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OF THESIS/TITRE DE LA THÈSE KILLING OF KLEBSIELLA PNEUMONIAE
PROMOTED BY INC N GROUP AND CLOSELY
RELATED PLASMIDS
UNIVERSITY CARLETON
DEGREE OF THESIS WAS PRESENTED/
DEGRÉ POUR LEQUEL CETTE THÈSE FUT PRÉSENTÉE M.Sc
DEGREE CONFERRED/ANNÉE D'OBTENTION DE CE GRADE 1981
SUPERVISOR/NOM DU DIRECTEUR DE THÈSE V. IYER

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KILLING OF KLEBSIELLA PNEUMONIAE PROMOTED BY
IncN GROUP AND CLOSELY RELATED PLASMIDS

by

Margarita Rodriguez Iglesias

A thesis submitted to the Faculty of
Graduate Studies and Research in partial fulfillment
of the requirements for the degree of
Master of Science

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May 4, 1981.

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ABSTRACT

E. coli strains carrying conjugative IncN plasmids have been shown to mediate killing of Klebsiella pneumoniae strain M5a1 during conjugation. Several procedures that prevented conjugation also prevented killing. Reduction of this killing ability upon treatment of the donor with nalidixic acid indicated that cell contacts between M5a1 and N^+ cells was an insufficient cause of killing. Some other aspect of the conjugation process, or events associated with the intracellular expression of the plasmid genome in M5a1 is required for killing. Plasmids of the groups P and W, related to the N group also kill M5a1 but to a lesser degree.

The basis of immunity to killing is the ability of the plasmid to induce a change in M5a1, which is expressed even upon curing of the plasmid.

The potential diagnostic use of the Kil^+ phenotype in the screening of N, P and W group plasmids in natural bacterial isolates is discussed.

ACKNOWLEDGEMENTS

I have not enough words to express my sentiments of gratitude to all those people who have given me help, encouragement and moments of their valuable time, making possible the realization and completion of this work.

I would like to express my special thanks to Dr. Iyer for providing me with a sterling example in work organization, for his wise suggestions and criticism throughout my work; as well as his help in the preparation of this thesis.

I would like also to thank Dr. Yamazaki and Dr. Neelin of the Department of Biology for their suggestions, advice and the critical reading of the first draft of this thesis.

The financial support that I received from Fundacion Gran Mariscal de Ayacucho is most gratefully acknowledged.

A TI TOÑITA, PORQUE TE
DEBO TODO CUANTO SOY

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LIST OF ABBREVIATIONS

The genetic abbreviations used are in accordance with the designations suggested by Novick et al. (1976).

A	Adenine
Ap ^r	Ampicillin
C	Cytosine
Col	Colicin plasmid
dNTP	Deoxyribonucleotide
E	Electrophoresis buffer
<u>E. coli</u>	<u>Escherichia coli</u>
EDTA	Ethylene diamine tetraacetate
Et Br	Ethidium bromide
F ⁺	F plasmid-bearing
F ⁻	Devoid of an F plasmid
G	Guanine
Hfr	Strain harbouring a conjugative plasmid integrated into the chromosome thereby promoting chromosomal transfer
Inc	Plasmid incompatibility group
K ⁺	Kill proficient strain
K ⁻	Kill deficient strain
<u>K. pneumoniae</u>	<u>Klebsiella pneumoniae</u>
λ	Bacteriophage lambda
u	Micro (10 ⁻⁶)

uCi	Microcurie
ug	Microgram
ul	Microlitre
NaI	Nalidixic acid
PA	Penassay agar
PB	Penassay broth
PLG	Polyethylene Glycol
r	Resistance
R ⁺	R plasmid-bearing
R ⁻	Devoid of R plasmid
SDS	Sodium Dodecyl Sulfate
Sm ^r	Streptomycin resistance (plasmid specified)
Sp ^r	Spectinomycin resistance
Str ^r	Streptomycin resistance (chromosomal specified)
T	Thymine
TE	Tris-HCl, EDTA buffer
Tra ⁺	Transfer proficient
Tra ⁻	Transfer deficient
TYS	Tryptone, Yeast extract, Sodium chloride
UV	Ultraviolet radiation
w/v	Weight per unit volume

INTRODUCTION

Plasmids are extrachromosomal elements consisting of covalently-closed circular double-stranded DNA. The first evidence about the presence of these elements in bacterial cells arose from genetic experiments in which it was shown that a bacterial strain was capable of transferring a genetic characteristic to another bacterial strain of the same species by conjugation (Løderberg et al., 1952; Hayes, 1953). In this instance, not only was the plasmid conjugative but it also conferred other distinct phenotypes to its host, phenotypes by means of which the presence and transfer of the plasmid could be traced. On the other hand, there are plasmids which are not conjugative and several that do not confer a recognizable phenotype to their host. Such plasmids have been called cryptic plasmids (Novick et al., 1976). Plasmids of the latter type are more difficult to detect and to study; therefore, the use of methods for their physical isolation, characterization and manipulation have become important.

DNA molecules have been isolated using the technique of sedimentation equilibrium in a density gradient of cesium chloride (CsCl), a low molecular weight and high density salt (Meselson et al., 1958). The DNA in this gradient will band at a position in the concentration gradient at which the density of the macromolecule equals the density of the solution (Freifelder, 1976).

Plasmid molecules which have the same G+C (Guanine+Cytosine) content as the chromosomal DNA, and therefore the same buoyant density, can be separated if the densities of the two DNAs are differentially altered by using intercalating dyes such as ethidium bromide or propidium diiodide (Hudson et al., 1968; Clewell and Helinski, 1969). The method takes advantage of the differences in the configuration of plasmid and chromosomal DNA upon isolation. Usually, some of the plasmid's DNA molecules can be released from the cells as covalently-closed circular structures, (Helinski and Clewell, 1971) whereas all of the chromosomal DNA and some of the plasmid DNA molecules breakdown forming linear or open circular molecules. Radloff et al. (1967) observed that linear and open circular molecules can bind more of the intercalating dyes than covalently-closed circular molecules, and because the buoyant density of the dye-DNA complex is inversely related to the amount of dye bound, plasmid DNA which is denser can be easily separated from less dense molecules (open circular and linear forms).

Although this method has been used often for the separation of plasmid DNA molecules from chromosomal DNA of the same density, it suffers from a major limitation that increases with the size of the plasmid. Large plasmids are more susceptible to breakdown in the extraction procedure. The introduction of even a single nick into one of the two

strands of DNA, alters its supercoiled nature and hence its buoyant density, thus making it more difficult to separate from chromosomal DNA. Such considerations make the quantitative estimation of the proportion of plasmid DNA ambiguous when this procedure is used. One approach that overcomes the above limitation is based on the principle of imparting a natural difference in density between plasmid and chromosomal DNA. Since the buoyant density of DNA in CsCl has been found to be a function of its G+C content (Sueoka et al., 1959) plasmid molecules with a 50% G+C content could be isolated in a CsCl gradient from bacterial species having a different average chromosomal G+C content e.g. Serratia marcescens 58% G+C (Marmur et al., 1961) or Proteus mirabilis 39% G+C (Falkow et al., 1964).

Bacterial plasmids are classified into different incompatibility or conjugative groups (Datta, 1975). These plasmids often determine conjugative systems that differ in their specificities and which can function independently of one another. Five such conjugative systems are now recognized: F, I, N, P and W (reviewed by Willetts, 1977; see also Morris et al., 1980). Each conjugative system is usually recognized and identified by means of bacteriophages that are specific for bacteria carrying plasmids of that group. It is thought (but not rigorously proven in every case) that all plasmids of a particular conjugative group determine the production of a specific cell surface

mating organelle called the 'sex pilus' which also provides sites for the attachment of particular phages. This may be the basis of the phage-specificity that is seen for each of the five groups.

Among the plasmids of these five groups, those of the groups N, P and W have some common features. Plasmids of all three groups are transferred at very high efficiencies on solid surfaces and poorly in liquid media (Bradley et al., 1980). Although each of these three groups can be distinguished by means of specific phages (Khatoon et al., 1972; Stanisich, 1974; Morris et al., 1980); there are also some bacteriophages whose host-range is broad enough to include bacteria carrying any one of these three groups, but not the other two groups F and I (Olsen et al., 1974; Bradley and Rutherford, 1975). Naturally occurring plasmids of the F and I group are usually repressed in expression of their transfer function, only a very small fraction of the population (about 10^{-5}) conjugating at one time. For these two systems, derepressed mutants had to be isolated and used in order to test for high efficiency transfer or sensitivity to their respective phages. The N, P and W group plasmids on the other hand are not repressed in this manner. The 'sex pili' that have been visualized and described on F⁺ and I⁺ bacteria are long and flexible, while those that have been described for N⁺, P⁺ and W⁺ bacteria are short and rigid (Brinton et al., 1964; Bradley et al., 1980). Taken together, the evidence that has been

summarized above suggests that the conjugation specified by N, P and W group plasmids, although distinguishable from one another, may be sufficiently similar to constitute one interrelated system.

In this laboratory an N incompatibility group plasmid was transferred to the well-known Klebsiella pneumoniae strain M5a1 (chromosomal DNA, 584 G+C; Hiles, B Sc. Honours thesis, Carleton University, 1979) in order to construct a suitable strain for the isolation and estimation of plasmid DNA (Iyer, personal communication). Although such a K. pneumoniae strain carrying the N group plasmid could be constructed, the experiment itself had yielded two unexpected results: the frequency of trans-conjugants was lower than was usually the case for matings involving N group plasmids and more important, the viability of the recipients had been reduced (Iyer and Messmer, personal communication). E. coli strains carrying conjugative plasmids are not normally expected to reduce the viability of the recipient strain in conjugation.

The novelty of this phenomenon involving M5a1, which is referred to as the 'killing phenomenon' and its possible relationship to conjugation was of interest. Studies on the nature and basis of this phenomenon were therefore undertaken. They are described and discussed in this thesis.

LITERATURE REVIEW

The basic observation presented in the 'Introduction' was that a strain of K. pneumoniae was killed during conjugation by a donor strain carrying an N group plasmid. Once the occurrence of the killing phenomenon was confirmed, several possible reasons for it could be proposed. These are listed and discussed in this chapter with an emphasis on the E. coli strain and its plasmids, since it was an E. coli strain which had been initially observed to be responsible for the killing phenomenon.

E. coli strains have been reported to promote killing of related strains by the following processes:

1. Production of antibacterial substances.
2. Production of a defective bacteriophage.
3. Zygotic Induction.
4. Lethal Zygotis.

1. Production of antibacterial substances

Antibacterial agents when produced by bacteria are substances excreted by them which are antagonistic to the growth of other bacteria. Antibiotics are a part of a vast range of natural products known as secondary metabolites, which are produced late in the growth phase, accumulated in substantial quantities and often excreted into the medium (Hammond and Lambert, 1978).

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Antibiotics vary in their chemical composition, and in their specific mechanisms of action. The taxonomic distribution of antibiotic-producing organisms is restricted to relatively few groups: 75% of the described antibiotics are produced by Actinomycetes; 20% are produced by a group of filamentous spore forming moulds (order Aspergillales), and only 5% of the known antibiotics are produced by bacteria; most by members of the genus Bacillus (Hammond and Lambert, 1978). On the other hand, bacteriocins are a large number of antibacterial substances which are produced by different bacterial genera, including Gram Negative as well as Gram Positive bacteria (for a review see Reeves, 1972).

Bacteriocins differ from antibiotics in their restriction of activity to strains of species related to the producing strain and particularly to strains of the same species. In a culture, the production of bacteriocins is confined to few organisms; nonetheless, their proportion can be greatly increased by treatment of the bacteriocinogenic organisms with DNA synthesis inhibitors such as ultraviolet light (Ozeki et al., 1959) and mitomycin C (Dewitt and Helinski, 1965). The production of bacteriocins can be detected on agar plates by allowing the bacteriocin-producing strain to grow to form colonies or streaks of growth, killing the cells with chloroform and overlaying the chloroform-killed stabs with a layer of agar containing a few drops of the sensitive strain. Under this condition,

the production of bacteriocin is indicated by areas of clearing in the place where the sensitive strain overlaps growth of the bacteriocin-producing strain (Fredericq, 1948; as cited by Reeves, 1972).

The best-studied group of bacteriocins are the colicins. These are proteins of a molecular weight in the range of 50,000 to 90,000. All colicins that have been described so far are specified by plasmids. These plasmids may be either conjugative or non-conjugative and when they specify colicin-production they are called colicinogenic factors or Col factors (Hardy, 1975). The presence of a Col factor in bacterial cells depends on their efficient replication and segregation (stability) in the cells. This statement implies that bacteriocins produced by different strains or species could be found in E. coli as long as the relevant plasmid could be maintained in the E. coli strain. The plasmid Clo DF13, is a good example. It produces a bacteriocin called cloacin, so called because it was first observed in Enterobacter cloacae (Stouthamer and Tieze, 1966). The term Clo factor was used for the corresponding plasmid. It was later found that Clo was stably maintained by E. coli and most subsequent studies on it have been carried out in an E. coli K-12 strain (Konings et al., 1977; Van Tiel-Menkveld et al., 1979). Had it been detected first in E. coli, it would have been called a Col factor and the bacteriocin, a colicin.

