutant R100-1 (Egawa and Hirota, 1962) were obtained from D. J. Finnegan.
[a] R100T^{-} is also transfer-defective.
[b] Meynell and Cooke (1969).

For this we used the method devised by Finnegan and Willetts (1971) to characterise their mutants of *Flac*. 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 4h 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 *fin*⁻ or 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 *fin* product and 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 3. Retransfer of derepressed plasmids from newly-infected R⁺ strains

R factor in donor strain	Intermediate strain		Frequency of transfer of donor R factor from newly-infected intermediate cells (as % of retransfer from the R⁻ intermediate JC5455)
	No.	R factor carried	
240*i*⁻1	38R444	334	100
240*i*⁻1	38R223	R100T⁻	0.2
R100-1	38R444	334	100
R100-1	38R447	240	2

The derepressed R factors retransfer from the R⁻ intermediate under the same conditions at a frequency of about 10^{-3} 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 *fin* product, the 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 P_R for inhibition of their transfer.

The assumption that 334 codes for a *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 μ2 of strains carrying derepressed mutants of 240 or of R100 (see Methods). Strains carrying both 334 and either of the *fin*⁻ mutants, 240*i*⁻1 or R100-1, were not visibly lysed by μ2 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 *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 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

Table 4. Sensitivity to male-specific phage μ^s

Derepressed R factor	Visible lysis with phage μ^s of K12 strains carrying	
	Derepressed mutant alone	Derepressed mutant and 334
240*i*⁻1	+	−
240*drp*1, 2, 4 to 8	+	+
R100-1	+	−

+ = visible lysis by μ^s; − = no visible lysis by μ^s.

results, by analogy with the conclusions drawn from the results obtained with 240*i*⁻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 these 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 R136*drd*H8, 240*drp*5, 240*drp*6 and 240*drp*8. Transfer of these four

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

Derepressed mutant of 240 (=R136) in donor strain JC6255	Frequency of transfer[a] of the donor plasmid from the newly-infected intermediate strain	
	38R223 (R100T⁻)[b]	38R626 (240T⁻)
R136*drd*H8	40	37
240*drp*5	56	65
6	33	84
8	26	57
1	5.9	2.3
4	6.7	5.4
2	0.2	0.2
7	0.6	0.3
240*i*⁻1	0.2	0.1

[a] Expressed as a percentage of the retransfer of the donor plasmid from the R⁻ intermediate 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 240*drp*1 and 240*drp*4. Both these mutants 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 240*i*⁻1, (which no longer codes for an active *fin* product). The third group of mutants, 240*drp*2 and 240*drp*7, 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 240*i*⁻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: R136*drd*H8, 240*drp*5, 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*⁻ (240*i*⁻1) (Grindley *et al.*, 1971); and those which

still repress F and are therefore *fin*$^+$ (240*drp*2 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 240*drp*2 and 240*drp*7, therefore, have the properties expected of 240 carrying a mutation in the *traP* gene.

The two mutants 240*drp*1 and 240*drp*4 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^{-1} in 30 min. If mutants 240*drp*1 and 4 coded for an altered *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 240*drp*1 and 4 are not caused by a quantitative difference in either inhibitor production or sensitivity of one of the mutant types *i*$^-$, *traP* or *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, 240*drp*1 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 240*drp* mutant. Any mutant which failed to produce this postulated third component of the transfer inhibitor would be classified as a *traP* mutant if retransfer were examined from newly infected cells also carrying the wild-type plasmid. Mutants classified as *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 R136*drd*H8, 240*drp*5, 240*drp*6 and 240*drp*8) R100T$^-$ is also transferred at a derepressed frequency.

The mutant 240*drp*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}).

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 *fi*$^+$ 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 R136 (= 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-K94, 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 (240*fi*$^-$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 (240*drp*2 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 (R136*drd*118, 240*drp*5, 6, and 8). These are analogous to the *traO* mutants of *Flac*.

It is difficult to explain the behaviour of two mutants, 240*drp*1 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 240*drp*2. No exclusion was shown in matings between strains carrying R1*drd*19 and either R100-1 or 240*drp*2 (although R1*drd*19 was excluded by a strain carrying R1*drd*19 itself).

