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Title of Thesis — Titre de la thèse

IncN group plasmids and plasmid regions determining the
killing of Klebsiella pneumoniae and of immunity to killing

University — Université

Carleton

Degree for which thesis was presented — Grade pour lequel cette thèse fut présentée

M.Sc.

Year this degree conferred — Année d'obtention de ce grade

1985

Name of Supervisor — Nom du directeur de thèse

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IncN GROUP PLASMIDS AND PLASMID REGIONS DETERMINING THE KILLING
OF KLEBSIELLA PNEUMONIAE AND OF
IMMUNITY TO KILLING

by

SANTOKH SINGH GILL, B.Sc. (Hon.)

A Thesis submitted to the Faculty of
Graduate Studies and Research in partial
fulfilment of the requirement for the
degree of Master of Science

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ABSTRACT

E. coli carrying conjugative IncN group plasmids caused the killing of Klebsiella pneumoniae upon mating with the latter.

In the prototype IncN plasmid, pCU1, two distinct regions called kilA and kilB, were needed for killing. These could be separated on two different plasmids and would still mediate killing but only under circumstances that allowed both of them to be transmitted into K. pneumoniae. Therefore, killing is most likely due to events occurring in K. pneumoniae during or after the plasmid has entered the cell. Furthermore, if a plasmid carrying one kil region was established in K. pneumoniae and the second region transferred into it, no killing was detected. This suggests that both kil regions or their products must act in concert.

K. pneumoniae strains that carry pCU1 were immune to this killing effect by a donor carrying pCU1. By using deletion derivatives of pCU1 and molecular clones containing parts of pCU1, one region conferring killing immunity (kim) was localized to a 4.5 kb region of the 39 kb parental plasmid. K. pneumoniae carrying plasmid vectors with only the kim region lost their immunity upon curing of these vectors. However, K. pneumoniae carrying the entire plasmid pCU1 or some of its derivatives did NOT

TABLE OF CONTENTS

Abstract	III
Acknowledgements	V
Dedications	VI
List of Figures and Tables	VIII
List of Abbreviations	X
INTRODUCTION	1

LITERATURE REVIEW

a. Bacteriocins and defective bacteriophages	6
b. Zygotic induction	10
c. Lethal zygotis	12
d. The <u>kil</u> and <u>kor</u> regions on the plasmid RK2	16

MATERIALS AND METHODS

Bacterial strains and plasmids	21
Media and chemicals	21
Mating procedures	28
Transformation	30
Extraction of plasmid DNA	31
Plasmid DNA manipulation	35
Electrophoresis of DNA	36
Isolation of total DNA	36
Curing of the plasmid pCU1 or its derivative from <u>K. pneumoniae</u>	38

Southern hybridization	39
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RESULTS

A. Detection of killing	43
B. Survey of naturally occurring IncN group plasmids for their ability to kill <u>K. pneumoniae</u>	48
C. Killing is a plasmid associated phenomenon	49
D. The requirement of the two <u>kil</u> regions to mediate killing	49
E. Effect of rearranging the two <u>kil</u> regions	51
F. Effects of the prior presence of the <u>kilA</u> or <u>kilB</u> region before the introduction of the second <u>kil</u> region by conjugation	59
G. A region associated with pCU1 that confers immunity to <u>K. pneumoniae</u> from killing by C600(pCU1)	62
H. Immunity which persists upon curing of the plasmid pCU1	69
DISCUSSION	84
REFERENCES	92

ABSTRACT

E. coli carrying conjugative IncN group plasmids caused the killing of Klebsiella pneumoniae upon mating with the latter.

In the prototype IncN plasmid, pCU1, two distinct regions called kilA and kilB, were needed for killing. These could be separated on two different plasmids and would still mediate killing but only under circumstances that allowed both of them to be transmitted into K. pneumoniae. Therefore, killing is most likely due to events occurring in K. pneumoniae during or after the plasmid has entered the cell. Furthermore, if a plasmid carrying one kil region was established in K. pneumoniae and the second region transferred into it, no killing was detected. This suggests that both kil regions or their products must act in concert.

K. pneumoniae strains that carry pCU1 were immune to this killing effect by a donor carrying pCU1. By using deletion derivatives of pCU1 and molecular clones containing parts of pCU1, one region conferring killing immunity (kim) was localized to a 4.5 kb region of the 39 kb parental plasmid. K. pneumoniae carrying plasmid vectors with only the kim region lost their immunity upon curing of these vectors. However, K. pneumoniae carrying the entire plasmid pCU1 or some of its derivatives did NOT

lose immunity upon curing of the plasmid. Two plasmid regions closely linked to kilA and kilB were required for this second type of immunity.

ACKNOWLEDGEMENTS

I am indebted to Bob Iyer for providing me an opportunity to widen my horizon in microbial genetics. I would like to thank him for his pragmatic and philosophical guidance.

I thank my advisors, Drs. Robert Watson and Hiroshi Yamazaki for their useful comments throughout my M. Sc. and reading the first draft of the thesis. I thank G. Selvaraj for reading the final draft of this thesis.

I extend my deepest appreciations to all the people, too numerous to mention, that I met for each has provided some moral support. In particular, I thank V. Thatte for his encouragement and numerous discussion.

For photography, I am indebted to Dr. Igor Livshitz.

I am grateful to Caroline Anne Dworschak for her continued moral support.

To my parents: Ajit Singh and Shiv Charen Kaur

and my niece: Rupri Cambow

" There is no worse tragedy
than what dies in a man
while he is still alive. "

A. Einstein

LIST OF FIGURES

Figure 1: Physical map of the plasmid pCU1	24
Figure 2: Appearance of the surviving colonies of <u>K. pneumoniae</u> following a mating experiment	45
Figure 3: The illustration of the qualitative 'spot' test used for the detection of the Kil phenotype	47
Figure 4: The pertinent regions on the plasmid pCU1 required for the Kil phenotype	52
Figure 5: The regions present on the derivatives of pCU1 which were used to determine the mode of action of <u>KilA</u> and <u>KilB</u>	56
Figure 6: A physical map of pCU1 showing the relevant phenotype for killing	60
Figure 7: The regions tested for the immunity phenotype	65
Figure 8: The regions on the pCU1 which conferred immunity to killing as determined by the qualitative test	67
Figure 9: The derivatives of pCU1 which were tested for their ability to confer permanent immunity to <u>K. pneumoniae</u>	75
Figure 10: Autoradiogram of the cured strains of <u>K. pneumoniae</u> using the probe ^{32}P -pCU1 DNA	79

LIST OF TABLES

Table I: Bacterial Strains used _____	22
Table II: Derivatives of pCU1 used _____	25
Table III: List of IncN plasmids used in detection of killing of <u>K. pneumoniae</u> _____	27
Table IV: Proficiency of conjugative transfer of pCU1 and its derivatives and their ability to mediate killing of <u>K. pneumoniae</u> _____	53
Table V: Frequency of transfer of plasmids from <u>E. coli</u> to <u>K. pneumoniae</u> in relation to the frequency of killing _____	57
Table VI: The effect on the <u>kil</u> phenotype of the prior presence of one <u>kil</u> region in <u>K. pneumoniae</u> before the introduction of the second <u>kil</u> region by conjugation _____	62
Table VII: Susceptibility of <u>K. pneumoniae</u> strains carrying different derivatives of pCU1 to killing by <u>E. coli</u> (pCU1) _____	66
Table VIII: The ability of different derivatives of pCU1 to confer killing immunity to <u>K. pneumoniae</u> by <u>E. coli</u> C600(pCU1) _____	68
Table IX: The ability of different derivatives of pCU1 to protect the host <u>K. pneumoniae</u> after plasmid curing _____	76
Table X: The susceptibility of plasmid cured strains of <u>K. pneumoniae</u> to killing by C600(pCU1) _____	78

List of Abbreviations

Amp	Ampicillin resistance (specified by the chromosome)
Ap	Ampicillin resistance (specified by the extrachromosomal element)
C	Celsius
Cm	Chloramphenicol
Col	Colicin plasmid
D	Donor
dATP	Deoxyadenosine triphosphate
dNTP	Deoxyribonucleotide triphosphate
E	Electrophoresis buffer
<u>E. coli</u>	<u>Escherichia coli</u>
EDTA	Ethyelene diamine tetraacetate
EtBr	Ethidium bromide
F +	Cell bearing an F plasmid
F -	Cell devoid of the F factor
Hfr	Strain carrying a conjugative plasmid intergrated into its chromosome
Inc	Plasmid Incompatibility group
Kil -	Kil deficient
Kil +	Kil proficient
km	Kanamycin
kb	Kilobase
<u>K. pneumoniae</u>	<u>Klebsiella pneumoniae</u>
mob	mobilization
min	minute

Nal	Nalidixic acid
PA	Penassay agar
PB	Penassay broth
PEG	Polyethylene glycol
r	resistance
R	recipient
R ⁺	Cell carrying an R plasmid
Rif	Rifampicin
SDS	Sodium Lauryl Sulphate
Sm	Streptomycin resistance (specified by the plasmid)
Sp	Spectinomycin
Str	Streptomycin resistance (specified by the chromosome)
Tc	Tetracycline
TE	Tris-EDTA buffer
Tn	Transposon
Tr ⁺	Transfer proficient
Tr ⁻	Transfer deficient
u	Micro
uCi	Micro curie
ug	microgram
ul	microlitre
UV	ultra-violet radiation
w/v	Weight per unit volume

INTRODUCTION

Plasmids are extrachromosomal, autonomously replicating DNA molecules which add substantially to the genetic capacity of their host. However, under most circumstances, plasmids are not essential for the successful growth and metabolism of the host (Elwell and Shipley, 1980). The ability of some bacterial strains to maintain and inherit plasmids for many generations indicates that plasmids are not usually deleterious to the host. Therefore, these genetic elements probably carry genes for some supplementary activity that allows their host to survive in adverse environments or permits their host to compete more successfully with other microorganisms of the same or related species (Elwell and Shipley 1980; Davis and Smith, 1978). Many plasmids code for supernumerary properties that are not so obviously related to the maintenance of the plasmid itself. Depending on the particular plasmid and bacterial host, these include resistance to or the production of antibiotics, toxin production, nitrogen fixation, synthesis of whole enzyme pathways and virulence. Currently, relatively few genes have been recognized for most large plasmids. This leaves a substantial amount of genetic material unaccounted for especially on large plasmids. Studies of plasmid gene expression using in vitro transcription and translation systems indicate that many more proteins are made than have been recognized in

vivo (Davis and Smith, 1978). It also needs to be pointed out that most studies on plasmid-determined functions have been conducted only in a single bacterial host such as E. coli. Mechanisms may exist for a plasmid function to be expressed in one host but not in another or for plasmid genes to be expressed with different consequences in different hosts (Selvaraj and Iyer, 1984; Guiney et al. 1984; Rodriguez and Iyer, 1981).

Another feature of the molecular genetics of plasmids that has so far received insufficient attention is that some aspects of the properties and behaviour of plasmid-carrying cells may well be under the joint control or influence of chromosomal and plasmid genes. There are suggestions of this in respect to functions involved in ~~SSNA~~ DNA replication, recombination, repair, and mutagenesis (Albert and Frey, 1970; Zeig et al., 1978; Hopkins et al., 1980; Pinney, 1978). This thesis concerns a novel plasmid-associated phenomenon that was reported a few years ago (Rodriguez and Iyer, 1981). When E. coli donors carrying the plasmid pCU1 or closely related plasmids were mated with a Klebsiella pneumoniae strain, there was a substantial reduction in the viability of the recipient. This intriguing and novel behaviour was referred to as the killing phenomenon (Rodriguez and Iyer, 1981). The mechanism by which a fraction of K. pneumoniae cells is killed upon mating or the mechanism by which a smaller

fraction escapes this effect is not known. A first and possibly useful step in understanding this phenomenon is to determine whether the entire plasmid is necessary for one or both of these effects or whether specific regions on the plasmid can be identified as being necessary for killing and for immunity to killing. Accordingly, the principal objective of this study has been to undertake a structural analysis of the DNA of a representative IncN group plasmid, pCU1, with a view to identifying such regions, if any. Before undertaking such an analysis, a large number of plasmids of this group were assembled and surveyed for this phenotype to ascertain (a) whether killing is a group-specific property or is a property unique to one or a few plasmids of the group and (b) if it is associated with any other plasmid phenotype. The study has led to the following conclusions:

(i) the Klebsiella Kil phenotype is a property shared by all plasmids of incompatibility group N, but only if they are conjugative; antibiotic-resistance genes present on these plasmids are irrelevant to the killing phenotype and the association of this phenotype with IncN group plasmids was probably established before the extensive use of antibiotics in the environment.