One interesting aspect of colicin studies is their mechanism of killing, which involves a sequence of molecular interactions, initiated by the adsorption of the colicin molecules to specific outer membrane protein receptors, and terminated by the direct interaction of the colicin with its particular target (Konisky, 1978). The receptors are utilized by the colicin molecules in order to overcome the physical barrier imposed by the outer membrane, and to gain access to the target. Even though the presence of the receptors render the bacteria sensitive to colicin action, the association of these receptors with other important membrane functions seems to be the selective pressure involved in their maintenance. Di Masi et al. (1973) have shown that the outer membrane receptor for all of the E group colicins is the same receptor that is utilized in vitamin B12 uptake. Colicin K receptor has been suggested by Hantke (1976) to be involved in nucleoside uptake; in the case of the colicin I receptor, there is sufficient circumstantial evidence to suggest that its physiological function relates to some aspects of iron metabolism (Konisky et al., 1976; Wayne et al., 1976).

The biochemical mechanism of inhibition is well known for some colicin molecules. Bowman et al. (1971) have shown that protein synthesis is blocked by the action of colicin E3 as a consequence of the cleavage of the 16S ribosomal DNA at the 3' terminus; Colicin E2 blocks cell division

and promotes DNA degradation by independent mechanisms (Beppu et al., 1972). Colicin E1 and K appear to act on the cytoplasmic membrane disrupting energized transport and oxidative phosphorylation (Tokuda and Konisky, 1978; Weiss and Luria, 1978).

2. Production of defective bacteriophages

There are some strains of bacteria which produce spontaneously, or upon induction, defective bacteriophages. The latter are defined as defective particles and contain either all or some phage components, but which fail to form plaques on any known host even at high multiplicities of infection (Garro and Marmur, 1971). Their overt action is so similar to the bacteriocins that defective bacteriophages have also been sometimes referred to in the literature as high molecular weight bacteriocins (Meynell, 1973). Examples of defective bacteriophages are the tail-like particles produced by some Pseudomonas strains called R-type pyocins (Bradley, 1967); the PBSX-like defective bacteriophages produced by Bacillus subtilis (Seaman et al., 1964), and the coliphage 15 from E. coli which was identified by Sandoval et al. (1965) as the antibacterial agent originally referred to as colicin 15.

Another type of phage component which can kill bacterial cells is the bacteriophage ghost. It has been shown by Herriott (1951) that osmotically shocked phages

from the T series (T1, T2 T6) retained the capacity to kill bacteria even though they had lost their infectivity. These empty protein coats inhibit a wide variety of host functions such as colony formation, galactoside induction, DNA synthesis, RNA and protein synthesis (Duckworth, 1970).

3. Zygotic Induction

This mechanism of killing was described by Jacob and Wollman (1961) using Hfr donors (an Hfr is a bacterial strain in which the plasmid F is inserted into the chromosome). In their experiments, an Hfr donor strain, having a lysogenic virus in its genome promoted the killing during mating of a non-immune (non-lysogenic) recipient strain. Transfer of the phage genome from donor to recipient was followed by an immediate induction of phage development in the recipient strain, resulting ultimately in lysis and release of phage particles. The induction occurred because the recipient did not have the repressor protein synthesized by the prophage in the donor which maintained it in an inert repressed state.

Plasmids may carry phages in a lysogenic state and if such a phage genome is transferred with the plasmid to a non-lysogenic recipient the phage could be induced to eventually lyse the cells (Havekes and Hoekstra, 1976).

4. Lethal Zygotis

Conjugation is a type of gene transfer which requires cell to cell contact (Davis, 1950). Of all the known types of gene transfer it is the most sophisticated since the process requires the coordinated synthesis and activity of a set of proteins by the bacterial plasmid in order to catalyze the transfer of genetic material (Achtman and Helmuth, 1974). Even though conjugation has been an important factor in the ecological distribution of plasmids among different bacterial genera, the mating process itself has been reported in some cases to be harmful to recipient strains. Iyer and Bhaskaran (1968) reported a lethal effect during conjugation when a donor strain of Vibrio El Tor was mated with strains of E. coli or Vibrio cholera on solid surfaces (Millipore filters, which was shown to be the best condition for transfer using Vibrio El Tor).

Ou and Anderson (1972) studying conjugation between Hfr and F⁻ strains by micromanipulation observed that bacteria which were in close contact produced a significant proportion of non-viable recipient cells. They also observed that after separating the male Hfr cells from mating pairs, some of the F⁻ recipients showed abnormal growth such as elongated cells and cells with abnormal cell-division patterns.

A lethal effect during conjugation was also reported by Gross (1963). In his experiments the viability

of the recipient was reduced in the mating experiments when high ratios of Hfr to F^- cells were used; the same phenomenon was further characterized and termed as lethal zygotis by Skurray and Reeves (1973a). In their experiments, the lethal effect of multiple matings between Hfr and F^- cells of E. coli was associated with the inhibition of macromolecular synthesis, failure of some active transport mechanisms and leakage of cytoplasmic components to the outside of cells.

There is no direct evidence in any conjugative system concerning how the DNA is being transported across the cell membrane during conjugation. However, some mechanisms have been speculated upon. In the F transfer system, it has been reported (Goldschmidt and Curtis as cited by Achtman and Skurray, 1977) that there is exchange of outer membrane proteins between mating cells which probably indicates membrane fusion. Skurray and Reeves (1973b) proposed that during mating, membrane rearrangements may occur in order to let the donor DNA into the recipient cell. The prolonged existence of those membrane changes in Hfr x F^- matings may result in excessive permeability of the membrane in the recipient which may be the cause of F-mediated lethal zygotis. The failure to observe lethal zygotis in F^+ x F^- mating was rationalized by Skurray and Reeves (1974) on the basis that in F^+ x F^- matings, the F plasmid-associated surface exclusion genes gain rapid entry

into and expression in the recipient, promoting active disaggregation and disrupting potentially lethal cell contacts. In Hfr x F⁻ matings on the other hand, the F-associated surface exclusion genes will be relatively delayed in their transfer and will often not be transferred on account of the spontaneous interruption of the mating process. This situation could thus promote the loss of viability of the recipient if there is a great excess of donors.

Recent observations by Ou (1980) have contradicted some of these earlier observations and shown that loss of viability of a F⁻ population can also occur when this population is mated with a great excess (200:1) of F⁺ cells or when the mating was conducted at relatively low F⁺ to F⁻ ratios (20:1) but in the presence of nalidixic acid. Nalidixic acid is an agent which prevents productive conjugation at some level following the formation of mating partners.

Bacteria cells which survived the killing effect produced by any one of the processes already described were shown to be immune. Immunity is a phenomenon which ensures the protection of an organism against factors usually biological in nature which are otherwise harmful to the organism. Among bacteria, immunity has been described for bacteriophages, for bacteriocins and for the loss of viability of F⁻ cells that occurs in lethal zygotis. In

all of these three cases, a study of immunity provided very useful insights into the mechanisms of killing which are different in each case. For example, a study of the basis of immunity to bacteriophage λ led to the discovery and eventual isolation and understanding of the λ repressor protein (Ptashne, 1971). In the case of the bacteriocin, colicin E3, immunity was caused by a highly acidic protein (determined by the Col E3 plasmid) which acted to inhibit the colicin E3 protein (Jakes et al., 1974; Sidikaro and Nomura, 1974). Immunity to lethal zygotis by Hfr or F⁺ strains resembles the phenomenon described in this thesis as it is also associated with mating. The known aspects of this phenomenon are therefore reviewed here in some detail.

In studies involving lethal zygotis promoted by F⁺ cells, it was found that when an F⁻ cell acquired an F plasmid by conjugation, it was thereafter immune to lethal zygotis; curing of the F plasmid by acridine orange from the immune cells rendered them subsequently sensitive to lethal zygotis. By using F transfer deficient mutants Skurray and Reeves (1976) concluded that the expression of immunity to lethal zygotis depends on two immunity genes that they designated; ilz A and ilz B. ilz A could be located within the F transfer region (a region of the F plasmid comprising a large cluster of genes that are required for the mating process; Willetts and Skurray, 1980), and ilz B, located outside the F transfer region.

tetracycline 20 ug/ml; streptomycin 50 ug/ml; kanamycin 50 ug/ml; spectomycin 50 ug/ml or 90 ug/ml (according to the strain for which it was intended) and nalidixic acid, 50 ug/ml. Antibiotics were donated by Bristol Meyers of Canada, Montreal and vitamins were purchased from Sigma Chemical Co.

R bottom and R top agar were used for phage assay (Miller, 1972). They contained per liter of distilled water: 10 g of Bacto-Tryptone, 1 g Bacto-Yeast extract, 8 g NaCl and Bacto-agar (1.2% for R bottom or 0.8% for R top). After autoclaving, 2 ml of sterile 1 M CaCl_2 solution and 5 ml of sterile, 20% glucose was added to these media.

The broth used to grow all the strains was TYS (Tryptone, Yeast extract, Sodium Chloride). This is basically Luria Broth (LB) described by Rosner (1972). The TYS designation was suggested by Selvaraj (M. Sc. thesis, Carleton University, 1979) because of the differences in the concentration of NaCl, given in recipes for LB in the literature (Miller, 1972; Rosner, 1972). TYS contains per liter of distilled water: 10 g Bacto-tryptone, 5 g yeast extract and 5 g sodium chloride; the pH of the medium was adjusted to 7.0 with 1 M NaOH. For the isolation of plasmid DNA, the bacterial culture medium used to grow the strain was Penassay Broth (Antibiotic medium 3; PB) supplemented with an antibiotic to which the strain was resistant.

MATERIALS AND METHODS

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study, their sources and relevant properties are listed in Table 1.

Bacteriophages

Bacteriophage IKE and PRD1 that are known to be specific for N Incompatibility Group plasmids were used. IKE was from this laboratory and PRD1 was secured from R. Olsen.

Media and chemicals

Penassay Agar (PA) was used as the organic solid medium in all the experiments. It was composed of 17.8 g/l of Antibiotic medium 3 (Difco Laboratories) and 1.5% (w/v) of Difco-Bacto agar.

The minimal medium used was that of Davis and Mingioli (1950), which was prepared by mixing pre-sterilized solutions of inorganic salts with sterilized water-agar (final agar concentration 1.5%); a pre-sterilized glucose solution (0.5%, (w/v)) was used as the carbon source. If required, the minimal medium was supplemented with Millipore filter-sterilized solutions of amino-acids, vitamins and antibiotics. The latter were added to give a final concentration of: amino-acids, 40 ug/ml; vitamin B₁ (thiamine) 5 ug/ml; thymine 10 ug/ml; ampicillin 30 ug/ml;

TABLE 1. A.

Bacterial Strain *

Strain	Relevant genetic markers **	Source or reference if any
<u>E. coli</u> K-12		
C600	Thr ⁻ Leu ⁻ Thi ⁻	Cohen <u>et al.</u> , (1972)
C600-Nal ^r	Thr ⁻ Leu ⁻ Thi ⁻ -Nal ^r	This study - spontaneous Nal-resistant mutant of C600
J5	Pro ⁻ Met ⁻	Datta, (1975)
J5-3 Nal ^r	Pro ⁻ Met ⁻ Nal ^r	Datta, (1975)
JE2571	Thr ⁻ Leu ⁻ Str ^r	Nishimura <u>et al.</u> , (1967)
CR34	Thr ⁻ Leu ⁻ Str ^r	Bonhoeffer, (1966)
C08	Thy ⁻ Arg ⁻ Met ⁻ Pro ⁻ Pur ⁻ Pyr ⁻ His ⁻ Nal ^r	This laboratory
WD00	Thy ⁻ Mal ⁻ Gal ⁻	Ogawa, (1975)
<u>K. pneumoniae</u>		
M5al	Amp ^r	MacNeil <u>et al.</u> , (1978)
M5al-Nal ^r	Amp ^r Nal ^r	This study - spontaneous Nal-resistant of M5al
M6, M7 and M8	Amp ^r	This study; M5al/pCU1 cured of its plasmid

TABLE 1. A. (Continued)

<u>S. typhimurium</u>	His ⁻	Sanderson, Salmonella stock center
LT2		
<u>Proteus mirabilis</u>		
MI3148	Nal ^r , Non-motile	Howe
<u>Pseudomonas aeruginosa</u>		
PA09505	Arg ⁻ Cat ^r Rif ^r	Stanisich

* Only plasmid-free bacterial strains are listed in this table. In addition, a large number of plasmid-carrying strains were constructed and used in this study. The nomenclature used in the text for such plasmid carrying strains is that of the corresponding plasmid-free strain with a slash followed by the name of the plasmid. For example, strain C600/pCU1 is a derivative of C600 carrying pCU1. The properties of the plasmids are shown in Table 1B.