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

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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_2 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 R1*drd*19 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, R136*drd*H8, 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 *fi*$^+$ 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, 1965*a*, *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 36 (= *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_2 (Dettori, Maccacaro & Piccinin, 1961). By contrast, 337 *S. typhimurium* clones that had received F-*lac* alone were resistant to μ_2, although K12 carrying F-*lac* only is sensitive to μ_2. 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 effected by the K determinant. The reactions of *S. typhimurium* and K12 lines with phage μ_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*

Cross: Donor	Cross: Recipient	Frequency of F-*lac* transfer	Sensitivity of donor to phage μ_2	Proportion of donor cells carrying F fimbriae (electron microscopy)
S. typhimurium KF-*lac*	× K12	1×10^{-3}	+	18/28 = 64 %
	× *S. typhimurium*	2×10^{-3}		
S. typhimurium F-*lac*	× K12	2×10^{-5}	−	1/36 = 2·8 %
	× *S. typhimurium*	2×10^{-5}		
S. typhimurium (K)⁻ F-*lac**	× K12	1×10^{-3}	+	21/47 = 44·7 %

* Resulting from spontaneous loss of K from *S. typhimurium* KF-*lac*.
+ = Visible lysis in spot tests with phage μ_2 on surface culture (Grindley *et al.* 1970);
− = No visible lysis with μ_2.

Table 2. *Reactions of* S. typhimurium *and K12 strains with male-specific phage* μ_2

Strains	Reaction with μ_2
S. typhimurium F-*lac*	−
S. typhimurium KF-*lac*	+
S. typhimurium (K)⁻ F-*lac**	+
S. typhimurium (KF-*lac*)⁻†	−
K12 F-*lac*	+
S. typhimurium	−
K12 F⁻	−

+ = Visible lysis with μ_2; − = No visible lysis with μ_2.
* 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 miscroscope (see Table 1). Specific adsorption of phage μ_2, 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 F
binants in which F-*lac* was r
tested retained the derepressi
overnight cross, the frequenc
8×10^{-3}. This frequency is ver
in overnight crosses to *S. typ*

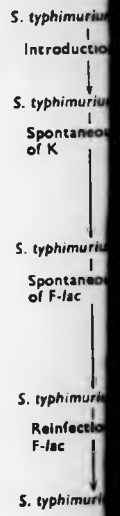

Fig. 1. Proportion of F-*la*
male-specific phage μ_2. **
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Cross		Frequency of F-*lac* transfer	Sensitivity of donor to phage μ_2	Proportion of donor cells carrying F fimbriae (electron microscopy)
ior	Recipient			
n KF-*lac*	× K12	1×10^{-3}	+	18/28 = 64 %
	× *S. typhimurium*	2×10^{-3}		
n F-*lac*	× K12	2×10^{-5}	−	1/36 = 2·8 %
	× *S. typhimurium*	2×10^{-5}		
m (K)⁻ F-*lac**	× K12	1×10^{-3}	+	21/47 = 44·7 %

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Table 2. *Reactions of* S. typhimurium *and K12 strains with male-specific phage* μ_2

Strains	Reaction with μ_2
S. typhimurium F-*lac*	−
S. typhimurium KF-*lac*	+
S. typhimurium (K)⁻ F-*lac**	+
S. typhimurium (KF-*lac*)⁻†	−
K12 F-*lac*	+
S. typhimurium	−
K12 F⁻	−

+ = Visible lysis with μ_2; − = No visible lysis with μ_2.
* Spontaneous loss of K.
† Spontaneous loss of both K and F-*lac*.

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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 2×10^{-1} in an overnight cross, the frequency of transfer of derepression was 4 % of 2×10^{-1}, that is, 8×10^{-3}. This frequency is very similar to that of K transfer from *S. typhimurium* KF-*lac* in overnight crosses to *S. typhimurium*, about 10^{-2}.

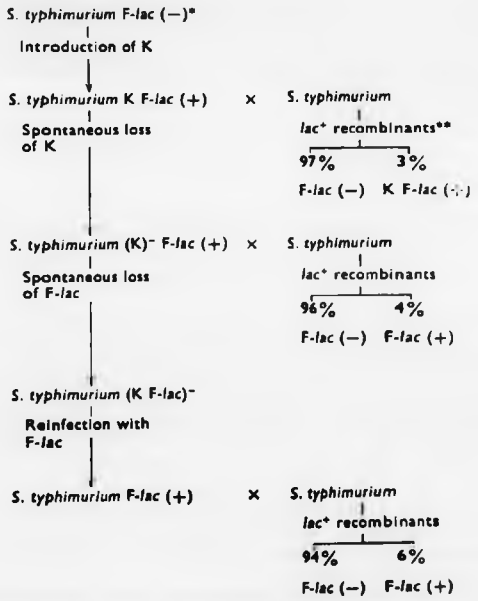

Fig. 1. Properties of F-*lac* and K in *S. typhimurium*. * (+); (−): reactions with malo-specific phage μ_2. ** F-*lac* was transferred at a frequency of *c.* 2×10^{-1} in overnight crosses in all cases.