For pCU1, a prototype IncN group conjugative plasmid that is being studied in detail (Kornaska-Kozłowska and Iyer, 1981; 1983; Kornaska-Kozłowska, Thatte, and Iyer; 1983;

and Thatte, V., Ph. D., 1984; Carleton University) (ii) there are at least two regions on the plasmid pCU1 that are needed to kill K. pneumoniae. By inference, analogous regions may be encountered in other plasmids of the group; (iii) to observe killing, both kil regions must be transferred simultaneously into K. pneumoniae, (iv) when a specific region of the plasmid which is separable from the above two kil regions is previously introduced and maintained by K. pneumoniae, it provides immunity to killing by the subsequent introduction of the two kil regions. Loss of a recombinant plasmid vector containing only this region results in the loss of immunity.

(v) the apparent loss of the entire plasmid pCU1 or some of its derivatives does not result in the loss of immunity. Two regions closely linked to kilA and to kilB were needed for this type of immunity that is distinguishable from the immunity conferred by kim.

Before describing and discussing the experiments on which these conclusions are based, I will briefly review observations that have been reported in the literature on the ability of bacterial populations carrying certain plasmids to kill other bacteria. This may provide a useful framework for my observations.

LITERATURE REVIEW

The basic observation presented in the Introduction was that a large fraction of the cells of a strain of Klebsiella pneumoniae was killed during or after conjugation with a donor strain carrying a transfer proficient N group plasmid. A small fraction of the cells survived even though they received the plasmid. The latter phenomenon is called immunity in this thesis. The mechanisms of these killing and immunity phenomena are unknown. However, several possible mechanisms could be considered based on observations reported in the literature.

Of these, the first is the production of an extracellular antibacterial compound such as an antibiotic the production of which is determined by the plasmid. A number of such bacterial inhibitors are known. The large majority are produced by either fungi or actinomycetes. However, inhibitors of this type as a rule have a host range that includes at least several species and genera of bacteria. Further and importantly, Rodriguez (M.Sc., thesis Carleton University, 1980) has shown that the killing phenomenon that is the subject of this thesis is not mediated by any substance that can diffuse from E. coli to K. pneumoniae. For these reasons, this mode of killing is not considered any further in this review. There are other modes of bacterial inhibition and killing

that can be plasmid mediated and for which there is precedent among the Gram negative bacteria. These are:

- (a) the production of bacteriocins and of defective bacteriophages,
- (b) zygotic induction of a latent bacteriophage,
- (c) lethal zygotis,
- (d) kil and kor genes of the plasmid RK2,

Information on each of these modes is reviewed with respect to the structural and functional organization of plasmid-borne genes determining killing, and immunity to killing, and the mechanism of killing and of immunity to killing, where this is known.

(a) Bacteriocins and defective bacteriophages

Bacteriocins are bactericidal substances, usually proteinaceous in nature. These are synthesized and often excreted by some strains of bacteria to which other strains of the same or related groups are susceptible (Reeves, 1972; Luria, 1977,). They are usually given names that indicate their origin; examples are megacin from Bacillus megaterium, subtilisin from Bacillus subtilis, colicins from E. coli, cloacin from Enterobacter cloaca (Amati and Ozeki, 1962, Dewitt and Helsinki, 1965, Coetzee, 1964). Colicins and cloacins are the best studied bacteriocins because it has been possible to relate studies with them to the wider corpus of knowledge of the molecular biology of E. coli and its close

relatives. All colicins (or cloacins) studied so far, are proteins specified by plasmids called Col factors. These factors fall into the same two classes as do antibiotic-resistance plasmids (R factors). Some are large (60 megadaltons or greater), and self-transmissible, while others are small, and not self-transmissible (Broda, 1979). New strains acquiring a Col plasmid produce a colicin identical to that of the original host. In addition, the host upon acquiring the Col factor resembles the noncolicinogenic parental strain in all respects except for the properties associated with the Col factor (Reeves, 1973). Further, plasmids coding for colicins also confer immunity against exogenous colicins of the same type. Immunity to colicins that act on intracellular targets is provided by cytoplasmic immunity proteins, which specifically inhibit the enzyme activities of their respective colicins (Konisky, 1980, Mock and Pugsley, 1982).

In well-characterized bacteriocin plasmids such as ColE1-K30, CloDF13 (Veltamp and Stuitje, 1981) ColE3-CA38 (Watson and Visentin, 1982) and ColN (Pugsley, 1984), the colicin and immunity genes are adjacent to each other, such that the immunity gene is near the 3'-terminus of the colicin gene. In the case of CloDF13, these genes allow a co-ordinated synthesis of both proteins from a single transcript (Oudega et al., 1982) whereas in ColE1, the

immunity gene although adjacent to the colicin producing gene, appears to be functionally independent and is thought to be transcribed in the opposite direction (Sabik et al., 1983). A third gene thought to be responsible for the lysis and death of the host cell also accompanies the genes which code for colicin and immunity production for the above mentioned Col factor (Watson et al., 1984; Jakes and Zinder, 1984). However, the mode of action of these lysis proteins remains to be elucidated. Genes with apparently similar functions exist, not only on other Col factors (Pugsley and Schwartz, 1983; Jakes and Zinder, 1984) but interestingly also on certain phages.

Immunity is distinct from resistance, which can arise due to alteration or loss of functional receptors for the colicins. Several receptors have been identified as proteins in the outer membrane (Hill and Holland, 1967; Jasper et al., 1972). Several types of colicin resistant mutants, which absorb colicins but are not killed by colicin have been isolated and characterized. For some of the Tol (tolerant) mutants it has been suggested that they affect the transport of the colicin from the receptor of the outer membrane to the biochemical targets inside the cell or that they affect the target itself (Luria, 1973; Nomura, 1967). The Tol mutations are in the chromosomal regions of E. coli and are distinct from the immunity genes that are plasmid-borne.

The production of colicins can be induced by treatment of bacteriocinogenic cells with agents known to induce the DNA repair system, such as mitomycin C or U.V. irradiation (Menkveld et al., 1979). They have traditionally been detected by the zone of clearing that colicinogenic colonies produce when overlaid with sensitive cells (Reeves, 1973). Colicins present a whole spectrum of behaviour involving different levels of the cell structure and also of biochemical reactions in the cell. As macromolecules, they pass across the cell membranes by special mechanisms. At least three fundamental steps are involved:

- (a) attachment of the colicin molecule to discrete and special receptors on the cell surface;
- (b) conversion of the colicin to a new state or its translocation to a new site by an energy-dependent process;
- (c) special interaction between the colicin and its biochemical target (Konisky, 1983, Garcia et al., 1982).

With respect to the specific interaction between the colicin and its biochemical target, three major types of mode actions have been recognized:

- (a) DNA degradation and the inhibition of cell division. An example is Colicin E2.
- (b) Inhibition of protein synthesis by cleaving the 16s ribosomal RNA. Thus, it is a nuclease acting on RNA

rather than DNA. Examples are colicin E3, ColD and cloacin CloDF13.

(c) Inhibition of macromolecular synthesis and also active transport of amino acids and B-galactosidase. Examples are colicins E1, K, Ia, A and S8.

Many organisms such as E. coli, Yersinia enterocolitica, Pseudomonas aeruginosa and Bacillus subtilis produce defective bacteriophages. The defective bacteriophages of B. subtilis were recognized as having properties of bacteriocins, while morphologically, they were identifiable as bacteriophage (Subbaih et al., 1965). These phages only contain host DNA and were detected by their ability to kill sensitive cells. Similar phenomena are likely to be found in other bacteria. These "bacteriocins" which are only identified by their biological rather than physiochemical properties may in fact be defective bacteriophages. A defective phage, which can kill without the normal infection of the bacteria would closely mimic the biological properties of bacteriocins (Reanney and Ackermann, 1979).

(b) Zygotic induction

Zygotic induction is an intriguing phenomenon which occurs following conjugation between Hfr donor cells (an Hfr cell is a bacterium in which an F factor is integrated into the chromosome) and F cells. The E. coli K-12 strains and most of its derivatives are

lysogenic for various bacteriophages; that is, the phage genome is carried in an inert form, the prophage, as part of the chromosome (Hayes, 1965). When the lysogenic Hfr strain is mated with an F^- cell which does not carry a prophage, transfer of the phage genome from the donor to the recipient is followed by an immediate induction of phage development in the recipient strain, resulting in the lysis and release of the phage particles. This is zygotic induction. Its cause is that the non-lysogenic cells lack the repressor protein synthesized by the prophage which maintains the inert state, so that the incoming prophage is expressed immediately upon entry into the non-lysogenic (or non-immune) cells (Fernstein and Low, 1982). Since all the zygotes which inherit the prophage are destroyed, those bacterial genes to which the prophage is linked on the donor chromosome are lost and fail to appear among the recombinants.

Low (1973) and Fernstein and Low (1982) observed similar results as above using E. coli carrying the rac locus. However, in the case of rac (recombination activation), no phage particles are produced upon induction. The rac 'prophage' could be a defective phage which has lost the part of the genome responsible for the production of phage particles (Fernstein and Low, 1982; Kaiser and Murray, 1979). Further, Kaiser and Murray (1982) have shown that many E. coli strains contain

sequences homologous to the rac locus which are close to the trp operon. The rac locus can be induced and lost from the chromosome. Such an event may have occurred in one of the ancestors of the strain AB1157 and also in the derivatives of C600 (Kaiser and Murray, 1982).

In zygotic induction the location of genes determining killing is determined by the site on the plasmid where the defective phage DNA is inserted. In principle, such a site may be unique as is the case of rac or not unique as in the case of bacteriophage Mu. As far as is known, immunity to this means of killing is determined only by the presence of another defective phage in the recipient DNA. However, and in principle, immunity requires only the presence of the immunity gene from a rac like phage.

(c) Lethal zygosis

Lethal zygosis is a phenomenon whereby up to 99% of the recipient F⁻ cells die within one to two hours when mated with a high multiplicity of Hfr cells (20:1::Donor:Recipient) (Gross, 1963; Skurray and Reeves, 1973; Skurray and Reeves, 1976). However, when F⁺ or F' cells are used as donors no lethal effect is observed (Skurray and Reeves, 1973; Ou, 1980) unless a higher multiplicity of 200:1 (D:R) is used. To observe this lethal effect, direct cell to cell contact is required such as is found in conjugation (Skurray and Reeves,

1976). Iyer and Bhaskaran (1968) observed results similar to the lethal zygotis of E. coli when a donor strain of Vibrio El Tor was mated with strains of E. coli or Vibrio cholera on a solid surface. When the donors were killed at high temperatures and then used in these mating experiments, no lethal effect was observed. This raises the possibility that the lethal effect observed in this case is a sequel to conjugation.

In lethal zygotis, the recipient F^- cells, after mating with Hfr cells, undergo a number of physiological changes in membrane permeability and transport. In addition, enzyme induction and DNA synthesis are also inhibited. According to Skurray and Reeves (1973), lethal zygotis is an extreme manifestation of events which normally occur during conjugation since the same but less dramatic effects are observed at low Hfr donor to recipient ratios (1:1). However, as the number of donors increase, the survival of F^- cells decreases. These observations suggest that the lethality observed results from multiple conjugations involving simultaneous mating between one F^- cell and several (Hfr) donor cells (Skurray and Reeves, 1973; Ou, 1980). One speculated reason for the absence of a lethal effect with F^+ or F' (ratio 20:1::D:R) is that they are able to transfer and express the immunity gene(s) earlier than Hfr cells (Skurray and Reeves, 1973; Ou, 1980). It has been observed that in F^+

X F^- matings, the killing of the recipients occurs only if F plasmid transfer is inhibited by an inhibitor of DNA transfer such as nalidixic acid (Nal) (Ou, 1980). The observation that at a low D:R ratio, F^- recipients are only killed when F^+ transfer is inhibited lends credence to a hypothesis that the F plasmid contains the gene(s) which expresses immunity to lethal zygosis (ilz). This result also supports the hypothesis that the primary cause for lethal zygosis is an extensive damage inflicted upon the recipient membrane by the mating donor (Ou, 1980). When the inhibitory effect of Nal is removed, F^+ donors show a dramatic decrease in killing, which is probably due to the entry and expression of the ilz gene(s) in the recipient. When Hfr donors are used, killing of the F^- cells is observed regardless of the presence or absence of Nal. However, in the absence of Nal, Killing is 16-fold greater (Ou, 1980). This suggests that the entry of DNA from Hfr donors aggravates the pre-existing damaged bacteria, causing extensive killing. The speculative reason for the ability of Hfr cells to kill F^- cells, (whereas F^+ or F' cells do not kill), is that the ilz gene(s) is located near the transfer (tra) genes and that in Hfr cells this ilz gene is transferred late (Broda, 1972). In contrast, in matings with F^+ or F' donors the ilz gene is transferred rapidly; its expression would render the recipients immune to lethal zygosis (Skurray

and Reeves, 1973; Ou, 1980). Recipients harboring an F factor exhibit Ilz^+ in matings with Hfr donors (Skurray and Reeves, 1976).