** Abbreviations: Pro, proline; His, histidine; Met, methionine; Thr, threonine; Leu, leucine; Thi, thiamine; Arg, arginine; Pur, purine; Pyr, pyrimidine; Nal, maltose; Gal, galactose; Sm, streptomycin; Nal, nalidixic acid; Cat, chloramphenicol; Rif, rifampicin; Amp, ampicillin; -, nutritional dependency; r, resistance,

TABLE 1. B.

Plasmids Used

Plasmid	Incompatibility		Relevant Markers	Source & Reference
	Group			
RM98	N	Ap ^r Sm ^r Sp ^r Tc ^r	Khatoon <u>et al.</u> , (1972)	
R46	N	Sm ^r Su ^r Sp ^r Tc ^r	Datta, (1975)	
R113	N	Tc ^r	Scavizzi, (1973)	
R199	N	Tc ^r Su ^r	Bannister, (1969)	
R205	N	Ap ^r Su ^r Tc ^r	Bannister, (1969)	
pTM558	N	Ap ^r Km ^r Sm ^r	Babudri & Monti-Bragadin, (1977)	
R48	N	Ap ^r Sm ^r Sp ^r Su ^r Tc ^r	Datta, (1975)	
R45	N	Ap ^r Su ^r Tc ^r	Datta, (1975)	
pDT200	N	Sm ^r Su ^r Tp ^r	Taylor <u>et al.</u> , (1980)	
pCU1	N	Ap ^r Sm ^r Sp ^r	Konarska-Kozłowska & Iyer, (1981a)	
pCU2	Unknown	Tc ^r Sm ^r	Konarska-Kozłowska & Iyer, (1981a)	
pCU4	N	Ap ^r Sm ^r Sp ^r Tc ^r	Konarska-Kozłowska & Iyer, (1981a)	
pCU7	N	Ap ^r Tc ^r	Konarska-Kozłowska & Iyer, (1981a)	
pCU8	N	Ap ^r	Konarska-Kozłowska & Iyer, (1981b)	

TABLE 1. B. (Continued).

pCU10, 11, 12, 13	N(Tra ⁻)	Ap ^r Sm ^r Sp ^r	Konarska-Kozłowska & Iyer (1981b)
pCU16	N	Ap ^r Sm ^r Sp ^r	Konarska-Kozłowska & Iyer (1981b)
pIE305, 330, 331	N	"Cryptic"	Tschape, (1977)
pIE512, 514	N	"Cryptic"	Tschape, (1977)
S-a	W	Cm ^r Kn ^r Sm ^r	Watanabe <u>et al.</u> , (1968)
R411	W	Su ^r Tp ^r	Datta & Hedges, (1972)
R388	W	Su ^r Tp ^r	Datta & Hedges, (1972)
RP4	P	Ap ^r Kn ^r Tc ^r	Datta <u>et al.</u> , (1971)
RK2	P	Ap ^r Sm ^r	Ingram <u>et al.</u> , (1973)
PRK229	P(Tra ⁻)	Pn ^r Tc ^r	Helinski, (1977)
RP1	P	Ap ^r Kn ^r Tc ^r	Stanisich
R386	FI	Tc ^r	Dennison, (1972)
R100-1	FII	Cm ^r Sm ^r Su ^r Tc ^r drd 1	Egawa & Hirota, (1962)
PAR132	FII	Tc ^r drd 1	Chandler <u>et al.</u> , (1977)
R64-11	I	Sm ^r Su ^r Tc ^r drd 11	Meynell & Datta, (1967)
R144-3	I	Kn ^r Tc ^r drd 3	Meynell & Datta, (1967)
RA3	U	Cm ^r	Aoki <u>et al.</u> (1979) as cited by Bradley <u>et al.</u> (1980)

TABLE 1. B. (Continued)

R446b	M	Sm ^r Tc ^r	Hedges & Datta, (1973)
R6K	X	Ap ^r Sm ^r	Kontomichalou, (1970)

* Abbreviations: Ap, ampicillin; Sm, streptomycin; Sp, spectinomycin, Tc, tetracycline; Cm, chloramphenicol; Su, sulfanilamide; Kn, kanamycin; Pn, penicillin; Tp, trimethoprim; Tra, transfer (conjugative); r, resistance; -, deficiency, drd, derepressed (for conjugal transfer).

tetracycline 20 ug/ml; streptomycin 50 ug/ml; kanamycin 50 ug/ml; spectomycin 50 ug/ml or 90 ug/ml (according to the strain for which it was intended) and nalidixic acid, 50 ug/ml. Antibiotics were donated by Bristol Meyers of Canada, Montreal and vitamins were purchased from Sigma Chemical Co.

R bottom and R top agar were used for phage assay (Miller, 1972). They contained per liter of distilled water: 10 g of Bacto-Tryptone, 1 g Bacto-Yeast extract, 8 g NaCl and Bacto-agar (1.2% for R bottom or 0.8% for R top). After autoclaving, 2 ml of sterile 1 M CaCl_2 solution and 5 ml of sterile, 20% glucose was added to these media.

The broth used to grow all the strains was TYS (Tryptone, Yeast extract, Sodium Chloride). This is basically Luria Broth (LB) described by Rosner (1972). The TYS designation was suggested by Selvaraj (M. Sc. thesis, Carleton University, 1979) because of the differences in the concentration of NaCl, given in recipes for LB in the literature (Miller, 1972; Rosner, 1972). TYS contains per liter of distilled water: 10 g Bacto-tryptone, 5 g yeast extract and 5 g sodium chloride; the pH of the medium was adjusted to 7.0 with 1 M NaOH. For the isolation of plasmid DNA, the bacterial culture medium used to grow the strain was Penassay Broth (Antibiotic medium 3; PB) supplemented with an antibiotic to which the strain was resistant.

Preparation of Bacterial Cultures

Bacterial cells were incubated in TYS broth, aerated by agitation in a reciprocating shaker overnight at 37° C. Logarithmic cultures were prepared by diluting the overnight culture 50-fold in fresh broth. The cultures were grown to an optical density (OD) of 0.30 to 0.35 at 590 nm (Gilford spectrophotometer, Stasar II; light path 10 mm). This OD value gave a viable cell count of approximately $2 \text{ to } 3 \times 10^8$ cells/ml. When stationary phase cultures were used, they were diluted 20-fold from the overnight culture and incubated at 37° C in the reciprocating shaker until maximum turbidity was reached.

Mating procedures

a. Spot mating - This procedure was used mainly for strain construction or for screening a large number of derivatives for the conjugal transfer property. An early stationary phase culture of the recipient (0.2 ml) was spread on the dry surface of an agar medium selective for the transconjugants (i.e. a medium on which neither donor nor recipient alone would grow). A drop of an exponential phase donor culture was spotted on the surface of the recipient-seeded agar plate and incubated for 24 to 48 hours at 37° C. Controls of donor and recipient alone were done every time. Transconjugants were purified twice to give single colonies on the selective media and then used to determine the presence

of the nutritional marker of the recipient before they were used in further experiments. When a large number of donor cells had to be tested, a 25-point inoculator designed for this purpose was used (Fig. 1). Each donor strain was incubated into one of the 25 individual compartments of a sterile plastic culture container (ELESA, Milano, Italy), where 0.5 ml of sterile TYS broth was added previously to each compartment. The cultures were incubated for 5 hours at 37° C. Using a sterile 25 multipoint inoculator, drops of donor cells were transferred, first to a PA plate to save them (master plate), then to different selective plates to confirm the markers on the plasmid and lastly to the selective plate where 0.2 ml of recipient had been previously spread. Controls were plated on the selective media for donor and recipient cells.

b. Filter mating - This was used for determining the frequency of transfer of a plasmid from R⁺ strains. An exponential-phase donor culture was mixed with a stationary-phase recipient at a donor to recipient ratio of 1:10. The mixture was immediately collected on a membrane filter (Millipore Corp.; diameter 45 mm, pore size 0.45 μ m) by suction. The membrane filter was placed with the collected bacteria on top in a pre-warmed (37° C) PA plate for one hour. After incubation, the cells were washed off the filter with 2 ml of sterile 0.85% saline by agitation in a Vortex mixer. In all cases, serial 10-fold dilutions of

FIGURE I

The multipoint replicator designed
and used for the rapid screening of
a large number of isolates.

- A. - Multipoint inoculator
- B. - Microculture container

the mating mixture were made in saline and 0.1 ml of the dilutions placed on the appropriate media for the selection of transconjugants. The plates were incubated for 24 to 48 hours (depending upon the media used) before colonies were counted.

Phage assay

Ike and PRD1-sensitivity was determined for all the strains carrying N group plasmids that were used in this study. To assay for phage-sensitivity, 1 ml of a late exponential-phase culture was mixed with 0.1 ml of a stock solution of the phages of known titre, and the mixture was incubated at 37° C for 20 minutes; at the end of this interval, molten R-top agar was added to the mixture, mixed gently and then poured into a fresh R-bottom agar plate. At least two different dilutions of the phage stock were plated.

Sometimes for screening purposes, a qualitative procedure was used to test for phage-sensitivity. An R-bottom agar plate was spread with 0.2 ml of a late exponential-phase culture of the strain to be tested and a drop of a high-titre phage preparation (approximately 10^{11} plaque-forming units) was spotted onto the seeded plate. A region of clearing where the phage drop was placed indicated sensitivity to the phage.

Assay for colicin production

R^+ and R^- strains were streaked in horizontal lines on a PA plate. After 24 hours of incubation at 37°C the cells were exposed to chloroform vapours for 20 minutes. The plate was incubated upside down with the lid off for 20 additional minutes. This step dissipates vapours which could remain on the plate. Since the chloroform lyses the cells (if colicins are produced) and releases the colicins, the dead cells and debris may also be removed from the plates before plating or streaking the sensitive culture. After cross-streaking the strain to be tested, the plates were then incubated for an additional 24 hours. This test has been used successfully in this and other laboratories to indicate colicin-producing strains of E. coli. In some experiments, where indicated, the cross-streaking was done without removing the chloroform-lysed cell debris.

Extraction of plasmid DNA

The procedure described as Method D, a modification by Michael Ashe (1980) of the procedure of Clewell and Helinski (1969) was used. 10 ml of an overnight PB culture of an R^+ strain was diluted to 1 liter in fresh sterile medium containing an antibiotic to which the plasmid conferred resistance. The culture was grown to stationary phase and the cells were pelleted by centrifugation at $10,000 \times g$ for 10 minutes at $0^{\circ} - 5^{\circ}\text{C}$ in a GSA rotor. The cells were

resuspended in 50 ml of cold 25% Sucrose, 0.1 M Tris-HCl (pH 8.0) on ice. 6 ml of a freshly prepared lysozyme solution (Sigma, 3 x crystallized lysozyme), 0.01 g in TE buffer (1 mM Tris-HCl pH 8.0, 1 mM EDTA (ethylene diamine tetraacetate)) was added to the cell suspension and maintained for 5 minutes on ice; then, 28 ml of 0.25 M EDTA pH 8.0 was added. The suspension was kept at 0° C (swirling occasionally) for another 5 minutes after which lysis was brought about by the addition of 50 ml of a detergent mixture (1% Brij-58, 0.4% sodium deoxycholate, 0.0625 M EDTA and 0.05 M Tris-HCl pH 8.0). After 5 minutes, the sample became relatively clear and viscous; it was then centrifuged at 4° for 25 minutes at 34,800 xg in an SS-34 rotor; this step normally pellets the cell debris and most of the chromosomal DNA, while the plasmid DNA is recovered in the supernatant. To this supernatant called "cleared lysate", NaCl was added to a final concentration of 0.5 M and polyethylene glycol (PEG 6,000) to a concentration of 10% (w/v). These were dissolved by gentle swirling and the solution stored overnight at 4° C. The precipitate that was formed was collected by centrifugation in a SS-34 rotor at 1,900 xg for 3 minutes and was dissolved in the minimal volume of TE buffer required.

Ethidium bromide (10 mg/ml stock solution in TE buffer) was added to the preparation to a final concentration of 500 ug/ml and solid CsCl was added until the solution

reached a refractive index of 1.380 - 1.385 (as determined by using a Bausch and Lomb refractometer). The sample was centrifuged in the SW-40 rotor of a Beckman Spinco ultracentrifuge at 270,000 xg for 48 hours at 15° C. After centrifugation, the plasmid DNA band was separated from the chromosomal DNA band by puncturing the tube with a 1 ml disposable hypodermic syringe and withdrawing the plasmid band under conditions of illumination with a long wave ultraviolet lamp.

Ethidium bromide was extracted from the DNA solution by adding CsCl-saturated isopropanol and discarding the alcohol layer which contained the ethidium bromide; this step was repeated several times until all visible traces of the dye were removed. To the resulting plasmid DNA solution (1 ml), 0.2 ml of TE buffer was added followed by 2 volumes of ethyl alcohol, mixed and stored overnight at -20° C. The DNA that was precipitated was sedimented by centrifugation in an SS-34 rotor at 1,900 xg for 6 minutes; the precipitation process was repeated to ensure the complete removal of CsCl. The precipitated DNA was finally dissolved in TE buffer. The approximate concentration of DNA in the sample was determined by measuring the optical density at 260 nm of a known dilution of the sample in the buffer. Knowing the absorbance of the sample, the approximate concentration could be calculated by the following relationship based on previous calibrations. $OD_{260 \text{ nm}} \text{ of } 1.0 = 50 \text{ ug/ml}$ (Davis et al., 1980).