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_2 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, $1{\cdot}8 \times 10^{-1}$, 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.* 1×10^{-2}).

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. Spontaneous 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 independent plasmids, co-transduction should be a very rare event. Bacteriophage P22 was grown on *S. typhimurium* KF-*lac*. The resulting phage preparation (titre *c.* 8×10^{10} p.f.u./ml) was sterilized with toluene, and 1 ml was mixed with an equal quantity of a culture of *S. typhimurium* F-*lac* (*c.* 5×10^8 organisms/ml). After 30 min at 37 °C the bacteria were washed and plated with selection for kanamycin resistance. Of 150 kanamycin-resistant transductant colonies tested, 16 (10·7 %) were sensitive to the male-specific phage μ_2. This co-transduction supports the suggestion that K and *der* are closely linked.

The possibility that *der* might affect derepressed factors other than F was explored. Two derepressed fi^+ R factors, R1*drd*19 and R136*drd*H8 (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 R136 resistance to tetracyclines only. R1 and R136 were originally identified in strains of *S. paratyphi B* phage type 3a var 4 and *S. typhimurium* phage type 29 respectively (E. S. Anderson & N. Datta, unpublished).

K12 carrying R1 or R136 is sensitive to μ_2. 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 μ_2, 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 μ_2, this lysis was more turbid than that on *S. typhimurium der* R1.

Transfer of R1 from μ_2-insensitive *S. typhimurium* R1 to *S. typhimurium* occurred at a repressed frequency of $2{\cdot}5 \times 10^{-4}$ in 2 h, and $1{\cdot}6 \times 10^{-3}$ overnight. The μ_2-sensitive line of *S. typhimurium* R1, in contrast, transferred R1 at a derepressed frequency of 10^{-1} in 2 h and $4{\cdot}5 \times 10^{-1}$ overnight. The derepression was not transferred, however, since all of 100 progeny tested from the overnight cross were insensitive to μ_2.

S. typhimurium der R1 transferred R1 to *S. typhimurium* at a frequency of $1{\cdot}5 \times 10^{-4}$ in a 2 h cross, and 3×10^{-1} overnight. Ten of 100 colonies from the overnight cross were sensitive to μ_2. 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 μ_2.

The presence of *der* in *S. typhimurium* therefore potentiated derepression of R1*drd*19, as it did of F-*lac* in that host. Since R136*drd*H8 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 R1*drd*19 are i^- mutants which do not synthesize repressor, but are sensitive to repressor in K12, while R136*drd*H8 is an o^c mutant which is insensitive to repressor in K12 (Fr
The possibility that *S. typhimuriu*
therefore be explored.

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[*Note added in proof.*] Recent experi
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We thank Dr Anne M. Field for c
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that *der* might affect derepressed factors other than F was explored.
f+ R factors, R1*drd*19 and R136*drd*H8 (Meynell & Datta, 1967) were
e will be referred to as R1 and R136 hereafter. R1 carries resistance to
ycin, streptomycin and sulphonamides, and R136 resistance to tetra-
and R136 were originally identified in strains of *S. paratyphi B* phage
d *S. typhimurium* phage type 29 respectively (E. S. Anderson & N.
ed).

1 or R136 is sensitive to μ_2. K12 R1 was crossed with *S. typhimurium*
m der, to yield *S. typhimurium* R1 and *S. typhimurium der* R1 progeny
y-nine of 50 colonies of *S. typhimurium* R1 gave no visible lysis with
S. typhimurium der R1 colonies gave good lysis with the phage. In the
himurium R1 which gave lysis with μ_2, this lysis was more turbid
yphimurium der R1.

from μ_2-insensitive *S. typhimurium* R1 to *S. typhimurium* occurred at a
ey of $2{\cdot}5 \times 10^{-4}$ in 2 h, and $1{\cdot}6 \times 10^{-2}$ overnight. The μ_2-sensitive line of
1, in contrast, transferred R1 at a derepressed frequency of 10^{-1} in 2 h
ight. The derepression was not transferred, however, since all of 100
he overnight cross were insensitive to μ_2.