Surface exclusion (Sfx) is a phenomenon whereby sex factor carrying cells are poor recipients in matings with donor cells carrying related sex factor.

Ou (1980) suggests that the sfx genes are the most likely candidates for the Ilz phenotype, since Sfx is a surface phenomenon and the sfx gene products are known to be associated with the bacterial membrane (Achtman et al., 1977). There are two known Sfx-determining genes, traS, whose product is associated with the inner membrane (Achtman et al., 1977; Minkley and Ippen-Ihler, 1977), and traT, whose product is associated with the outer membrane (Achtman et al., 1977; Achtman et al., 1977b).

It appears that the ilz genes may play a role in repairing the damaged membrane. These ilz genes are thought to be expressed soon after transfer into the recipient and to function in membrane repair. With time, they elicit surface exclusion to prevent further mating. Since the presence of Nal inhibits the transfer of F plasmid, no repair can be carried out. There is a difference in the degree of killing observed in mating with the two Sfx mutants. Each retains some membrane repair ability (Ou, 1980). Furthermore, damage of the inner membrane, where the traS product has been shown to

be localized, may be more detrimental to the bacteria (Ou, 1980). Thus, the mechanism of Sfx is proposed to have two components:

- (a) a contribution from traT, which acts primarily to inhibit the formation of stable mating aggregates;
- (b) a contribution of traS, which inhibits DNA transfer and plays only a minor role in inhibiting formation of mating aggregates (Achtman et al., 1977).

However, and despite these speculations, it is emphasized that to date, the molecular mechanism of Sfx is still obscure.

(d) The kil and kor regions on the plasmid RK2

The plasmid RK2 belongs to the Incompatibility (Inc) group P. Members of this group have a broad host range and are reported to be transferred to and maintained by virtually all Gram negative bacteria tested (Smith and Thomas, 1984). This may be indicative of strategies for transfer, maintenance and gene expression. The initial observations were that RK2 possesses a number of genetic loci (called kil regions) which could not be cloned in the absence of other loci (called kor regions for kil override). It was also shown that each kor region could act in trans to suppress the effect of a specific kil region (Figurski et al., 1982; Smith and Thomas, 1983; Young et al., 1984). To date, there are 4 such kil regions, each with its corresponding kor region. The

inability to clone kil regions in the absence of its complementary kor region could be due to:

- (i) inhibition of replication of the plasmid; or
- (ii) inhibition of colony formation by the cells.

To distinguish between these two possibilities, the kil region was linked to a plasmid which does not require DNA polymerase I for plasmid replication; while the corresponding kor region was inserted into a ColEI-like plasmid whose replication is dependent upon DNA polymerase I. For each kil region, the plasmid containing it and its corresponding kor plasmid was first established at the permissive temperature in a polA (ts) E. coli, a mutant which is temperature sensitive for DNA polymerase I. Once established, they were plated at 42°C (the nonpermissive temperature) on media, without selection for plasmid.

If the plasmid containing kor is required to provide a function which allows replication or maintenance of the kil plasmid, colonies formed at 42°C should be devoid of both plasmids. In contrast, if the kor function is involved in preventing kil-mediated cell death, no colonies should form at 42°C (Figurski et al., 1982). For kilA, kilB and kilC, the results supported the latter mechanism.

Since colonies did form at the nonpermissive temperature with kilD under selective and nonselective conditions, no loss of viability occurs as is the case for

kilA, kilB and kilC. However, the colonies which did form with kilD at the nonpermissive temperature and on selective media for the kilD plasmid, were smaller than those in the nonselective medium and in addition did not grow when subcultured in a medium containing an antibiotic resistance to which was determined by the plasmid (Smith and Thomas, 1983).

The korA and korB regions and probably also korD were found to be highly conserved in many IncP plasmids by DNA-DNA hybridization at high stringencies of hybridization. These regions were also found to be specific to IncP plasmids. Plasmids from 19 other Inc groups tested did not show cross-hybridization with DNA probes containing korA and korB. Although kil and kor regions are ubiquitous in IncP plasmids, no specific role has been assigned to them. It has been suggested that although nonessential and potentially harmful to E. coli, they may represent functions necessary for their existence in other hosts. An example of this is a particular host range mutant described by Cowan and Krishnapallai (1982). The mutant was generated by a Tn7 transpositional insertion into R18, which is identical to RK2 (Burkardt et al., 1979). This mutant was isolated in P. aeruginosa. Although this plasmid mutant is capable of being transferred between P. aeruginosa strains, it could not be transferred to E. coli C by either mating or

DNA-mediated transformation. The parent R18 plasmid could be transferred by both of these methods. The Tn7 insertion in R18 mapped in kilB.

Barth (1979) described another host range mutant of RP4 with a Tn76 insertion in the korB region of the plasmid. It was observed that this mutant is highly unstable in E. coli and has a tendency to generate spontaneous deletions. The deletions most likely remove kilB which otherwise would be lethal to the cell in the absence of its korB. In addition, this mutant could not be transferred to P. aeruginosa, although, it could be transferred to S. typhimurium, P. mirabilis,

S. marcescens, K. aerogenes, and E. coli. Inability of this mutant to transfer to P. aeruginosa is related to its korB region as shown below. Although the smallest genetic region required for plasmid maintenance in E. coli for RK2 is defined as a vegetative replication origin (ori RK2) and a gene specifying a positively required trans acting product (trfA), the same is not true for the host P. aeruginosa. Thomas et al. (1982) have demonstrated that in addition to ori RK2 and trfA, a third region containing korA, korB and korD (called trfB) is required. This region has to be physically linked regardless of the orientation to oriT and trfA. In further support of this, a mutant of RP4 which is temperature sensitive for maintenance in P. aeruginosa but not in E. coli,

physically maps within the trfB region.

These observations raise the possibility that kil and or kor regions may be somehow related to providing functions for the maintenance of plasmids in bacteria other than E. coli, the host in which most studies have been conducted.

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 I to Table III respectively.

Media and chemicals

Penassay Agar (PA) was used as the organic solid medium in all experiments. It was composed of 17.5g/l of Antibiotic Medium 3 (Difco Laboratories) and 15.0g/l of Difco-Bacto Agar. The minimal medium used was that of Davis and Mingioli (1950), which was prepared by mixing presterilized (autoclaved) solutions of inorganic salts with sterilized water and agar (final concentration 1.5%). Glucose 0.5% (w/v) was used as the carbon source. When required, the minimal medium was supplemented with Millipore-filter-sterilized solutions of amino acids, vitamins, and antibiotics. These were added to give a final concentration (ug/ml) of: L-amino acids, 40, vitamin B1 (thiamine), 5, thymine, 10, ampicillin (Ap) 30, chloramphenicol (Cm) 30, kanamycin (Km) 50, nalidixic acid (Nal) 100, spectinomycin 50 or 70, (depending on the strain) streptomycin (Sm) 50, tetracycline (Tc) 10 and Rifampicin was prepared fresh in methanol and used at 100 ug/ml.

The L-broth used to grow all the strains.

TABLE 1
BACTERIAL STRAINS USED

Plasmid free bacteria strains	Relevant phenotype	Source or derivation
<u>E. coli</u>		
AB1527	His ⁻ Leu ⁻ Pro ⁻ Thi ⁻ Thr ⁻	A. J. Clark
AB1528	As above and Nal ^r	Spontaneous Nal mutant of AB1527
C600	Leu ⁻ Thi ⁻ Thr ⁻	Cohen <u>et al.</u> (1972)
CSR603	Arg ⁻ Leu ⁻ Pro ⁻ Thi ⁻ Thr ⁻ Phr ^S Uvr ^S Str ^r	Sancar and Rupp (1978)
HB101	Leu ⁻ Pro ⁻ Thi ⁻ Rec ⁻ Str ^r	Bolivar <u>et al.</u> (1977)
GS500	As above and Rif ^r	Spontaneous Rif of HB101
W1485	Sup42	Bachmann (1972)
<u>Klebsiella pneumoniae</u>		
M5a1	Amp ^r	MacNeil <u>et al.</u> (1978)
Mna1	Amp ^r Nal ^r	Spontaneous Nal of M5a1
IRAN	Amp ^r Nal ^r Rif ^r	Spontaneous Rif of Mna1
S1, S2, S3	Amp ^r	M5a1(pCU1) cured of its plasmid
S4	Amp ^r	M5a1(pCU167) "
S5	Amp ^r	M5a1(pCU66) "
S6	Amp ^r	M5a1(pCU184) "

S7	Amp ^r	M5a1(pCU10) "
S8	Amp ^r	M5a1(pCU16) "
S9	Amp ^r	M5a1(pCU109) "
S10	Amp ^r	M5a1(pCU11) "
S11	Amp ^r	M5a1(pCU13) "
S12	Amp ^r	M5a1(pCU101) "
S13	Amp ^r	M5a1(pCU401) "

Other Bacterial
Strains

<u>Salmonella</u> <u>typhmurium</u>	His ⁻	Sanderson, Salmonella center
<u>Pseudomonas</u> <u>aeruginosa</u>	Leu ⁻ Rif ^r Nal ^r	This Lab
<u>Achromobacter</u> <u>xylosoxidans</u>	Rif ^r Nal ^r	This Lab
<u>Agrobacterium</u> <u>tumefaciens</u>	Rif ^r Nal ^r	This Lab

a. A large number of plasmid-carrying strains were constructed and used in this study. The nomenclature of such plasmid carrying strains is that of the corresponding plasmid free strain followed by the name of the plasmid, enclosed by brackets. For example, strain C600(pCU1) is a derivative of C600 carrying the plasmid pCU1. The phenotypes of the plasmids are shown in Table 11 and Table 111.

b. Abbreviations

Arg, Arginine; His, Histidine; Leu, Leucine; Met, Methionine; Pro, Proline; Pyr, Pyrimidine; Thi, Thiamine; Rec, Recombination deficient; Nal Nalidixic acid Rif, Rifampicin; Sm, Streptomycin; Ap, Ampicillin, r, resistant, - dependent, Phr, photoreactivation; Uvr, ultraviolet sensitive.

TABLE 11

The derivatives of pCU1 used

Derivative	Relevant Markers								Source	Map position (kb coordinates on pCU1, see Fig.1)	
	Ap	Cm	Km	Sm	Sp	Ike	PRD1	Mob			Tra
pCU1	r	s	s	r	r	s	s	+	+	Konarska-Kozlowska and Iyer (1983)	parental plasmid
pCU10	r	s	s	r	r	r	r	+	-	"	5.1-33.0
pCU11	r	s	s	r	r	r	r	+	-	"	0.0-27.0
pCU12	r	s	s	r	r	r	r	+	-	"	0.0-28.0
pCU16	r	s	s	r	r	s	s	+	+	"	27.6-0.0 & 0.0-25.0
pCU45	s	r	s	s	s	r	r	-	-	"	
pCU46	s	r	s	s	s	r	r	-	-	"	
pCU51	s	s	r	s	s	r	r	-	-	Thatte (1984)	0.2-21.0
pCU53	s	r	s	s	s	r	r	-	-	"	27.0-3.0
pCU54	r	s	s	r	r	r	r	-	-	"	21.0-20.0
pCU55	s	r	s	s	s	r	r	-	-	"	11.5-15.7
pCU57	s	r	s	s	s	r	r	-	-	"	0.0-3.2
pCU58	s	s	s	r	r	r	r	+	-	"	27.7-24.0 & 5.1-8.3
pCU59	r	s	r	s	s	r	r	-	-	"	0.4-7.2
pCU101	s	r	s	s	s	s	s	+	+	"	27.0-3.2
pCU109	s	r	s	s	s	s	s	+	+	"	27.0-11.2
pCU401	r	s	r	s	s	s	s	+	+	"	31.4-21.0

PCU400	s	r	s	s	s	r	r	-	-	"	12.2-21.0
PCU107	r	s	r	r	r	s	r	+	+	"	31.6*
PCU104	r	s	r	r	r	s	r	+	+	"	31.4*
PCUCC	r	s	r	r	r	s	r	+	+	"	31.6*
PCU404	r	s	r	s	s	r	r	+	-	This study	7.0-11.5

Abbreviation: Ap,apicillin; Cm,chloramphenicol; Km,kanamycin;
 Mob,mobilization; Sm,streptomycin; Sp,spectinomycin; +,presence of
 phenotype; -,absence of phenotype. * Position of Tn5.