In vitro mutagenesis of plasmid DNA

The procedure used for the in vitro mutagenesis of pCU1 plasmid DNA was that of Hashimoto and Sekiguchi (1976). Plasmid DNA was isolated as described in the previous section. A solution of 20 ug of plasmid DNA in 1 ml of TE buffer was mixed with a solution of hydroxylamine (0.4 M) in 0.05 M sodium phosphate buffer (pH 6.0), 1 mM EDTA. The mixture was incubated at 37° C for different intervals after which samples were withdrawn and diluted 5-fold in TE buffer (pH 8.0). After dilution, the samples were dialyzed for approximately 30 hours against 0.02 M Tris-HCl (pH 8.0), 1 mM EDTA, 0.02 M NaCl in the cold. The dialyzed DNA was used to transform E. coli strain C600.

The inactivation of the transforming activity of the DNA by hydroxylamine as a function of time was then followed. The sample treated in this manner for 40 hours gave a 60-fold reduction in the frequency of transformants as compared to untreated DNA. This sample was used in further experiments intended to obtain transformants containing mutated plasmid DNA. The choice of hydroxylamine as the mutagen was made because it is known that under these conditions, it will produce a specific base-pair transition, from G:C to A:T (Freese et al., 1961; Tessman, 1968).

Transformation procedure

The procedure used was that of Cohen et al. (1972). An overnight culture of strain C600 was diluted 100-fold in TYS and regrown to an optical density of 0.85 at 590 nm (Gilford spectrophotometer, Stasar II). The cells were chilled on ice for 15-20 minutes and sedimented at 12,000 xg for 8 minutes. The pellet was resuspended in 0.5 volumes of 10 mM NaCl solution. The cells were pelleted again and resuspended in half the original volume of chilled 0.03 M CaCl_2 kept at 0° C for 20 minutes, sedimented and resuspended in 0.1 of the original volume of the 0.03 M CaCl_2 solution.

Chilled hydroxylamine-treated DNA (0.1 ml) was added to 0.2 ml of the competent CaCl_2 treated cells and after an additional incubation for 60 minutes at 0° C, the mixture was subjected to a heat pulse at 42° C for 2 minutes. The mixture was then quickly chilled and diluted 10-fold in prewarmed (37° C) TYS broth. After incubation for 1 hour at 37° C, the mixture was plated on different kinds of media selective for transformants. Transformant colonies selected for one plasmid-associated marker were then tested (by replication) for other plasmid-specific markers.

Restriction endonuclease digestion

The reaction mixture for endonuclease digestion contained 1 or 2 ug of the DNA sample in the TE buffer, an

appropriate amount of restriction endonuclease (in order to ensure complete digestion), an appropriate volume of a 10X restriction endonuclease buffer and distilled water to make up a final volume of 20 ul. The reaction mixture was incubated for 3 to 5 hours at 37° C to ensure complete DNA digestion.

Bgl I and Pvu II, the enzymes used for digestion, were purchased from New England Bio-Labs. The enzyme digestion buffers were prepared as 10X stock solution as recommended by the manufacturer. Bovine serum albumin (BSA) was purchased from Sigma; Dithiothreitol (DTT) was purchased as Cleland's Reagent from Sigma Chemicals and 2-mercaptoethanol was purchased from BDH biochemicals.

Electrophoresis of DNA fragments

Electrophoresis of DNA samples was performed on vertical gels of 0.85% agarose. The agarose was dissolved in 100 ml of E buffer (prepared as 20X stock solution, 0.4 M Tris base, 0.2 M Na-acetate, 0.01 M EDTA; at pH 7.9 adjusted with glacial acetic acid (Tanaka *et al.*, 1976) by autoclaving or heating it in a microwave oven. Ethidium bromide was added to the agarose at a final concentration of 0.4 ug/ml. Before assembling the vertical slab gel apparatus, the back glass plate was spread with a thin layer of 0.1% agarose solution and dried in a microwave oven to prevent the gel from slipping when placed in

the electrophoresis chamber. After pouring the agarose in the gel apparatus, 10 sample wells were constructed by inserting a teflon comb into the molten agar. The dimension of the gel slab was 12 cm x 10 cm x 3 mm.

The digested DNA sample (20 ul) was mixed with 10 ul loading buffer solution (30% w/v glycerol, 0.05% Bromophenol blue, 5 mM EDTA) and applied to the well with a 50 ul Hamilton syringe. Electrophoresis was carried out at a constant voltage of 2 V/cm. The gels were photographed using a polaroid MP 4 vertical camera under long wave UV light.

Curing of pCU1 Plasmid from M5a1 by Ethidium Bromide

Ethidium bromide was reported by Bouanchaud et al. (1969) to be a useful agent in eliminating R factors from different bacterial genera. 0.1 ml of a 10^{-5} dilution of a M5a1/pCU1 overnight culture was added to 5 ml of TYS broth containing 180 ug/ml of ethidium bromide (minimal inhibitory concentration, determined previously). The culture was incubated overnight in the dark. Serial dilutions were plated on PA plates and further tested for loss of antibiotic markers (Sp^r and Sm^r) by replicating colonies on PA plates containing the antibiotics and observing those that did not grow.

Colony-filter hybridization

Derivatives of M5a1/pCU1 which were suspected to be cured of plasmid pCU1 were examined for the presence of the plasmid using colony hybridization with radioactively labelled plasmid DNA.

1. Colony lysis and DNA denaturation

The procedure used was a modification of the one described by Grunstein and Wallis (1979).

The putatively cured derivatives of M5a1/pCU1 were grown overnight in TYS broth in a microculture container. M5a1/pCU1 and M5a1 wild type were included as controls. An inoculum of each culture that was grown in this manner was transferred by the multipoint inoculator on a PA plate (reference plate) and on a membrane filter of nitrocellulose (Schleicher & Schnell) which was previously placed on a fresh PA plate. The plate was incubated at 37° C until a considerable colony growth could be observed on the filter surface. The filter was transferred to a square petri-dish containing filter paper (Whatman 3 MM) soaked in 0.5 M NaOH. Once the colonies were lysed completely (7 minutes approximately), the filter was transferred to a second petri-dish containing filter paper soaked in 1 M Tris-HCl (pH 7.5) for 1 minute, and finally to a third petri-dish containing filter paper soaked in 0.5 M Tris-HCl, 1.5 M NaCl, pH 7.5. The filter was left

in this solution until the colonies had a dry appearance. Before changing the filter from one solution to the next, the excess liquid was removed by placing the filter on pieces of a dry (Whatman 3 MM) filter paper. Finally, the filter was baked at 80° C in vacuo for 1.5 hours to bind the denatured DNA to the nitrocellulose filter.

2. α -³²P Labelling of pCU1 plasmid DNA by nick translation

pCU1 DNA was labelled radioactively by the in vitro nick translation reaction (Kelly et al., 1970), as developed by Rigby et al. (1977). The nick translation buffer which contained 50 mM Tris-HCl (pH 7.4), 5 mM MgCl₂ and 1 mM mercaptoethanol, 50 ug/ml bovine serum albumin was prepared as a 10X concentrated solution. Each dNTP (minus the dNTP containing ³²P) was prepared as a 0.1 mM combined stock solution and added to a final concentration of 0.18 nmoles. dCTP, dTTP and dGTP (Grade I) were purchased from Sigma, (α -³²P) dATP (with an activity of 800 Ci/mmol and at a concentration of 13 nmol/ml) was purchased from New England Nuclear Corp. DNA polymerase I was purchased from Boehringer-Mannheim.

To 100 ul of sample 1 ug of pCU1 DNA in TE buffer was added along with 2 units of DNA Polymerase I (grade I), 1 ng of DNaseI, 10 ul of the 10X buffer and sufficient sterile H₂O. The reaction was incubated for 10 hours at 15° C and stopped by the addition of 0.5% SDS and 0.01 M EDTA.

3. Recovery of Labelled DNA

The (α -³²P) labelled DNA was separated from unincorporated dNTP's by gel filtration. The sample was loaded carefully in a siliconized 7 cm x 0.6 cm Sephadex G-50 (medium) column, which was pre-equilibrated with 10 mM Hepes buffer (Sigma), 0.01% SDS. This buffer was also used to elute the DNA from the column. Aliquots of 3 drops were collected in disposable plastic scintillation vials (Packard) and counted in ³H channel utilizing Cerenkov radiation of a Beckman Scintillation counter. Radioactivity (cpm) in each fraction was plotted and the fractions containing the first peak of activity were pooled and used in the following step.

4. DNA Hybridization

DNA hybridization was performed as described by Wahl et al. (1979). The membrane filter was placed in a heat-sealable plastic bag containing 10 ml of a pre-hybridization solution. The composition of this solution was as follows: 50% formamide (99%, A.C.S. grade Matheson, Coleman & Bell); 5X concentrated SSC (0.15 M NaCl; 0.015 M Na₃ Citrate); 5X concentrated Denhardt's reagent (0.02% (wt/vol) each of bovine serum albumin, polyvinylpyrrolidone, and Ficoll (MW 400,000)); 50 mM sodium phosphate (pH 6.5); 1% glycine; 250 ug/ml of sonicated, denatured salmon sperm DNA (Sigma) per ml. The plastic bag was incubated overnight

at 42° C. Before hybridization, the prehybridization buffer was poured off the bag. The composition of the hybridization buffer was as follows: 50% formamide, 5X concentrated, SSC; 5X concentrated, Denhardt's reagent; 20 mM sodium phosphate (pH 6.5); 100 µg/ml denatured salmon sperm DNA and 10% sodium dextran sulfate 500 (Pharmacia) freshly prepared as a 50% (w/v) solution. 9 ml of this hybridization buffer was added to the bag, wetting the paper thoroughly, 1 ml was heated to 65° C and added to the probe. The probe DNA was denatured by heating it at 95° C for 10 minutes and cooling it in an ice-water bath. The denatured probe was added to the heated hybridization buffer, mixed vigorously, and added to the bag. The bag was sealed, the solution mixed thoroughly and incubated for 16 hours.

The membrane filter was washed three times with 250 ml portions of 2X concentrated SSC, 0.1% SDS for 5 minutes each at room temperature and then washed twice with 250 ml portions of 0.1X concentrated SSC, 0.1% SDS for 5 additional minutes each at 42° C, the membrane filter was dried at 37° C and exposed for 3 hours to X-ray film (Kodak, XRP-1) for autoradiography at -90° C, by using a Du Pont Cronex Hi-Plus screen.

R E S U L T S

A. Initial observations

The initial observations on the killing of K. pneumoniae strain M5a1 (to be described under B) were made with a strain of E. coli K-12 called C08/RM98 which was believed to carry a single plasmid called RM98 (Khatoon et al., 1972). Recent studies on the plasmid DNA composition of this E. coli strain have shown that it is composed of two different molecules that are physically and genetically unrelated to one another (Konarska-Kozłowska and Iyer, 1981a). One of these plasmids was designated as pCU1 and the other as pCU2 (CU = Carleton University). Strains have been obtained carrying either one of these plasmids alone. In initial experiments to test the ability of these strains to kill M5a1, it was found that the strain carrying pCU1 alone or pCU1 and pCU2 did so, while the strain carrying only pCU2 did not kill M5a1. It was therefore concluded tentatively that the killing ability of the E. coli strain was a function which requires the presence of pCU1.

B. Detection of Killing

Killing of M5a1 by a pCU1⁺ E. coli strain could be detected in several ways.

1. An E. coli strain carrying pCU1 and the K. pneumoniae strain M5a1 were each grown to exponential phase in TYS broth and mixed at an E. coli to K. pneumoniae ratio

of 20. The mixture was collected by suction on a sterile cellulose membrane filter (Millipore HA Type, 0.45 μ m porosity) and incubated at 37⁰ C with the cells facing up, on a prewarmed PA plate. After an increasing period of incubation the cells were washed off from the filter with sterile 0.85% saline; 0.1 ml of appropriate dilutions of the suspension were then plated on glucose minimal agar without any antibiotic, a medium permitting all surviving M5a1 cells to grow. In most experiments, the E. coli strains were auxotrophic mutants so that their growth was restricted on the minimal media plate. In some experiments, a nalidixic acid-resistant spontaneous mutant of M5a1 was used. In this case, nalidixic acid was added to the selection media at a concentration of 50 μ g/ml. Two types of controls were used; a mixture of M5a1 cells and the R⁻ E. coli strain or M5a1 alone with enough broth to equal the volume used in the mixture.

A typical result of such an experiment is shown in Figure 2. It can be seen that under these conditions M5a1 cells rapidly lost their viability until about 95% of the cells were killed. In different experiments, the number of M5a1 cells that were killed in this manner varied from 90 to 99%. A further feature of the results of these experiments is that about half of the colonies that survived on minimal agar were abnormal in morphology (Figure 3).

FIGURE 2

Loss of viability of Klebsiella pneumoniae, M5a1 as a function of mating with the E. coli strain C600/pCU1 carrying a conjugative N group antibiotic-resistance plasmid. A donor to recipient ratio of 20 was used in this experiment. Each value represents the average of three experiments.