R1 transferred R1 to *S. typhimurium* at a frequency of $1{\cdot}5 \times 10^{-1}$
10^{-1} overnight. Ten of 100 colonies from the overnight cross were
er was transferred by R1 at a frequency of about 10^{-2}, similar to
F-*lac*.

nsferred from K12 to *S. typhimurium* and *S. typhimurium der*, all
36 and *S. typhimurium der* R136 progeny tested (20 of each) gave

S. typhimurium therefore potentiated derepression of R1*drd*19,
ost. Since R136*drd*H8 is already derepressed in *S. typhimurium*,
its state of derepression was affected by *der*.

hat F and R1*drd*19 are *i*− mutants which do not synthesize
to repressor in K12, while R136*drd*H8 is an *o*^{c} mutant which

is insensitive to repressor in K12 (Frydman & Meynell, 1969; Meynell & Cooke, 1969). The possibility that *S. typhimurium* actively represses both F and R1*drd*19 must therefore be explored.

Alternatively, *S. typhimurium* may be (passively) unable to express the derepressed state of F-*lac* and R1*drd*19 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* R1*drd*19 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 R1*drd*19 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 o^c mutants of F-like plasmids. This work is being prepared for publication.

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SHORT PAPER

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

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SUMMARY

Derepressed mutants of F-like transfer factors, isolated by mutagenesis, were characterized as repressor-minus (i^-) or operator-constitutive (o^c). Mutants of the i^- class are derepressed in K 12 but repressed in *Salmonella typhimurium*. They are derepressed in *S. typhimurium* by a kanamycin resistance determinant carrying a locus *der*, described previously. Most o^c mutants of F-like factors are derepressed in both K 12 and *S. typhimurium*. However, one mutant of F-*lac* was o^c in K 12 but was repressed in *S. typhimurium*. It was derepressed by *der*. Repression by *S. typhimurium* is different from that by fi^+ 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 36 (= *S. typhimurium*) on an F-*lac* factor and on the derepressed F-like R factors R1*drd*19 and R136*drd*H8. *S. typhimurium* carrying either F-*lac* or R1*drd*19 showed poor donor ability and was insensitive to the male-specific phage μ_s; that is, the factors were repressed in the *S. typhimurium* host. In contrast, R136*drd*H8 remained derepressed in *S. typhimurium*. The fact that F and R1*drd*19 are repressor-minus (i^-) mutants while R136*drd*H8 is an operator-constitutive (o^c) 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 o^c 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 R1*drd*19 (Smith *et al.* 1970). K could also be transferred by either F-*lac* or R1*drd*19 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 R1*drd*19 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 o^c mutants of F-*lac*. It should be borne in mind that F-*lac* is i^-, so that o^c mutants of F-*lac* are both i^- and o^c. 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* K 12 (= K 12) 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 M 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 μ_2. To determine whether the derepressed R factors were i^- or o^c, they were transferred into a K12 strain carrying the factor 782, which also confers resistance to kanamycin. Factor 782 is incompatible with the fi^- I-like factor Δ 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 fi^+, 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^c. 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^c mutants, we also obtained i^- mutants in this way.

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

Ref. no.	Resistance*	Derepressed mutants	
		i^-	o^c
334†	ACSSu	.	334o^c1
R 1†	ACKSSu	R 1*drd*19	.
240‡	T	240i^-1 and 2	240o^c1 to 8
F-*lac*§	.	F-*lac*	F-*lac*o^c1 to 3

* A, Ampicillin; C, chloramphenicol; K, neomycin–kanamycin; S, streptomycin; Su, sulphonamides; T, tetracyclines.

† R factors 334 and R 1 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^c mutants of F-*lac* were isolated by a modification of the method of Frydman *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*-Str^r* 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*-Nal^r recipient strain 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 μ_2. The majority of colonies tested were sensitive to phage μ_2 in spite of the presence of the fi^+ factor 782, and therefore carried F-*lac* o^c mutants. The three F-*lac*o^c mutants studied were isolated in independent derepression experiments. They were separated from factor 782 by interrupted crosses into K12F$^-$-*lac*-Str^r.

* Str^r = streptomycin-resistant mutant; Nal^r = nalidixic acid resistant mutant.

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 ed colonies were detected by their sensitivity to phage μ_2. To determine repressed R factors were i^- or o^c, they were transferred into a K12 strain ctor 782, which also confers resistance to kanamycin. Factor 782 is ith the fi^- I-like factor Δ of Anderson & Lewis (1965). Strains carrying agate the I-specific phage If1. However, 782 represses the fertility and age sensitivity of strains carrying the F factor; that is, it is fi^+, in spite of 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 , it is useful for testing the repressor sensitivity of derepressed F-like essed mutants which were repressed by factor 782 were designated i^-, t remained derepressed were designated o^c. The results were confirmed by effect of the derepressed mutants on the fertility of HfrH. Although direct repression after mutagenesis yields mainly o^c mutants, we also obtained this way.