TABLE 111

IncN group plasmids of diverse origin tested as part of this study or by Rodriguez and Iyer (1981) for their ability to mediate killing of K. pneumoniae. (Procedure 2 as in Materials and Methods).

Plasmid	Ap	Cm	Hg	Km	Sm	Sp	Su	Tc	Source	Reference
R395	r	r	s	s	r	r	r	s	<u>P. rettgeri</u>	Coetzee <u>et al.</u> (1972)
R390	r	r	s	s	r	s	s	s	"	"
R701	r	s	s	s	r	r	s	s	<u>Proteus sp.</u>	Hedges (1972)
R348	s	s	s	s	r	s	s	r	<u>E. coli</u>	Bannister (1969)
R15	s	s	r	s	r	s	r	s	<u>P. vulgaris</u>	Datta and Hedges (1971)
N3T	s	s	s	s	s	s	s	r	<u>P. mirabilis</u>	Hedges (1975)
R760a	r	s	s	r	r	s	s	s	<u>P. morganii</u>	Hedges <u>et al.</u> (1972)
R393	s	s	s	s	r	s	s	s	"	"
R461	r	r	s	s	r	r	r	r	"	"
R448	r	r	s	s	r	r	r	s	"	"
R476	s	s	s	r	s	s	s	s	"	"
R465	s	s	s	s	r	r	r	s	"	"
R447b	r	s	s	r	s	s	s	s	"	"
R447b-1	r	s	s	s	s	s	s	s	"	"
R497	r	s	s	r	r	s	s	s	"	"
pCF290	s	s	s	s	r	s	r	s	<u>E. coli</u>	Tardif and Grant (1980)
pMUR547					nil				<u>Shigella sp.</u>	Murray Collection
pMUR274					nil				"	via Datta and

pCU1	r	s	s	s	r	r	s	s	s	<u>S. typhimurium</u>	Konarska-Kozłowska and Iyer (1981)
R45	r	s	s	s	s	s	r	r	s	"	Datta (1975)
R40	s	s	s	s	r	r	r	r	s	"	"
R40	r	s	s	s	r	r	r	r	s	"	"
P110	s	s	s	s	s	s	s	r	s	"	"
P100	s	s	s	s	s	s	r	r	s	<u>S. paratyphi</u>	Scavizzi (1973)
R205	r	s	s	s	s	s	r	r	s	<u>S. typhi</u>	Dannister (1969)
PT1550	r	s	s	r	r	s	s	s	s		Babudri and Monti-Dragocin (1970)
PLT200	s	s	s	s	r	s	r	s	r		Taylor et al. (1970)
p12005					nil						Tschape (1977)
p12030					nil						"
p11001					nil						"
p12512					nil						"
p12514					nil						"
Other Plasmids Used											
pNCYC104	s	r	s	s	s	s	s	s	r		Cohen et al. (1972)
pK2004	r	s	s	r	s	s	s	s	r		Kahn et al. (1979)

Abbreviations: Ap, Ampicillin; Cm, Chloramphenicol; Hg, Mercury; Km, Kanamycin; Sm, Streptomycin; Sp, Spectinomycin; Su, Sulphonamide; Tc, Tetracycline; Tp, Trimethoprim; r, Resistance; s, sensitivity. All plasmids also conferred sensitivity to group specific bacteriophages IRe and PFD1.

L-Broth contains per litre of distilled water: 10 g Bactotryptone, 5 g yeast-extract, and 5 g sodium chloride at pH 7.0. For the isolation of plasmid DNA, the bacterial culture medium used to grow the strain was Penassay Broth (Antibiotic medium 3; PB) supplemented with the antibiotic (half of the concentration which was used for solid media) to which the strain was resistant.

Mating Procedures:

(a) Spot Mating

An early stationary phase culture of the recipient (0.2 ml) was spread on the surface of a predried agar medium selective for the transconjugants (i.e. a medium on which neither donor nor recipients alone would grow). A drop (0.05 ml) of an exponential phase donor culture was spotted on the surface of this recipient-seeded agar plate and incubated for 24 to 48 hours at 37°C. As controls, donors and recipients were tested for growth on the selective media each time. Transconjugants that arose on this plate were purified twice to give single colonies on the selective media and then used to determine the presence of the nutritional or other unselected marker of the recipient before they were used in further experiments.

(b) Filter Mating

This procedure was used for determining the frequency of transfer of a plasmid from an R⁺ strain. In

addition, it was used to determine the efficiency of killing of Klebsiella pneumoniae strains by potential donor strains. An exponential-phase donor culture was mixed with an exponential-phase recipient culture at a ratio of 20:1. The mixture was immediately collected on a membrane filter (Millipore Corp., diameter 45mm, pore size 0.45 μ m) by suction. The membrane filter was placed with the collected bacteria on top, on a pre-warmed (37°C) PA plate. After 1 hour of incubation, the cells were washed off the filter with 2 ml of sterile 0.85% saline by agitation in a Vortex mixer. Serial dilutions were made in saline, and 0.1 ml of the dilutions were spread on the appropriate media for the selection of transconjugants. The plates were incubated for 24 to 48 hours (depending upon the media used) before the colonies were counted.

Transformation

The procedure used was that of Cohen et al. (1972). An overnight culture of the strain used was diluted either 50 or 100-fold in LB supplemented with an appropriate antibiotic where this was desired (for example, when the strain carried an antibiotic-resistance plasmid). The cells were regrown to an optical density of 0.6-0.8. The cells were chilled for about 20 mins on ice and sedimented at 12,000 xg for 10 mins. The pellet was resuspended in 0.5 volume of cold 10 mM NaCl. The cells were pelleted again and resuspended in 0.5 volume of cold

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and kept on ice for 20 mins , sedimented and resuspended in 0.1 of the original the 30 mM CaCl_2 solution. To 0.2 ml aliquots of competent cells, the desired DNA was added , whereas the control was devoid of exogenous DNA. After an additional incubation for 60 mins at 0°C, the mixture was subjected to a heat pulse at 37°C for 5 mins. The mixture was chilled for 10 mins and then diluted 5-fold in LB. After incubation for 1 hour at 37°C, the mixture was plated on different kinds of media selective for transformants. Transformant colonies selected after 24 to 48 hours of incubation at 37°C were then checked for the desired properties.

Extraction of Plasmid DNA

For the extraction of plasmid DNA one of three methods was used depending on the amount and purity of DNA desired.

Method 1:

A modification (outlined by V. Thätte, personal communication) of the procedure described by Clewell and Helinski (1969) was used. An overnight culture of an R strain which had been grown in the presence of the antibiotic to which the plasmid conferred resistance was pelleted by centrifugation at 10,000 xg for 10 mins at 0°C-5°C in a GSA Sorvall rotor. The cells were resuspended in 20 ml of cold 25% sucrose in 10 mM Tris.HCl (pH 8.0). 5.0 ml of a freshly prepared solution of

lysozyme in TE buffer (1 mM Tris.HCl pH 8.0, 1 mM EDTA (ethylene diamine tetraacetate) was added to the cell suspension and mixture was placed on ice. After 5-10 mins; 10 ml of chilled 250 mM EDTA (pH 8.0) was added. The mixture was kept at 0°C with occasional swirling for an additional 5-10 mins after which lysis was brought about by the addition of 10 ml of a detergent mixture (1% BriJ-58, 0.4% sodium deoxycholate, 60 mM EDTA and 50 mM Tris.HCl pH 8.0). With E. coli cells, the samples became relatively viscous and clear within 10 mins. With K. pneumoniae, the sample had to be incubated for 20-30 mins at 37°C for viscosity to be apparent. At this point, the sample was centrifuged at 4°C for 30 mins at 34,800 xg in an SS-34 rotor; this step normally pelleted the cell debris and most of the chromosomal DNA while the plasmid DNA was recovered in the supernatant. To this supernatant, called the "cleared lysate", NaCl and polyethylene glycol (PEG 6,000) were added to a final concentration of 3.0% and 10% (w/v) respectively. The mixture was gently swirled to dissolve the solute and this was stored anywhere from 4 hours to overnight during which time DNA precipitation had occurred. This precipitate was collected by centrifugation in a GSA rotor at 10,000 xg for 5 mins and the DNA pellet was dissolved in TE buffer. Ethidium bromide (EtBr) made in TE buffer was added to the preparation to a final concentration of 500 ug/ml and

5

solid cesium chloride (CsCl) was added until the solution reached a refractive index of 1.386-1.390 (as determined by using a Bausch and Lomb refractometer). The sample was centrifuged in a Ti75, Ti50 and or VTi80 rotor of a Beckman Spinco ultracentrifuge at 270,000 xg for approximately 48 hours at 15° C (in the Ti50 or Ti75) or at 300,000 xg for 4 hours (in the VTi80). After centrifugation, the plasmid DNA band was removed by puncturing the tube with a 1.0 ml disposable hypodermic syringe and withdrawing the plasmid band under illumination with a long wave ultraviolet lamp. The EtBr was extracted from the DNA solution by adding CsCl-saturated isopropanol and discarding the alcohol layer which contained the EtBr; this step was repeated several times until all visible traces of this dye were removed. To this aqueous layer, 1 volume of cold TE buffer, followed by 2 volumes of cold ethyl alcohol were added; this was gently mixed and was stored at -20° C to precipitate the DNA. The DNA was sedimented by centrifugation at 3,000 xg for 15 mins. The DNA pellet was vacuum dried for 10 min and was dissolved in 0.2 ml of sterile TE.

Method 2

For the isolation of small plasmids required for restriction analysis of genetic transformation, the rapid boiling method of Holmes and Quigley (1981) was employed.

5.0 ml of an overnight culture grown in LB with the appropriate antibiotic (where desired), was centrifuged in a Sorvall table top centrifuge at 3,000 xg for 10 mins. The pellet was resuspended in 0.35 ml of cold STET (8% Sucrose, 5% Triton X-100, 50 mM EDTA, 50 mM Tris.HCl pH 8.0) and transferred to a microfuge tube. A 25 ul aliquot of a freshly prepared lysozyme solution (10 mg/ ml in TE buffer) was added; this was mixed gently, and immersed in a boiling water bath for about 40 seconds. The resulting lysate was spun at top speed (15,000 xg) in an Eppendorf microfuge for 15 mins. The pellet was removed with a sterile toothpick, and an equal volume of cold isopropanol was added and the mixture was left for 30 mins to 1 hour at -80 °C. The precipitated DNA was collected by centrifugation at 15,000 xg for 15 mins and washed with 70% cold ethyl alcohol. The pellet was vacuum dried and dissolved in 30 ul of TE.

Method 3

This method was that described by Birnboim and Doly (1979). 5.0 ml of an overnight culture was grown with the appropriate antibiotic at 37 °C. The cells were pelleted and resuspended in ice cold solution I (The composition of solution I is 50 mM glucose, 10 mM EDTA, 25 mM Tris.HCl pH 8.0). An aliquot of 25 ul of freshly prepared lysozyme (2 mg/ml) was added, this was gently mixed, and left on ice for 10-15 mins. After this time, 200 ul of a lysing

opened, the pre-hybridization buffer was poured off; and 9.0 ml of hybridization solution was added wetting the filter paper thoroughly; the hybridization solution contained the following: 50% formamide, 5X SSC, 5X Denhardt's reagent, 20 mM sodium phosphate (pH 7.0), 100 ug/ml denatured salmon sperm DNA and 10% sodium dextran sulfate 500 freshly prepared as a 50% (w/v) solution. One ml of this was heated to 65°C and added to the probe. The probe DNA was denatured by heating it at 95°C for 10 mins and cooling it in an ice-water bath. The denatured probe was added to the heated hybridization buffer, mixed gently but thoroughly and added to the bag. The bag was sealed, the solution mixed thoroughly and incubated overnight at 42°C.