FIGURE 3

Abnormal appearance of surviving colonies
of Klebsiella pneumoniae, MSa1 following mating.
The figure on the top illustrates normal colonies
and the one on the bottom, abnormal colonies.
Magnification: 5X

2. 0.2 ml of an exponentially-growing TYS broth culture of M5a1 was spread on glucose minimal agar and allowed to dry. Drops of exponentially-growing TYS cultures of the auxotrophic *E. coli*/pCU1 strain and a plasmid-free strain (otherwise isogenic to the pCU1⁺ strain) were spotted on the seeded plate. The plate was incubated at 37° C for 24 hours after which there was a clear zone of inhibition (lack of growth) in the area where the pCU1⁺ strain was spotted (Figure 4A, Right). No killing was observed where the drop of the isogenic plasmid-free strain was placed (Figure 4A, Left). This qualitative test (referred to as the 'spot test' in the rest of the thesis) was found to be reproducible, reliable and very useful when a large number of strains had to be tested.

C. The need for viable cells of the pCU1⁺ strain to detect killing

The published literature on bacterial killing phenomena indicates that killing mediated by antibiotics or colicins may serve as a precedent for the kind of effect that was observed with the pCU1⁺ strain and M5a1. The following observations, however, indicated that this was unlikely.

1. Cell-free culture-filtrates of the pCU1⁺ strain did not reduce the viability of M5a1. TYS broth cultures of pCU1⁺ strain in either exponential or stationary phase were centrifuged to remove most of the cells, or in another

FIGURE 4

Illustration of the results of the 'spot test' used for the detection of the killing phenotype.

- A. from left to right: J5 control (R^- strain); phenotype of a strain carrying an N group plasmid (pCU1); phenotype of a strain carrying a W group plasmid (S-a).
- B. example of the screening procedure used to detect Kil^- mutants of pCU1. The arrow indicates the position of a potential Kil^- mutant. $\blacktriangle$ indicates the result with a control R^- E. coli strain.

A

B

case, filtered through a Millipore filter (0.45 μ m porosity). Such supernates or filtrates were then tested on M5a1 seeded plates and found to be unable to kill M5a1.

2. A test for colicin production was performed as described in MATERIALS AND METHODS. It proved to be negative. The pCU1⁺ strain and the isogenic plasmid-free strain were streaked on PA plates. After 24 hours incubation at 37⁰ C, the cells were killed by exposing them to chloroform vapours. The chloroform-killed cells were then cross-streaked with an M5a1 culture, or the growth of the killed cells was removed with a sterile spatula before cross-streaking the M5a1 culture. In both cases there was no inhibition of the growth of M5a1.

3. pCU1⁺ cultures were exposed to 60⁰ C or to short-wave (254 nm) ultra-violet irradiation under conditions that reduced viability to $<10^{-6}$. These treated cultures were then used to spot a M5a1 seeded plate. Following both of these treatments, killing was abolished. These results indicate that living cells are needed for killing. UV irradiation is also known to often induce latent bacteriophages when their genomes are present in the cells. The absence of killing, under these conditions, suggests that this does not occur.

D. Conditions which promote bacterial mating are also those under which killing was detected

pCU1 is a conjugative plasmid of the Incompatibility group N. One of the properties of this group of conjugative

plasmids is that bacteria harbouring them mate poorly in liquid media but very efficiently on solid surfaces. The difference in mating efficiency under these two conditions can be as high as a thousand fold when E. coli strains are used as donors (Dennison and Baumberg, 1975b; Bradley et al., 1980; Konarska-Kozłowska and Iyer, 1981a).

Table 2 shows the results of liquid and solid mating when the E. coli strain C600-Nal^r and M5al-Nal^r were used as recipients. When M5al-Nal^r was used as the recipient, the mating efficiency was greater on a solid surface (membrane filter) as compared to liquid mating when little transfer was detected. However, even in membrane-filter matings, the number of transconjugants with M5al-Nal^r as the recipient was not as great as when E. coli was used as the recipient. One reason for this could be that with M5al-Nal^r, a fraction of potential transconjugants are killed. In matings between C600/pCU1 and M5al-Nal^r in liquid media, no killing of M5al could be detected even under conditions where the donor to recipient ratio was as high as 100. Thus, it is only under the conditions where bacterial mating is optimal that killing can be detected. Although a very low frequency of killing (<1%) would not have been detected by any of the three methods we have described, high efficiency killing occurs under the conditions that promote high efficiency mating.

TABLE 2

Transfer Frequency of pCU1 plasmid to E. coli and M5al under different mating conditions.

Recipient Strain	Selective drug	Conjugal transfer frequency *	
		membrane mating	liquid mating
<hr/>			
<u>E. coli</u>			
C600-Nal ^r	Nal+Sp	53	2 x 10 ⁻³
<u>K. pneumoniae</u>			
M5al-Nal ^r	Nal+Sp	5 x 10 ⁻² (70% killing)	1 x10 ⁻⁸ (no detectable killing)

* Matings were performed at a donor to recipient ratio of 1:10. The mating mixture was incubated at 37°C for 1 hour in a 50 ml flask (without shaking) for liquid mating and on a millipore membrane which was placed on a fresh PA plate, for membrane matings. After 1 hour incubation, the mixture was disrupted, and suitable dilutions were plated on the selective media containing Nal (50 ug/ml) and Sp (50 ug/ml). The frequency of transfer was calculated as the number of transconjugants obtained per '100 donors'.

The percentage of killing shown is the average of two experiments.

In other experiments, instead of premixing C600/pCU1 and M5a1 and collecting them on one membrane-filter surface, they were first collected separately on two separate filters. The charged surfaces were then brought in apposition to one another and placed on Penassay agar for 30 minutes at 37° C. Under these conditions, 60% of the M5a1 population were killed. If a third uncharged filter was 'sandwiched' between the two charged surfaces, preventing a mating reaction, no killing was detected.

E. Basis of the abnormal colony-morphology of survivors.

As described previously and in Figure 3, about half of the M5a1 cells that survived a mating reaction to form colonies on glucose minimal agar were often abnormal in their colony morphology. Since in such experiments both donor and recipients were spread on the minimal agar plates and donors are in excess of the recipients, it is likely that matings can continue on these plates even though the donor population is prevented from growing to a size at which their colonies are visible. The abnormal morphology of M5a1 could be a consequence of such matings occurring on the agar surface. Bouck and Adelberg (1970) have shown that genetic transfer from a nalidixic acid sensitive male strain stops immediately at any time during mating upon nalidixic acid treatment. It has been also recently demonstrated that nalidixic acid can inhibit mating between C600/pCU1 and other E. coli strains on solid surfaces

(Gill, BSc. Honours thesis, Carleton University, 1980).

To test whether plate-mating could be the basis of abnormal colony development, a spontaneous nalidixic-acid resistant mutant of M5a1 was isolated. This mutant was then used in an experiment in which it was mixed with C600/pCU1 (this strain was nalidixic-acid sensitive) on a membrane filter and incubated on a PA plate for 30 minutes at 37° C after which the M5a1-Nal^r survivors were assayed on: a) glucose minimal media containing Nal (50 ug/ml); b) glucose minimal media. Under both conditions of assay, the viability of M5a1-Nal^r was reduced to the same extent but only the survivors on the glucose minimal media without Nal were abnormal in appearance. This supports the notion that abnormal colony morphology is a consequence of mating events occurring on the surface of the selective media.

F. Conditions that inhibit the conjugative transfer of N group plasmids also inhibit the Killing of M5a1

In the previous section (E) it was shown that upon exposure of M5a1 or M5a1-Nal^r to E. coli carrying pCU1, about 5% of the M5a1 cells survived to form colonies on glucose minimal agar and that about half of these colonies were abnormal in morphology. Such abnormal colonies did not arise when the surviving colonies were selected on glucose minimal agar containing Nal. Nalidixic acid is known to inhibit DNA synthesis in bacteria by acting on subunit A of DNA gyrase (Morrison and Cozzarelli, 1979),

an enzyme that is essential for DNA replication in E. coli (Drlica and Snyder, 1978). During bacterial conjugation mediated by the plasmid F, DNA replication occurs as part of the DNA transfer process and nalidixic acid is also known to inhibit this process (Bouck and Adelberg, 1970; Harada and Mitsuhashi, 1977). Furthermore, experiments in this laboratory have indicated that nalidixic acid also inhibits the conjugal transfer of pCU1 and other IncN group plasmids (Gill, BSc. Honours thesis, Carleton University, 1980).

When a qualitative killing test was done on M5a1-Nal^r with the nalidixic acid-sensitive E. coli C600/pCU1 on glucose minimal agar containing Nal (50 ug/ml), there was an observable reduction in killing as compared to control plates of minimal agar that did not contain Nal.

A more quantitative experiment was performed next. The nalidixic acid-sensitive E. coli C600/pCU1 was pretreated with Nal (100 ug/ml) for 2 minutes before filter-mating and the filter used was presoaked with Nal solution (100 ug/ml). After the mixture was collected on top of this filter, it was placed on a fresh PA plate containing Nal (50 ug/ml) for incubation. Survivors were selected on glucose minimal media containing Nal (50 ug/ml). Under this experimental condition, the frequency of killing was 40% while with untreated donors and in the absence of nalidixic acid it was 96%. In the same experiments the mixtures were also plated on appropriate media to determine the frequency

of M5a1 transconjugants. With the nalidixic acid-treated donor the frequency of transfer was 4×10^{-4} and with the untreated mixture, the frequency was 3×10^{-2} . Thus, under these experimental conditions, the killing effect could not be completely abolished presumably because transfer could not be completely prevented. Since Nal acts at the level of conjugal DNA transfer replication and not at an early step, this indicates that killing of M5a1 by C600/pCU1 requires more than mere cell to cell contact between C600/pCU1 and M5a1. Either the transfer process itself or some event(s) following transfer must be responsible for the loss of viability of the M5a1 recipients.

G. Ability of pCU1 to promote killing after transfer to different hosts

From its original E. coli host, plasmid pCU1 was transferred by conjugation to a number of other E. coli K-12 strains (J5, WD00, JE, CR34) and also to a strain of Salmonella typhimurium. All of such derived strains displayed the killing phenomenon. Furthermore, an M5a1 cell which survived to form a colony and now contained pCU1 could kill the M5a1-Nal^r strain (provided nalidixic acid was absent during mating) just as effectively as the original C600/pCU1. These observations indicated that killing was a pCU1-specified property.

H. Survey of bacterial plasmids for their ability to mediate killing of M5a1

E. coli K-12 strains carrying plasmids from each one of the five group of plasmids F, I, N, P and W and some other plasmids of undesignated conjugative system(s) belonging to the incompatibility group IncM, IncU and IncX were tested for their ability to transfer the plasmid to an E. coli strain and to M5a1, and to kill M5a1. The results of this survey are shown in Table 3. It can be noticed from these results that all strains carrying N group plasmids tested (with the exception of pTM558) had the ability to kill M5a1. The inability of pTM558 to promote killing is correlated with its low frequency of transfer to both E. coli and M5a1. N plasmids are transferred stably to M5a1 at a frequency that is less than to E. coli (Table 3). This is a result that would be expected if some potential transconjugants were killed. Plasmids from the P and W groups were found to transfer as efficiently as N group plasmids, although the efficiency with which they killed M5a1 was clearly less. This implies that the basis of the relatively poor killing efficiency of P and W group plasmids is not simply poor mating efficiency. In the case of plasmid pRK229, killing was not observed. This is correlated with its transfer-deficient phenotype. Figure 4A illustrates the differences observed between the degree of killing promoted by N and W group plasmids when they were used in the 'spot test'.

TABLE 3

Tests of the ability of E. coli K-12 strains carrying different conjugative plasmids to kill Klebsiella pneumoniae M5a1

Donor strain of plasmid*	Conjugative group	Conjugal transfer frequency** (% of donor) to <u>E. coli</u> K-12	to <u>K. pneumoniae</u> M5a1	Fraction (%) of M5a1 cells killed upon mating on a filter ***
CO8/RM98	N	46	0.60	98
CO8/R46	N	10	0.17	92
CO8/R113	N	10	0.11	90
CO8/R199	N	26	0.38	88
J5/R205	N	23	0.57	63
J5/pTM558	N	0.22	3×10^{-3}	0
J5/R48	N	10	0.2	93
J5/RP4	P	31	17	20
C600/RK2	P	20	14	12
C600/pRK229	P (Tra ⁻)	0	0	0
J5/S-a	W	10	0.10	20
J5/R386	F	1.9	4×10^{-4}	0
J5/R100-1	F	3.5	1×10^{-4}	0

TABLE 3 (Continued)

LC524/PAR132	F	16	4.7×10^{-2}	0
J5/R64-11	I	16	0.1	0
J5/R144-3	I	0.23	3.7×10^{-3}	0
C600/pCU2	Unknown	0.1	1×10^{-2}	0
JE2175/RA3	Unknown	3.4	1.6	0

* In addition to the N and W plasmids shown here other N group plasmids (R45, PDT200) and the W group plasmids (R411, R388) tested have been found to kill M5a1 in the 'spot test' (RESULTS B.2 page 39). There was no killing when the plasmids R446-b (IncM) and R6K (IncX) were tested.