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

		Derepressed mutants	
no.	Resistance*	i^-	o^c
††	ACSSu	.	334o^c1
†	ACKSSu	R1*drd*19	.
‡‡	T	240i^-1 and 2	240o^c1 to 8
lac§	.	F-*lac*	F-*lac*o^c1 to 3

illin; C, chloramphenicol; K, neomycin–kanamycin; S, streptomycin; Su, ; T, tetracyclines.

334 and R1 were isolated from a strain of *S. paratyphi B* BB7268. Factor 334 is ensitive segregant of the original R factor which carried the resistances Pitton & Anderson, 1970).

240 was freshly isolated from its wild host strain of *S. typhimurium* 3M4466. An n (E. S. Anderson and N. Datta, unpublished) was designated R136 (Meynell &

factor of Jacob and Adelberg (1959), supplied by Professor W. Hayes.

ants of F-*lac* were isolated by a modification of the method of Frydman K12F-*lac* carrying 782 was treated with EMS or NG as described above. then incubated overnight in nutrient broth and crossed in a ratio -Str^r* recipient carrying 782. Mating was interrupted at 30 min, mating mixtures were diluted into 100 ml of broth containing in. After 3 h incubation at 37 °C, 1 ml quantities were subcul- inimal medium containing 500 μg streptomycin/ml and with carbon source. The mixture was allowed to grow overnight. re then crossed in a ratio of 20:1 with a K12F$^-$-*lac*-Nal^r reci- . After 30 min, suitable dilutions were plated on MacConkey l of nalidixic acid; the plates were incubated overnight at olonies were then purified and tested with male-specific f colonies tested were sensitive to phage μ_2 in spite of the 2, and therefore carried F-*lac* o^c mutants. The three F-*lac*o^c ted in independent derepression experiments. They were interrupted crosses into K12F$^-$-*lac*-Str^r.

istant mutant; Nal^r = nalidixic acid resistant mutant.

With the exception of R1*drd*19, 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 μ_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 R1*drd*19 and 240i^-1 and 2 confer sensitivity to phage μ_2 on K12F$^-$, and are repressed by factor 782 in the same host. They do not significantly reduce the fertility of HfrH. The o^c mutants, in contrast, while conferring μ_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*

Derepressed factor	Sensitivity to μ_2 of K12 carrying the factor	Sensitivity to μ_2 of K12 carrying the factor & 782	Transfer frequency of *pro* from HfrH + factor (HfrH = 1)
334o^c1	+	+	0·001
R1*drd*19	+	−	0·5
240o^c1 to 8	+	+	0·07
240i^-1 and 2*	+	−	0·5–1
F-*lac*o^c1 to 3	+	+	.
F-*lac*	+	−	.

+, Visible lysis with μ_2; −, no visible lysis with μ_2.

* The derepressed mutants 240i^-1 and 2 gave good lysis with μ_2 only when grown without shaking. Consequently all μ_2 phage sensitivity tests of strains carrying these factors were carried out on unshaken cultures.

Table 3. *Sensitivity to phage μ_2 of* Salmonella typhimurium *and* S. typhimurium *K der carrying derepressed factors*

Derepressed factor	Type in K12	Sensitivity to μ_2 In *S. typhimurium*	Sensitivity to μ_2 In *S. typhimurium* K *der*
334o^c1	o^c	+	NT
R1*drd*19	i^-	−	+
240o^c1 to 8	o^c	+	NT
240i^-1 and 2	i^-	−	+
F-*lac*o^c1	o^c	−	+
F-*lac*o^c2 and 3	o^c	+	NT
F-*lac*	i^-	−	+

NT, Not tested; +, visible lysis with μ_2; −, no visible lysis with μ_2.

All the derepressed mutants were transferred into *S. typhimurium* and the resulting progeny were tested with phage μ_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^c R factor mutants and two of the three F-*lac*o^c mutants were derepressed in *S. typhimurium*, although one mutant of F-*lac* which was o^c in K12 was repressed in *S. typhimurium*. All the factors repressed in *S. typhimurium* alone were derepressed when that host carried *der*.

When the *fi*+ 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 μ_2. Thus, *der* has no influence on the repressor activity of an *fi*+ R factor. The repressor activity of *S. typhimurium* on *i*− derepressed mutants, which is reversed by *der*, is therefore different from that of the *fi*+ 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 the *fi*+ repressors encoded by 334 and 782, 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 *fi*+ 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 R1*drd*19 is transferred to *S. typhimurium*, although the majority of recombinant colonies are resistant to μ_2, 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 clones, reinfection with F-*lac* or R1*drd*19 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^c 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^c 1 has a mutation in the operator rendering it insensitive to the *fi*+ 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^c 1 is reversed by *der*, as would be expected. These results suggest that o^c 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^c 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^c in one host, may be repressed by another.