The membrane filter was washed thrice with 250 ml portions of 2X SSC and 0.1% SDS for 5 mins at room temperature and then washed twice with 250 ml portions of 0.1X SSC and 0.1% SDS for 5 mins at 42°C. The filter was air dried and exposed to X-ray film (Kodak, XRP-1) for autoradiography at -90°C, using a Du Pont Cronex Hi-Plus screen.

A

B

otherwise specified).

The total DNA was isolated in one of two ways. The first method, was outlined by M. Konarska-Kozłowska (personal communication). This method was as follows. A 200 ml overnight culture was sedimented at 8,000 $\times g$ for 10 mins. The cells were resuspended in 1.0 ml of 25% sucrose, and 1.0 ml of lysozyme was added, and this was maintained on ice for 5-10 mins. Thereafter 1.0 ml of cold 250 mM EDTA (pH 8.0) was added and chilled for an additional 10 mins with occasional swirling. The lysis of the cells was brought about by the addition of 2.0 ml of detergent mixture. This viscous mixture was diluted to 12 ml with TE buffer and to this mixture, CsCl was added such that the refractive index was between 1.386-1.390. The subsequent steps were the same as for plasmid DNA isolation.

The second method was described by Meade et al. (1982). A 5 ml culture was pelleted and washed in an equal volume of cold TES (same as TE but with the addition of 150 mM NaCl). The cells were pelleted and resuspended in 5.0 ml of cold TE, to which 0.5 ml of freshly prepared lysozyme (2 mg/ml in TE) was added. This was left at room temperature for 15-20 mins. To lyse the cells, 0.6 ml of cold Sarkosyl-Pronase (100 mg/ml of Sarkosyl and 5 mg/ml of Pronase made in TE) was added and this was maintained at 37°C for 1 hour. The DNA was extracted twice with an

equal volume of phenol saturated with TE. The aqueous (viscous) layer was gently removed to which an equal volume of cold ethyl alcohol was added. The two layers were gently mixed. The DNA was immediately precipitated and was spooled out. It was washed with 70% alcohol, air dried and dissolved in sterile TE.

The latter method, although more laborious, involved less time and DNA could be prepared and used the same day.

Curing of the plasmid pCU1 or its derivatives from
K. pneumoniae

Although several agents have been reported to be used for curing plasmids, we desired to avoid their use, as such agents are known to cause several types of mutations. An overnight culture was grown in LB with the antibiotic selective for the plasmid. This was subcultured in antibiotic-free media and regrown to a cell density of approximately 10^6 cells/ml. Serial dilutions of the culture were made in saline and plated on an antibiotic free medium. The colonies that arose were tested for the loss of antibiotic markers associated with the plasmid by replicating colonies on PA plates containing the antibiotics and observing the absence of growth on PA plates with the antibiotic. The corresponding colony which grew on a PA plate was purified and checked for the appropriate phenotype.

The strain was also confirmed to have lost the plasmid by using the rapid plasmid DNA isolation procedure followed by CsCl-Ethidium bromide gradient centrifugation.

Southern hybridization

Derivatives of K. pneumoniae which were cured of pCUI or its derivatives, were examined for the integration of all or part of pCUI into the genome of the cured derivatives of K. pneumoniae, using the Southern hybridization procedure with radioactively labelled (α -³²P) pCUI DNA as described in steps 1-4.

1. Denaturation and transfer of DNA from an electrophoresis gel to nitrocellulose filter

The method used was as described by Wahl et al. (1979). A gel of 0.7% agarose containing fractionated DNA was photographed as described and exposed to U.V. light for approximately 10 mins to ensure sufficient breakage of DNA strands for their efficient diffusion from the gel. Subsequently, the gel was immersed in 500 ml of a denaturation buffer (DB=0.4 M NaOH and 0.8 M NaCl). The denaturation was allowed to occur with gentle agitation for 30 mins. After this, the DB was decanted and 500 ml of neutralizing buffer (0.5 M Tris.HCl, 1.5 M NaCl, pH 7.5) was placed on the gel for 30 mins. After this step, the DNA was transferred overnight from the gel to a nitrocellulose filter using 20X SSC (150 mM NaCl, 15 mM Na

citrate ,pH 7.5) as described by Southern (1975). The next day the outline of the gel was traced with a pencil on the nitrocellulose filter. The gel was rephotographed to make certain that DNA transfer had occurred. The filter was washed with 3X SSC, and gently rubbed with a gloved hand to remove any residual agarose particles, which otherwise would give rise to a high background noise upon autoradiography. In the next step, the filter was air-dried for 1 hour and then baked, at 80 °C in vacuo for 2 hours to permanently bind the single stranded DNA to the nitrocellulose paper.

2. Radioactive labelling of plasmid DNA

Labelling of plasmid DNA with (α -³²P)dATP was performed by the in vitro nick translation reaction of (Kelly et al., 1970) as described by Rigby et al. (1977). The reaction mixture was prepared as 10X concentrated solution which was composed of (50 mM Tris-HCl pH 7.4, 5 mM MgCl₂ and 10 mM mercaptoethanol, 50 ug/ml BSA), 5 ul of dNTP (which were prepared as a 1.0 mM combined stock solution and added to a final concentration of 180 pMoles), 1 ul of 10 mg/ml DNase I (Grade I, Boehringer-Mannheim) and 2 ug of (α -³²P)dATP (New England Nuclear Corporation specific activity of .1mCi in 0.07 ml H₂O). Distilled sterile water was added to achieve a final volume of 100 ul. To this 100 ul, 1 ug of pCU1 DNA was added, along with 2 units of DNA polymerase I. The

reaction mixture was incubated for 10-15 hours at 15 C and the reaction was stopped by the addition of 0.5% SDS and 10 mM EDTA.

3. Recovery of labelled DNA

The (α - ^{32}P) labelled DNA was separated from unincorporated dNTP's by gel filtration. The sample was carefully loaded onto a siliconized 7 cm x 0.6 cm Sephadex G-50 column, which had been pre-equilibrated with elution buffer (10 mM Hepes buffer, pH 6.5; 0.01% SDS). Aliquots of 3 drops were collected in scintillation vials and counted in the ^3H channel (of a Beckman Scintillation Counter) utilizing Cerenkov radiation. Radioactivity (CPM) in each fraction was plotted and the fractions containing the first peak were pooled and used in the following step.

4. DNA hybridization

DNA hybridization was performed as described by Wahl et al. (1979). The nitrocellulose filter was placed in a heat sealable plastic bag containing 10 ml of prehybridization solution 50% formamide (99%, A.C.S. grade, Matheson, Coleman and Bell), 5X SSC, 5X Denhardt's reagent (0.02% w/v each of BSA, polyvinyl pyrrolidone, and Ficoll); 50 mM sodium phosphate pH 6.5, 1% glycine, 250 ug of alkali denatured salmon sperm DNA. The plastic bag was sealed and incubated overnight at 42 C. The bag was

opened, the pre-hybridization buffer was poured off; and 9.0 ml of hybridization solution was added wetting the filter paper thoroughly; the hybridization solution contained the following: 50% formamide, 5X SSC, 5X Denhardt's reagent, 20 mM sodium phosphate (pH 7.0), 100 ug/ml denatured salmon sperm DNA and 10% sodium dextran sulfate 500 freshly prepared as a 50% (w/v) solution. One ml of this was heated to 65°C and added to the probe. The probe DNA was denatured by heating it at 95°C for 10 mins and cooling it in an ice-water bath. The denatured probe was added to the heated hybridization buffer, mixed gently but thoroughly and added to the bag. The bag was sealed, the solution mixed thoroughly and incubated overnight at 42°C.

The membrane filter was washed thrice with 250 ml portions of 2X SSC and 0.1% SDS for 5 mins at room temperature and then washed twice with 250 ml portions of 0.1X SSC and 0.1% SDS for 5 mins at 42°C. The filter was air dried and exposed to X-ray film (Kodak, XRP-1) for autoradiography at -90°C, using a Du Pont Cronex Hi-Plus screen.

RESULTS

A. Detection of killing

The killing of K. pneumoniae by E. coli strains carrying the plasmid pCU1 was detected by either one of the two ways described. The filter mating method was used whenever a quantitative measure of killing was desired; the spot test was used when a large number of strains needed to be tested for this phenotype. The survivors, using the quantitative method, are shown in Figure 2. In an experiment with E. coli carrying pCU1, the number of K. pneumoniae cells that were killed ranged from 70% to 99%. This is in agreement with the observations of Rodriguez (M.Sc. thesis, Carleton University, 1980).

The results of a typical 'spot' test are illustrated in Figure 3. The survivors from a quantitative experiment show two types of colony morphology on glucose minimal agar. Since in such experiments both donors and recipients were spread on the minimal agar plates and the 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 colony morphology of K. pneumoniae could be a consequence of such matings occurring on the agar surface. This reasoning was supported by experiments in which a nalidixic acid (NAL)-resistant recipient K.

Fig.2: Colonies of K. pneumoniae survivors after a mating experiment with C600 and C600(pCU1). The normal colonies after mixing with C600 are illustrated in A and abnormal colonies after mating with C600 (pCU1) are illustrated in B. The magnification of A and B are approximately equal.

.3

A

B

Fig.3: Illustration of the result of the qualitative 'spot' test used for the detection of the killing phenotype. From left to right: A. C600(R⁻ strain); B. phenotype of a strain carrying an IncN plasmid (pCU1); C. phenotype of a strain carrying a plasmid of IncW which has been shown to kill to a lesser degree than IncN(Rodriguez and Iyer, 1981).

(Courtesy of Rodricuez, 1.1980)

pneumoniae was used and the mating mixture spread on glucose minimal agar containing NaI. In this situation, the surviving colonies were not abnormal. Nalidixic acid has been shown to prevent the conjugal transfer of IncN group plasmids (Gill and Iyer, 1981).

B. Survey of naturally occurring IncN group plasmids for their ability to kill K. pneumoniae

A large number of different wild type IncN group plasmids were assembled from a wide range of hosts (see Table 111) and surveyed for their ability to mediate the killing of K. pneumoniae. Eighteen of these plasmids were surveyed as part of this study, and in an earlier study Rodriguez and Iyer (1981) surveyed a different set of plasmids. All the plasmids tested, with the exception of pTM558, had the ability to kill K. pneumoniae. The inability of pTM558 to promote killing is correlated with its low frequency of transfer to both E. coli and Klebsiella pneumoniae (Rodriguez and Iyer, 1981). In addition, all the plasmids that mediated killing showed a concomitant transfer of the plasmid-borne markers. Therefore, it is concluded that the Kil phenotype is a general property of all transfer proficient IncN group plasmids regardless of their origin.

C. Killing is a plasmid associated phenomemon

When the plasmid pCUI or its derivatives, were transferred from their original E. coli host by conjugation to a number of other E. coli strains (GS500, C600, CSR6013) and also to Agrobacterium tumefaciens, Achromobacter xylooxidans and Pseudomonas aeruginosa caused these latter strains to mediate killing of K. pneumoniae. Furthermore, a K. pneumoniae cell which survived to form a colony and upon acquiring pCUI, could kill derivatives of K. pneumoniae, just as efficiently as the original E. coli donor carrying the plasmid. This leads one to conclude that killing is a plasmid-borne characteristic. However, E. coli donors carrying pCUI do not kill other E. coli strains tested (W1485, GS500, and AB1157) as determined by the qualitative method.

D. The requirement for two kil regions to mediate killing

From the previous two sections, the main conclusion was that killing is a general property of all transfer proficient plasmids belonging to the IncN group. Further, a plasmid, pTM558 with a reduced transfer, (a 200x fold decrease in transfer compared to pCUI) did not mediate killing. This observation raised the possibility that killing ability may be specified by a gene or genes contained within the transfer region. This is substantiated since upon mutagenesis of the plasmid pCUI, Kil^+Tra^+ and Kil^-Tra^+ mutants were found but none were

Kil⁺Tra⁻ out of the 1900 colonies screened (Rodriguez, M. Sc., Carleton University, 1980). Thatte and Iyer (1983) have defined and cloned the entire pCU1 transfer system using insertion and deletion derivatives of the parental plasmid, pCU1. When the entire transfer system (19.4 Kb) was cloned into the multicopy plasmid, pACYC184, the transfer frequency of the hybrid, pCU101, was comparable to the parental pCU1 (using the E. coli as a recipient). However, pCU101 did not mediate killing of K. pneumoniae, and the surviving K. pneumoniae colonies appeared normal. When an additional 3.0 Kb region, adjacent to the transfer region, between the coordinates 9.0 to 12.0 (see Fig.4), was present as is in the hybrid, pCU109, the degree of killing by E. coli carrying this hybrid plasmid was comparable to that mediated by pCU1. Concomitant with the killing by E. coli donors carrying pCU109 was the appearance of two types of surviving colonies of K. pneumoniae as illustrated in Fig.2.