** Matings were performed by mixing exponential-phase donor and stationary phase recipients at a ratio 1:10. The mixture was incubated on a millipore filter placed on PA at 37°C for one hour. Transconjugants were selected on the appropriate media. The frequency of transfer was calculated as the number of transconjugants obtained per '100 donors'.

*** For the estimation of killing, exponential-phase donor and recipient cultures were mixed at a ratio of 20:1. The mating mixture was incubated for thirty minutes at 37°C. Survivors were scored on glucose minimal agar, where the auxotrophic donors were counter-selected.

Plasmids of the FI, FII and I ∞ incompatibility groups did not mediate any detectable killing (Table 3). Plasmids from the incompatibility groups U (RA3), M (R446-b) and X (R6K) also did not show any detectable killing.

The results of this survey have shown that the ability to kill M5a1 is a general property of all conjugation-proficient N group plasmids. The survey has also lent some support to the belief that plasmids of the N, P and W groups are physiologically related, as the latter two groups also kill M5a1, although to a lesser extent.

I. Studies on mutants of pCU1

As a result of other studies in this laboratory plasmid pCU1 is at present the best characterized plasmid of the group N. Several spontaneously occurring mutants of the plasmid arising through physical rearrangements of the DNA of this plasmid have also been characterized and were available. Some of these plasmid-derivatives had also lost other plasmid-associated phenotypes. Correlation, if any, between these phenotypes and killing ability could therefore be sought. The results of these experiments are shown in Table 4. It can be noticed that reduction in the transfer proficiency of the plasmid carrying strain also resulted in reduction of the killing ability (pCU7 and pCU10 to pCU13).

TABLE 4

Test of the ability of E. coli K-12 strain carrying plasmid pCU1 or its derivatives to transfer the respective plasmid by conjugation and to kill Klebsiella pneumoniae, M5al

Plasmid	Origin of Plasmid *	Phenotypes other than Kil phenotype (a)	Kil phenotype (b)
pCU1	Parental plasmid	Ap ^r Sm ^r Sp ^r Ike ^s Tra ⁺	+
pCU4	Insertion of a fragment from pCU2 into pCU1	Ap ^r Sm ^r Sp ^r Tc ^r Ike ^s Tra ⁺	+
pCU7	Insertion of a fragment from pCU2 into pCU1 plus deletion of Sm and Sp region	Ap ^r Tc ^r Ike ^s Tra ⁺	+
pCU8	Deletion of Sm and Sp region	Ap ^r Ike ^s Tra ⁺	+
pCU10 to pCU13	Deletion mutants generated in vitro using Sma I, Kpn I and <u>Sal</u> I restriction endonucleases	Ap ^r Sm ^r Sp ^r Ike ^s Tra ⁻	-
pCU16	Deletion generated in vitro using Hind III restriction endonuclease	Ap ^r Sm ^r Sp ^r Ike ^s Tra ⁺	+

TABLE 4 (Continued)

(a) Transfer phenotype was analyzed by a spot test and also quantitatively by filter mating as described in Materials and Methods. The frequency was calculated as the number of transconjugants obtained per 100 'donors'.

Tra^+ phenotype = (transfer 0.1 - 0.5 of pCU1)

Tra^- phenotype = transfer not detected ($<10^{-8}$)

(b) Kil phenotype was analyzed by the 'spot test' and quantitatively as described in RESULTS (B.1).

Kil^+ (20% of M5a1 killed in contrast to 90 - 99% when pCU1 was used).

* pCU1 and its derivatives were described by Konarska-Kozłowska and Iyer (1981 a); (1981 b).

J. Isolation and properties of plasmid mutants by
in vitro mutagenesis

pCU1 DNA was isolated as described under MATERIALS AND METHODS. It was then used to transform the E. coli strain C600. The transformants were selected on PA plates containing spectinomycin (50 ug/ml). A large number of Sp^r transformants were then screened for changes, if any, in other phenotypes. Such an experiment was necessary as a control because pCU1 DNA is known to undergo spontaneous changes (see previous section) and the transformation process itself may enhance or select for such spontaneous events. The results of such events could then be mistaken for spontaneous point mutants. 1,600 transformants were tested for all of the known pCU1-determined phenotypes including killing ability. All these transformants were indistinguishable phenotypically from the original strain C600/pCU1. While establishing the above, the same plasmid DNA preparation was exposed to hydroxylamine as described under MATERIALS AND METHODS. The DNA preparation that was treated for 40 hours with hydroxylamine was used to transform competent cells of strain C600. The frequency of transformation was 60-fold less as compared to the frequency obtained with the untreated DNA. Transformants were selected on PA plates containing spectinomycin (50 ug/ml). From this experiment, 1,900 Sp^r transformants were screened for other pCU1-associated phenotypes, such as: antibiotic

resistance, conjugal transfer and killing ability.

Figure 4B page 40 shows an example of the screening used to select kil^- mutants, on a glucose minimal agar plate seeded with M5a1.

From the 1,900 Sp^r transformants analyzed, 147 were deficient in their ability to kill M5a1 (this phenotype is abbreviated kil^-) the rest of the transformants were kill proficient (kil^+). A further classification of the 147 kil^- mutants on the basis of other phenotypes associated with them is shown in Table 5. These results indicate that there is a very high correlation between the loss of killing ability and the loss of transfer property.

The in vitro mutagenesis of DNA with hydroxylamine is known to produce a specific type of base substitution mutation in the direction of G:C to A:T (Freese et al., 1961; Tessman, 1968). Under the conditions that were used for in vitro mutagenesis, the probability of two independently arising hydroxylamine-induced mutations was expected to be very low (Hashimoto and Sekiguchi, 1976). Using this procedure and pCU1 DNA 147 kil^- mutants were obtained upon screening 1900 Sp^r transformants (Table 5). No $kil^+ Tra^-$ mutants were isolated. Rarely some mutants that were apparently $kil^- Tra^+$ were detected (Group III, Table 5), but it will be shown in the following section that most of these were slightly transfer deficient.

TABLE 5

Classification of the Kil^- mutants
on the basis of other phenotypes
associated with them

Group	Phenotypes other than Kil^- *	Number of mutants in each group
I	Tra^- , Sp^r Ap^r Sm^r	131
II	Tra^- , Sp^r	12
III	Tra^+ , Sp^r , Ap^r , Sm^r	4

* The different plasmid-associated phenotypes were analyzed by spot tests, using the multipoint inoculator (Figure 1). Drops of the Kil^- strains were spotted on the respective antibiotic-media plates for determining antibiotic-resistance and on a suitable recipient-seeded plate for determining the transfer phenotype.

Tra^- , transfer deficient in E. coli matings (frequency: 10^{-2} to 10^{-8}).

Tra^+ , transfer proficient in E. coli matings (frequency: 5 to 60%).

Ap^r , ampicillin resistance; Sp^r , spectinomycin resistance;
 Sm^r , streptomycin resistance.

K. Further studies on selected Kil⁻ mutants

As indicated in (J) above, the predominant class of mutants to emerge from this screening was Kil⁻ Tra⁻ (Group I). The transfer frequency of this group was determined by spot mating first, and several of them were then analyzed quantitatively in order to see the degree of transfer deficiency to E. coli. It was as low as 10^{-2} to 10^{-8} of the parent strain. Such a correlation between the loss of transfer and killing ability has also been observed in independent studies on conjugal transfer (Thatte, personal communication). Most of the group I mutants have been found to carry plasmids with DNA of the same length as pCU1 DNA and with no gross dissimilarity to this parental plasmid. When group II mutants were examined for plasmid DNA, 4 out of 12 did not show any detectable plasmid band in the ethidium bromide-CsCl gradient. In the context of the present study they were of no further interest. Presumably they arise by a rare recombination event which transposes the Sp^r gene onto the chromosome.

The 4 rare mutants of Group III were of special interest because in the initial screening they alone suggested that the Kil phenotype could be mutationally dissociated from the Tra phenotype. The transfer proficiency and the Kil⁻ phenotype of the four mutants were analyzed quantitatively by filter mating. There was no detectable killing of M5a1 when they were used as donors in membrane

filter matings under the conditions described in RESULTS (B.1 page 35). The transfer proficiency of these strains was estimated by membrane filter mating (MATERIALS AND METHODS, page 21). Both E. coli and K. pneumoniae (M5a1) strains were used as recipients, and matings with the same recipient and the parental C600/pCU1 were always conducted concurrently. In addition, the donors were examined for their sensitivity to the bacteriophages IKe and PRD1, two bacteriophages that are known to be specific for the IncN group of conjugative plasmids (Khatoon et al., 1972; Olsen et al., 1974). The results of these experiments are shown in Table 6.

Plasmid DNA was also extracted and purified from each mutant and from C600/pCU1. Each of the DNA samples was digested completely with the restriction endonucleases Bgl I and Pvu II and the preparation electrophoresed, stained and photographed as described in MATERIALS AND METHODS. Figure 5 and Figure 6 display the resulting pattern for each plasmid DNA. In the following, the properties of these four mutants and their plasmids are described and discussed separately. It should be noted that all four mutants are only slightly deficient in conjugation (0.2 to 0.9 of C600/pCU1 as compared to 0.0001 for most Group I mutants).

C600/pCU21

This mutant is the only one that has a conjugation proficiency that is the same or nearly the same as that of

FIGURE 5

Pvu II Restriction endonuclease cleavage
pattern of pCU1 and (Tra⁺ kil⁻) derivatives of pCU1.

- A. Pvu II digest of pCU1
- B. Pvu II digest of pCU23
- C. Pvu II digest of pCU24
- D. Pvu II digest of pCU22
- E. Pvu II digest of pCU21
- F. Hind III digest of λ (control)

( ) indicates the position of the alteration of gel
pattern in pCU23 DNA.

( ) indicates the position of the alteration of gel
pattern in pCU21 DNA.

A B C D E F

FIGURE 6

Bgl I Restriction endonuclease cleavage
pattern of pCU1 and (Tra⁺ Kil⁻) derivatives of pCU1.

- A. Bgl I digest of pCU1
- B. Bgl I digest of pCU23
- C. Bgl I digest of pCU24
- D. Bgl I digest of pCU22
- E. Bgl I digest of pCU21

(>) indicates the position of the alteration of gel
pattern in pCU23 DNA.

(►) indicates the position of the alteration of gel
pattern in pCU21 DNA.

Bgl I

TABLE 6

Relative conjugation proficiency of Group III mutants, and their sensitivity to bacteriophages Ike and PRD1

Donor strain	Relative transfer frequency to*			Sensitivity to bacteriophages	
	<u>E. coli</u> C600-Nal ^r	<u>E. coli</u> J5	<u>K. pneumoniae</u> M5a1	Ike	PRD1
C600/pCU21	1	0.9	182	+	+
C600/pCU22	0.5	0.9	0.3	+	+
C600/pCU23	0.2	0.3	0.2	-	-
C600/pCU24	0.2	0.4	0.2	-	-

* Transfer frequency was measured by mixing exponential-phase donors and stationary recipient cultures at 1:10 ratio. The mixture was incubated at 37° C for 1 hour on a millipore filter placed on fresh PA plates. Transconjugants were selected on selective media containing spectinomycin (50 ug/ml).

Relative transfer frequency = frequency of transfer of mutant plasmid/frequency of transfer of pCU1 from C600/pCU1.

The frequency of transfer of pCU1 to the 3 recipients was as follows: C600, 60%; J5, 53%; M5a1, $4 \times 10^{-2}\%$

the parental strain C600/pCU1. It has also retained its sensitivity to IKe and PRD1. In conjugation experiments with M5a1, it is transferred more efficiently than is pCU1 from C600/pCU1. This seems consistent with it being a Kil^- mutant, as potential transconjugants would not be killed in this case. However, the increase in transfer efficiency to M5a1 (182-fold increase, Table 6) is greater than would be expected for this reason alone.

The existence of this mutant suggests that killing and transfer functions can be dissociated by mutation. Further studies on C600/pCU21 have revealed that the strain may have suffered more than one mutational lesion. In matings between this strain and M5a1 (but not with this strain and E. coli strains), the frequency of Sm^R transconjugants was 5000-fold less than Sp^R transconjugants. When such M5a1/pCU21 transconjugants that were $Sp^R Sm^S$ were purified and 'back-crossed' to E. coli C600-Nal^R strain, all Sp^R transconjugants expressed Sm^R . Thus the Sm^R gene was present on pCU21 when this plasmid was resident in M5a1. It was also observed that M5a1/pCU21 was resistant to bacteriophages IKe and PRD1 but the E. coli transconjugants arising from the 'back-cross' were sensitive to both phages. It seems clear that the mechanisms of expression of some genes in pCU21 are affected in the background of M5a1 cells but not in E. coli cells.