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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-12F$^+$, 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 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 with the F factor and F*lac* (27). Watanabe et al. (29) showed that pairs of "fertility inhibition$^+$" (*fi*$^+$) R factors were incompatible but that *fi*$^+$ and *fi*$^-$ plasmids were stably maintained in the same cell. Studies on the *fi*$^-$ I-like plasmid, Δ, and its derived R factor, T-Δ (4), showed that these two plasmids were incompatible (1). Incompatibility has also been demonstrated for nontransferring 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 phages μ2 and fd or the I-specific phage If1. 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-12F$^+$. 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, φ2 (11), and the I-specific phage, If1 (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. Broth cultures of donor and recipient strains containing about 2×10^8 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 on to nutrient agar containing the relevant antibiotics. Strains which were stable for the resistances of the

TABLE 1. *Standard strains of Escherichia coli K-12*

No.	Description[a]
14R525	K-12λ[−] prototrophic Nal[r]
40R850	14R525 carrying F[+]
34R649	K-12F *lac* Str[r]
30R893	K-12F*lac*T[+]
32R1000	K-12HfrH Nal[r]

[a] Nal[r], Nalidixic acid-resistant mutant; Str[r], streptomycin-resistant mutant.

[b] The F factor from strain 58-161 (20).

[c] F*lac*T is a recombinant between F*lac* and the tetracycline (T) resistance marker of the I-like R factor T-Δ (4, 6).

two plasmids were tested in crosses with K-12F (34R649) 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, μ2, and the female-specific phage, φ2. K-12F[+] or Hfr strains are fully sensitive to μ2 but restrict visible lysis by φ2. Strains that have lost F are, like the K-12F[−] control, resistant to μ2 but fully sensitive to φ2. 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 μ2 or the I-specific phage If1 was tested as described previously (15).

DNA reassociation experiments. The DNA of each plasmid was labeled with ^{3}H-thymidine, extracted with sarkosyl lysis, and separated from chromosomal DNA by caesium chloride-ethidium bromide density gradient centrifugation, as described earlier (17). The methods of preparing the unlabeled DNA from plasmid-bearing strains and of measuring reassociation of denatured DNA from different plasmids have also been described (17).

RESULTS

The four R factors listed in Table 2 have already 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 μ2 or the I-specific phage, If1. The R factors were transferred into HfrH, and the resulting strains were tested for sensitivity to phage μ2. 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 TP116 and TP117 have already been published (16).

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^{-3} or as low as 10^{-7}.

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, μ2, and the female-specific phage, φ2. The progeny were also tested for the

TABLE 2. *H group R factors*

Plasmid no.	Drug resistance[a]	Species of original host and place of origin	Phage type of original host strain	ERL reference no.
TP116	CSSu[c]	*Salmonella typhi* Spain 1969	C1	1T3616
TP117	T	*S. typhimurium* England 1961	1a var 2	1M1407
TP123	CSSuT[c]	*S. typhi* Mexico 1972	Degraded Vi	1T4739
TP124	CSSuT[c]	*S. typhi* India 1972	D1-N	1T4754

[a] Symbols for drug resistances: C, chloramphenicol; S, streptomycin; Su, sulphonamides; T, tetracyclines.

[b] Strains carrying the R factors TP116, 123, and 124 are also resistant to spectinomycin. This suggests that these plasmids code for an adenylate synthetase which inactivates both streptomycin and spectinomycin (7). In this paper we use the symbol S for resistance to both streptomycin and spectinomycin.

[c] See Anderson and Smith (5).

TABLE 3. *Incompatibility of*

Plasmid in donor strain	Plasmid in recipient strain	Selected marker	
TP116 (CSSu)	TP117 (T)	C	
TP116 (CSSu)	TP124 (T)	C	
TP123[a] (T)	TP116 (CSSu)	T	
TP123[a] (CSSu)	TP117 (T)	C	
TP123[a] (CSSu)	TP124[a] (T)	C	
TP124[a] (CSSu)	TP117 (T)	C	

[a] Unstable, rapidly segregati

[b] Appropriate segregants of T used for these experiments. Th result of spontaneous loss of re

[c] Stable hybrids.

selected and any unselected (Table 4).