In addition to the cloning data which defines this kil region, (henceforth designated as kilB) to the coordinates 8.2-11.2 Kb, there are ~~three~~ insertion mutants of pCU1 (pCU167, pCU184, and pCU66) which are Kil⁻ (Kil deficient). These insertions were isolated using the transposon (Tn5), which specifies resistance to the antibiotic, kanamycin. Using the appropriate restriction enzymes, these insertions were mapped at the opposite end

of the tra region from the 3 Kb region defined earlier, at the positions 31.4, 31.6 and 31.2 (Fig. 1) (Thatte, V., Ph.D. Dissertation, Carleton University, 1984). These insertion mutants showed a slight reduction (3 to 4 fold) in transfer to E. coli recipients when compared to pCU1 (Table IV). However, the inability of these mutants to kill K. pneumoniae could be complemented by a plasmid containing this region (designated as kilA), provided it could be mated efficiently into K. pneumoniae. In this circumstance, the mutant plasmid was still transferred at the slightly reduced frequency. The combined data from the analysis of insertions (in kilA) and the clones, pCU101(kilA) and pCU109(kilA and kilB), indicate that there are two separate regions on pCU1 which are required to maximally mediate killing of K. pneumoniae. In addition, none of the several transfer defective insertion mutants that map in or at different parts of the plasmid tra regions, mediate killing (Thatte, V., Ph.D. Dissertation, Carleton University, 1984). Some of these tra mutants do not produce N pili, the cell surface organelles implicated in conjugation, while others do.

E. Effect of rearranging the two kil regions

The mechanism(s) by which donors carrying pCU1 kill K. pneumoniae is not understood.

Fig.4: The pertinent regions on the plasmid required for the *Kil* phenotype. The arcs outside the circle represent regions cloned into the vector, pCYC104, pCU101, and pCU109). The *Kil⁻* insertions are shown on the left side enclosed in a box at 31.5kb on the plasmid.

TABLE IV

Proficiency of conjugative transfer of pCU1 and its derivatives and their ability to mediate killing of *K. pneumoniae*. The figure across from this (Fig.4) indicates the regions of pCU1 included in the derivatives.

Plasmid in <u>E. coli</u>	Frequency of Transfer to ^a <u>E. coli</u>	<u>K. pneumoniae</u>	% Killing of ^b <u>K. pneumoniae</u>
pCU1	2×10^{-1}	1×10^{-2}	9×10^1
pCU101	4×10^{-1}	7×10^{-3}	2×10^1
pCU109	7×10^{-1}	3×10^{-5}	9×10^1
pCU167	5×10^{-2}	2×10^{-2}	2×10^1
pCU184	7×10^{-2}	2×10^{-2}	2×10^1

(a). The transfer frequency was measured under the same conditions as those used for killing and is expressed as the fraction of donors capable of transferring the plasmid. The donor to recipient was 20:1

(b). The killing frequency was calculated from the fraction of survivors which had survived after mating for 1 hr with *E. coli* (pCU1) donor. With *E. coli* donors carrying the vector or devoid of the plasmid, no killing was detected. The killing of *K. pneumoniae* by C600(pCU1) ranged from 70% to 99% (20 experiments). With C600(pCU101) or C600(pCU167) this value ranged from 0%-20% (3 experiments). Since these donors failed to give a positive reaction in the qualitative test, this range of values was considered to be kill deficient and so stated in the rest of this thesis.

In this and the following section experiments were designed to shed some light on the possible mode of action of kilA and kilB. Experiments were conducted to determine whether killing could occur if the two kil regions were placed on different compatible plasmids. However, before such experiments could be performed, the kilB region had to be cloned onto a compatible plasmid. The plasmid pMK2004, has a ColE1 replicon (Kahn et al. 1978) which was found to be compatible with both pCU1 and pACYC184 (p15A type replicon).

From the studies of Thatte (1984), it is known that kilB on pCU1 is flanked by restriction enzyme sites, for BglII. The plasmid pMK2004 has a unique site for the BamHI within the region which specifies resistance to tetracycline. Cleavage with BamHI yields DNA fragments with protuding 5' cohesive termini identical to those generated by BglII (5'-GATC-3'). These can be ligated together to form a hybrid site that is not recognized by either enzyme (Maniatis et al., 1982). The DNA samples were digested and ligated as described in Materials and Methods. The ligation mixture was transformed into E. coli strain C600; the colonies that arose were screened for sensitivity to tetracycline and resistance to kanamycin and ampicillin which were specified by pMK2004. Insertion at the BamHI site in pMK2004, inactivates the region on pMK2004 which specifies resistance to

tetracycline. One advantage of using the enzyme BglII for cloning the kilB region was that the fragment containing kilB, generated by digestion with BglII, contained a region called mob. This mob region facilitated screening for the kilB clone; as described. Mobilization (or plasmid donation) is a process whereby a self transmissible plasmid like pCU1 provides trans-acting factors that can mobilize a non self-transmissible plasmid for transfer but only provided the latter has a mob region, recognized by these trans-acting factors. The colonies which were sensitive to tetracycline but resistant to kanamycin or ampicillin were pooled and used in a triparental mating (as described in Materials and Methods). If the mob region was cloned, it would mediate the transfer of the pMK2004 associated markers, using the helper plasmid pCU1, whereas pMK2004 or any derivative of it that did not include mob of pCU1 would not be transferred. Such Mob⁺ clones were found and their restriction pattern verified. Mob⁺ derivatives that carried kilB were used to construct E. coli carrying this derivative and pCU101, the self-transmissible derivative of pCU1 that carried kilA but not kilB. Such E. coli strains and others illustrated in Fig.5 were tested for their ability to kill K. pneumoniae. Results obtained with several such constructed strains are shown in Fig.5.

plasmid	coordinates (kb)	KluA	Tra	mob	KluB	Klu phenotype
pCU101	278-82	■	—	○	—	—
pCU109	278-112	■	—	○	■	+
pCU167	± 314	■	—	○	■	—
pCU10	51-33.9	■	—	○	■	—
pCU11	0-278	—	—	○	■	—
pCU51	82-21	—	—	—	■	—
pCU53	273-3.3	■	—	—	—	—
pCU58	277-34 51-82	■	—	○	—	—
pCU57	0-82	—	—	○	—	—
pCU404	70-11.5	—	—	○	■	—
pCU101	278-82	■	—	○	—	+
pCU10	51-33.9	■	—	○	■	+
pCU101	278-82	■	—	○	—	+
pCU11	0-278	—	—	○	■	+
pCU101	278-82	■	—	○	—	+
pCU404	70-11.5	—	—	○	■	+
pCU101	278-82	■	—	○	—	—
pCU51	82-210	—	—	—	■	—
pCU167	± 314	■	—	○	■	—
pCU57	00-82	—	—	○	—	—
pCU167	± 314	■	—	○	■	+
pCU58	277-340 51-82	■	—	○	—	+
pCU167	± 314	■	—	○	■	—
pCU53	273-3.3	■	—	—	—	—

Fig.5: This figure illustrates the relevant regions required for killing that were present in different clones or deletion derivatives of pCU1. The Kil phenotype was determined using the qualitative and quantitative tests. The ∇ indicates insertion in KilA.

TABLE V

Frequency of transfer of plasmids from E. coli to K. pneumoniae in relation to the frequency of killing.

Plasmids in the <u>E. coli</u> donor	Frequency of Mobilization or Transfer ^a of the plasmid		
	Cm	Km	Sp
pCU1	-	-	1×10^{-2}
pCU101	7×10^{-3}	-	-
pCU109	2.5×10^{-5}	-	-
pCU167	-	-	2×10^{-2}
pCU101	2×10^{-3}	-	-
pCU10	-	-	1×10^{-2}
pCU101	2.7×10^{-3}	-	-
pCU11	-	-	1×10^{-2}
pCU101	1×10^{-3}	-	-
pCU51	-	10^{-6}	-
pCU101	1×10^{-3}	-	-
pCU404	-	2.7×10^{-2}	-
pCU167	-	2.7×10^{-3}	2×10^{-2}
pCU58	2.5×10^{-3}	-	-

a. the frequency of transfer or mobilization is expressed per 100 donors.

which also shows diagrammatically, the regions present on these plasmids. Earlier experiments (section D) had suggested that the introduction of either kilA alone (present on pCU101) or kilB alone (present in pCU167) was not sufficient to give a Kil^+ phenotype even though in E. coli to E. coli matings the transfer frequencies were not drastically affected. The results in Figure 5 show that any combination of plasmids carrying kilA and kilB between them will cause the killing of K. pneumoniae provided both have mob associated with them. If either kilA or kilB was associated with a plasmid that is incapable of being transferred to K. pneumoniae, no killing was observed.

The plasmids have not recombined through homologous recombination to mediate killing. This is apparent since the plasmids are transferred or are mobilized at different frequencies (Table V). Further, when the surviving colonies of K. pneumoniae were examined for the presence of the markers associated with the plasmids, the following was observed: of the colonies which were tested from glucose minimal agar, >90% had the markers of pCU1 or its derivatives whereas the marker of the p15A replicon was rarely found. If colonies were screened from minimal agar plus spectinomycin (pCU1 marker); 41% had Cm, the marker associated with the p15A replicon; if the colonies were screened from minimal agar containing Cm, >90% also had the unselected marker associated with the pCU1

derivatives. The different frequencies of loss of these two markers from K. pneumoniae suggests that they were present on separate plasmids. All donors which were Kil^+ gave rise to two types of K. pneumoniae survivors, the normal type and the abnormal type (Fig.2). Thus, kilA and kilB regions can mediate a Kil^+ phenotype even if these 2 regions are on separate plasmids in the donors E. coli strains.

F. Effect of the prior presence of kilA or kilB regions in K. pneumoniae before the introduction of the second kil region by conjugation.

From section E it is apparent that the kil regions can act in trans and kill K. pneumoniae but only provided that both kilA and kilB are simultaneously delivered by conjugation into K. pneumoniae.

If this is the case, one could ask whether killing could occur if one kil region was present in K. pneumoniae prior to the introduction of the second kil region by conjugation. K. pneumoniae strains carrying kilA or kilB were constructed by transformation. These strains were used in mating experiments with E. coli donors carrying different conjugative derivatives containing either kilA

~~or kilB.~~

Fig. 6: Physical map of pCU1 containing the relevant regions for killing. The arcs outside the circle represent regions cloned onto the vector pK2004. pCU53 and pCU101 pCU53 is a *KpnI* deletion derivative of pCU101. In addition pCU53 has *Kil⁺* but no *lob* region. pCU58 has *Kil⁺* and *lob* but is *tra⁻*, whereas pCU101 is *Kil⁺*, *lob⁺*, and is *tra⁺*. The region present in pCU404 has *lob⁺* and *tra⁺*. It was cloned onto pK2004. The *Kil⁺* insertion mutant, pCU167 *Tn5* in *kilA*, is *tra⁺* and has *Kil⁺*.

TABLE VI

The effect on the Kil phenotype of the prior presence of one kil region in K. pneumoniae before the introduction of the second kil region by conjugation.

Region present in <u>K. pneumoniae</u>	Region introduced by conjugation	Overall phenotype
<u>kilA</u> (pCU53 or pCU58)	<u>kilB</u> (pCU167)	-
<u>kilB</u> (pCU404)	<u>kilA</u> (pCU101)	-
<u>kilA</u> or <u>kilB</u>	<u>kilA</u> and <u>kilB</u>	+
nil	<u>kilA</u> and <u>kilB</u>	+

a. The qualitative and the quantitative tests were used to determine the overall phenotype. -, no killing was observed, +, killing was observed.

Table IV shows that if kilA (in pCU58 or pCU53) or kilB (in pCU404) was present prior to the introduction by conjugation of the second kil region, kilB (in pCU167) or kilA (in pCU101) respectively no reduction in the viability of K. pneumoniae occurred. However, when the recipients which had acquired the second kil region, were purified and themselves used as donors these newly constructed donors mediated killing of a nalidixic acid-resistant derivative of K. pneumoniae. The inability of E. coli donors carrying pCU101 or pCU167 to kill K. pneumoniae containing kilB (as in pCU404) or kilA (as in pCU53 or pCU58) was not due to immunity to killing by strains carrying the entire plasmid pCU1 because K. pneumoniae carrying either kilA or kilB was susceptible to killing by E. coli containing pCU1 (see section G).