Figures 5 and 6 (slot E) show the Pvu II and Bgl I generated restriction pattern of the DNA of this plasmid. They should be compared to slot A in both figures that represents the wild type pCU1 DNA. It is clear that in pCU21 DNA there is an alteration in the gel pattern (fragments Pvu II-4 and Bgl I-4). Using the fragments generated from bacteriophage λ DNA upon Hind III digestion as molecular weight standards (Murray and Murray, 1975) the deleted portion that could account for the difference in mobility of fragment Bgl I-4 could be estimated to be about 0.2 Md (data not shown). These properties of C600/pCU21 DNA prevents one from ascribing the loss of the Kil^- phenotype to either a hydroxylamine-induced base-pair transition or the above deletion. Since hydroxylamine is not known to induce a deletion and pCU1 is known to undergo spontaneous deletions, it would seem more likely that pCU21 has either only a deletion or a deletion and a base-pair transition. The relationship between such changes in DNA and the loss of expression of some genes is also not clear. However, regardless of whether pCU1 has suffered a single or double mutation to give rise to pCU21, this mutation(s) affects the killing phenotype without markedly affecting the transfer of the plasmid and expression of the Sp^r marker.

C600/pCU22

In matings with E. coli, this mutant had a frequency of transfer in the range of 0.5 to 0.9 of the parental pCU1 plasmid. Like pCU21 it was also sensitive

to both N-specific phages (Table 6). However, unlike C600/pCU21, the transfer frequency to M5a1 was also reduced and to a greater extent than to E. coli. The restriction endonuclease-patterns of the DNA of this plasmid (slot D, Figures 5 and 6) was similar to that of pCU1 DNA (slot A, Figures 5 and 6), showing no evidence of any gross changes. The properties of this mutant are thus consistent with its having arisen only by a base-substitution induced by hydroxylamine treatment. This mutation resulted in a slight effect on conjugation (0.5 to 0.9 relative to wild type pCU1 in E. coli crosses; in crosses with M5a1, these relative values were 0.3).

C600/pCU23

As in the case of C600/pCU21, pCU23 has also undergone gross structural aberrations. There is an addition of genetic material that is visible in the first Pvu II-fragment (Figure 5, slot B) and in the 9th Bgl I-fragment (Figure 6, slot B). The same kind of uncertainty, as observed for pCU21 therefore exists in relating observed phenotypic changes in this strain to a specific mutation(s) in its plasmid.

C600/pCU24

The restriction pattern of pCU24 DNA suggests that like pCU22, it has not undergone any structural changes (slot C, Figures 5 and 6). Like pCU22, transfer

efficiency is reduced slightly, but unlike C600/pCU22, this strain is resistant to both N-specific phages.

The properties exhibited by these four mutants will be discussed further and in a broader context under DISCUSSION.

L. Circumstances that provide M5a1 with immunity to killing by strains carrying pCU1 or related plasmids

In the present study it was observed that M5a1 cells which survived the killing effect of E. coli/pCU1 during mating and also acquired the plasmid, could express all the plasmid-associated characteristics such as: resistance to antibiotics, sensitivity to the bacteriophages IKe and PRD1, transfer of the plasmid to other strains by conjugation and the ability to kill M5a1-Nal^r upon a second round of mating. These results suggested that all or most of the genes borne by the pCU1-plasmid were expressed in the host M5a1. Another observation was that the presence of the plasmid rendered M5a1 resistant to killing by pCU1 or by other N group plasmids such as R113 and R199.

Several derivatives of M5a1 carrying different plasmids from the groups N, P and W were constructed in this study and they were all examined for their immunity to killing by E. coli strains carrying N, P and W group plasmids. The results are shown in Table 7 from which the following conclusions are derived:

TABLE 7

Immunity* of M5a1/R- strains to <u>E. coli</u> strains** carrying the same or closely related plasmids		<u>E. coli</u> strains carrying the plasmids					
M5a1-derived strains	(Inc group)***	pCU1		S-a		R388	
		(Inc N)	(Inc N)	(Inc W)	(Inc W)	(Inc W)	(Inc P)
M5a1/pCU1	N	+	+	-	-	-	-
M5a1/pCU21	N	-	-	-	-	-	-
M5a1/pCU22	N	+	+	-	-	-	-
M5a1/pCU23	N	+	+	-	-	-	-
M5a1/pCU24	N	+	+	-	-	-	-
M5a1/S-a	W	+	+	+	+	+	-
M5a1/ RK2	P	-	-	+	+	+	+
M5a1/nil		-	-	-	-	-	-

* Immunity was assayed by the 'spot test' as described in RESULTS B. 2 (page 39).

+ Immune (no killing detected)

- Not immune (inhibition of growth in the spotted area)

** The E. coli strains used were all derivatives of strain C600 differing from one another only in the plasmid they carried; pCU21 to pCU24 are the mutant derivatives of pCU1.

*** Incompatibility group of the plasmid present in the bacteria.

1. M5a1 carrying any one of these plasmids is always immune to E. coli carrying the same plasmid.
2. With wild-type plasmids, this immunity extends to other plasmids in the same incompatibility group but not always to plasmids in the two other related plasmid groups. (For example, M5a1/pCU1 is immune to E. coli/R113 but not to E. coli/S-a).
3. M5a1 carrying pCU21, the Kil⁻ mutant derivative of pCU1 does not express immunity to E. coli carrying the parental plasmid pCU1 or to the other N group plasmid R113.
4. M5a1/S-a is immune not only to E. coli/S-a or the other W group plasmid R388 but also to E. coli carrying the N group plasmids pCU1 or R113.
5. M5a1/RK2 was not only immune to E. coli carrying RK2 or the other P group plasmid PP4 but also to the W group plasmids S-a and R388.

These observations will be considered under DISCUSSION after some further experiments relating to immunity are described.

M. Elimination of pCU1 or pCU21 from their respective M5a1 hosts and phenotypic characteristics of the derivatives

As shown earlier (Table 7) M5a1/pCU1 was immune to E. coli/pCU1. It was of interest to know whether the

presence of the plasmid in M5a1 was needed for the expression of immunity. M5a1/pCU1 was cured of its plasmid by using ethidium bromide as described in MATERIALS AND METHODS. The frequency of curing varied from 5 to 20% in four independent experiments.

M5a1 was considered to be probably cured on the basis of loss of all plasmid-associated markers (Sp^r , Sm^r , IKe^s , $PRD1^s$, Kil^+). Three of the cured strains (M6, M7, M8) were also tested for the possible presence of the plasmid DNA, by extracting their DNA and centrifuging it in a cesium-chloride/Et Br gradient as described in MATERIALS AND METHODS. M5a1/pCU1 DNA was extracted as a control and it was the only extraction where two distinguishable bands were observed in the gradient. No plasmid band was observed in these three supposedly cured strains.

pCU21 was also cured from M5a1 with ethidium bromide at a frequency of 6%. In this case, the only marker that could be tested was sensitivity to spectinomycin (sensitivity to IKe , $PRD1$ and Sm^r are not well-expressed with this plasmid derivative).

M5a1 strains cured of pCU1 (30 strains including M6, M7, M8) were used to determine whether they were sensitive to C600/pCU1. The results indicated that all of them retained immunity. M5a1 strains cured of pCU21 (6 strains) did not retain immunity.

Several possible explanations for these results were considered:

1. Heterogeneity in the M5a1 Population

Although colonies or cells of M5a1 appeared morphologically uniform, it was possible that a fraction of this population was sensitive to C600/pCU1 and a fraction resistant before this population was exposed to C600/pCU1. If this were the case, only these naturally immune strains would be the ones capable of acquiring the plasmid. Upon curing the plasmid, these strains would continue to be immune. In order to test this possibility 250 independent M5a1 colonies were tested for their sensitivity to C600/pCU1. All of them were found to be sensitive. Since the frequency of immune derivatives of M5a1 that arise upon mating with C600/pCU1 is much greater than $1/250$ (about $1/5$), this excludes heterogeneity in the population prior to mating as the basis of immunity.

2. Mutation to immunity induced by ethidium bromide

The possibility that immunity to killing was due to Et Br treatment was excluded by examining 250 additional M5a1 colonies which arose as survivors following this treatment (same conditions as the cured experiment). None of these was resistant to C600/pCU1.

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3. Integration of all or part of the plasmid into the M5a1 chromosome

Although it was shown that cured derivatives of M5a1/pCU1 no longer carry supercoiled plasmid DNA, the plasmid or some region of the plasmid could have been integrated into the M5a1 chromosome. This event may have led to immunity to killing in the same way as Hfr strains of *E. coli* are immune to acquiring an F plasmid. To test this possibility, pCU1 plasmid DNA was isolated and purified. It was then radioactively labelled with the nick translation procedure (Rigby *et al.*, 1977) as described in MATERIALS AND METHODS. The radioactive DNA was purified from the unincorporated α -³²P ATP by gel filtration. It was then used after denaturation as a hybridization probe in a colony-hybridization procedure. Colonies of the cured strains were grown and lysed on a nitrocellulose membrane filter, where the DNA was fixed by treatment with 0.5 Tris-HCl, 1.5 M NaCl (pH 7.5) buffer followed by baking at 80° C under vacuum for 2 hours. Hybridization was performed following the method described by Wahl *et al.* (1979). Along with the cured strains on the nitrocellulose filters, M5a1/pCU1 and M5a1 were included as controls.

The hybridization results are shown in Figure 7-A. It can be noticed that only M5a1/pCU1 (●) hybridizes with the probe while the cured strains and M5a1 (✱) do not. These results support a conclusion that all or most of the

FIGURE 7

Autoradiographs of colony hybridization involving ^{32}P -labelled pCU1 DNA as a radioactive probe and putative plasmid-cured derivatives of M5a1/pCU1 and M5a1/pCU21

- A. M5a1/pCU1 derivatives cured from pCU1 plasmid.
 10^6 cpm of pCU1 probe hybridizing with M5a1/pCU1 ($\oplus$).
($\star$) hybridization background (pCU1 probe and M5a1 wild type).
- B. M5a1/pCU21 derivatives cured from pCU21 plasmid.
 10^6 cpm of pCU1 probe hybridizing with M5a1/pCU1 ($\oplus$) and M5a1/pCU21 ($\bullet$).
($\star$) hybridization background (pCU1 probe and M5a1 wild type).

A

B

plasmid DNA is eliminated from the cells. It is possible, however, that only a small region of the pCU1 DNA (below the level of detection by colony hybridization) was integrated into the M5a1 chromosome. It should be noted, however, that no other plasmid-specified phenotype was expressed by the cured derivatives. Figure 7-B shows the results of a similar colony hybridization experiment with the cured derivatives of M5a1/pCU21. A similar conclusion concerning curing of pCU21, can be drawn from this result. Unlike M5a1/pCU1, M5a1/pCU21 was not immune to killing by C600/pCU1. Thus pCU21, only when present in M5a1, conferred three phenotypic differences: loss of immunity against killing, partial suppression of streptomycin-resistance, sensitivity to the two phages (IKe and PRD1). All of these three differences could conceivably be the manifestation of a single mutational difference between the two plasmids; a mutation which interferes indirectly with the expression of these three phenotypes; the fact that suppression of streptomycin-resistance and phage-sensitivity is only partial suggests that this may be the case.

DISCUSSION

For convenience, this discussion is organized in three parts. Parts A and B deal respectively with the Kil⁺ phenotype and immunity to killing. They will attempt to interpret the results that have been presented and will be speculative. Part C is a discussion of the potential practical use of the phenomenon.

A. The Kil phenotype and its nature

This study arose from the chance observation that conjugation between an E. coli strain carrying the plasmid pCU1 and the Klebsiella pneumoniae strain M5a1 results in the loss of viability of a major fraction of the population of M5a1. pCU1 is a conjugative plasmid of the incompatibility group IncN. One major conclusion of this study is that this effect is a general phenomenon involving all naturally occurring IncN plasmids that are conjugative. This phenotype has been abbreviated as Kil⁺ (Killing). On surveying other naturally-occurring plasmids, two other groups of plasmids IncP and IncW were also found to promote killing of M5a1 but to a degree that was about 0.25 of that promoted by IncN plasmids (Table 3). These effects are clearly plasmid-associated because the successful transfer of such a plasmid to a series of other plasmid-free and Kil⁻ strains always resulted in the latter acquiring the Kil⁺ phenotype. Furthermore, the Kil⁺ property was expressed by other species of bacteria besides E. coli provided they were

carrying the appropriate plasmid. Plasmids from the IncFI, FII, I, M, U and X groups (Table 3) did not mediate any detectable killing.

The mechanisms by which the IncN, P and W plasmids mediate this killing is not known. Since it is a general property of all IncN group plasmids that is shared to some extent by IncP and IncW group plasmids, it is most likely that some feature that is common to all the three groups of plasmids is associated with the killing phenomenon. Such common features of the plasmids are, therefore, considered.

Antibiotic-resistance plasmids in general specify the following three types of functions (Jacob et al., 1977): (a) resistance to antibiotics or other bacterial inhibitors, (b) plasmid replication, segregation and the related phenomenon of plasmid incompatibility and (c) conjugative ability and the related property of sensitivity to bacteriophages that are plasmid group-specific.