Transfer of the plasmids TP124 to K-12F[+] resulted the F factor in the great ma progeny of these crosses in w resistant to phage μ2 but full would be expected. In contr of TP116 into K-12F[+] did ment of F. Lines carrying were stable and transferred TP116 to K-12F[−] in 1-h cro

In a separate experiment transferred to K-12F[+], 16 tested still carried F and the all these lines had lost the cline resistance marker of resistance to chloramphen and sulphonamides. The were F[−] and carried all the of TP124. An exconjugant T[−] segregant of TP124 was orange to eliminate the F f had been confirmed, the transferred to K-12F[+]. The fully sensitive to phage μ2, was no displacement of F. which determines incompa thus been lost with the te marker. This T[−] segregant

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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, $\mu 2$, and the female-specific phage, $\varphi 2$. The progeny were also tested for the

TABLE 2. *H group R factors*

Plasmid no.	Drug resistance*	Species of original host and place of origin	Phage type of original host strain	ERI reference no.
TP116	C88u*	*Salmonella typhi* Spain 1969	C1	1T3616
TP117	T	*S. typhimurium* England 1961	Ia var 2	1M1407
TP123	C88uT*	*S. typhi* Mexico 1972	Degraded Vi	1T4739
TP124	C88uT*	*S. typhi* India 1972	D1 N	1T4754

* Symbols for drug resistances: C, chloramphenicol; S, streptomycin; Su, sulphonamides; T, tetracyclines.

† Strains carrying the R factors TP116, 123, and 124 are also resistant to spectinomycin. This suggests that these plasmids code for an adenylate synthetase which inactivates both streptomycin and spectinomycin (7). In this paper we use the symbol S for resistance to both streptomycin and spectinomycin.

‡ See Anderson and Smith (5).

TABLE 3. *Incompatibility of group H plasmids*

Plasmid in donor strain	Plasmid in recipient strain	Selected marker	Progeny carrying both resistance markers after selection for the incoming R factor	Progeny retaining both resistance markers after further selection (%)
TP116 (CSSu)	TP117 (T)	C	0/5	0.5[a]
TP116 (CSSu)	TP124 (T)	C	0/10	1.0[a]
TP123[b] (T)	TP116 (CSSu)	T	0/10	0
TP123[b] (CSSu)	TP117 (T)	C	0/10	100 (0)
TP123[b] (CSSu)	TP124[b] (T)	C	2/10	100 (0)
TP124[b] (CSSu)	TP117 (T)	C	2/10	100 (0)

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

selected and any unselected resistance transfer markers (Table 4).

Transfer of the plasmids TP117, TP123, 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 lost were resistant to phage μ2 but fully sensitive to phage f2, as would be expected. In contrast, the introduction of TP116 into K-12F⁻ did not cause displacement of F. Lines carrying both these plasmids were stable and transferred F independently of TP116 to K-12F⁻ in 1-h crosses.

In a separate experiment in which TP124 was transferred to K-12F⁻, 16 of 30 exconjugants 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 14 lines were F⁻ and carried all the resistance transfer markers of TP124. An exconjugant line carrying R' F⁻ and a T⁻ segregant of TP124 was treated with acridine orange to eliminate the F factor. After loss of F had been confirmed, the CSSu R factor was transferred to K-12F⁻. The progeny were still fully sensitive to phage μ2, indicating that there was no displacement of F. The region of TP124 which determines incompatibility with the F has thus been lost with the tetracycline resistance marker. This T⁻ segregant of TP124 was, however, 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 TP123 or TP124 in 1-h crosses. *Flac*T (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 in the recipient strains. In contrast with the T⁻ segregants of TP124 which were compatible with F as described above, these spontaneous T⁻ segregants of TP123 and TP124 displaced F when they were transferred to K-12F⁻.

There was no surface exclusion of *Flac*T by strains carrying TP116, TP123T⁻, or TP124T⁻. The frequency of transfer of *Flac*T to these strains was approximately 2×10^{-1} 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 *Flac*T were stable and showed independent transfer of *Flac*T to K-12F⁻. When strains carrying TP123T⁻ were superinfected with *Flac*T, the resulting progeny carried both plasmids. Segregation tests on these lines, however, showed no loss of TP123T⁻, but *Flac*T was lost from most of the lines, although a minority (about 10%) were stable for both plasmids and transferred them independently into new recipients. No spontaneous loss of *Flac*T was detected in the control experiment in which this plasmid was transferred to K-12F⁻.

The findings with TP124T⁻ and *Flac*T were identical with those described for TP123T⁻ and *Flac*T.