G. A region associated with pCU1 that confers immunity to K. pneumoniae from killing by C600 (pCU1)

It has been shown that if a K. pneumoniae strain carries the plasmid pCU1, it is immune to killing by E. coli carrying the same or any other N group plasmid (Rodriguez, M., M.Sc. thesis, Carleton University, 1980). This suggests that immunity is a consequence of K. pneumoniae having acquired the plasmid. Several possible reasons for this immunity can be envisioned (for example, plasmid incompatibility, plasmid surface exclusion). We define plasmid-dependent immunity as the immunity acquired

by K. pneumoniae upon receipt of a plasmid and which requires the continued presence of the plasmid. In the experiments described in this section, we sought to determine whether all or only part of the plasmid is required for this immunity and if the latter is the case, where this region is in relation to other plasmid-associated regions.

This phenotype was tested by the following sequence of experiments (a) introducing a plasmid to be tested into K. pneumoniae (by transformation or conjugation), purifying the resulting strain on a medium selective for the presence of the plasmid, (b) from each such constructed strain, deriving a secondary strain from which the plasmid had been eliminated (as described in Materials and Methods) and (c) comparing such constructed pairs of strains one with and one without the plasmid for their susceptibility to killing by E. coli carrying pCU1. A strain that was immune to killing when it carried a plasmid but which lost this immunity upon plasmid curing was considered to carry a plasmid which included an immunity region. Recombinant plasmids and deletion derivatives spanning the entire pCU1 physical map were tested in this manner. Fig.7 shows the regions present on these tested plasmids. Table VII shows the results with K. pneumoniae strains carrying these plasmids using the qualitative test. It can be concluded that the only

region on pCU1 that is necessary to confer immunity to K. pneumoniae from killing by C600(pCU1) is a region between the coordinates 11.5 to 15.7 Kb, that is common in the derivatives pCU11, pCU51, pCU406 and pCU55. Table VIII (and Fig.8) shows the results of experiments on immunity using the quantitative test for killing. The recipient ability of these strains to pCU1 from E. coli C600(pCU1) was also determined in these experiments. From Table VIII, the following are apparent: (1) the K. pneumoniae strains carrying plasmids with the 11.5 to 15.7 kb coordinate region of pCU1 showed an eight-fold increase in survival frequency when compared to K. pneumoniae devoid of the plasmid; (ii) curing of K. pneumoniae carrying any of these derivatives resulted in a killing frequency similar to the parental plasmid-free strain, and (iii) K. pneumoniae strains carrying the plasmids with this immunity region showed better recipient ability for pCU1 from C600(pCU1) presumably because all recipients of the plasmids and not merely the fraction that survive, produce colonies. This ability was lost concurrently with curing of the plasmid. The strains carrying the derivatives which conferred immunity to killing showed no difference in growth rate or colony morphology when compared to the susceptible strains.

TABLE VII

Susceptibility of K. pneumoniae strains carrying different derivatives of pCU1 to killing by E. coli (pCU1).

Plasmids in ^a <u>K. pneumoniae</u>	Susceptibility ^b to killing
pCU11	-
pCU45	+
pCU46	+
pCU51	-
pCU53	+
pCU55	+
pCU57	+
pCU58	+
pCU59	+
pCU404	+
pCU406	-

a. Fig.7 across this page for the regions included in these derivatives.

b. Tests were performed using the qualitative test, +, susceptible to killing, -, resistant to killing.

Fig.8: The derivatives of pCU1 which conferred immunity by C600(pCU1) as determined by the quantitative test. pCU404 and pCU53 do not confer immunity but were used to determine if they affected the recipient ability when present in *K. pneumoniae*. The derivatives pCU53 and pCU55 represent regions cloned into pACYC104, and pCU404 contains the region cloned into pMK2004. The derivatives pCU51 and pCU406 are deletion derivatives of pCU1.

TABLE VI

The ability of different derivatives of pCU1 to confer immunity to killing to K. pneumoniae from killing by E. coli C600 (pCU1). In addition, the conjugal recipient ability of these derivatives to pCU1 is also shown.

Plasmids in	Range of Survival Frequency	Recipient ability
<u>K. pneumoniae</u>	(%) of <u>K. pneumoniae</u> strains	relative to
	with plasmid	plasmid-free
	carried strain	<u>K. pneumoniae</u>

nil	1-20	-	1
pACYC184	10	ND	ND
pCU55	65-100	1-20	3-80
pCU51	70-100	1-20	4-30
PCU406	60-100	1-20	3-50
PCU404	1-20	1-20	.06-1
PCU53	1-20	1-20	.06-1

H. Immunity which persists after curing of the plasmid pCU1

During the course of the studies on immunity to killing that are described in section G it was concluded that if K.pneumoniae carried the recombinant plasmid pCU55, which contains a single region of the plasmid pCU1 (coordinates 11.5-15.5kb), it was immune to killing by E. coli strains carrying pCU1. Curing of the hybrid plasmid resulted in the loss of immunity. This suggested that since immunity to killing was conferred by a single region of the plasmid, immunity would also be lost concomitant with the loss of the plasmid pCU1. An unexpected observation was made, that this was not the case; that is, the loss of pCU1 or some derivatives of it other than pCU55, was not always accompanied by the loss of immunity. The basis for this phenomena is not understood and as far as we are aware, even the observation that immunity is retained despite the apparent loss of the plasmid has no precedent in the literature on plasmid specified immunity. It is an unexpected and a novel phenomenon, the basis of which is unknown and which clearly deserves much more study than has been possible as part of this thesis. In this section the existence of this phenomenon is established. Experiments are also described which lead to two conclusions: (a) the plasmid does not act as a selective agent to yield resistant strains that pre-existed in the population and (b) only a part of pCU1

DNA, if any, could have been retained by the immune strains cured of the plasmid state. Different possibilities are discussed.

1. Description of the phenomenon

In tests for the killing of K. pneumoniae, a general observation that was made (Rodriguez and Iyer 1981; this thesis) was that in a given experiment, a small proportion of the K. pneumoniae cells always survived. Such survivors could arise if only a fraction of K. pneumoniae cells in the population did not make contact with the donors carrying the plasmid. This is likely because in any given experiment, there were some colonies (approximately 1%) which did not have the plasmid markers. Some of the colonies tested which had the pCUI plasmid markers were immune to killing. Fifty independent strains each spontaneously cured of pCUI markers were isolated (as described in Materials and Methods). All of these strains were immune to killing by C600(pCUI). All fifty strains were prototrophic, like the parent strain. Since these resistant colonies arose at a high frequency and were phenotypically indistinguishable from the K. pneumoniae strain before it mated, it was reasoned that E. coli donors carrying pCUI may be acting as selective agents to select pre-existing resistant cells out of the K. pneumoniae population. To test this possibility, a K. pneumoniae population was plated out to give a large

number of discrete colonies which were used concurrently in a killing experiment with C600(pCU1). The frequency of killing in this experiment was 99.1%. Thus, if resistant cells pre-existed, they would constitute a fraction of approximately only 9 per 1000 cells. 2,600 individual discrete colonies were used to restart fresh cultures and used for a killing experiment. All 2,600 cultures were sensitive. It was calculated that if resistant cells were present in the population of K. pneumoniae, they would be present at a frequency of 6×10^{-14} .

It was concluded that this explanation was very unlikely. This conclusion is also supported by the finding that when K. pneumoniae is cured of the hybrid plasmids carrying a particular region of pCU1, such cured strains are no longer resistant (see subsequent pages).

2. Plasmid region(s) involved in this type of immunity

In order to determine whether a specific part or parts of the plasmid was necessary for this type of immunity, different deletion derivatives of pCU1 were introduced by either transformation or conjugation into K. pneumoniae. These derivatives are shown in Figure 9.

Each strain was then cured of its particular plasmid to give a corresponding set of cured strains. The derivatives which were immune to killing whether or not the plasmid was present are listed in Table IX. Further,

the quantitative data is presented in Table X. The immune strains show a higher survival frequency and the recipient ability is improved at least by a 100X fold. From the curing data the following was observed. The large hybrid plasmid, pCU101 (Kil^-), when cured from *K. pneumoniae* did not confer immunity, whereas the larger hybrid plasmid, pCU109 (Kil^+) which contained an additional 3 kb, of PCU1 DNA did confer complete immunity even upon curing. These results indicate that there is a region responsible for this type of immunity in the 7.2 to 11.5 kb region of pCU1. However, the deletion derivative pCU11 and pCU13 (0.0-28.8 kb coordinates shown in Figure 1) which have this region do not confer immunity upon curing. This indicates that a second region (coordinates 28.8-39.0 kb) is also required for this type of immunity. pCU53, a hybrid carrying this region (coordinates 27.5-3.5 kb) without the 7.2 to 11.5 kb region does not confer immunity. Both of these regions therefore appear to be necessary for this type of immunity. As shown in Figure 9 these 2 regions have been further narrowed down to a 31.2 to 33.8 kb region and a 7.2 to 11.5 kb region. It should be noted that these two regions are the same as those defined as the kilA and kilB region respectively. However, the Kil^- insertion mutations pCU167, pCU66 and pCU184 that mapped at approximately 31.6 kb did not inactivate this immunity. Therefore the Kil phenotype can

be inactivated without inactivating this immunity and the 31.2 to 33.8 kb region can be separated by mutation into two functional regions. In this region, mutations that inactivate immunity without inactivating Kil have not been isolated. It is possible that the sequence required for Kil is contained within a larger region required for immunity. This is supported by the following data; the derivative pCU401 confers upon the host permanent immunity, even though it is kil deficient. Further, any Tn5 insertion which maps clockwise to 32.0kb is Tra⁻. Therefore, the sequences required for immunity may also form a part of tra. These sequences required extend up to 33.8kb since the derivative pCU10 also confers immunity after curing.

3. Effect of re-arrangement of the two immunity regions

K. pneumoniae strains were constructed such that the two immunity and (kil) regions were present on different replicons (pCU11 and pCU53, or, pCU51 and pCU53). When such strains were cured, all 10 cured strains tested were susceptible to killing. Thus it appears that, unlike the Kil phenotype which persisted even when these regions are on separate plasmids, this immunity requires both of these regions to be present on the same plasmid.

4. No part of pCU1 is detected in the cured strains

The possibility that in these immune and

plasmid-cured strains part of the plasmid had been integrated into the chromosome of K. pneumoniae was examined. Genomic DNA was isolated (as described in Materials and Methods) from several immune and nonimmune strains and prepared for Southern hybridization (Materials and Methods p 39-42). The plasmid pCU1 was radioactively labeled (Materials and Methods p41-42) and used as a probe. Figure 10 shows the results of hybridization. The cured strains showed no DNA- DNA hybridization, whereas, K. pneumoniae carrying pCU500 which contains 4.2kb of pCU1 DNA showed hybridization. Therefore, within these limits no part of pCU1 could have been integrated into the bacterial chromosome. It should be noted however that the positive control (pCU500) was present in multiple-copies whereas, the region of pCU1 which may possibly have been integrated into K. pneumoniae would be present in only a single copy.

TABLE IX

THE ability of different derivatives of pCU1 to protect the host K. pneumoniae after plasmid curing

Plasmid present in <u>K. pneumoniae</u>	Property of the strain * after curing of the plasmid
--	--

pCU10

-

pCU11

+

pCU13

+

pCU16

+

pCU101

+

pCU109

-

pCU167

-

pCU401

-

* This test was done using the qualitative test, as described in materials and methods. The Fig.9 across this Table illustrates the regions present in the various derivatives. -, resistant to killing; +, sensitive to killing

TABLE X

The susceptibility of plasmid-cured strains of K. pneumoniae to killing by C600(pCU1).

Strain	Plasmid present ^a in the strain before curing	% Survival	Range of frequency % of transfer of pCU1
<u>K. pneumoniae</u>	nil	1-20	.001-.02
S1	pCU1	80-100	.1-2
S2	pCU167	80-100	.2-2
S3	pCU10	75-100	.1-4
S4	pCU16	90-100	ND
S5	pCU109	90-100	.4-2
S10	pCU11	1-20	.01-.02
S11	pCU13	ND	ND
S12	pCU101	1-20	ND
S13	pCU401	80-100	.5-3

a. The curing was done as described in Materials and Methods p. 38.