For the following reasons, it is unlikely that genes specifying antibiotic resistance are directly involved with the killing phenomenon: (1) the antibiotic-resistance genes encountered among N, P or W group plasmids can also be encountered among other plasmid groups, members of which do not mediate the Kil^- phenotype, (2) the pattern of antibiotic-resistance associated with Kil^+ plasmid of the groups N, P or W is variable, and most

antibiotic-resistance genes could be deleted from a Kil^+ plasmid (pCUI) with no loss of the Kil^+ phenotype and (3) naturally-occurring conjugative and Kil^+ plasmids that do not confer antibiotic-resistance have been encountered. There is, thus, no indication that resistance to any one or more antibiotics is conserved or confined to plasmids of these three groups with which the Kil^+ phenotype is associated.

The mechanisms determining intracellular incompatibility between any two plasmids of one incompatibility group are not well-understood. However, studies with some model plasmid systems such as the classical bacterial sex factor F or F-like plasmids have indicated that the basis of incompatibility is likely to be plasmid DNA replication, plasmid DNA copy control and/or plasmid segregation during cell division (Novick, and Hoppensteadt, 1978). Whatever the details of the molecular basis of plasmid incompatibility, it is a property that has proved to be useful in grouping naturally-occurring plasmids (Datta, 1977). It is on the basis of plasmid incompatibility tests that N, P and W plasmids (and other plasmids) have been grouped into separate incompatibility groups. This is possible presumably because the replication, copy control or segregation specificities of these three groups are different from one another. Nevertheless, plasmids

of all three groups mediate the killing phenomenon. This makes it unlikely that components of incompatibility are directly related to the Kil⁺ phenotype.

For the following reasons, a plasmid gene or genes associated with bacterial conjugation are attractive candidates to consider as being associated with the Kil⁺ phenotype.

1. Independent of the killing phenomenon, there is evidence which suggests that conjugative systems specified by these three groups of plasmids are interrelated. For these three plasmid groups, two types of plasmid-specific bacteriophages exist: (a) bacteriophages which have a narrow specificity, infecting only strains carrying plasmids that belong to a single group from among N, P or W plasmids (Khatoon et al., 1972; Stanisich, 1974; Morris et al., 1980), (b) bacteriophages that have a broader host-range to include strains carrying plasmids from any of the groups N, P or W but not other groups (Olsen et al., 1974; Bradley and Rutherford, 1975). Stanisich (1974), Dennison and Baumberg (1975a) and Thatte (personal communication) have observed that mutations which abolish or reduce the conjugative ability of strains carrying plasmids from any of these groups can simultaneously abolish sensitivity to these phages, implying that sensitivity to the phages is related to conjugation.

2. Bacteria carrying these plasmids have been observed to have short rigid pili (hair-like cell surface organelles) (Bradley, 1980). By analogy with the F-like plasmids that specify long flexible pili, it has been suggested that these short rigid pili may function as organelles that are needed for a step in the mating process (Bradley et al., 1980). There is some evidence that the plasmid-specific phages attach to these specific pili and this may be the basis of the plasmid-specificity of these phages (Bradley, 1979). These observations, particularly the existence of phages such as PRD1 which will infect E. coli strains carrying plasmids from any one of the groups N, P or W, have suggested that the conjugative systems determined by each of these three groups may have overlapping and interrelated specificities; that is they comprise an interrelated conjugation system. The fact that it is these three plasmid groups and no other that mediated the killing of M5a1, suggests that killing may in some way have a basis in the functioning of common or related genes comprising these three conjugative systems.

Yet other alternatives that one might consider are that some other unknown and conserved genetic region on these plasmids is responsible for the phenomenon or that each plasmid group or plasmid utilizes a different mechanism of killing. The hypothesis concerning the possible involvement of genes and mechanisms promoting conjugation was the most attractive to pursue. This

possibility is therefore discussed further.

At the outset, it must be emphasized that the data presented here show that with the possible exception of rare mutants, any process that reduces the frequency of conjugal transfer of the plasmid into M5a1 also reduces or abolishes killing. This could be either because killing is intimately associated with some aspect of the conjugative process or that conjugation is relevant only insofar as it provides an efficient way of transferring a potentially lethal gene into M5a1.

For N group plasmids such as pCU1, it has been shown that nalidixic acid inhibits conjugation at a step after the formation of mating partners (Gill, BSc. Honours thesis, Carleton University, 1980). However, in matings between M5a1 and C600/pCU1 (Results, F), nalidixic acid did not promote the killing of M5a1 but reduced it. This result therefore indicates that cell-to-cell contact is insufficient for killing to be expressed.

If the latter stages of conjugation are not mandatory for killing and the role of conjugation is merely to transfer a potentially lethal gene into M5a1, any other method of transferring the plasmid into pCU1 should have the same effect. A direct examination of

this question has not been possible because any other method that has been used to transfer a plasmid into M5a1 is less efficient than conjugative transfer by several orders. Since killing is detected as the fraction of recipients that fail to give rise to colonies it would be important to ensure that all or most recipients and not just a small fraction receive the plasmid. Because of this technical difficulty, the question was addressed indirectly. If genetic regions determining conjugation and killing ability are independent of one another, it should be possible to inactivate frequently the killing ability without reducing conjugative ability..

As pointed out in RESULTS (J), a large proportion (131/135) of the Kil⁻ plasmid mutants showed a greatly reduced (10^{-2} - 10^{-8}) conjugative ability. With three plasmid mutants (pCU22, pCU23, pCU24; Table 6) no killing could be detected but there was only a slight reduction in conjugal transfer ability to either E. coli or to M5a1.

Relative plasmid transfer ability in E. coli x E. coli matings can provide a measure of the degree to which a mutation has affected conjugation proficiency. By this criterion, the relative transfer frequency of

these 5 plasmids was in the range of 0.2 to 0.9 and the absolute frequencies about 9% to 48%. With IncA group plasmids that transfer at such frequencies or even lower frequencies, killing of M5a1 could be detected (Tables 3, 4). This suggests that the absence of killing with pCU22, pCU23 and pCU24 cannot be attributed only to their slight transfer deficiency. In matings with M5a1 on the other hand, a mutant plasmid that is Kil^- may be expected to give rise to a relatively larger number of trans-conjugants as no potential transconjugants are expected to have lost their viability. In fact (Table 6), this is not the case. The relative transfer frequencies of these three plasmids are less and not more than that of pCU1. Presuming that these three plasmids are the result of single mutational events (which may not be the case), this implies that each mutation has three distinct effects: abolishing the ability to mediate killing of M5a1, reducing conjugal transfer to E. coli and reducing conjugal transfer to M5a1. pCU23 and pCU24 (but not pCU22) have also lost their ability to confer sensitivity to the phages IKe and PRD1, properties known to be associated with transfer-deficiency.

The mutant plasmid pCU21 is considered separately because it was the only one of the Kil^- mutant plasmids that was transferred as efficiently as pCU1 in E. coli x E. coli matings. This observation had suggested that transfer-proficiency and the Kil^- phenotype could be

mutationally dissociated. For this reason, this plasmid was selected for further studies. In matings with M5a1, the transfer frequency of this plasmid was much greater than that of pCU1 (182 fold, Table 6): This degree of increase is quantitatively more than can be accounted for only by the Kil⁻ phenotype (95% of M5a1 recipients are killed usually by C600/pCU1). This implies that the mutation in pCU21 had an opposite effect to the mutations in pCU22, pCU23 and pCU24. It promotes a more efficient mating with M5a1. Thus, in every case where a mutant plasmid with a Kil⁻ phenotype was isolated there was a concurrently demonstrable change in conjugal transfer proficiency suggesting that these two phenotypes are in some way physiologically associated.

The experiments with nalidixic acid have shown that the mere formation of mating partners is an insufficient cause of killing and that killing is mediated by events that occur during or after the entry of the plasmids. Such events occur probably with any conjugative plasmid from the three groups. The experiments with the four mutants of pCU1 lends some credence to a hypothesis that it is those bacterial mating functions concerned with the stabilization of mating pairs or the transit and establishment of the plasmid in the recipient that are physiologically related to the killing phenomenon.

B. Immunity to Killing

As a rule, when an E. coli strain carries any conjugative plasmid it is immune to stable infection by the same or closely related plasmid. This property is a plasmid-specified function called superinfection immunity and it has at least two components: one which operates to prevent the entry of the second plasmid into the cell and hence called entry exclusion or surface exclusion and another which operates intracellularly after the second plasmid has gained entry into the cell carrying the first plasmid. The latter component is called intracellular incompatibility or simply incompatibility.

If one or both of these processes occur in K. pneumoniae M5a1, a plasmid-carrying derivative of M5a1 may be expected to express resistance to killing promoted by the same or closely-related plasmid i.e. immunity to killing may simply be a consequence of the general phenomenon of plasmid superinfection inhibition. In RESULTS (L), this expectation was seen to be fulfilled. An M5a1-derivative carrying a wild-type N, P or W group plasmid was not susceptible to killing by E. coli strains carrying a plasmid of the same group. However and in addition, these three plasmid groups could be arranged in the sequence shown in Figure 8, such that N group plasmids were immune only to N group plasmids, P group plasmids

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were immune to both P and W group plasmids and W group plasmids were immune to W and X group plasmids. There is thus a predictable cross-reacting pattern of immunity to killing. At least in the case of M5a1/pCU1, the basis of immunity is the ability of the plasmid to induce an irreversible change in M5a1, one that is not lost upon eliminating the plasmid state. The nature of this irreversible genetic change is of considerable interest as it appears to occur with a high frequency. The Kil⁻ mutant plasmid pCU21 was unable to induce such a change; that is M5a1/pCU21 was not immune to killing. It is possible that the lack of immunity in this strain is the indirect consequence of a plasmid mutation that affects the expression of several apparently unrelated functions. This is suggested by the observation that IKe-sensitivity, PRD1 sensitivity, and streptomycin-resistance are partially suppressed in this strain but not in M5a1/pCU1. Since E. coli strain C600/pCU21 expresses all these functions, suppression must require this mutation as well as other unspecified factors within M5a1.

C. Potential diagnostic use of the Kil⁺ phenotype

Several epidemiological studies of antibiotic-resistance plasmids have revealed that conjugative plasmids of the IncN group are frequently isolated from many species of Enterobacteriaceae and in many parts of the world, (Anderson, 1977; Coetzee et al., 1972). Since bacterial

conjugation mediated by this group is so efficient, this is probably the means of dispersal of this group of plasmids in bacterial populations. As pointed out earlier, there are also indications in the literature that two other groups of plasmids P and W are related to N group plasmids in their conjugative systems. If this is correct and N, P and W constitute an interrelated system the importance of these plasmids would even be greater since their host range extends beyond the Enterobacteriaceae to many species of bacteria (Olsen and Shipley, 1973; Tardif and Grant 1980).

In many parts of the world with urban populations and where it is feasible, it is common practice to monitor the prevalence of the different groups of plasmids. Both antibiotic-resistant and antibiotic-sensitive, naturally occurring isolates may be monitored in this manner, the latter because of evidence suggesting that there exists in nature a pool of plasmids that do not initially have antibiotic-resistance genes but which acquire such genes from other genomes (viruses, chromosomes, other plasmids) by recombination events such as transposition (Mitsuhashi et al., 1977). This latter group of plasmids have been referred to as 'cryptic' conjugative plasmids or as 'unloaded plasmids' and in situations that require the examination of large numbers of cultures as in epidemiological studies, simple tests for their detection would be extremely valuable. A test that is often used is

sensitivity to bacteriophages that are specific for bacteria carrying conjugative plasmids of particular groups (e.g. bacteriophage IKE for N group plasmids). For the detection of bacteria carrying otherwise cryptic conjugative plasmids, such tests have the disadvantage that the plasmids have often to be transferred to standard bacterial hosts before the phage-sensitivity test can be done. In the absence of selectable genetic markers on a plasmid, this becomes time consuming and expensive.

The discovery that a wide range of bacteria carrying any of the N, P and W groups of plasmids kill MSa1 (Table 3) has suggested a relatively much simpler and less-expensive test that is easily adapted to mass-screening programmes. This test is as described previously in RESULTS (page 39, 'spot test') except that the test strain may have no known plasmid-markers conferring resistance to antibiotics or other inhibitors.

To test the usefulness of this method, a number of natural bacterial isolates that were known to carry N group plasmids (on the basis of IKE-sensitivity and the plasmid Incompatibility test) but which were not resistant to any tested antibiotic (Tschape, 1977) were secured and tested in this manner. All of the isolates tested gave a clearly positive reaction as in Figure 4A. The simultaneous presence in the same strain of other plasmids

that by themselves do not confer a Kil^+ phenotype, did not interfere with the reaction (except plasmids of the group X). Various E. coli, Salmonella typhimurium, Proteus mirabilis and Pseudomonas aeruginosa strains, carrying N, P or W groups of plasmid gave positive results. Within the limits of the number of isolates tested these results have thus shown that this very simple and economical test can be very useful. It should encourage a more extensive investigation of its usefulness and limitations if any. Following our description of this procedure (Rodriguez and Iyer, 1981) and the provision of strains, such tests are currently being conducted with naturally occurring isolates in Athens, Bristol, Caracas and Maracaibo.

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