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

TABLE 4. *Transfer of H group R factors to K-12F⁻*

Plasmid in donor strain	Plasmid in recipient strain	Selected marker	Progeny carrying both F and the incoming plasmid
TP116	F⁻	C	30/30
TP117	F⁻	T	0/30
TP123	F⁻	C	0/30
TP124	F⁻	C	0/30[a]

[a] A separate experiment with TP124 gave different results from those shown in the Table (see text).

TABLE 5. *Homology between the F factor and H group plasmids*[a]

Unlabeled DNA from strains bearing plasmids	Labeled plasmid DNA			
	F	TP116	TP117	TP124
F	100	0	0	0
TP116	1	100	2	4
TP117	1.5	3	100	87
TP123	0	0.9[b]	98[b]	95
TP124	3.7	2	95	100

[a] The values indicate the degree of reassociation at 75 C of ^{3}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 μm with a standard deviation of 1.0. This is equivalent to a mol wt of 59 × 10^6 assuming that 1 μm = 2.07 × 10^6 daltons (24). Molecular weights of the group H plasmids, calculated by the same method, are as follows: TP116, 144 × 10^6; TP117, 112 × 10^6; TP123, 123 × 10^6; TP124, 120 × 10^6 (17).

[b] 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; TP116; and TP117, 123, and 124. This suggests that these groups are basically unrelated to each other.

In the DNA reassociation experiments, about 45% of ^{3}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 R 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 F*lac*T was introduced into lines carrying the spontaneous T$^-$ segregants of TP123 and 124, 90% of the progeny subsequently lost F*lac*T. About 10% of the progeny of these crosses, however, were stable F*lac*T/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 (personal communication), who showed that a CSSuT resistance factor caused displacement of F. This R 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

the effect of introducing TP117 TP124 into K-12F$^+$, which result F factor, superinfection of Hfr str H group factors yielded stable hy ably the chromosomal integrati lines protects it from "displace group H plasmids.

The displacement of F by ce may result in misinterpretation bition tests where K-12F$^+$ is use R27 (25) was isolated from the sa *typhimurium* (1M1407) as TP originally described as *fi*$^+$, but it its host to support multiplica I-specific phages. Since the *fi* apparently determined by transf K-12F$^+$ and testing the resultin F-specific phage, it seems proba our results with TP117, that R27 F rather than inhibited its fe ments on the inhibition of transf led Harden and Meynell (19) strains carrying R27 had F-like R100 serotype although they demonstrate F-specific phage strains carrying R27. Our resul indicate that it is an *fi*$^-$ H group minimal homology with F or wi factor (17). This suggests that drawn from serological studies Meynell may be erroneous.

DNA reassociation experime that there is very little homology the four group H plasmids. The group H factors to displace F ca be explained in terms of genetic displacement of a plasmid of on factor belonging to another grou reported by Coetzee et al. (10) plasmid, R394, isolated from *P* dislodged resident R factors of t further experiments showed th plasmids could stably coexist an independently. When R394 wa strains carrying N group plasmi ance markers from both classes also observed.

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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 *fi*$^+$, but it did not enable its host to support multiplication of F- or I-specific phages. Since the *fi* 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 antiserum led Harden and Meynell (19) to suggest that strains carrying R27 had F-like fimbriae 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 *fi*$^-$ 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 reassociation 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 Coetzee 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.

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Chloramphenicol Resistance in the Typhoid Bacillus

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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, 1965), and Greece and Israel (Kontomichalou, 1967; Sompolinsky *et al.*, 1967). The resistance in the last two instances was caused by a transferable extrachromosomal 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 μg/ml with the technique routinely used in 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 μg/ml. The other Chilean culture belonged to Vi—phage type A, and its chloramphenicol M.I.C. was 12·5 μg/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 μg/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 E1, 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 μg/ml, chloramphenicol 250 μg/ml, and tetracyclines 62·5 μg/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 3×10^{-3} to the type strains of Vi—types A and E1, 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 C1 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^{-4}) into *E. coli* K12, and thence at only about 10^{-6} into Vi—types A and C1 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 C1 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 μg/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 μg/ml.

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This R factor transfers from the resistant *S. typhi* to *E. coli* K12 at a frequency of about 10^{-4}, and from *E. coli* K12 to Vi—type A of *S. typhi* at about 10^{-6} in overnight crosses. Like the CSSu factor described above, which came from Spain, it is *fi*⁻ 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 *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 *S. typhi* originated in one of the non-pathogenic enterobacteria.

The ultimate appearance of epidemic strains of *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 *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 *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 *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 *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.

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