Fig. 10: Hybridization of ^{32}P -labelled oCU1 DNA probe to various K. pneumoniae(pCU1) cured strains, lanes A-C K. pneumoniae(pCU500); lane D, K. pneumoniae carrying less than 0.2kb of pCU1 DNA. Lane E shows hybridization with the DNA from the wild type K. pneumoniae.

did not mediate killing whereas pCU109, carrying all of the region present in pCU101, and an additional 3 kb from pCU1, did. This 3 kb region present in pCU1 and pCU109, but not in pCU101 was therefore defined as the kilB region. Transposon (Tn5) insertions in a region of pCU1, quite separate from kilB also inactivated the Kil phenotype without inactivating the Tra phenotype. Therefore, there are two physically separate regions on pCU1 both of which are needed for the Kil⁺ phenotype. In addition, Thatté (Ph.D., Thesis Dissertation, Carleton University, 1984) has shown that any one of a large number of insertion mutations that abolish Tra and which map into any one of the eight known Tra complementation groups, also abolish killing. One possible explanation for these observations is that kilA and kilB have to enter K. pneumoniae in order to express the Kil⁺ phenotype. When either of these two kil regions was present in E. coli as part of a nontransmissible and nonmobilizable plasmid, no killing occurred. Furthermore, both kil regions had to be delivered at about the same time into K. pneumoniae for the latter to be killed. The prior presence of either kil region followed by the delivery of the the second kil region by conjugation did not lead to killing. However, when the same recipient (carrying either the kilA or kilB region) was mated with E. coli (pCU1), killing, of K. pneumoniae occurred.

DISCUSSION

The killing of K. pneumoniae was first reported by Rodriguez and Iyer (1981). One major conclusion of that study and the present one is that killing is a general property of all naturally occurring, conjugative IncN group plasmids. This phenotype is plasmid associated since the successful transfer of the IncN plasmid to a series of other plasmid free and Kil⁻ strains always resulted in the latter acquiring the Kil⁺ phenotype. The Kil phenotype was shown to be expressed in Salmonella typhimurium and Proteus mirabilis (Rodriguez, M.Sc. Thesis, Carleton University). This observation was extended to other hosts such as Agrobacterium tumefaciens, Achromobacter xylooxidans and Pseudomonas aeruginosa which had the acquired plasmid. Conditions which do not favor optimum mating such as liquid mating do not cause killing. A natural plasmid, pTM558 which showed a 200 fold decrease in transfer as compared to pCU1 did not promote detectable killing. Since the killing phenotype is always associated with the presence of IncN plasmids that are transfer proficient in conjugation, two possibilities arise that may not however be mutually exclusive. Killing may be specified by a gene(s) contained within the tra region. Secondly, efficient conjugation is relevant only insofar as it is a means of delivery of lethal genes into K. pneumoniae. A fully transfer-proficient hybrid, pCU101,

did not mediate killing whereas pCU109, carrying all of the region present in pCU101, and an additional 3 kb from pCU1, did. This 3 kb region present in pCU1 and pCU109, but not in pCU101 was therefore defined as the kilB region. Transposon (Tn5) insertions in a region of pCU1, quite separate from kilB also inactivated the Kil phenotype without inactivating the Tra phenotype. Therefore, there are two physically separate regions on pCU1 both of which are needed for the Kil⁺ phenotype. In addition, Thatte (Ph.D., Thesis Dissertation, Carleton University, 1984) has shown that any one of a large number of insertion mutations that abolish Tra and which map into any one of the eight known Tra complementation groups, also abolish killing. One possible explanation for these observations is that kilA and kilB have to enter K. pneumoniae in order to express the Kil⁺ phenotype. When either of these two kil regions was present in E. coli as part of a nontransmissible and nonmobilizable plasmid, no killing occurred. Furthermore, both kil regions had to be delivered at about the same time into K. pneumoniae for the latter to be killed. The prior presence of either kil region followed by the delivery of the the second kil region by conjugation did not lead to killing. However, when the same recipient (carrying either the kilA or kilB region) was mated with E. coli (pCU1), killing, of K. pneumoniae occurred.

This suggests a mechanism whereby kilA and kilB are coordinately and transiently expressed in K. pneumoniae. Alternately, products of the kilA, and or kilB region/or both are delivered into K. pneumoniae during the mating reaction. KilA::Tn5 mutants were shown to be recessive and complemented by the wild type region. No mutants of kilB were isolated. These two possibilities could, in principle, be discriminated from one another by transforming K. pneumoniae with the plasmid DNA carrying one or both of these two kil regions. Unfortunately, DNA-mediated transformation of K. pneumoniae is not yet sufficiently reproducible or efficient enough to permit such tests. The possibility that a gene or genes from the tra region is required in addition to kilA and kilB is not excluded.

The efficiency of killing exhibited by C600(pCU1) and C600(pCU109) is approximately the same (Table IV), although in pCU109, the copy number of the vector onto which the Tra and Kil were cloned is about three fold greater than that of pCU1. It has been shown that the amount of some of the gene products produced by a cell can be raised by increasing the copy number of the plasmid harbouring the gene (Uhlin et al., 1979). This gene dosage effect of pCU109 was observed in an increased number of pili produced by E.coli(pCU109), in comparison to E.coli(pCU1) (Thatte, Ph.D., Thesis Dissertation, Carleton

University, 1984).

Several possible mechanisms of killing that were discussed in the Literature Review can be considered for this Kil phenotype. Rodriguez (M. Sc. Thesis, Carleton University, 1980) has shown that killing is not mediated by any substance which diffuses from E.coli to K. pneumoniae and that viable E. coli cells carrying the appropriate plasmid are needed for killing. In addition, a test for bacteriocin production proved negative. Therefore, other possible methods by which pCUL could kill K. pneumoniae were considered. Some of the features of killing described here are shared by the phenomenon of lethal zygosis that was considered in the Literature Review. However, lethal zygosis occurs between E. coli strains and is a phenomenon that does not require plasmid DNA entry into the recipient; it occurs even when plasmid transfer is inhibited by nalidixic acid. In contrast, nalidixic acid or any process that inhibits plasmid DNA transfer also inhibits the killing of K.pneumoniae. This supports the belief that mere cell-to-cell contact between cells carrying an IncN plasmid and K. pneumoniae is not sufficient for the latter to be killed. Singleton (1983) has proposed that during solid surface mating involving IncN plasmid donors, the donor and the recipient cells are pressed together. Since the structure of IncN pili is short and rigid (Bradley, 1980), during such mating these

pili are thought to puncture the recipient cell envelope and cause cell death. This hypothesis is not supported by the experiments that were described. A number of Tra⁻ insertion mutants of pCU1 which produced pili were found to be Kil⁻. Tra⁺ Kil⁻ mutants have also been found.

E. coli(pCU1) could kill K. pneumoniae by a phenomenon analogous to zygotic induction. Presently, the possibility that the kil regions are derived from a cryptic prophage is not excluded. It is possible that such a cryptic prophage associated with pCU1 is induced in K. pneumoniae. This hypothesis would require such a cryptic prophage to be present in all or most IncN, IncP, and IncW group plasmids.

Plasmids of the IncP and IncW have been reported to kill K. pneumoniae but only at 25% of the efficiency shown by IncN plasmids (Rodriguez and Iyer, 1981). Recently, Figurski et al. (1982) have illustrated that the IncP plasmid RK2 possesses a number of loci which kill E. coli. The lethal effects of these kil determinants can be suppressed by the presence of kor regions. In the case of pCU1 two kil regions are required simultaneously to kill K. pneumoniae. This killing can be prevented by the presence of the kim region. The first indications of the presence of RK2 kil regions was the inability to clone certain regions in the absence of other regions. This could not be tested for pCU1 due to the inefficiency of

transformation of K. pneumoniae. This could be circumvented by cloning kilA and kilB of pCUI into a phasmid (phage-plasmid) cloning vector, which could exist either as plasmid or as a packaged phage particle. The region for the maturation of phage particles would be provided by a helper phage. P4-hybrid phages have been shown to allow efficient and synchronous transfer of genetic material in K. pneumoniae. In addition, they allow stable propagation and expression of DNA inserts (Ou and Ausubel, 1980; 1984). This would also clarify if killing mediated by pCUI is subsequent to or during the conjugation process. Whether any of the RK2 kil loci are the same as those involved in killing K. pneumoniae is not known. At present, it is not possible to ascribe any function(s) other than killing of K. pneumoniae to the kil regions of pCUI. These regions are not essential for replication in E. coli K-12. However, other IncN plasmids have these kil regions as shown by (i) their ability to mediate killing and (ii) by DNA-DNA hybridization (Konarska-Kozłowska and Iyer, 1983). In addition the kil genes probably evolved early and prior to the use of antibiotics since the plasmids pMUR574 and pMUR257 which were collected in early 1900's (from Murray's collection via Datta and Hughes, 1983) were capable of killing K. pneumoniae. One explanation for the ubiquity of these regions in the IncN group is that there is a need for

these plasmids to retain these regions. Although Kil⁻ mutants can be isolated (pCU167, pCU66, and pCU184), the survival of these regions in nature suggests that it is preferable to keep them. In general, plasmid functions that are highly conserved within an incompatibility group are concerned with replication and, to a lesser extent transmissibility (Figurski et al., 1982). kilA and kilB do not map within the regions required for pCU1 maintenance in E. coli (Thatte, Ph.D., Thesis Dissertation, Carleton University, 1984). However, the kil regions of pCU1 do flank the ends of the transfer region. Whether, this is fortuitous or has some significance remains to be seen. Therefore, it is interesting to speculate that the kil regions of pCU1, although seemingly nonessential in E. coli may provide auxiliary functions for their existence in other hosts.

The K. pneumoniae survivors from a mating experiment with C600(pCU1) could have arisen by two means. First, the K. pneumoniae did not have an opportunity to meet its potential killer C600(pCU1) or the K. pneumoniae has survived despite acquiring the plasmid, pCU1. Both cases were found to be true; first, some surviving colonies that were tested did not have the plasmid associated with them. Such cells were in the minority and their colonies were susceptible to subsequent killing in a second round of mating. Secondly, all survivors which did

have the plasmid were immune to killing by C600(pCU1). Plasmid DNA isolated from surviving K. pneumoniae, when examined by restriction analysis had not undergone any internal rearrangement or deletions. These survivors which had acquired the plasmid in turn killed a Nal- resistant derivative of K. pneumoniae as efficiently as C600(pCU1).

In an attempt to determine whether there was a specific plasmid region responsible for the immunity, different derivatives of pCU1 were introduced into K. pneumoniae. Only some of these conferred a form of immunity that was lost when the plasmid was cured from the cells. This region called kim was physically distinct from kilA and kilB. The Kim phenotype required the continuous presence of these derivatives and was lost concomitantly with the loss of the plasmids. This type of immunity is different from the immunity for lethal zygotis. Immunity to lethal zygotis (Ilz) is reported to be due to a surface exclusion phenomenon. Surface exclusion is a phenomenon whereby sex factor carrying cells are poor recipients in mating experiment with donor cells carrying a related sex factor. However, the K. pneumoniae strain carrying any one of these derivatives which confer immunity is a better recipient than K. pneumoniae devoid of a plasmid. Hence surface exclusion and intracellular incompatibility do not seem to play a role in the Kim phenotype. The smallest cloned region which exhibits immunity is approximately 4.5

kb. However, since no Tn5 insertion in this clone that inactivated the Kim phenotype could be isolated, the region may in fact be much smaller.

The mechanism(s) by which Kim acts is not known. It could act by several modes: (i) a product of the gene could inactivate a kilA-kilB complex, (ii) it could modify the site of action of the presumed complex,

(iii) it could prevent the coordinated expression of kilA and or kilB regions that were postulated to be necessary for killing.

In addition to the plasmid dependent immunity, there is another type of immunity which persists after curing of the plasmid. For this type of immunity two regions are required each of which is closely linked to kilA and kilB. However, there are insertion mutations in kilA which inactivate the Kil phenotype without inactivating immunity. There also exists a deletion derivative (pCU401) which provides this type of immunity to K. pneumoniae without providing (to E. coli or K. pneumoniae) the Kil phenotype. On the pCU1 map, this immunity region maps adjacent to kilA and overlaps with some tra genes. In contrast, it has not been possible to resolve the 3kb kilB region into separate sub-regions determining the killing and immunity phenotypes. Extended efforts in this direction and the sequencing of kilA and kilB (currently in progress) may shed further light on

this phenomenon. Within the limits of the hybridization experiments described, no part of pCU1 was detected in the cured and immune K. pneumoniae strains. The sensitivity of these experiments need to be improved but one other possibility is that the plasmid pCU1 may provide some function to a defective indigenous Insertion Sequence like element or "inverton" which is causing this type of immunity.

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